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MSS. should be sent in **duplicate** only after careful revision and correction, otherwise they are liable to be refused. Authors are asked especially (*a*) to verify the proper logical arrangement and sequence of their headings and sub-headings, and (*b*) to maintain uniformity in the use of contractions, capitals, italics, etc. A short summary of the salient points of the paper should be placed at the end.

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The following are some of the abbreviations in use :—

Amer. J. Hyg.
Ann. Trop. Med. Parasit.
Brit. Med. J.
Bull. Ent. Res.
Bull. Soc. Path. Exot.
C. R. Soc. Biol.
Geneesk. Tijd. Ned.-Ind.
Ind. J. Mal.
Ind. J. Med. Res.
J. Mal. Inst. Ind.
Jl. R. A. M. C.
J. Trop. Med. Hyg.
Meded. Volk. Ned.-Ind.
Rec. Mal. Surv. Ind.
Rev. App. Ent.
Ric. Malariol.
Trans. Roy. Soc. Trop. Med. Hyg.
Trop. Dis. Bull.
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ERRATA

Indian Journal of Malariology, **7**, 1, *March* 1953.

Siddons, L. B. SCREENING OF ANTIMALARIAL COMPOUNDS IN MICE WITH *PLASMODIUM BERGHEI* INFECTION.

Page 46, Table II, Reference number Q 11, Column 2,
read 2-methyl-4-(2 : 5-dichlorobenzenesulphonyl)-
for 2-methyl-4-2 : 5-(dichlorobenzenesulphonyl)-

Page 47, Table III, Reference number B 201, Column 2,
read 4-nitro-4-
for 4-nitro-4-

Ramakrishnan, S. P. STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948. VIII. The course of blood-induced infection in starved albino rats.

Page 54, para 4, line 2, *read* 6 rats
for 16 rats

Indian Journal of Malariology, 7, 1, *March* 1953.

MALARIA IN THE MALDIVE ISLANDS.

BY

M. O. T. IYENGAR,

M. I. MATHEW,

AND

M. A. U. MENON.

(March 31, 1953.)

DURING January to March 1951 when the authors were engaged on an investigation on filariasis in the Maldives, they made certain observations on the incidence of malaria, which are recorded in this article. The Maldives, situated in the equatorial region of the Indian Ocean, south of the Laccadives and south-west of Ceylon, are an archipelago of tiny coral islands, about 2,000 in number, of which only about 200 are inhabited. A description of these islands is given in Iyengar (1952).

The authors visited several islands in four atolls, namely Male, Haddumatti, Suvadiva and Addu, in the southern part of the Maldives. Cases of clinical malaria were observed to occur in most of the villages visited. The incidence of such cases was particularly high in the villages of Addu, the southernmost atoll of the Maldives.

In ten villages, children under 12 years of age were examined for splenic enlargement. The results are furnished in Table I. In most of these villages, the spleen rate was high; in seven out of the ten villages examined, the spleen rate was higher than 50 per cent. Malaria appeared to be highly endemic in most of the villages visited.

TABLE I.

Results of the examination of children for enlarged spleen.

Atoll.	Village.	Number of children examined.	Number with enlarged spleen.	Spleen-rate, per cent.
Male	Hulule	20	14	70·0
	Wilingili	5*	2	40·0
Haddumati	Medawa and Barisulu	38	20	52·1
Addu	Hitadu	44	25	56·8
	Maradu	51	26	51·0
	Feidu	27	11	40·7
	Gan	40	10	25·0
	Holedu	48	28	58·3
	Midu	28	16	57·1

*Wilingili is a village with a small population; a larger number of children was not available for examination in this village.

The malaria parasite rate among children, 2 to 11 years of age, was determined in one village, namely Maradu in Addu Atoll. Out of 51 children examined—both thin and thick smears being examined—23 were found to be positive for malaria parasites, giving a parasite rate of 45·1 per cent.

The relative incidence of the different species of malaria parasites, based on the examination of blood smears from 98 children from the villages of Addu Atoll, is presented in Table II.

TABLE II.

Results of the examination of blood smears taken from children in Addu Atoll.

Number examined.	Number positive.	CLASSIFICATION.				
		Falciparum.	*Vivax.*	*Malariæ.*	Mixed *falciparum* and *vivax.*	Mixed *vivax* and *malariæ.*
98	44	6	10	26	1	1

It is of interest to note that *Plasmodium malariæ* is the predominant species in these villages. The number of *P. malariæ* infections recorded was much greater than the other two infections, *P. vivax* and *P. falciparum*, taken together. Of the

44 positives recorded, *P. malariæ* occurred in 27 cases, *P. vivax* in 12 and *P. falciparum* in 7.

The Maldives have a poor anopheline fauna. The individual islands are very small in area and lack natural breeding places such as marshes, ponds and depressions. Only two species of *Anopheles* were recorded during this investigation, namely *A. subpictus* and *A. tessellatus*. The former was of rare occurrence and was recorded from a single locality, Gadu Island in Haddumatti Atoll. *Anopheles tessellatus*, on the other hand, was found to occur in all the inhabited islands that were visited. It breeds extensively in wells and to a small extent in step-wells. The villages have a large number of wells,—shallow wells, about 3 feet in diameter, dug in the coral-gravel soil. The subsoil water is close to the ground surface, at a depth of 3 to 4 feet. Almost every house has a well, wells being the only source of fresh-water supply. At the time of this investigation, which was a comparatively dry season in the Maldives, *A. tessellatus* was found breeding in 27·9 per cent of the wells (177 out of 635 examined). The breeding incidence is likely to be even higher during the rainy season, May to August.

In comparison with wells, the extent of *A. tessellatus* breeding in step-wells was low, evidently because the water in step-wells was often grossly contaminated. This mosquito was found breeding only in 4·2 per cent of the step-wells (28 out of 659 examined). There are no other situations on these islands, besides the wells and the step-wells, where *A. tessellatus* could breed.

Anopheles tessellatus was found to be the malaria vector in the Maldives. The authors examined 22 specimens of this species caught from dwelling houses. Of these, one showed heavy sporozoite infection.

Covell (1944) had previously reported the finding of two gland infections and four gut infections from among 160 specimens of *A. tessellatus* examined from certain islands of the Maldives.

The adults of *A. tessellatus* rest entirely indoors. Their favourite resting place is the lower part of the walls, close to the flooring, in the dark corners of the huts.

SUMMARY.

Malaria appears to be highly endemic in the villages of the southern atolls of the Maldive Islands. In the majority of the ten villages examined, the spleen rates ranged between 40 and 60 per cent. The parasite rate in children, which was determined in one village, Maradu, was 45·1 per cent. Quartan is the predominant infection in the Maldives; this infection outnumbers the combined number of tertian and subtertian infections.

Anopheles tessellatus is the vector of malaria in the Maldives. Out of 22 specimens examined, one was found naturally infected, and showed a heavy sporozoite infection. This species, which is widely prevalent in the inhabited islands of the Maldives, breeds extensively in wells and to a small extent in step-wells.

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Indian Journal of Malariology, **7**, 1, *March* 1953.

THERAPEUTIC TRIAL OF PYRIMETHAMINE (DARAPRIM) IN HUMAN MALARIA.

BY

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(March 10, 1953.)

PYRIMETHAMINE (Daraprim), a product of the Welcome Laboratories of New York and London, has a chemical structure as 2 : 4-diamino-5-4 chlorophenyl-6-ethylpyrimidine and is available in compressed tablets of 25 mg. each.

The manufacturers claim that comparatively small doses are sufficient to control acute attacks of malaria.

Therapeutic trials of the drug were organized in a few villages in Uttar Pradesh, Terai where transmission was in progress, and also in the Colonisation Hospital at Rudrapur during the later part of October 1952. The following five regimes were adopted for administration of the drug:—

Regime I.—A single dose of 2 tablets (50 mg.) once only.

Regime II.—A single dose of 2 tablets (50 mg.) on two consecutive days.

Regime III.—One tablet (25 mg.) once a day only.

Regime IV.—One tablet (25 mg.) twice daily for one day.

Regime V.—One tablet (25 mg.) twice daily for two consecutive days.

These regimes refer to adult doses. Proportionate doses were administered to children.

A total of 86 microscopically diagnosed cases were placed under the different regimes. In view of predominance of *P. falciparum*, the number of *P. vivax* treated was only ten (Table I). There were also a few cases with mixed infection of *P. vivax* and *P. falciparum*.

As a rule, patients were discharged only when they were afebrile and no asexual parasites were detectable in the peripheral blood. Thereafter, in most cases blood smears were collected daily for a period of 8 to 10 days for "follow up" observation.

As active transmission was in progress in the area, further observation was considered to be unnecessary.

TABLE I.

Species of plasmodia and distribution of cases as per regimes.

Regime.	*P. vivax.*	*P. falciparum.*	*Mixed.*	Total
I	2	12	1	15
II	3	16	...	19
III	1	13	1	15
IV	...	13	1	14
V	4	17	2	23
Total ...	10	71	5	86

The effect of the drug on the course of the disease is shown in Table II.

TABLE II.

Relief of clinical symptoms (within hours) after treatment with pyrimethamine in P. vivax *and* P. falciparum *cases.*

Regime.	*P. vivax.*						*P. falciparum.*							
	Total number of cases.	Number afebrile initially.	Number of cases with fever.	24	48	72	Total number of cases.	Number afebrile.	Number of cases with fever.	24	48	72	96	120
I	2	1	1	...	1	...	12	...	12	7(53·3)	...	2(81·8)	3(100)	...
II	3	1	2	2	...	...	16	3	13	8(61·5)	5(100)	...	...	...
III	1	...	1	1	...	...	13	2	11	7(63·6)	2(87·7)	...	1(90·0)	1
IV		None					13	3	10	7(70·0)	...	2(90·0)	...	1
V	4	3	1	...	1	...	17	4	13	9(69·2)	3(90·2)	1(100)	...	1

Figures in bracket indicate percentage.

Clinical symptoms.—In *P. vivax* infection, it was observed that 5 out of 10 cases had fever at the time treatment was commenced, and in all of them, irrespective of the dose, relief was attained within 48 hours. None of the other cases showed any clinical manifestation at the time treatment was commenced.

Early recrudescence was not encountered in any of these cases. Out of 71 cases of *P. falciparum* infection, 59 cases had fever at the time of treatment. A few of them were of hyperpyrexial bilious or algid type and they were placed under Regimes II and V. Prompt relief of clinical symptoms was observed in all cases. Further, under the two regimes, in all cases, either of the severe or ordinary type, patients became afebrile within 72 hours. But under the other regimes, similar results were encountered in 81 to 100 per cent of cases during the same period.

Irrespective of the regimes, in some cases after initial relief of clinical symptoms, patients developed fever again after 24 to 48 hours even though parasitæmia was considerably low. This feature was more evident under Regime IV where 4 out of 13 cases behaved in a similar manner. In one case, the drug had no effect whatsoever.

Three out of twelve cases who were initially afebrile, developed fever 24 to 48 hours after the treatment was commenced (2 under Regime V and 1 under Regime III).

All the cases with mixed infection who had fever initially, were relieved of clinical symptoms within 24 to 48 hours.

Asexual parasites.—As will be observed from Table III, in all the 7 cases under Regimes II and V, clearance of asexual parasites in *P. vivax* infection was obtained within 48 hours whereas the same was observed in two cases under Regime I within 72 hours. The only one case, treated as per Regime III, was free from parasites within 24 hours.

TABLE III.

Clearance of asexual parasites (within hours) after treatment with pyrimethamine in P. vivax *and* P. falciparum *cases.*

Regime.	*P. vivax.*				*P. falciparum.*				
	Number of cases.	24	48	72	Number of cases.	24	48	72	96 or over
I	2	...	...	2	12	6 (50·0)	4 (83·3)	2 (100)	...
II	3	...	3	...	16	5 (31·2)	4 (59·2)	7 (100)	...
III	1	1	...	...	13	6 (46·1)	4 (76·9)	2 (92·2)	1
IV	None	...	...	...	13	4 (30·7)	2 (46·0)	3 (69·7)	4
V	4	1	3	...	17	11 (64·7)	6 (100)	...	...

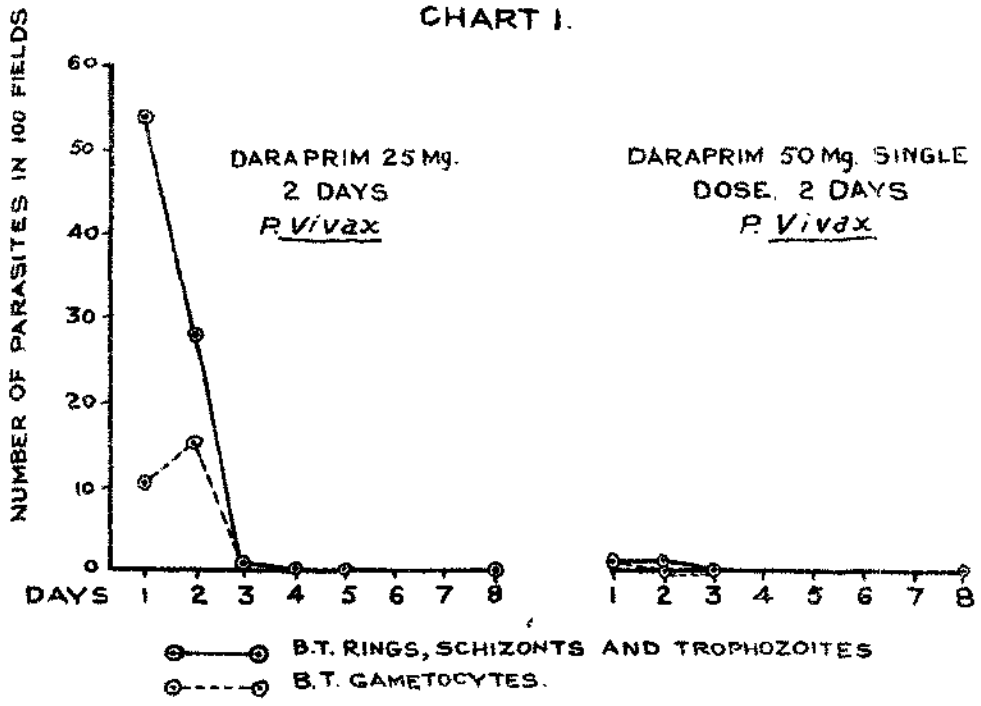
However, in *P. falciparum* infection the response was not the same under Regimes I, II and V. Parasite clearance was attained in all cases within 72 hours

and between 59 and 100 per cent of cases within 48 hours. The rate of clearance was found to be still lower under Regimes III and IV where only 46 and 76 per cent of cases respectively had parasite clearance within 48 hours, and 69·7 and 92·2 per cent within 72 hours. In several cases (4 out of 13 under Regime IV and 1 case under Regime III) parasitæmia was observed beyond this period and in some cases beyond 96 hours. The overall picture shows that the best results were obtained under Regime V whereas Regimes I and II are the second best.

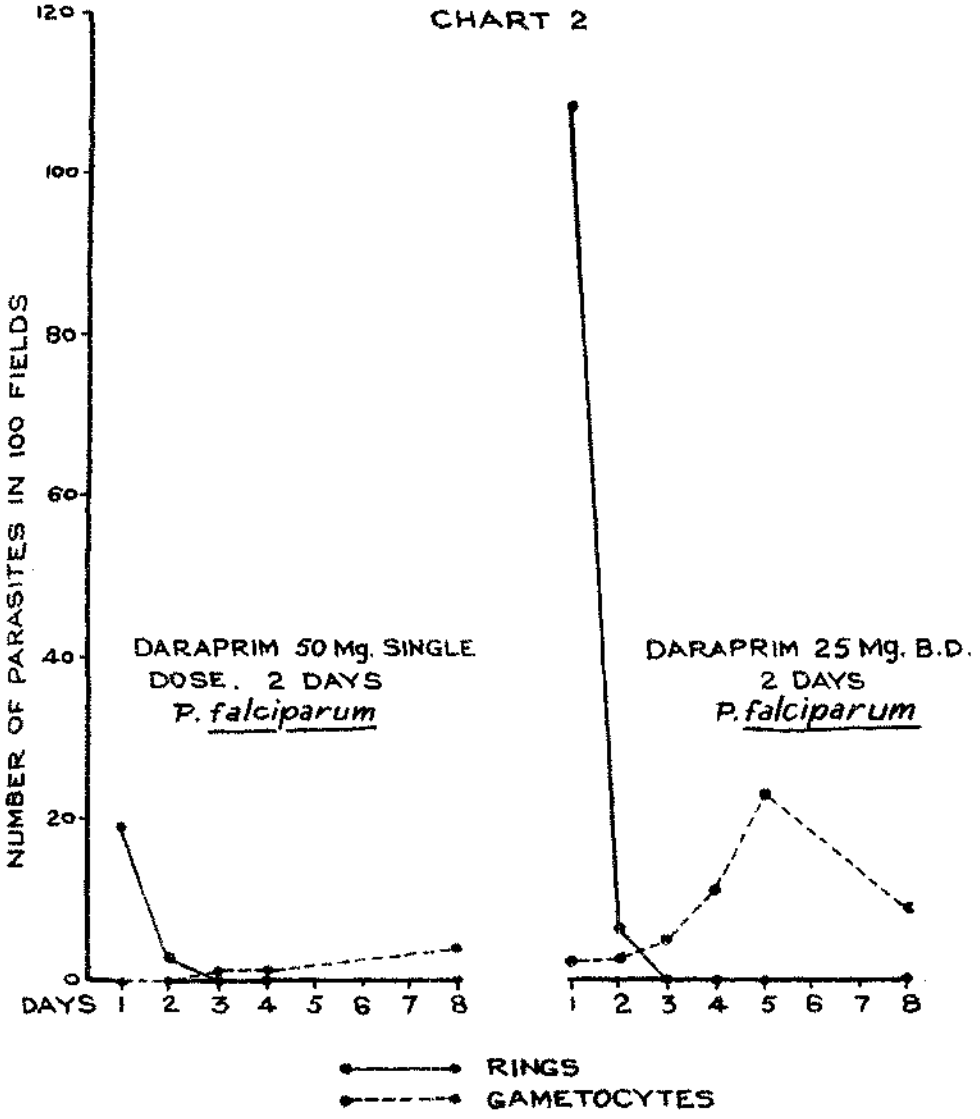
Parasite clearance in the cases with mixed infection was attained in all cases between 48 and 72 hours, but none within 24 hours. In one case under Regime I, ring forms of *P. falciparum* were not detectable until after 48 hours.

CLEARANCE OF GAMETOCYTES.

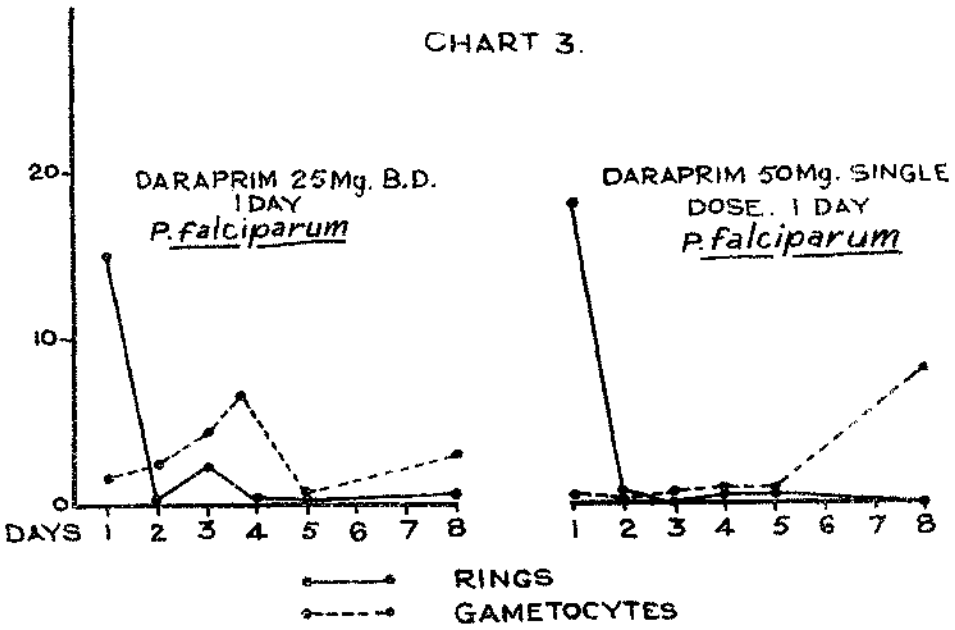
In *P. vivax* infection, gametocyte clearance was as rapid as the asexual forms (Chart 1) whereas in *P. falciparum* infection the crescents, detected initially, persisted throughout the period of observation in most cases, irrespective of the regime of treatment. In some cases where no gametocytes were found initially or only a few in number, these appeared progressively and reached a stage of heavy parasitæmia even when no asexual forms were detectable (Charts 2 and 3). However, in a few cases some of the micro- and macro-gametocytes were found in various stages of degeneration. Besides, there were a few cases of atypical forms



somewhat resembling those of the Abyssinian strain described by Brumpt (1949). Immature forms of the crescents, soon after the beginning of treatment, was a common feature.



In *mixed* infections, gametocytes of *P. vivax* disappeared promptly whereas crescents persisted.



DISCUSSION.

In *P. vivax* and *mixed* infections, relief of clinical symptoms and clearance of asexual parasites were fairly rapid; similar to that observed by Jaswant Singh, Ray, Basu and Misra (1952) in the series of cases who had previous attacks of malaria. But in fresh cases they reported that the response of the plasmodium to the drug was tardy in spite of two doses of 25 mg. and at least in one case recourse to other antimalarials had to be taken.

As regards the regimes of treatment in the present series, a critical analysis is not possible as the number per regime is far too small. However, it may be said that in none of the cases did the fever last beyond 72 hours nor was any asexual parasites detected.

Although McGregor and Smith (1952) and Schneider *et al.* (1952) reported excellent results in *P. falciparum* cases with a single dose of the drug varying from 25 to 100 mg., the results observed in the present series cannot be called as encouraging. Besides a frank case of failure, in several cases early recrudescence was found to be a common feature. In some cases fresh bouts of fever were observed in patients who became afebrile after 24 to 48 hours after the treatment began although parasitæmia was invariably low. In this respect, the behaviour of pyrimethamine in some cases appeared to be somewhat similar to proguanil. Similar observations have been recently made by Chakravarty and Chaudhuri (1953) who reported several cases of failures and early recrudescence.

However, in the majority of the cases in the current studies, relief of clinical symptoms and clearance of asexual parasites were observed within 72 hours, similar to that reported by Jaswant Singh, Ray, Misra and Basu (1952) who have reported only 2 cases of failures out of 84 cases treated with a dose of 25 mg. on two consecutive days. Further, an encouraging feature of pyrimethamine treatment was that a few cases responded remarkably well to the drug. It should be pointed out at this stage that all such cases were placed along with some others under Regimes II and V where drug was administered on two consecutive days. Undoubtedly these two regimes proved more satisfactory than the others as 90 to 100 per cent of the cases were afebrile within 48 hours. Cases under Regimes III and IV responded rather poorly. Regime I showed somewhat intermediate result.

Thus the overall picture appears to be that in *P. falciparum* infection only higher doses responded well, but even then, compared to antimalarials like chloroquine or camoquin, action of pyrimethamine appears to be somewhat slower.

In view of the very satisfactory results reported by McGregor and Smith (*loc. cit.*) and Schneider *et al.* (1952), and comparatively less encouraging observations made in the present series, it may be argued that the difference in the reaction is attributable to the strain differences. Coggeshall (1952) observed that it is too commonly believed that any standard form of therapy is sufficient. This is far from true. It is essential to know not only the species involved but also the strains.

Besides the strain differences, recently certain new factors have also come to light. Jaswant Singh, Ray, Basu and Nair (1952), and Schmidt and Genther (1953) have shown the existence of cross resistance between proguanil and pyrimethamine in *P. knowlesi* and *P. cynomolgi* respectively. Gilroy (1952), Hay Arthur (Goodwin, 1952), and Robertson, Davey and Fairley (1952) reported that in areas where the population was previously treated with proguanil, pyrimethamine failed to respond well.

It is difficult to assess whether such factors were operative in the present series to explain for the tardy action in some cases. But in view of the present popularity of proguanil amongst the masses, it should not be surprising that at least a few of the cases had been taking proguanil whenever they had febrile attacks.

The remarkable increase in the gametocyte count in some cases is another interesting factor and these observations are similar to those observed by Jaswant Singh, Ray, Misra and Basu (1952).

SUMMARY.

A total of 86 cases (10 *P. vivax*, 71 *P. falciparum*, 5 *mixed*) were treated with pyrimethamine under five different regimes.

Cases with *mixed* and with *P. vivax* infections were promptly relieved of fever and asexual parasites.

In *P. falciparum* infection, although majority of the cases were afebrile within 72 hours, in some cases the reaction of the plasmodium to the drug was tardy. There was one case of failure and several cases of early recrudescence.

Regimes II and V showed the best results.

Gametocytes of *P. vivax* responded as promptly as the asexual forms. But the crescents were mostly refractory and in some cases there was considerable increase (absolute) in the gametocyte count.

ACKNOWLEDGMENT.

The authors thank the Welcome Laboratories Ltd., for adequate quantities of pyrimethamine (Daraprim) supplied through the Director, Malaria Institute of India. They also wish to express their appreciation of guidance and help rendered by the Director, Malaria Institute of India.

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EFFECT OF PYRIMETHAMINE IN HUMAN MALARIA (SUPPRESSIVE TREATMENT).

PART III.

BY

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JASWANT Singh, Ray, Basu and Misra (1952) and Jaswant Singh, Ray, Misra and Basu (1952) have already reported their observations on the effects of pyrimethamine in the treatment of acute attacks of *P. vivax* and *P. falciparum*. Cases with latter infection were treated at two centres in U.P., Terai. In the same area certain groups of the population were placed on suppressive treatment during 8 to 10 weeks when transmission of malaria was active. The present paper records the results with pyrimethamine in suppressing overt attacks.

Prior to commencement of treatment, a survey of spleen and parasite rates was undertaken as an initial measure on which selection of the population was mainly based. Where the rates were high the whole population was placed under suppressive treatment, whereas in villages with relatively lower rates, only school children were treated and not the entire population. The neighbouring villages were kept under observation for comparison.

Subsequent surveys were carried out, once when the investigation was half way through, once soon thereafter, and for the last time 10 weeks after the termination of suppressive treatment. The second and fourth surveys were of check nature and their data have not been included in Table I. During the period (December to February) between the two last surveys, transimssion, if any, is believed to be at its minimum. For parasite survey, J. S. B. stain (Jaswant Singh and Bhattacharji, 1944) was used throughout these studies.

The number of anopheline species in the area exceeds a dozen. *A. culicifacies* and *A. fluviatilis* have been incriminated as responsible for transmission which goes on throughout the year in varying degrees. The intensity is the highest during August to October and lowest during December to February. *A. fluviatilis* has been incriminated as the vector species during the pre-monsoon (March-June) and post-monsoon periods (October and November) while *A. culicifacies* is the chief vector during the monsoon period from July to September (Issaris, Rastogi and Ramakrishna, 1953).

On two occasions during 1952 (August and September), *A. culicifacies* was found infective (Ramakrishna, 1952). In October, sporozoites were detected in two *A. fluviatilis*, one from Manpur (Experimental Centre III, Table I), a village chosen for suppressive treatment, and the other from a neighbouring area (Chakrabarti, 1952; Ramakarishna, 1952).

A total of 304 persons at four experimental stations were placed on suppressive treatment. Of these, 92 were school children 4 to 12 years of age from two villages. During the initial survey in Experimental Station I, 35 out of 55 students were attending the school. Out of these, 9 gave history of recent attacks of fever and in some spleen was palpable. The large number of absentees were said to be mainly due to illness (fever). The rest (212) consisted of the entire population of two villages of all age groups including infants. Population of the two villages which served as comparison stations (1 and 2, Table I) consisted of about 176 persons of different age groups.

Some individuals under the experimental group were treated with one tablet of 25 mg. base of pyrimethamine (Regime I) as an adult dose while the others received double the dose (Regime II). Children, between 3 and 12 years, received one-half tablet (12·5 mg. base) and those below 3 years one-quarter tablet (6·25 mg. base) under Regime I. For corresponding age groups in children the doses under Regime II were one tablet and one-half tablet respectively. In all cases the drug was administered under the direct supervision of at least one of the authors who had ensured in each case that the drug was actually swallowed. Children who could not swallow were made to chew it up and as pyrimethamine is without bitter taste no difficulty was encountered.

Weekly records were carefully kept in each case and those who missed the drug on any particular occasion were marked 'A' (absent). Such cases were fewer in experimental centres I to III as they were usually traced by domiciliary visits. Further, those who were not traceable on any particular day were sought out and treated the following day.

After cessation of suppressive treatment weekly visits were made in every village for ten weeks and blood smears of any one complaining of fever during the week were examined, for patent parasitæmia.

The spleen and parasite rates prior to commencement and just after termination of the suppressive treatment, in the four experimental stations have been shown in Table I. Along side, similar data in the two comparison areas during the corresponding periods have also been included.

TABLE I.

Spleen and parasite rates in experimental and comparison areas.

Station.	Number of persons.	Regime.	Spleen rate.		Parasite rate.		Remarks.
			Before treatment.	On termination of treatment.	Before treatment.	On termination of treatment.	
Experimental I	55*	I	19·1	3·6	14·7	0	
II	37*	I	16·2†	10·7	13·5	0	
III	58	I	36·2†	28·0	20·7	0	
IV	154	II	25·0	13·7	24·3	0	
Total	304						
Comparison 1	55	No anti-malarials administered	27·2	34·5	32·7	41·8	
2	121		10·0	12·4	17·0	26·4	
Total	176						

*School children.

†In majority of cases, spleens were fairly enlarged and hard, and many of them had *P. vivax* infection.

Dramatic reduction in parasitæmia was evident during the survey carried out between the 5th and 6th week after commencement of suppressive treatment and by the end of 10 weeks parasite rates in all experimental centres were nil. On the other hand in the comparison areas far from any reduction, the rates were somewhat higher than observed during the initial survey.

While reduction in spleen rate in Experimental Centre I amongst the school children was quite appreciable, response in the two other centres under Regime I was not so satisfactory. In Experimental Centre IV, the spleen rate was reduced by about 50 per cent at the termination of the suppressive treatment. In this connection it may be mentioned that both in Experimental Stations I and IV, during the initial survey enlarged spleens were usually soft and just palpable in majority of the cases. Blood examination revealed predominance of *P. falciparum* infection.

There was only one case of overt malaria under Regime I in Experimental Centre III. A woman aged 20 years had taken the drug regularly every week for 4 weeks. In one case under Regime II crescents were detected during the second parasite survey, *i.e.* 5 weeks after commencement of treatment.

There were two more cases of acute attacks of malaria under Regime II but they had missed their weekly doses in two consecutive weeks.

The general health of the population placed under suppressive treatment was found to be considerably improved. This was particularly so in school children and school register showed higher attendance. Prior to commencement of treatment, average attendance in the schools in experimental centres was

between 60 and 70 per cent as against 90 to 95 per cent during and after the suppressive regime. Similar results were observed in Experimental Centre IV, where before the treatment quite a number of villagers were unable to go for harvesting and were attending the local dispensary. But since the treatment began no case of fever reported to the dispensary, and almost every one was actively engaged in harvesting or tilling.

Conditions in the comparison villages continued to be the same throughout and a number of villagers were found to be suffering from malaria every time the stations were inspected.

DISCUSSION.

Vincke (1952) reported satisfactory results in a group of school children placed on suppressive treatment with pyrimethamine administered as a single weekly dose of 25 mg. base irrespective of age for a period of about 10 weeks. Subsequently, Coatney (1952) demonstrated that a weekly dose of 3·12 to 25 mg. base effected complete suppression in sporozoite-induced infection with *P. vivax* (Chesson strain) in human volunteers.

From the data on the current investigations presented earlier, it was evident that active transmission was in progress in the area selected for suppressive treatment. Before drug administration was commenced it was observed that the parasite rates at the different stations were fairly high particularly so at Experimental Station IV and Comparison Station I (Table I). Moreover, at this stage many people were actively suffering from malaria. Rapid reduction of parasite rates within a few weeks after the commencement of treatment demonstrates the effective suppressive action of pyrimethamine. Parasite rates in the experimental centres after termination of treatment, were nil in all the experimental groups, while they were as high as 41·8 and 26·4 per cent in the comparison groups. This is understandable as the trials were carried out during heavy transmission when the parasite index would normally be expected to show a progressive rise.

Reduction in spleen rates was more significant in Experimental Centres I and IV than that observed in Experimental Centres II and III. Explanation might be sought in the type of infection and enlargement of spleen observed at the different centres. In Experimental Centres II and III, it was found that spleens were mostly large and hard indicating infections of long standing. During the initial survey, *P. vivax* was found to be the predominant species. On the other hand in Experimental Centres I and IV there was a predominance of *P. falciparum* infection and in most cases spleens were soft and just palpable, and as such, they were more amenable to treatment than the other type encountered.

Coatney (*loc. cit.*) reports that as small a dose as 3·12 mg. was effective in suppressing *P. vivax* infection caused by the bites of 10 infected *A. quadrimaculatus*. But in the present series a dose of 25 mg. base did not suppress *P. falciparum* infection in the case who had been taking the drug regularly for 4 weeks. As no such breakthrough occurred under Regime II it may be interpreted that either the dose adopted in Regime I was insufficient in this particular case, or the quantum of infection which occurred in nature was of a more severe nature than that induced

by mosquito bites in the series observed by Coatney (*loc. cit.*). It would, however, indicate that, though, quite encouraging results were attained with the doses adopted under Regime I there might be occasional cases of genuine break-through. In this respect Regime II appears to be the better of the two but adoption of this regime would automatically increase the cost *per capita*.

Parasite rate was observed to be *nil* at all experimental stations except at IV where the rate was 3·3 per cent even 10 weeks after discontinuation of treatment and this indicates prolonged suppressive effect of pyrimethamine.

As only a small dose of the drug is required for suppressive treatment it is presumed that the price structure would be proportionately lower than other antimalarials commonly used for this purpose. In that event suppressive treatment, whenever considered necessary, would undoubtedly be economically advantageous if pyrimethamine is used. But at this stage a word of caution may not be considered out of place. Recently it has been shown by Schmidt and Genther (1953) that resistance to pyrimethamine occur with relatively great frequency and rapidity in plasmodia like *P. cynomolgi* in *rhesus* monkeys and *P. gallinaceum* in fowls. Further, it has been observed that a strain of plasmodium which has acquired resistance to proguanil is also refractory to pyrimethamine and *vice versa* (Jaswant Singh, Ray, Basu and Nair, 1952; Robertson, Davey and Fairley, 1952; Schimdt and Genther, 1953).

In view of these observations the authors are not quite clear at the moment as to what would be the rôle of these two compounds in suppressive treatment in malaria although it is likely that they would be comparatively cheaper than the drugs of the 4-aminoquinoline series like chloroquine or camoquin.

SUMMARY.

Two regimes were adopted for suppressive treatment in an area where active transmission was in progress. A weekly dose of 25 mg. base of pyrimethamine for an adult and proportionate dose for children was administered to 150 in one group and double the dose to 154 in another group for a period of 8-10 weeks. In the two comparison areas no antimalarials were used.

Initial survey showed high parasite rate in all and fairly so in some areas. During subsequent surveys after the treatment began there was marked reduction in parasitæmia and the rate was nil 10 weeks after commencement of treatment and continued to be so upto 10 weeks after termination of the course. Spleen rate was considerably lowered at least in two centres.

In the comparison areas both spleen and parasite rates continued to be high.

There was one case of genuine break-through under Regime I but none under Regime II although crescents were detectable in one case 5 weeks after treatment. In this, there was general improvement in health of the population placed under suppressive treatment, increase in school attendance, reduction in dispensary figures from these areas, as well as incentive to work amongst adult population.

In the comparison areas there was hardly any appreciable change in the general health and number of acute attacks was quite high.

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4-AMINOQUINOLINES IN THE SINGLE DOSE TREATMENT OF MALARIA.

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CONVENTIONAL treatment of malaria usually requires administration of an antimalarial in single or divided doses for periods ranging from three to ten days. While such regimes may be convenient particularly in hospitals and urban areas, they have been found by experience, to be difficult and irksome under rural conditions in India.

On the basis of preliminary reports on the effectiveness of proguanil in relatively small doses, extensive field and hospital investigations were undertaken in India in 1946 and one of the regimes adopted, was 0·3 gm., administered as a single dose to cases attending some rural dispensaries in Baluchistan. Although relief of clinical symptoms was observed in over 80 per cent of cases, early recrudescence was a common feature (Afridi *et al.*, 1947). The regime, therefore, fell far short of expectations.

On the other hand, preliminary reports on single dose treatment with amodiaquine (camoquin) proved highly effective with comparatively low relapse rates (Simeons and Chhatre, 1947; Halawani *et al.*, 1948; Patel and Mehta, 1948). Chaudhuri and Chakravarty (1948) commenting on the single dosage treatment with amodiaquine observed that the drug is "ideally suited for rural conditions". As regards chloroquine, though extensive trials had been undertaken in the past and volume of literature is available on the subject, reports on large scale use of this drug in a single dose are comparatively few, and it has not been possible to evaluate the relative merits of amodiaquine and chloroquine, when administered in a single dose.

In order to assess the rôle of single dose treatment, extensive hospital and field trials were initiated in various rural areas of India during 1952 using* resochin, nivaquine and amodiaquine.

*Resochin was received through the courtesy of Bayer Laboratories, Leverkusen, Germany. Nivaquine and amodiaquine tablets were received through the courtesy of May & Baker and Parke, Davis & Co., Bombay, respectively.

The present report records the findings of investigations conducted during active transmission season, at two centres in Uttar Pradesh. One of these was established at the rural dispensary of Maswasi in Sowar Tehsil of Rampur District while the other was in Gadarpur in the district of Naini Tal.

All fever cases reporting at the dispensaries were subjected to blood examination and diagnosed microscopically. Two hundred and sixty one positive cases were treated. Some of them were treated with a single dose of 600 mg. of resochin (chloroquine diphosphate), nivaquine (chloroquine sulphate) or amodiaquine, while others with 400 mg. of resochin or amodiaquine.

The criterion of activity was the rate of clearance of asexual parasites from the peripheral blood. As the cases were from widely distributed areas with difficult means of communication, no attempt was made to record the temperature though it was done in those who reported to the dispensaries on the following days. But comparatively such cases were few, as by their very nature the villagers, when free from fever, were reluctant to attend dispensaries instead of harvesting. It was, therefore, necessary to make frequent domicilliary visits.

For rapid microscopic diagnosis, J.S.B. stain (Jaswant Singh and Bhattacharji, 1944) was found extremely useful. In view of the rapidity with which thick and thin smears could be stained, an amazingly large number of cases could be examined daily at the dispensaries and in the villages. Chiefly for study of relapse rates right through the period of 3 to 5 months until the beginning of the spring transmission, blood smears were examined at interval of 10 days of practically all cases present at each visit.

The distribution of cases under the different regimes, types of infection and the clearance of asexual parasites are shown in Tables I and II.

TABLE I.

Rate of asexual parasite clearance under 600 *mg. dosage schedule.*

Type of infection.	Drug.	Number of cases.	Clearance (expressed in percentage within hours.)		
			24	48	72
P. vivax	Amodiaquine	24	87·0	96·7	100·0
	Resochin	24	66·6	100·0	...
	Nivaquine	29	65·5	100·0	...
	Total	77			
P. falciparum	Amodiaquine	56	73·2	95·2	100·0
	Resochin	41*	70·0	100·0	...
	Nivaquine	25	48·6	100·0	...
	Total	122			

*One patient had scanty infection of *P. vivax* as well.

In *P. vivax* infection, the rate of asexual parasite clearance in cases treated with resochin or nivaquine was found to be similar. During the first 24 hours amodiaquine seemed to act somewhat quicker than the other two. But by 48 hours, the clearance rate was 100 per cent in all cases treated with chloroquine diphosphate or sulphate as against 96·7 per cent under amodiaquine.

In *P. falciparum* infection, the rate of clearance within 24 hours, under amodiaquine and resochin, was more or less similar. Nivaquine appeared to act slower than the other two. But by 48 hours asexual parasites had disappeared from peripheral blood in all cases treated with resochin or nivaquine as against 95·2 per cent of cases under amodiaquine treatment.

Table II shows the effects of 400 mg. of amodiaquine and of resochin in *P. vivax* and *P. falciparum* infections :—

TABLE II.

Rate of asexual parasite clearance under 400 mg. dosage.

Type of infection.	Drug.	Number of cases.	Clearance (expressed in percentage within hours.)		
			24	48	72
P. vivax	Amodiaquine	9	66·6	100	...
	Resochin	6	50·0	100	...
	Total	15			
P. falciparum	Amodiaquine	22	76·3	86·5	100
	Resochin	25	52·0	96·0	100
	Total	47			

The number of *P. vivax* cases was comparat vely small in this series, and the difference in the rate of parasite clearance is not statistically so significant. More important point is that even with this small dose complete parasite clearance *within 48 hours was attained in all cases.*

Reaction of 400 mg. of amodiaquine on *P. falciparum* infection during the first 24 hours was surprisingly similar to that observed with the higher dosage (Table I). But the subsequent clearance rates showed slight superiority of the latter, and slower action of resochin in the lower dosage becomes quite apparent.

Effects of these antimalarials against the gametocytes have been summarized in Table III.

TABLE III.

Effect on gametocytes.

Type of infection.	Drug.	Dosage (mg.)	Number of cases showing gameto-cytes.	Total number of cases.	Clearance within hours.				
					24	48	72	96	120 or over.
P. vivax	Amodiaquine	600	14	24	4	9	1	...	...
	Resochin		11	24	5	5	1	...	...
	Nivaquine		17	29	4	12	1	...	...
	Amodiaquine	400	7	9	3	4	...	...	...
	Resochin		4	6	...	3	1	...	...
P. falciparum	Amodiaquine	600	14	55	3	4	5 (85·7 per cent)	...	1
	Resochin		10	58	1	2	1 (40·0 per cent)	...	6
	Nivaquine		12	46	1	3	1 (33·3 per cent)	...	7
	Amodiaquine	400	8	23	3	2	1 (75·0 per cent)	...	2
	Resochin		6	19	...	1	1 (33·4 per cent)	...	4

The striking feature is the clearance of gametocytes of *P. vivax* in 100 per cent of cases within 72 hours irrespective of the drug and the dosage used. But during the same period clearance of crescents occurred in 85·7 and 75·0 per cent of cases treated with amodiaquine in 600 and 400 mg. doses respectively. The rate was slower still with resochin (40 per cent) or nivaquine (33·3 per cent) even in the higher dosage schedule. In the majority of cases treated with chloroquine salts, gametocytes of *P. falciparum* presisted for 120 hours or more.

Relapse rates.—Out of 261 cases treated, parasitic relapse was detectable in only four. Three of those had initially *P. falciparum* infection and were treated with 600 mg. of nivaquine. In 6 to 8 weeks after the treatment the same species was again detectable. In the course of routine blood examination during the follow-up period, *P. vivax* was encountered after 30 days in the case of mixed infection previously treated with 600 mg. resochin.

DISCUSSION.

Powerful schizonticidal action of antimalarials of the 4-aminoquinoline series like chloroquine (resochin, aralen) and amodiaquine (camoquin, cam-AQI) has been amply demonstrated by many workers. After a systematic study of several synthetic antimalarials carried out during 1946-48 against simian malaria, Jaswant Singh, Ray and Nair (1949) reported that parasite clearance from the

peripheral blood occurred earlier with chloroquine and amodiaquine than with others. But in those trials, the drugs were administered in divided doses for several days, as was tried in human malaria with amodiaquine by Halawani and Baz (1950) ; Halawani *et al.* (1948) ; Chaudhuri (1948) ; Chaudhuri and Chakravarty (1948); and with chloroquine by Most *et al.* (1946); Halawani *et al.* (1948); Chaudhuri *et al.* (1948); Butts (1950); Jaswant Singh *et al.* (1951) and others.

Simeons and Chhatre (1947), Mein (1951), Chaudhuri and Chakravarty (*loc. cit.*), Patel and Mehta (1948) and Hoeckenga (1950 : 1951) reported on single dose treatment with amodiaquine and potentiality of this compound became recognizable. Concerning its administration, the World Health Organization (1950) in a communication summarizing reviews on literature on amodiaquine, reported that " the best results are reached with the single dose schedule which terminates the acute attacks earlier than the other schedules, gives a lower relapse rate and is easier to control and administer, particularly in rural areas ". A single dose of 600 mg. (0·6 gm.) was recommended as an adult dose.

In some of his series Chaudhuri *et al.* (1948) had tried a single massive dose (1·5 gm. base) of chloroquine and subsequently Chaudhuri *et al.* (1950) also reported satisfactory results on 25 cases treated with a single dose of 500 mg. (0·5 gm.) of chloroquine.

In the present studies it may be observed that in *P. vivax* infection, the overall speed of action of chloroquine salts in 0·6 gm. dosage is more or less similar to that of amodiaquine, although during the first 24 hours the latter acts somewhat quicker. With chloroquine salts, there was clearance of parasites within 48 hours in all cases, against 96·7 per cent in the series treated with amodiaquine. Only in this respect, the former may be considered slightly better. Action of resochin and nivaquine was found to be almost identical and there is little to choose between the two. Although the number of cases treated under the lower dosage (0·4 gm.) is not large, during the first 24 hours amodiaquine gave a slightly higher percentage of parasite clearance.

In *P. falciparum* infection, action of amodiaquine and resochin was rather similar during the first 24 hours, but the sulphate salt (nivaquine) seemed to act slowly. This is not clearly understood unless this difference can be attributed to possible difference in the rate of absorption of sulphate and diphosphate. During the subsequent hours, speed of action of all the three seemed to be similar as observed in *P. vivax* infection.

Significant difference in reaction to amodiaquine and resochin was noticeable when the dose was reduced to 400 mg. Both were found to be comparatively slow acting as compared to higher doses. Further, during the first 24 hours resochin acted appreciably slowly than amodiaquine.

Gametocytocidal action of these antimalarials in *P. vivax* is fairly rapid and is somewhat comparable to that against the asexual forms. In all cases and under all regimes clearance of asexual and gametocyte stages was attained within 72 hours. These results are somewhat similar to those observed by Chaudhuri (1948); Halawani and Baz (1950), Van der Walle (1949) and Jaswant Singh *et al.* (1951). But the difference in action against the crescents is at once noticeable. Crescents presisted in some cases for 120 hours or over although the rate of disappearance

within 72 hours was much higher with amodiaquine than under the chloroquine regimes. Mein (1951) reported that in 30 per cent of cases with crescents, amodiaquine did not clear the gametocytes as against 75 per cent under chloroquine regime reported by Jaswant Singh *et al.* (1951).

Thus it would be seen that though action of these antimalarials on the crescents is tardy as compared to the asexual forms, amodiaquine is somewhat more effective than resochin or nivaquine.

It has been mentioned earlier that in three cases (under 0·6 gm. nivaquine), *P. falciparum* was detectable again 6 to 8 weeks after the initial attack. Since transmission of malaria was considered to be at its minimum during the latter part of November and no case of parasitæmia under amodiaquin or resochin was detected at about that time or later, one may be led to believe that the three cases under nivaquin had recrudescence. Similarly as only *P. vivax* was detectable in the case of mixed infection it is likely that it was a case of relapse. Even if all the four are frank cases of recrudescence or relapse it should be considered that the rate (1·5 per cent) is remarkably low.

Thus single dose treatment with any of the 4-aminoquinolines is not only very effective, both in the rate of clearance of asexual parasites and low relapse rate, it has an added advantage of convenience. Further, supervision during drug administration is automatically cut down to a minimum. The authors are of the opinion that this regime is obviously the method of choice in the treatment of malaria patients particularly in rural areas with difficult means of communication and where the population is reluctant to visit dispensaries frequently when they are free from any clinical symptoms.

As regards the choice between the three 4-aminoquinoline compounds, it may be said that for all practical purposes they are equally efficacious in 0·6 gm. dosage, as in 95 to 100 per cent of cases, irrespective of the type of infection, there was complete clearance of asexual parasites within a period of 48 hours, and there was not a single case of failure in the present series.

SUMMARY.

In one series, 77 cases of *P. vivax* and 122 of *P. falciparum* were treated with 0·6 gm. of resochin, nivaquine or amodiaquine. In the second series 62 cases (*P. vivax* 15, *P. falciparum* 47) received treatment with 0·4 gm. of resochin or amodiaquine.

Asexual parasite clearance within 72 hours occurred in all cases irrespective of the type of infection, the antimalarial used or the dose.

Clearance rate observed during the first 48 hours showed that with a dose of 0·6 gm. of resochin or nivaquine no parasites were detectable in any of the cases infected with *P. vivax* or *P. falciparum* as against 96·7 and 95·2 per cent of cases respectively under amodiaquine. But during the first 24 hours action of amodiaquine was found somewhat quicker than the chloroquine salts, particularly so in respect of *P. falciparum* cases treated with nivaquine.

In *P. falciparum* cases, both resochin and amodiaquine in the 0·4 gm. dosage acted slower than in cases treated with 0·6 gm.

All drugs acted promptly against gametocytes of *P. vivax* but action against crescents was slower, particularly so in those treated with resochin or nivaquine.

Parasitic relapse was detectable only in 4 out of 261 cases during an observation period of 3 to 5 months.

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SUPPRESSIVE TREATMENT WITH AMODIAQUIN.

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BASED on the various reports and observations, Jaswant Singh (1951) had suggested that for suppressive treatment with amodiaquin the dose of 0·3 gm. once a week or 0·6 gm. fortnightly should be effective. In the autumn of 1952 several field investigations were undertaken in certain areas in U.P., Terai, which are known to be highly malarious. One of these investigations was in relation to the determination of the efficacy of the drug as a suppressant and also to evaluate the merits of the two different regimes.

From entomological data it was evident that active transmission was in progress in the area as has been indicated by Jaswant Singh, Misra and Ray (1953) in another report. Selections of the villages were made after an initial survey of the spleen and parasite rates. Communication facilities were taken into consideration in deciding the regimes to be adopted in the different areas. Two villages near the main road with a total population of 161, were selected for suppressive treatment with a dose of 0·3 gm. base (for adults) administered once a week (Regime I). A group of farmers consisting mainly of adult population of 125 in a village in the interior with difficult communication (particularly so during the monsoon and post-monsoon period) were placed under Regime II (0·6 gm. once a fortnight).

Besides, two other villages situated in between the two areas with a total population of 176 were selected for comparison. The actual distance from the experimental centres, to the comparison villages, as the crow flies, is less than a mile.

Subsequent spleen and parasite surveys of all the inhabitants mentioned above were undertaken, once 4 to 5 weeks after the treatment was begun in the experimental areas, once soon after cessation of treatment at the end of 10 weeks (end of November) and for the last time by the middle of February (ten weeks after treatment was stopped). The last one was a sample survey and the data has not been included in the Table. Besides, domicilliary visits were made once a week in each area to determine if any of the villagers had fever during the week. Blood smears were examined of every patient complaining of fever or malaise.

All personnel above 12 years of age received the adult dose of 0·3 gm. (1½ tablet) once a week under Regime I. Children between 4 and 12 years received 0·2 gm. (1 tablet), those from 1 to 4 years were given 0·1 gm. (½ tablet) and those below one year had 0·05 gm. (¼ tablet). Under Regime II double the dose was administered for the different age groups but only once a fortnight. Those who missed the drug on any particular day, attempts were made to treat them on the following day. It was, therefore, possible to ensure regular drug administration to most cases except those who were temporarily away.

RESULTS.

Effects of suppressive treatment as reflected on the spleen and parasite rates are indicated in Table I, along with the findings on the comparison areas, during the corresponding periods. For convenience, the villages have been represented as experimetal I, II and III and Comparison Centres as 1 and 2.

TABLE I.

Spleen and parasite rates just prior to commencement of suppressive treatment and soon after cessation.

Centre.	Regime.	Population.	Spleen rate.		Parasite rate.		Remark.
			Before suppressive treatment.	Soon after cessation of treatment.	Before suppressive treatment.	Soon after cessation of treatment.	
Experimental I	I	65	20·0	6·1	29·2	0	
II	I	96	38·0	14·5	38·5	1·04	
III	II	125	*9·6	3·0	*14·4	0	
Total		286					
Comparison 1	No anti-malarials administered.	55	27·2	35·4	32·7	41·8	
2		121	10·0	12·4	17·0	26·4	
Total		176					

* Refers to adult spleen and parasite rates.

During the initial survey, in most cases spleens were found to be just palpable, soft and tender. Parasite rates were found to be high and very much so in Experimental Centres I and II and Comparison Centre I where nearly 30 per cent of the population showed asexual parasites in their blood. *P. falciparum* was the predominant species. Besides, several patients were found to be actually suffering from malaria in every village and they were diagnosed microscopically. Incidence of malaria was so high in Experimental Centre III, that every week large number of labourers were being repatriated to their own villages. This resulted in depletion of the force as it was not being augmented at the same rate as they were being sent out. Shortly before the initial survey some of the labourers were sent out on account of severe infection. *Prima facie* the spleen and parasite rates as shown in the Table do not appear to be alarming. This is because of dilution with fresh labour imported from outside stations. But those in whom parasites were detectable, had clinical symptoms as well. In the normal course of events they too would have been repatriated within a few weeks.

Comparison between the data observed during the first and third survey shows a striking reduction in the spleen and parasites more so in the latter. Within 10 weeks, spleen rates in the experimental centres were reduced by 2·6 to 3·2 times as against an overall rise by 1·2 times at the comparison centres. Further, while parasite rate shows a dramatic reduction to zero per cent at two centres and 1·04 per cent in another the rates were increased to 41·8 and 26·4 per cent from 32·7 and 17·0, respectively, at the two comparsion centres.

In none of the experimental groups who had taken the drug regularly under the authors' direct supervision was there any overt attack of malaria. But there were several cases amongst those who had missed the drug on a few consecutive weeks or irregularly or in some of those infants whose mothers insisted that they would personally administer the drug when they went home.

There were 5 such cases in Experimental Centre II and two in Centre III. Experimental Centre I being a small village it had been possible to ensure drug administration to all by domicilliary visits and in no case asexual forms were detectable after treatment was begun. However, during the second and third survey (10 weeks after cessation of treatment) crescents were detectable in five cases. In 3 of these no parasites, asexual or crescents, were detectable initially.

Besides frequent visits to these centres, counter check was kept at the nearby dispensary for any case reporting for fever. While none was found to do so after treatment began in the experimental centres, several cases reported daily from each of the comparison centres. The villagers in these two areas continued to be ill till almost the middle of December as against a community rendered apparently healthy and active in the experimental centres. Another striking feature was that no repartriation of infected labourers at Experimental Centre III was considered necessary as, soon after treatment was begun, all remained apparently healthy throughout.

During the sample survey in February, 10 weeks after cessation of treatment, *P. falciparum* rings were detected in one of 55 cases in Experimental Centre III, which could be interpreted as an evidence of transmission.

DISCUSSION.

In a well controlled investigation, Coatney *et al.* (1950) demonstrated that amodiaquin was highly effective in suppressing *P. vivax* (Chesson strain) infection induced experimentally through bites of infected *A. quadrimaculatus* to human volunteers and that the results were similar to those observed with chloroquine.

From the data available during the preliminary survey in the present investigations, it is evident that active transmission was in progress and a near epidemic condition existed in the area at the time the suppressive treatment was begun. Further, from the spleen and parasite rates at the comparison centres taken at a time when suppressive treatment had just been concluded in the experimental areas, it would be observed that the brunt of the transmission was heavy on the community. As such the rapid decline in the rates in the experimental centres has been interpreted to be due to the effective suppressive action of amodiaquin.

Further it is observed that those who were either irregular or missed the drug on a few consecutive weeks, had overt attacks of malaria. There were five such cases. In a similar study on proguanil and chloroquine, Ray (1948) had illustrated in Tables XII and XIII the various types of irregularity which may cause overt attacks. Such irregularity was also encountered in the present investigation and overt attacks were to be found only amongst these.

The appearance of gametocytes (crescents) during treatment in some cases in whose blood no parasites were detectable initially, might indicate latent or fresh infection with *P. falciparum*. But in view of the suppressive action of the drug, asexual forms were not detected nor was there any clinical manifestation. This observation confirms that amodiaquin is not effective against the crescents as recently reported by Jaswant Singh, Ray and Misra (1953).

As regards the regimes, a weekly dose of 0·3 gm. was found to be equally effective as 0·6 gm. fortnightly in spite of heavy transmission season. Further, there was no case of genuine breakthrough under the two regimes.

Thus though the cost remains the same, Regime II appears to have definite advantage over the other as administration and supervision costs are cut down by 50 per cent.

Chaudhuri *et al.* (1952) and Chaudhuri and Chakravarty (1952) have lately made similar observations but under lower dosage regimes like 0·2 gm. weekly and 0·4 gm. fortnightly.

The lowering of the dosage schedule will no doubt reduce the cost of treatment but the present authors are unable to give a definite opinion whether these regimes would be as effective in areas where heavy transmission with *P. falciparum* infection is in progress as was experienced during the current studies. But in view of the efficacy of amodiaquin it would be worthwhile if similar investigations with varying doses are taken up in areas having different epidemiological problems.

SUMMARY.

A weekly dose of 0·3 gm. of amodiaquin was administered to the population (161) of two villages for a period of 10 weeks during heavy transmission. In another area closeby but with difficult communication a dose of 0·6 gm. was administered once a fortnight to 125 people during the same period.

Two neighbouring villages were kept for comparison where no antimalarials were administered.

Spleen and parasite surveys were carried out initially before commencement of suppressive treatment and twice later.

While there was progressive rise in the rates at the comparison centres there was a striking reduction in all the experimental centres. During the ten weeks a total of 7 cases of malaria were detected out of a total population of 287. Most of these cases took the drug irregularly.

Both the regimes proved equaly effective. In view of less frequent drug administration required as per Regime II, it will be found advantageous in areas where communication difficulties exist.

ACKNOWLEDGMENT.

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ASSAY OF ANTIMALARIALS AGAINST THE SPOROGONY CYCLE OF *P. GALLINACEUM*.

PART I.

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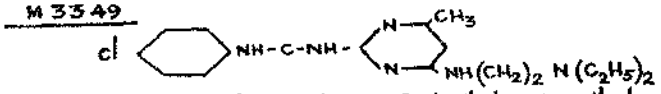
ORDINARILY, antimalarials are tested against the developmental phases of plasmodia in the vertebrate hosts. Terzian(1947)evolved a technique to determine the efficacy of compounds against the sporogony cycle of malaria parasites in the insect host. Subsequently, Terzian and Weathersby (1949) reported that certain concentrations of proguanil in glucose solution inhibited the growth of oöcysts of *P. gallinaceum* in *Ædes ægypti*, and that development of sporozoites was prevented.

The authors employing similar techniques in the evaluation of M. 3349, usually known as proguanil precursor, proguanil and pyrimethamine, have presented their observations in this paper.

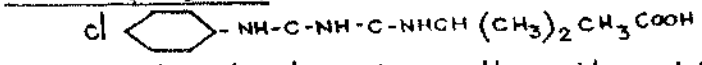
The chemical formulæ of the 3 compounds are shown below and the structural relationship has been discussed elsewhere.

M 3349



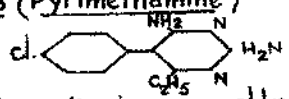
(2-P-Chlorophenyl guanidino - 4β di ethyl amino ethyl amino - 6 methyl Pyrimidine)

M 4888 (Proguanil)



(N_1-P-chlorophenyl-N5-isopropyl biguanide acetate)

50-63 (Pyrimethamine)



(2:4 diamino - 5p-chlorophenyl - 6 ethyl Pyrimidine)

MATERIALS AND METHODS.

Plasmodium and the vertebrate host.—A strain of *P. gallinaceum* maintained in these laboratories by continued blood passage through white leghorn and Rhode Island red fowls, was used for these studies.

Insect host.—Batches of freshly hatched *Ædes ægypti* from the colony were kept in a series of Barraud cages and after being kept starved overnight were allowed to imbibe solutions of glucose (4 per cent) or of varied concentrations of antimalarial drugs under trial in 4 per cent glucose for a period of 2 to 3 days. Subsequently, the mosquitoes were again starved overnight and then fed on fowls showing fair number of gametocytes. Those which were found fully engorged with blood were transferred back to their respective cages and allowed to imbibe the same solutions, provided prior to infective blood feed throughout the period of observation and kept there till dissected.

Nutrient and antimalarial drugs.—The standard nutrient used for the batches of mosquitoes which served as comparison groups was freshly prepared 4 per cent solution of glucose.

After determining the maximum strength of antimalarials which the mosquitoes could tolerate, concentrations ranging from 1·0 to 0·001 per cent (in one case up to 0·0001 per cent) of the base content in 4 per cent glucose solution, were used for the experimental groups. The mosquitoes imbibed the various solutions from soaked cotton pads kept both on the top and at the bottom of cages. To avoid degradation of the drugs, if any, fresh solutions were made every day and the pads were changed both morning and evening.

The concentrations of drugs were calculated in terms of grammes per 100 c.c. of glucose solution.

DISSECTION.

The day of infective blood feed was considered as zero day. Days of drug administration prior or subsequent to the feed were expressed as minus or plus (day) respectively. Sample dissections from each batch were commenced from plus 3 to 5 day and continued thereafter to plus 12 to 15 day till either sporozoites were observed or all mosquitoes had been dissected whichever was earlier.

When oöcysts were detectable whether in the comparison or experimental batches, the size expressed in microns and number per gut were recorded. On detection of sporozoites on the first day during sample dissection, 5 to 10 mosquitoes (calculated according to the rate of infection) out of the same batch were allowed to feed on a normal fowl. These mosquitoes were then dissected and sporozoites were inoculated intravenously to the same fowl. A mixture of serum and normal saline was used for inoculation. On detection of sporozoites in a batch for 2 to 3 consecutive days, further dissection was discontinued.

RESULTS.

The investigations were carried out in several series. For actual assay of the compounds over 5,000 *Ædes ægypti* were hatched, fed on fowls, and reared. Several thousands more were used for preliminary trials to determine the highest

concentration of a drug which could be tolerated by the mosquitoes. The number of mosquitoes which were dissected for examination of gut and gland was 680.

Preliminary trials showed that a concentration of proguanil or pyrimethamine higher than 1·0 per cent was not well tolerated by the mosquitoes as determined by comparison of the mortality rates in the experimental and comparison groups. A concentration of 1·0 per cent of M 3349 proved too toxic for *Ædes ægypti* and all died by plus 5 day as against 5 to 7 per cent of the comparison group.

PROGUANIL.

Details of observations have been shown in Table I from which it is evident that infection of gut was detectable in mosquitoes of all batches throughout the period of dissections from plus 3 to plus 13 day, but the average size and the number of oöcysts per gut varied in different batches. On plus 6 day when sporozoites were first detectable in the salivary glands of mosquitoes from the comparison group and also in those allowed to imbibe on 0·001 per cent proguanil solution, the average size and the number of oöcysts per gut were highest in the comparison group and lowest in the group kept on 1·0 per cent proguanil solution. In the experimental groups the size of the oöcysts seemed to vary inversely to the concentration of the drug; higher the concentration, smaller the size of the oöcysts.

TABLE I.

Gut and gland infectivity rate in Ædes ægypti *after allowing them to imbibe different concentrations of proguanil.*

Number of days after infective blood feed.	Control.			0·001 per cent.			0·01 per cent.			0·1 per cent.			1·0 per cent.		
	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.
3	5	0	4	5	0	2	5	0	3	5	0	3	5	0	3
4	5	0	5	5	0	3	5	0	3	5	0	4	5	0	3
5	5	0	3	5	0	3	5	0	2	5	0	2	5	0	4
6	5	3	4	5	2	3	5	0	1	5	0	1	5	0	2
7	5	3	2	5	4	0	5	0	2	5	0	1	5	0	2
8	5	3	4	5	4	3	5	0	3	5	0	2	5	0	4
9	5	3	2	5	3	2	5	0	3	5	0	1	5	0	1
10	5	4	2	5	4	1	5	0	2	5	0	2	1	0	0
11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
12	...	...	...	5	3	0	5	0	0	3	0	3	...	...	...
13	...	...	...	...	...	...	5	0	2	6	0	1	...	...	...

No sporozoites were detectable in the group of mosquitoes fed on 0·01 to 1·0 per cent solution of drug up to plus 13 day whereas on plus 6 day gland infection was well established both in the comparison group as well as in the one allowed to imbibe 0·001 per cent solution. To test the viability, sporozoites were inoculated to normal fowls and observation was continued till patent infection was established. In all cases sporozoites were found to be viable.

PYRIMETHAMINE.

Infection of the gut was well established by plus 6 day in all batches of mosquitoes, except in those fed on 1·0 per cent solution of pyrimethamine. Details have been shown in Table II. In the last batch mentioned no infection of the gut or gland was detectable at any stage. Before the appearance of sporozoites in any batch the average number of oöcysts per gut and the size were lower in the experimental than in the comparison groups. Sporozoites were first detectable on plus 9 day both in the comparison group and in those fed on 0·001 and 0·0001 per cent solution. In none others, sporozoites were found up to plus 15 day.

TABLE II.

Gut and gland infectivity rate in Ædes ægypti *after allowing them to imbibe different concentrations of pyrimethamine.*

Number of days after infective blood feed.	CONTROL.			0·0001 PER CENT.			0·001 PER CENT.			0·01 PER CENT.			0·1 PER CENT.			1·0 PER CENT.		
	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.
3	5	0	4	...	...	...	...	...	...	5	0	2	5	0	0	5	0	0
4	5	0	4	...	...	...	...	...	...	5	0	2	5	0	1	5	0	0
5	10	0	6	5	0	0	5	0	1	5	0	1	5	0	1	5	0	0
6	10	0	4	5	0	1	5	0	0	5	0	0	5	0	0	5	0	0
7	5	0	0	5	0	0	5	0	2	...	...	...	...	...	...	...	...	...
8	2	0	0	5	0	0	5	0	0	...	...	...	...	...	...	...	...	...
9	5	5	4	5	2	0	5	1	0	5	0	0	5	0	0	5	0	0
10	...	...	...	4	1	0	5	1	0	5	0	1	5	0	1	5	0	0
11	...	...	...	5	4	1	5	2	0	5	0	1	5	0	0	5	0	0
12	...	...	...	...	...	...	...	...	...	5	0	0	5	0	0	5	0	0
13	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
14	...	...	...	...	...	...	...	...	...	5	0	0	5	0	0	5	0	0
15	...	...	...	...	...	...	...	...	...	5	0	0	5	0	0	5	0	0

M. 3349.

Infections of gut and gland were established in both comparison and experimental groups. Sporozoites were first detectable on plus 8 day in the comparison group against on plus 9, plus 10 and plus 13 day in groups of mosquitoes fed on 0·001, 0·01 and 0·1 per cent solutions respectively. There seemed to be no significant difference in the size or number of oöcysts per gut amongst the various batches of mosquitoes.

Patency was established in all fowls inoculated with sporozoites from the various batches of mosquitoes.

TABLE III.

Gut and gland infectivity rate in Æedes ægypti *after allowing them to imbibe different concentrations of proguanil precursor* (*M.* 3349).

Number of days after infective blood feed.	CONTROL.			0·001 PER CENT.			0·01 PER CENT.			0·1 PER CENT.			1·0 PER CENT.		
	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.	Number dissected.	+ gland.	+ gut.
3	...	...	...	...	...	...	...	...	...	...	...	...	None out of 414 mosquitoes placed on 1·0 per cent solution diet survived beyond seven days on account of the toxic effect of the drug. As such, no relevant data could be obtained.		
4	5	0	4	5	0	2	6	0	5	...	...	...			
5	10	0	8	5	0	4	5	0	2	5	0	2			
6	10	0	8	5	0	5	5	0	2	5	0	2			
7	10	0	10	5	0	4	5	0	5	5	0	3			
8	10	0	5	5	0	2	5	0	3	5	0	5			
9	10	2	8	5	2	4	5	0	5	5	0	4			
10	10	6	9	18	12	...	5	3	4	5	0	5			
11	5	0	4	...	...	...	...	...	...	5	0	4			
12	10	4	3	...	...	...	...	...	...	5	0	3			
13	14	8	6	...	...	...	...	...	...	5	0	3			
14	...	...	...	...	...	...	...	...	...	5	1	4			
15	3	3	0	...	...	...	...	...	...	6	6	...			

DISCUSSION.

It has been reported that there is no morphological change in the gametocytes after proguanil administration to vertebrate host (Black, 1947), but there is reason to believe that they, especially the macrogametocyres, are affected in such a way that the sporogony cycle is inhibited "till all the proguanil is excreted"

(Findlay, 1951). That the drug actually inhibits the development of sporozoites for some days after treatment, has been amply demonstrated in respect of *P. falciparum* (Shute and Maryon, 1948; Mackerras and Ercole, 1948; Ramakrishnan, Young *et al.*, 1953); *P. knowlesi* (Jaswant Singh, Ray and Ramakrishnan, 1951) and *P. gallinaceum* (Ramakrishnan, Ray *et al.*, 1952). Recently somewhat, similar observations have been made on pyrimethamine by Shute (1952).

The current investigation may be considered a step further in a way that instead of administration of the antimalarials to the vertebrate host, the mosquitoes were allowed to imbibe these before and after blood meal from gametocyte carriers (*P. gallinaceum* in fowls). Proguanil was found to be effective, in that, none of the mosquitoes dissected showed infection of the gland when allowed to imbibe concentrations of the drug varying from 0·01 to 1·0 per cent. However, oöcysts were found in all batches though their number per gut and size varied according to concentration of proguanil.

Terzian and Weathersby (1949) made similar observations with a concentration of 0·01 per cent proguanil solution, though they had not been able to observe sporozoites with the lower dose of 0·001 per cent. In the present series development of infection in the gut and gland of mosquitoes kept on the same concentration (0·001 per cent) was unhampered similar to that seen in the comparison group.

The findings on the series of mosquitoes allowed to imbibe different solutions of pyrimethamine were similar to those observed in respect of proguanil. Concentrations of 0·001 and 0·0001 per cent were found to be inadequate to inhibit the development of oöcysts or sporozoites.

On the other hand, M. 3349 was found to be ineffective in all concentrations up to 0·1 per cent and too toxic in higher strength. In this respect the drug behaved in a manner similar to mepacrine (Terzian and Weathersby, 1949).

Although M. 3349 is called 'proguanil precursor' and has somewhat structural similarity, action on the sporogony phase was found to be quite different. But since like mepacrine, M. 3349 has also an amino-alkylamino side chain attached to a heterocyclic ring, the similarity of action of both these compounds may be justified. Similarity in the biological response to proguanil and pyrimethamine may be due to a greater structural similarity between these two compounds (Falco, 1951; Jaswant Singh *et al.* 1951).

It is, therefore, interesting to note that these two compounds which are effective against pre-erythrocytic phase of *P. gallinaceum* (Jaswant Singh *et al.* 1952 *a* : *b*) should also affect the sporogony cycle; in other words, they have prophylactic action both against vertebrate and invertebrate hosts.

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SCREENING OF ANTIMALARIAL COMPOUNDS IN MICE WITH *PLASMODIUM BERGHEI* INFECTION.

BY

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(Enquiry on the Chemotherapy of Malaria under the Indian Council of Medical Research at the Haffkine Institute, Parel, Bombay.)

(March 31, 1953.)

THE present paper describes some preliminary results obtained in the screening of antimalarial compounds which the author was privileged to carry out on behalf of the Department of Chemotherapy of the Haffkine Institute, Bombay, under Dr. K. Ganapathi. The compounds tested included standard antimalarials, others previously described but not yet tried against *Plasmodium berghei*, and new compounds designed and synthesized by N. J. Sardesai, M. V. Shirsat, K. Ganapathi and M. H. Shah with a view to elucidating the complex relationship existing between chemical structure and plasmodicidal activity. Some new compounds tested have already been described in the literature, while the synthesis of others will be presented in detail in forthcoming publications.

The procedure for testing compounds in the present investigation, adapted from the methods of Coatney and Sebrell (1946) and Curd, Davey and Rose (1945), had already been decided on, when the papers by Goodwin (1948), Schneider, Decourt and Montezin (1949), Thurston (1950) and Hill (1950), all dealing with chemotherapeutic studies on *P. berghei*, were received here. While differing in details as to the dose of infection, route of administration of drugs and schedule of treatment, all these studies suggest that *P. berghei* has been accepted as a new test organism for evaluating antimalarial activity. The view of Mudrow-Reichenow (1951) that this parasite is not suitable for screening of compounds for ultimate use in human malaria in view of its much greater resistance to quinine than some avian species hitherto employed with such success in the search for new and more potent drugs, is perhaps a little extreme, and contrasts with the view that *P. berghei* resembles the human plasmodia and *P. knowlesi* in its sensitivity to sulphadiazine (Thurston *loc. cit.*; Ramakrishnan, Krishnaswami and Satya Prakash, 1951).

MATERIALS AND METHODS.

The strain of *Plasmodium berghei* was very kindly provided by Col. Jaswant Singh on two occasions; first in 1950 and again in 1951 after a severe epizootic of *Salmonella typhi murinum* among the mice belonging to this unit had necessitated

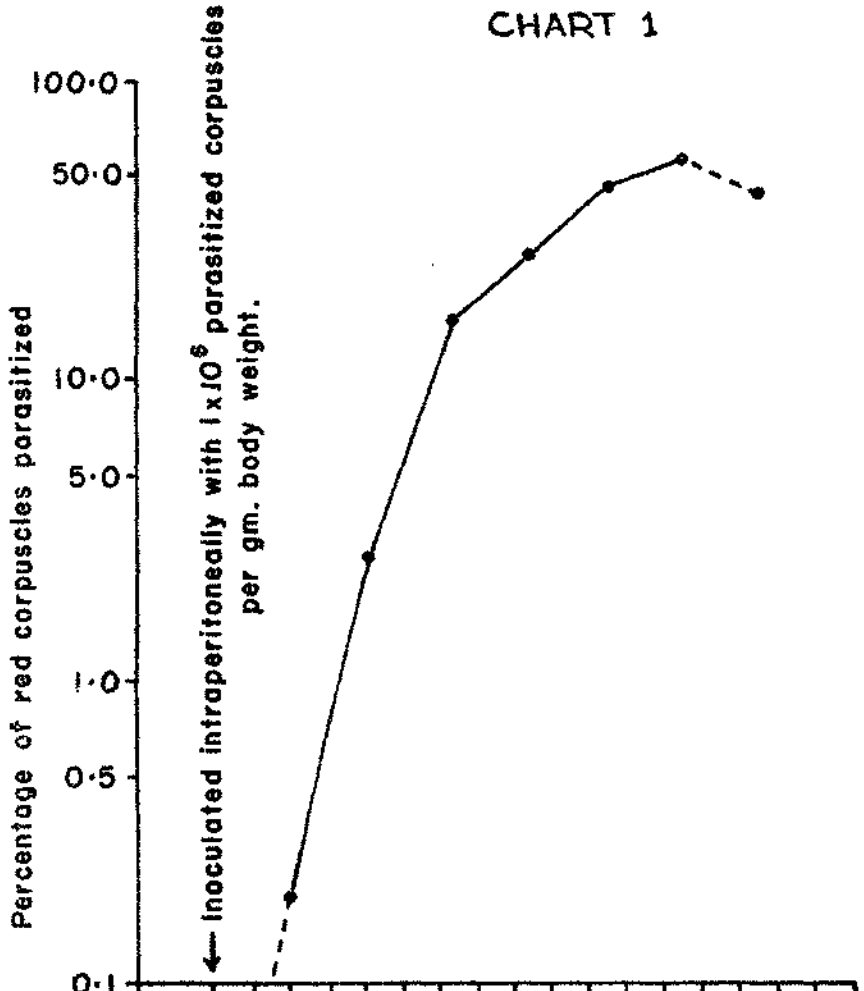
the destruction of all survivors, including strain animals, in the larger interest of the Institute's herd.

Haffkine inbred mice of both sexes, aged 6-7 weeks and weighing 16-24 gm., were used throughout this investigation. The weight range is a little greater than usually employed in such work and mice of 22-24 gm. were admitted to complete test groups which usually consisted of 5 mice each. The average weight of the groups in a test series was approximately equal. The groups were serially numbered and individual mice were given a distinctive colour.

The standard inoculum for both strain passage and test infections consisted of 20 million parasitized red corpuscles, suspended in 0·1 c.c. of citrated blood diluted to the required degree with normal saline, for a 20 gm. mouse. The dose was adjusted for individual mice by 0·01 c.c. for a variation of 2 gm. in body weight. The intraperitoneal route of inoculation was used throughout. The infected blood for inoculation was drawn by cardiac puncture or from a tail vein, depending on the number of mice to be inoculated, on the fourth or fifth day of infection—always on the fifth day for a test infection—in a donor having in most instances not less than 20 per cent of the red corpuscles parasitized. The dilution factor was determined from the total red-corpuscle count and the infection rate of the red corpuscles. The inoculations were performed within half an hour of drawing the infected blood. The curve of infection for a group of five untreated mice inoculated by these methods is illustrated in Chart 1 and data for several untreated control groups from test series are included in Table I. Parasites were usually demonstrable without much difficulty in stained thin blood films prepared from a tail vein about two hours after inoculation, when the first routine examination of all test animals was made. Animals not showing infection were excluded from the test. The infections increased in intensity virtually in geometric progression during the first three days, with a retardation in the rate of multiplication of the parasites after the fourth day as peak numbers are approached on the sixth or seventh day. The mean survival period of these untreated mice is 6-10 days and very few indeed linger on to die in the second or third week of infection.

Treatment was given orally in the form of solutions or acacia suspensions of the compounds by means of a blunted wide-bore hypodermic needle attached to a 1 c.c. tuberculin syringe and passed gently into the oesophagus. The daily dose was divided into two equal doses given at 9 a.m. and 5 p.m. respectively, each consisting of 0·2 c.c. of the preparation for a 20 gm. mouse, adjusted for each animal according to its daily weight. The full treatment comprised a half daily dose at 5 p.m. on the day of inoculation after the first blood examinations have established patency and 3 full daily doses on the next three days. The quantity of the compound contained in the dose for the initial trial was either the maximum tolerated dose or an approximation to it, determined in similar preliminary 4-day tests for chronic toxicity, or some multiple of the minimum effective dose (M.E.D.) or other significant dose of a closely related standard antimalarial if direct comparison was considered adequate. If a compound showed activity in the initial trials the dosage in subsequent trials was varied to determine important levels of drug activity such as the M.E.D., the minimum suppressive dose and the minimum curative dose, the criterion for the two latter dosages being a response in 75 per cent or more of the animals.

CHART 1



Day of infection	1	2	3	4	5	6	7	8	9
Percentage r.b.c. parasitized	<0·01	0·2	2·7	17·5	28·7	51·6	63·8	50·8	
Mice alive	5	5	5	5	5	5	5	1	0
Mice dead	0	0	0	0	0	0	0	4	5

Course of Plasmodium berghei infection

TABLE I.

The activity of some standard antimalarial compounds against Plasmodium berghei.

Compound.	Daily dose of base in mg./kg. for 3½ days.	Number of mice: infected and fully treated/inoculated.	EFFECT ON PARASITÆMIA.					Remarks.
			INHIBITION.		SUPPRESSION.		ERADICATION.	
			Percentage of r.b.c. parasitized on 5th day (geometric mean and S.E.)	Survival period in days: (average and S.E.)	Number of mice: responded/treated.	Duration in days.	Number of mice: responded/treated.	
1.	2.	3.	4.	5.	6.	7.	8.	9.
Quinine sulphate	55	3/5	6·44±1·70	12	0/3		0/3	
	75	4/5	3·55±1·65	12	0/4		0/4	
	Controls	5/5	18·25±2·02	10·0±2·0				
	60	5/5	1·76±1·05	17·0±3·2	0/5		0/5	In 4 other tests at 60 mg./kg., there was 75 per cent reduction in parasitæmia in 3 tests and 73 per cent reduction in 1 test, as compared with the controls. M.E.D.=60 mg./kg.
	75	5/5	1·83±1·02	17·8±3·2	0/5		0/5	
	Controls	2/2	27·74±3·70	6·5±0·3				
	75	5/5	<0·2	17·8±2·7	0/5		0/5	Fifth day counts included 1 "negative". G. M. not calculated.
	Controls	5/5	17·90±3·76	6·4±0·3				
	90	4/4	1·88±0·69	16·7±5·1	0/4		0/4	
	Controls	2/4	36·29±3·80	8·5±0·2				
	150	5/5	<0·2	22·2±8·2	0/5		0/5	
	Controls	5/5	33·75±3·13	9·0±1·7				
	400	5/5	0.00	32	5/5	10, 11, 13, 45	0/4	One mouse died "negative" on the 10th day. Sub-inoculation from mouse with latent infection of 45 days was "positive" and re-inoculation resulted in a protracted infection of 60 days' duration with counts over 40 per cent r.b.c. parasitized.
	Controls	5/5	35·04±2·86	6·0±0·0				
Proguanil hydrochloride	4·5	5/5	9·02±3·24		0/5		0/5	Marked inhibitory effect of 9 mg./kg. was confirmed in several tests.
	9	5/5	<0·2	14·6±1·9	1/5	9	0/5	
	Controls	5/5	23·86±1·49	7·8±0·4				

TABLE I—(*Concld.*)

Compound.	Daily dose of base in mg./kg. for 3½ days.	Number of mice: infected and fully treated/inoculated.	EFFECT ON PARASITÆMIA.					Remarks.
			INHIBITION.		SUPPRESSION.		ERADICATION.	
			Percentage of r.b.c. parasitized on 5th day (geometric mean and S.E.)	Survival period in days: (average and S.E.)	Number of mice: responded/treated.	Duration in days.	Number of mice: responded/treated.	
1.	2.	3.	4.	5.	6.	7.	8.	9.
Proguanil hydrochloride	7	4/4	5·77±4·92	18·2±1·5	0/4		0/4	M.E.D.; Q.E.=8·6 M.E.D. confirmed in 4 trials.
	Controls	4/4	38·85±2·93	8·0±1·3				
	20	5/5	0·00	>34	2/5	11, 14	3/5	One mouse died "negative" on the 14th day from an injury. Three cured mice died with high parasitæmia within 7 days of re-inoculation on the 45th day. Sub-inoculations were "negative".
	Controls	5/5	18·57±2·31	6·2±0·2				
	20	4/4	0·00	>137			4/4	
	Controls	5/5	18·02±1·89	8·6±1·6				
	36	3/5	0·00	47			3/3	Toxic dose, 2 mice died. Sub-inoculations from 2 'cures' negative for 12 days when recipients died. Re-inoculation of 3 'cures' gave 5th day counts of 14·5—30 per cent r.b.c. parasitized and survival periods of 17, 22 and 120 days.
	Controls	5/5	35·04±2·86	6·0±0·0				
Chloroquine diphosphate	2	4/4	14·14±2·41	13·2±1·6	0/4		0/4	
	Controls	2/4	36·29±3·80	8·5±0·2				
	3	4/4	0·00	16·0±5·2	0/4		0/4	M.E.D.; Q.E.=20. Confirmed in several tests.
	Controls	3/3	28·27±1·99	8·7±0·3				
Sulphathiazole	0·1	3/5	30·76±4·68	8·6±1·1	0/3		0/3	
	Controls	5/5	34·99±3·67	10·2±7·5				
	0·4	5/5	19·32±7·94	8·0±1·3	0/5		0/5	
	Controls	5/5	30·37±3·70	7·4±0·9				
	1·6	5/5	13·01±0·96	11·6±2·5	0/5		0/5	
	Controls	3/5	15·50±1·69	7·0±0·0				
	3·2	5/5	0·56±1·02	15·4±3·2	0/5		0/5	M.E.D.; Q.E.= Approx. 20.
	Controls	5/5	40·67±3·26	9·2±3·2				

TABLE II.

Compounds tested against P. berghei *and found inactive.*

Reference number.	Compound.	Daily dose of base in mg./kg. for 3½ days.
	QUINALDINES, ETC.	
MHS 5	4-(*p-aminoanilino*)-8-*methoxyquinaldine hydrochloride*	100
MHS 9	4-(*p-acetoaminoanilino*)-8-*methoxyquinaldine hydrochloride*	100
MHS 7	4-(*p-acetoaminoanilino*)-6-*chloroquinaldine*	100
MHS 17	4-(*p-aminoanilino*)-6-*chloroquinaldine*	400
MHS 15	4-amino-6-methoxy-quinaldine	400
MHS 12	*m*-chloroaceto-acetanilide	800
MHS 18	*m-tolylguanidine sulphate*	400
MHS 19	6-*chloro*-8-*nitroquinoline*	400
MHS 20	4-(*p-guanidinoanilino*)-7-*chloroquinoline*	200
	QUINOLINE-SULPHONES.	
Q 2	4-(*p-aminobenzenesulphonyl*)-7-*chloroquinoline*	50*
Q 9	2-*methyl*-4-(*p-chlorobenzenesulphonyl*)-6-*chloroquinoline*	50*
Q 11	2-*methyl*-4-2 : 5'-(*dichlorobenzenesulphonyl*)-6-*chloroquinoline*	50*
Q 15	2-*methyl*-4-(*p-chlorobenzenesulphonyl*)-6-*methoxyquinoline*	50*
Q 29	2-(2' : 5'-*dichlorobenzenesulphonyl*)-4 : 6-*dimethylquinoline*	50*
	SULPHONAMIDES.	
B 203	*p*-cyanophenyl-methylsulphone	125-250
MN 53	N^1-*p-chlorophenyl*-N^1-*isopropyl sulphanilamide*	200
	PYRIMIDINES.	
BNP VI 72/24	N^3-*phenyl*-4-*amino*-6-*oxy*-2-*thiopyrimidine*	400
BNP VI 72/25	N^3-*p-chlorophenyl*-4-*amino*-6-*oxy*-2-*thiopyrimidine*	800
BNP VI 72/30	N^3-*o-anisyl*-4-*amino*-6-*oxy*-2-*thiopyrimidine*	1600
BNP VI 72/32	N^3-*p-anisyl*-4-*amino*-6-*oxy*-2-*thiopyrimidine*	100
	NITROBENZOIC ACID ESTER.	
MV 6	2-methoxy-4-nitrobenzoic acid methyl ester	800

New compounds in italic type.

*Arbitrary dose, *cf.* m.e.d. of chloroquine 3 mg./kg. Dose of other compounds in this table is the approximate M.T.D.

SN numbers: MHS 15=9862-1121; MHS 12=7264-572; both were inactive against avian malarial parasites (Wiselogle, 1946).

TABLE III.

The activity of test compounds which inhibited the multiplication of Plasmodium berghei.

Reference number.	Compound.	Daily dose of base in mg./kg. for 3½ days.	EFFECT ON PARASITÆMIA					Remarks
			SUPPRESSION.			ERADICATION.		
			Number of mice responded/treated.	Duration (days).	Minimum dose for 3-day effect in 75 per cent of animals. (mg./kg.)	Number of mice responded/treated.	Minimum dose for cure of 75 per cent of animals. (mg./kg.)	
1.	2.	3.	4.	5.	6.	7.	8.	9.
MHS 6	2-anilino-6-methoxylepidine hydrochloride.	200	0/5			0/5		Slight activity.
MHS 8	4-(*p*-*N*¹-*Isopropylthiowed-phenyl*) *amino-7-chloroquinoline.*	100	5/5	7	50	0/5		M.E.D. =25 mg./kg.; Q.E. =2·4.
MHS 21	4-*sulphathiazolyl-7-chloroquinoline*	800	4/5	3–4		0/5		
	p-aminophenylmethylsulphone hydrochloride.	412	5/5					M.E.D. =41·2 mg./kg. ; Q.E. =1·4.
	p-aminophenylethylsulphone hydrochloride.	417·5	4/5	3–5	417·5	0/5		Toxic to R.B.C.
	p-aminophenyl-n-propyl-sulphone hydrochloride.	422·5	0/3			0/3		Toxic to R.B.C.
	p-aminophenyl-n-butyl-sulphone hydrochloride.	420·5	0/5			0/5		Toxic to R.B.C.
B 201	4-nitro-4-aminophenyl-sulphide	32	2/5	7	8	3/5		Toxic to R.B.C., M.E.D. =3 mg./kg. ; Q.E. =20.
B 202	2 : 5-dihydroxy-4-amino-diphenylsulphone.	878–1756	5/5	5–10		0/5		M.E.D. =200 mg./kg. ; Q.E. =0·3.
	SULPHANILAMIDES.							
MN 1	N¹-*p*-methoxyphenyl sulphanilamide.	800	4/5	14		0/5		
MN 3	N¹-*p*-ethoxyphenyl sulphanilamide.	800	4/4	7		1/4		
MN 7	N¹-*p*-chlorophenyl sulphanilamide.	800	5/5	6–21		0/5		
MN 10	N¹-*p*-aminophenyl sulphanilamide.	400			20	5/5	200	M.E.D. =4 mg./kg. ; Q.E. =15.
MN 60	N¹-*m*-chloro-phenyl-sulphanilamide.	800	5/5	4–7		0/5		Toxic to R.B.C.

TABLE III—(*Concld.*)

Reference number.	Compound.	Daily dose of base in mg./kg. for 3½ days.	EFFECT ON PARASITÆMIA.					Remarks.
			SUPPRESSION.			ERADICATION.		
			Number of mice: responded/ treated.	Duration (days).	Minimum dose for 3-day effect in 75 per cent of animals. (mg./kg.)	Number of mice: responded/ treated.	Minimum dose for cure of 75 per cent of animals. (mg./kg.)	
1.	2.	3.	4.	5.	6.	7.	8.	9.
MN 61	N^1-*o*-chlorophenyl-sulphanilamide.	800	5/5	4–7		0/5		
MN 62	N^1-2 : 5-dichlorophenyl sulphanilamide.	800	2/5			0/5		
MN 63	*N^1-2 : 4-dichlorophenyl sulphanilamide.*	800	0/5			0/5		
MN 12	N^1-phenyl sulphanilamide.	800	4/5	14		1/5		Toxic to R.B.C.
MN 58	N^1-benzyl sulphanilamide.	800	3/5	6–14		2/5		
MN 51	*N^1-phenyl-N^1-isopropyl sulphanilamide.*	400	0/5			0/5		
MN 54	*N^1-p-methoxyphenyl-N^1-isopropyl sulphanilamide.*	800	5/5	4–7		0/5		
MN 56	*N^1-p-ethoxyphenyl-N^1-isopropyl sulphanilamide.*	1600	5/5	4–8		0/5		
MN 5	N^1-*p*-hydroxyphenyl sulphanilamide.	1600	2/4	13		2/4		
MN 52	*N^1-phenyl-N^1-isopropylacetyl sulphanilamide.*	800	0/5			0/5		
MN 55	*N^1-p-methoxyphenyl-N^1-isopropyl-acetyl sulphanilamide.*	800	0/5			0/5		
MN 57	*N^1-p-ethoxyphenyl-N^1-isopropyl acetyl sulphanilamide.*	800	5/5	4–7		0/5		
	SULPHATHIAZOLES.							
	5-ethyl sulphathiazole.	3·2	0/5			0/5		Tested at M.E.D.
	5-isopropyl sulphathiazole.	1·6	0/5			0/5		Tested at M.E.D.

Survey numbers : B 202 = SN 1159-853; MN 1 = SN 237-773; MN 7 = SN 2492-769; MN 10 = SN 260-807; MN 60 = SN 8145-769; MN 61 = SN 8146-769; MN 62 = SN 8619-769; MN 12 = SN 276-767; MN 58 = SN 2529-703; MN 5 = SN 275-773; only MN 10 and MN 58 were active against avian malarial parasites (Wiselogle, 1946).

On the fifth day of the test and the fourth morning after the day of inoculation, thin blood films were prepared from all animals in a test series, stained and examined, and the infection rate of the red corpuscles determined from a random sample of 500 cells. A 75 per cent reduction in the parasitæmia of a drugged group as compared with the parasitæmia of the untreated group of the same series denotes activity in the drug. The parasitæmias are compared by the geometric means of the individual counts (Marshall, 1946). Slight activity is attributed to a compound for a reduction of less than 75 per cent but more than 50 per cent. Infection rates below 1 infected cell per 500 were recorded as $< 0{\cdot}2$ per cent. A 'negative' result (0·00 per cent) is based on an examination of not less than 200 fields of a thin film and confirmed by examination of the greater part of a thick film. If a drug shows activity, the animals concerned are examined twice weekly from the 7th day. Suppression of an infection is indicated by a 'negative' finding on the 5th and 7th day of the test. Animals remaining 'negative' for 30 or more days were tested for eradication of infection by sub-inoculation of 0·2 c.c. of blood into a clean mouse and re-inoculation with the standard dose of infection.

Field's stain was mainly employed in this investigation for staining blood-films. It gave excellent results with thin films fixed by either methyl or ethyl alcohols, and the staining time for such films is about 20 seconds.

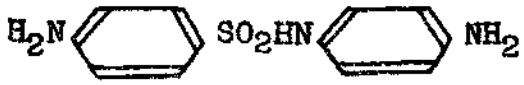
RESULTS.

Data on the activity of some standard reference antimalarials are presented in Table I. The data are drawn largely from actual test series in which unknown compounds were screened. This selection of data serves the double purpose of indicating the reaction of the test organism to established drugs and of illustrating the test infections which the unknown drugs were called upon to inhibit. The M.E.D. of quinine and the dose used for calculating quinine equivalents (Q.E.) was placed at 60 mg./kg. Quinine controlled the infections to a limited degree and failed to suppress them except at very high dosage levels. Proguanil is about nine times as efficient as quinine and effected several cures at 20 mg./kg. and higher but toxic doses. Chloroquine and sulphathiazole are twenty times as active as quinine.

Of the test compounds, the quinaldines, which included some new compounds (Ganapathi and Shah, 1951 : 1951*a*), a guanidine-quinoline, a chloroacetoacetanilide, a tolylguanidine, a nitroquinoline, a nitrobenzoic acid ester and four thiopyrimidines, all tested at near maximum tolerated doses, were inactive. Some quinoline sulphones had no inhibitory action at a dose several times higher than the M.E.D. of chloroquine. All these inactive compounds are listed by full name in Table II.

Compounds showing definite activity and those believed to be active but which also exhibited concomitant toxic effects on the red corpuscles are listed in Table III. A thiourea derivative of a 4-aminoquinoline, compound No. MHS 8 (Ganapathi and Shah, 1951*a*), acted as a suppressant, and its Q.E. is 2·4. The 7-chloroquinoline with a substituted sulphathiazolyl ring, Ref. No. MHS 21 (Ganapathi and Shah, 1951*a*), showed a limited degree of activity at a comparatively high dosage and was, therefore, not given further trials to determine its

M.E.D. The sulphones tested are probably active, but toxic side-effects made proper evaluation difficult. One of these compounds, No. B 201, was tested in four sub-divided doses of the presumed minimum curative dosage, but the toxic effects were not eliminated. The sulphanilamides (new compounds are from unpublished work of Sardesai, Shirasat and Ganapathi) were active as a class without being unduly toxic. As it was not practicable to determine the M.E.D. of each of these compounds, selection for further study was done on the basis of curative effect at high dosage. Compound No. MN.10 was thus selected and its Q.E. determined to be 15. Activity appears to be enhanced by substitution of NH_2 in the para position :



(MN. 10)

DISCUSSION.

The minimum dose of quinine base effecting a 75 per cent reduction in the parasitæmia of chicks infected with *P. gallinaceum*, the most widely used test organism for screening programmes, is 32 mg./kg. (Coatney and Sebrell, 1946). *P. berghei* is thus about twice as resistant to quinine as the avian parasite. Most authors, to whose chemotherapeutic trials against *P. berghei* reference was made in the introduction to this report, have noted that this parasite is more resistant to quinine than the avian plasmodia commonly used for such work. The quinine equivalents of proguanil and chloroquine obtained in the present investigation are quite similar to the values recorded by Goodwin (*loc. cit.*), Thurston (*loc. cit.*) and Hill (1950). The quinine equivalent of sulphathiazole, approximately 20, is, however, totally unlike the figure of 1,000 given by Hill (*loc. cit.*). This discrepancy is evidently not accounted for by the higher level of activity selected by Hill as the criterion for the M.E.D. Tests with sulphadiazine are in progress and the results obtained will be compared with Hill's and other workers' findings to ascertain if the discrepancy extends to this compound. It may be noted that the 5-isopropyl derivative of sulphathiazole is more active than the parent compound.

None of the active test compounds proved superior to the corresponding reference drug. Thus MHS. 8 is less active than chloroquine. The M.E.D. of sulphadiazine is not greater than 1 mg./kg. (studies as yet incomplete), so that the active sulphanilamides, represented by MN. 10 for comparative purposes, are less effective than sulphadiazine under similar test conditions, the comparison being restricted for the present to the M.E.D. of the compounds.

Some of the compounds have been screened against avian plasmodia (Wiselogle, 1946); these compounds, with their ' Survey Numbers ', are indicated in the foot-notes to Tables II and III. Only MN. 10 and MN. 58 showed moderate activity against the avian parasites.

In passing, the amenability of blood-induced *P. berghei* infections to eradication by a number of standard drugs, including suppressive-type drugs, is worth noting. Thurston (*loc. cit.*) found that mepacrine readily produced cures. Baldi

and Della Rocca (1951) credit chloroquine and camoquin as being efficiently curative drugs. In the present investigation, proguanil cured 90 per cent of animals at doses of 20 mg./kg. and 36 mg./kg., though the latter was definitely toxic. From this chemotherapeutic aspect, *P. berghei* reacts to antimalarials in a manner resembling blood-induced infections of *P. vivax* and *P. falciparum* rather than blood-induced infections of avian species of *Plasmodium*. The mechanism of cure is, however, apparently different in *P. berghei* and the human forms. In the latter, it is now generally believed that exo-erythrocytic parasites are lacking after blood inoculation, thus explaining the well-established fact that blood-induced infections are easily cured as compared with sporozoite-induced infections. In *P. berghei*, exo-erythrocytic forms have been reported by Van den Berghe, Vincke and Chardome (1950) and Garnham (1951 : 1951*a*), though in different types of host cells respectively. If these late exo-erythrocytic forms are essentially parasites of cells of the hæmopoietic system, as the discussion by Garnham (1951*a*) appears to suggest, this may explain the vulnerability of the parasite to antimalarial agents, since such host cells would sooner or later be released into the blood stream.

SUMMARY.

The methods adopted for screening antimalarial compounds against blood-induced infections of *Plasmodium berghei* in a strain of inbred mice are described.

The results of testing some standard antimalarial drugs and a number of other compounds, some new and others known but untried against *P. berghei*, are recorded.

The minimum dose of quinine base effecting a 75 per cent reduction in the parasitæmia of the treated group as compared with the parasitæmia of the untreated control group in a five-day test, and the dose used for calculating quinine equivalents, is 60 mg./kg. The quinine equivalents of proguanil, chloroquine and sulphathiazole were thus found to be 8·6, 20 and 20 respectively.

Several test compounds, notably sulphanilamide derivatives, were found to be active, some even effecting cures, but none appeared to be outstanding on reference to an appropriate standard drug.

In respect of amenability to cure by suppressive-type drugs, blood-induced *P. berghei* infection resembles blood-induced *P. vivax* and *P. falciparum* infections rather than avian species. The mechanisms of cure in the rodent and human infections are, however, probably different.

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STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

*VIII. The course of blood-induced infection in starved albino rats.

BY

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(March 15, 1953.)

The Indian Famine Commission (1898) reported that in the opinion of the medical officers, the prevalent fever was ordinary malaria, which, though it attacked all classes more or less, was specially fatal in the case of those who had suffered from privation. Christophers (1910 : 1911) in his studies on malaria in the Punjab, observed that in epidemics, the highest mortality occurred among the poorest classes, and that, of the twelve epidemics of malaria which devastated the Punjab in the later half of the nineteenth and early years of the current century, four followed seasons of famine or acute scarcity. At the same time, he domonstrated an equally high correlation between fever and rainfall and emphasized the excess of rainfall as a causative factor of epidemics. Gill (1928 : 1935), as a result of experience of malaria epidemics in the Punjab and Ceylon, also strongly emphasized the importance of economic stress as a factor in the causation of endemic and epidemic malaria. Williams (1940) considered that nutrition was of the greatest importance in the reaction to malaria, particularly in children.

The converse view in regard to nutrition and malaria has been equally prevalent. James (1926) was of the opinion that malaria affected the healthy and strong equally as the weak and sickly, and that economic conditions had no influence on the prevalence of malaria. Ross (1929) emphasized that nutritional status of the human hosts had nothing whatsoever to do with the course of malaria in them. Sinton (1935*a* : 1935*b* : 1936) pointed out that malaria may itself be the direct cause of poverty and insufficient diet.

Hacket (1937) reviewed various opinions and remarked that the absence of experimental data made the discussion of the question a mere battle of opinions. His own view was that malaria was a cause of poverty and under-nutrition.

While a large number of investigators have interested themselves in the nutrition of the vertebrate host in relation to the extent of its hospitality to malaria

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parasites, only few investigations on human beings have been carried out in the field. Observations on the ability of parasites to grow in totally starved laboratory animals like monkeys were those of Geiman and Mackee (1948) who found that when monkeys were starved for 24 to 48 hours, *P. knowlesi* infection was strikingly ameliorated. Likewise they found that when fasting was started simultaneously with inoculation of animals with parasites, only a few organisms appeared in the blood during the period of continued fast. As soon as the animal was given a full diet, the parasites grew normally.

The present work was carried out in order to determine the host-parasite relationship between albino rats and *P. berghei* when the animals were starved of all food except water. The data presented here served as base line to further observations on host-parasite relationship, when the hosts were under different nutritional states; these will appear in subsequent publications.

MATERIAL AND METHODS.

Two strains* of *P. berghei* immunologically identical with each another (Krishnaswami *et al.* 1953) were used in these experiments. Parasite density per 10,000 erythrocytes in the donor was determined and its blood drawn into a syringe which contained a known quantity of 2 per cent citrate saline. The contents of the syringe were further diluted in order that one million parasites would be contained in 0·1 c.c. of the liquid which was the standard dose of inoculation given by the intraperitoneal route.

Stained thin blood smears were examined daily and if positive the parasites were counted against 10,000 erythrocytes. Slides were declared negative when no parasites were seen in at least 100 oil immersion fields.

Albino rats of known ages (six to eight months old) were purchased from other laboratories† ; 16 rats (two months old) were from the colony maintained at the Institute.

A preliminary trial indicated that uninfected rats could be starved with and without water for about 20 and 10 days respectively. Based on this, the standard period of starvation for these experiments was fixed as 10 days. Unless indicated, animals were given access to water in a bottle at all times. The animals serving as controls for the starved ones were on a standard‡diet given once a day on an *ad lib* basis.

*Strain I was received in 1950 by the kind courtesy of Professor E. Brumpt, Paris. Strain II was received from London through the courtesy of Brigadier J. S. K. Boyd.

†Central Drug Research Laboratory, Lucknow, and Nutrition Research Laboratories, Coonoor.

‡The standard diet contained :—

Pea nuts	...	8 parts
Whole wheat flour	...	70 ,,
Skimmed milk powder	...	16 ,,
Dry brewers yeast	...	2 ,,
Table salt	...	1 ,,
Calcium carbonate	...	1 ,,
Shark liver oil	...	2 ,,
		100

In addition to the above, each rat received 2 gm. germinated Bengal gram a day.

Forty adult rats were divided into eight groups each of which contained 5 animals. Six groups were experimental while the remaining two were controls. The former were starved from 5 to 10 days at different intervals during the observation period and the standard inoculum of one million parasites was given to the animals on the first day of the experiment in all cases except two where it was given on the sixth day of starvation. One control group was fed on standard diet throughout the period while the second was totally starved of both food and water. The daily average parasitæmia against 10,000 erythrocytes was determined and the results are shown in Table I.

RESULTS.

The parasitæmia in the control group on standard diet showed a gradual increase followed by decline, lasting for 15 days. The group totally starved of solid food as well as water, did not show any patent parasitæmia in the peripheral blood on any of the eight days during which all of them died of starvation.

Fasting for five and ten days after inoculation seemed to inhibit parasite multiplication to a very great extent. In the former which were fed from the sixth day, the parasites became patent once again on the thirteenth day and lasted for 10 days. The animals in the latter group were fed from the 11th day onwards, but the parasites did not re-appear during the observation period of sixteen days.

The group, inoculated after 5 days of fast which continued upto the tenth day, never showed patent parasitæmia either during the fast or the sixteen days of subsequent feeding.

Five days fast alternating twice with a similar period of feed in between and subsequent continuous feeding showed an interesting pattern of patent parasitæmia. The parasites were few during the fast periods and were not patent in the intervening period of 5 days of food. Parasites re-appeared on the twelfth day of uninterrupted feed which commenced on the 16th day of inoculation. They were patent for eleven days after which the infection became latent.

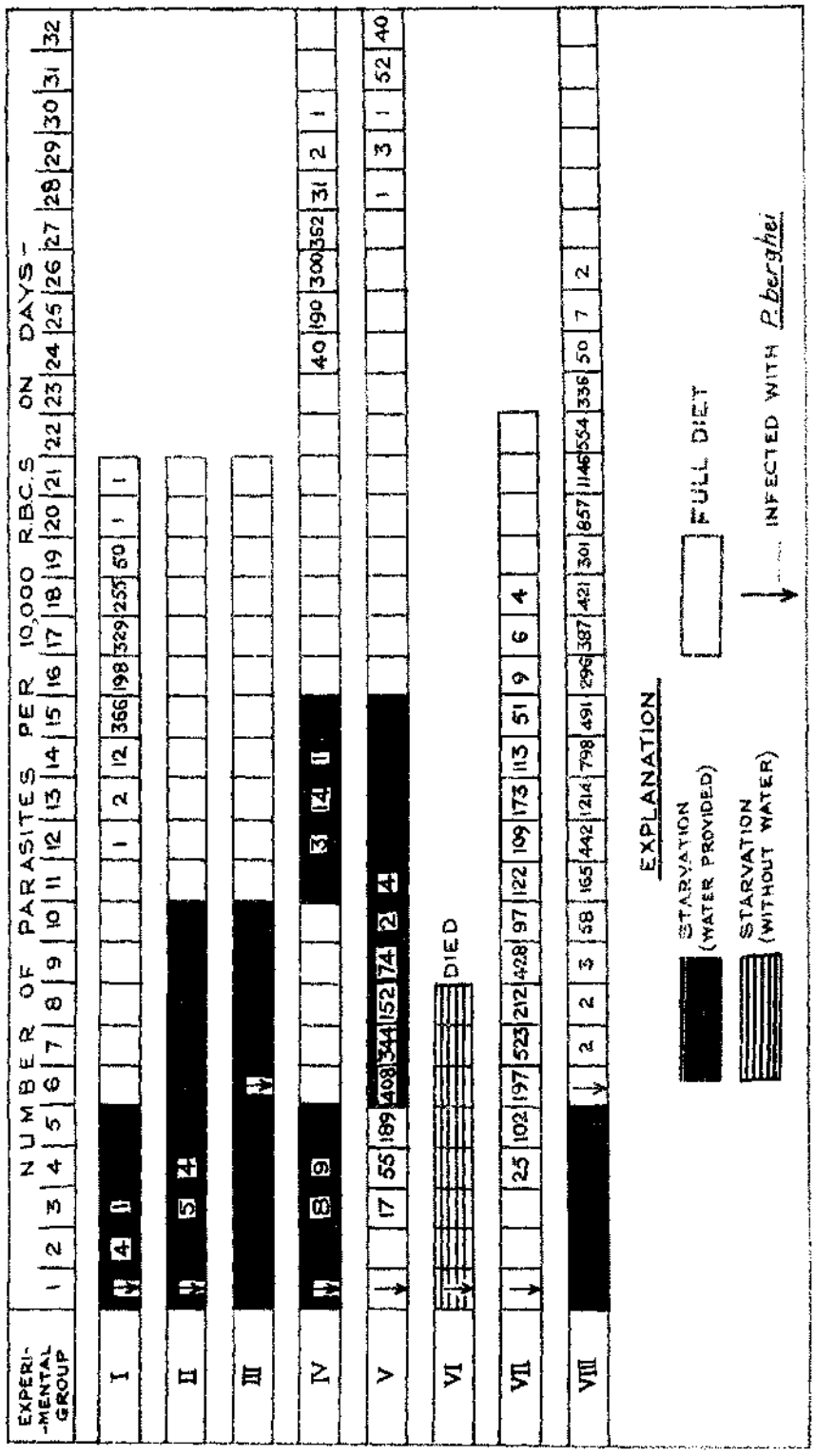
The group that was fasted for five days, infected and fed from the 6th day onwards, developed a normal pattern of parasitæmia except that in the first few days parasite multiplication was slower than in the control group. Death occurred due to acute infection on the 6th day in one and 13th day in two. The remaining two developed chronic infection after 16 and 20 days of patent parasitæmia.

In the group that fasted 10 days from the 6th day of inoculation, the parasitæmia which was on the ascent till then showed a decline rather abrputly. It became patent once again on the 13th day of subsequent feeding and lasted for 6 days in one of the animals. The second patent period was short and the parasite multiplication poor.

To confirm the effects of 10 days starvation after inoculation the experiment was repeated in three animals. The results were different from the first and the patent parasitæmia was higher and of longer duration. The only explanation seemed to be that these animals were two months of age and were chosen inadvertently. The experiment was repeated. Three more animals, two months

TABLE 1.

DAILY AVERAGE PARASITAEMIA IN ALBINO RATS DURING STARVATION.



old, and two groups of three adult animals, six months old, were inoculated and fasted for 10 days. For each of the latter groups two more animals were fed on standard diet and served as control. The results confirmed the previous findings as well as indicated that effects of starvation were influenced by the age of the animal as shown in Table II.

TABLE II.

DAILY AVERAGE PARASITAEMIA IN STARVING ALBINO RATS OF DIFFERENT AGES.

Trial No.	Age in months	No. of animals	Parasites per 10000 R.B.C.s on days following infection									
			1	2	3	4	5	6	7	8	9	10
1	2	3	2	12	29	28	2	D				
2	2	3	3	8	24	39	57	22	D			
3	6	3	21	1	Subinoculation of all three animals positive.							
4	6	2	2	10	108	390	630	468	549	732	513	209
5	6	3	21	*Subinoculation positive of one animal. The other two became positive on 7th and 11th day of feeding.								
6	6	2	2	19	140	185	450	342	343	480	668	366

EXPLANATION

STANDARD DIET

D = DEATH

STARVATION WITH WATER

With a view to determine changes in the formed elements of blood during starvation, total erythrocyte, leucocyte and differential leucocyte counts were made on two clean animals on eight consecutive days from commencement of starvation. There seemed to be a hæmoconcentration in the first three days of starvation after which the erythrocytes and leucocytes were less than at commencement. The average counts are shown in Table III.

TABLE III.

Day of starvation.	Erythrocytes per c.mm. in million	Leucocytes per c.mm.	Differential leucocyte count, per cent.				
			Polymorphs.	Lymphocytes.	Eosinophils.	Basophils.	Monocytes.
1	7·33	12,440	34	63	0	0	3
2	8·9	13,000	24·5	74·5	0	0	1
3	9·7	20,680	34	60	0	0	6
5	6·2	11,400	36·5	60	0	0	3·5
6	6·4	11,160	35·5	58	0	0	6·5
7	6·5	11,760	33·5	60·5	0·5	0	5·5
8	5·4	11,780	33·5	59	0·5	0	7·0

Leucocytosis with a relative increase of lymphocytes seemed to be the initial effect of acute starvation. A similar blood picture was reported (Ramakrishnan, Satya Prakash and Krishnaswami, 1953) in chronic malaria of rats with subpatent parasitæmia. It was, however, not possible to conclude whether the lymphocytæmia was responsible to any extent for the extremely low numbers of parasites present in the peripheral blood of starving animals.

DISCUSSION.

The results of experiments on the course of infection of *P. berghei* in fasting albino rats confirmed the findings of Geiman and Mackee (*loc. cit.*) in fasting monkeys infected with *P. knowlesi*. As already stated starvation for five days rendered conditions partially inhospitable to parasites, as shown by their re-appearance during subsequent feeding. When the starvation was extended for 10 days from inoculation, subsequent feeding did not cause the parasites to re-appear indicating that the parasites could not survive that period of fast.

A likely explanation for the observation seemed to be that when the host was starved, some nutritive principles essential to the parasites were depleted from the peripheral blood. Such was found to be the case by Mackee and Geiman (1948) and Geiman and Mackee (*loc. cit.*).

A second explanation was that ketonæmia or ketosis and allied phenomena either alone or in addition to the above was responsible for the inhospitable conditions to parasites when the host was starved. The development of ketosis during

complete absence of food for a period as short as two or three days in man has been recognized for a long time (Keys, Brozek, Henschel, Mickelsen and Taylor 1950). The same authors on the subject of response to infection in human starvation considered that it was unthinkable that starvation should be without any influence on chemical characteristic of the limiting membranes which in their turn must profoundly affect the amounts and the rate of penetration of the infecting organism. It is presumable in the present case that such a hypothesis is untenable inasmuch as the organism seemed to successfully pass through peritoneal as well as the capillary lining and was patent in the peripheral circulation though only in small numbers and for short time during starvation.

The results indicated that although *P. berghei* depended primarily on the erythrocyte, factors in the plasma were equally important for its survival and multiplication. The degree of inhospitality for *P. berghei* exhibited by starving adult rats was considerably less in the case of younger ones under identical conditions. The explanation for this is not known.

Response to antigenic stimulus of *P. berghei* seemed to be present even under conditions of starvation of the host animal. In animals which were submitted to two five-day periods of starvation intervened by a similar period of feeding, the patent parasites were few in number during the two starvation periods. The number of parasites in circulation must have been considerably less during the long sub-patent period of 8 days subsequent feeding. The fact that at the end of the period parasites became patent again only for a short duration and were not present in as large numbers as in controls showed that a certain degree of immunity must have been acquired during sub-patent period which included two intervals of starvation.

SUMMARY.

Starvation of rats for five days from the day of inoculation rendered conditions partially inhospitable for *P. berghei*. When the starvation was extended for 10 days after inoculation, the parasites did not appear in the peripheral blood during subsequent feeding.

The age of the animal seemed to influence the effects of starvation of the host on *P. berghei*.

The possible explanations for the observations are discussed.

ACKNOWLEDGMENT.

Help of Shri J. Mitroo, Laboratory Attendant, in the care and maintenance of animals, is acknowledged.

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STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

*IX. Effect of milk diet on the course of blood-induced infection in albino rats.

BY

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(March 31, 1953.)

In an earlier publication, Ramakrishnan (1953) reported that starvation of the host was inimical for the multiplication of *P. berghei*. This paper deals with the influence of an exclusive milk diet as compared to standard diets.

MATERIAL AND METHODS.

A strain† of *P. berghei* maintained in albino rats by blood passage was used in the experiments. The dose of inoculum was invariably one million parasites per rat given by the intraperitoneal route. Thin blood smears of all animals were examined daily and parasites were enumerated against 10,000 erythrocytes. Slides were declared negative when no parasites were seen in at least 100 oil immersion fields.

Albino rats of both sexes and known ages were used. Most of them were from the colony maintained at the Institute while some were obtained from the Central Drug Research Laboratories, Lucknow. The animals were housed in special individual cages and had no access to their excreta at any time.

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†Received by the courtesy of Professor Emile Brumpt, Paris, in 1950.

Two experiments were carried out. In the first, the effects of milk and balanced diets were compared and in the second the effects of uninterrupted milk diet were compared with those of milk for the first 10 days of infection followed by control diets. Standard commercial brands of powdered milk* were used in both the experiments and the animals were pre-conditioned for one week on milk in the case of experimental groups and on standard diet in the case of controls. Two types of diets (Nos. 1 and 2)† of the same calorific value were used for the control groups, one contained whole milk (Klim) as part of balanced diet while the other was a vegetarian diet without milk.

Certain difficulties were experienced during the second experiment when reconstituted Ostermilk was provided to the animals in bottles in accordance with the feeding technique described by Maegraith, Deegan and Jones (1952). In spite of the fact that bottles were sterilized daily and filled with fresh milk twice durine the day, it was found that the cream separated and floated on top and was not accessible to the animals. In some cases the milk curdled and blocked the tubes obstructing the free flow. The curdling of milk was no doubt due to the high temperatures prevalent in Delhi. The bottles were inspected frequently during the day and as far as possible it was ensured that the animals were getting the milk. The technique of feeding was not changed, however, as the intention was to adopt the same feeding technique as described by Maegraith *et al.* (*loc. cit.*).

CRITERIA FOR ASSESSMENT OF RESULTS.

For purposes of this investigation only the acute primary stage of infection was considered. Four indices were selected to assess and compare the effects of the diets on the course of infection during the acute phase. They were (1) duration of patent parasitæmia, (2) peak parasitæmia, (3) day of peak and (4) average daily parasitæmia. To allow for any individual variations, averages were determined for each group of animals.

RESULTS.

Parasites were patent for 15 to 16 days in animals on milk diet in both experiments, and showed an ascending phase, a peak and decline. But, as compared to animals on a balanced diet inclusive of milk, all the four indices were appreciably low. In the two experiments, three out of four animals on diet No. 1 died of acute infection while none exclusively on milk died. Results of the first experiment (Table I) were confirmed by the second (Table II).

*KLIM in the first and OSTERMILK in the second experiment.

† DIET NO. 1.

Whole wheat flour	... 72	parts.
Klim powder	... 23	,,
Brewers yeast (dry)	... 3	,,
Calcium carbonate	... 1	,,
Common salt	... 1	,,
	100	

DIET NO. 2.

Rice flour ...	... 77	parts.
Pea nuts ...	... 18	,,
Brewers yeast (dry)	... 3	,,
Calcium carbonate	... 1	,,
Common salt ...	... 1	,,
	100	

TABLE I.

Course of P. berghei *acute infection in five to six weeks old albino rats fed on milk and balanced diets.*

Number of animals.	Pre-inoculation diet for one week.	Post-inoculation diet.	PRIMARY PARASITÆMIA (AVERAGES).			
			Duration in days.	Daily parasitæmia per 10,000 erythrocytes.	Peak per 10,000 erythrocytes.	Day of peak.
5	Klim	Klim	15	205	428	8
4	Diet No. 1	Diet No. 1	25*	463	1325	9

*Three animals died on the 13th, 15th and 17th days from acute disease.

Note.—Klim was reconstituted into a thin paste with water and provided in small metal cups.

TABLE II.

Course of P. berghei *acute infection in eight to nine weeks old albino rats fed on milk and milkless diets.*

Group.	Number of animals.	Pre-inoculation diet for one week.	Post-inoculation diet.	PRIMARY PARASITÆMIA (AVERAGES).			
				Duration in days.	Daily parasitæmia per 10,000 erythrocytes.	Peak per 10,000 erythrocytes.	Day of peak.
1	3	Ostermilk	Ostermilk	16	206	506	6
2	3	Ostermilk	Ostermilk for 10 days following inoculation; diet No. 2 from 11th day.	17	233	532	9
3	3	Diet No. 2	Diet No. 2	18	419	1370	7
4	3	Colony diet.	Ostermilk for 10 days from inoculation and diet No. 2 from 11th day onwards.	19	431	1431	5
5	5	Diet No. 1	Diet No. 1	20*	593	1798	12

*One animal died on the 15th day from acute infection.

Note.—Ostermilk was reconstituted as per directions and were provided in bottles.

Effect of a week's pre-conditioning on milk was clearly brought out by comparing the indices of the two groups (2 and 4 of Table II) one of which was pre-conditioned on milk while the other was given colony* diet during the same period. In all other respects the two groups were treated identically, and were given milk exclusively for 10 days after inoculation and from the 11th day onwards they were both on diet No. 2. Pre-conditioning on milk resulted in lowering of the indices appreciably. Parasitæmia in the control groups (Groups 3 and 5 of Table II) fed throughout on diets No. 1 and 2 was similar to that in group 4 which was not pre-conditioned on milk.

It appeared that when rats were fed exclusively on milk, conditions were not optimum for the multiplication of *P. berghei* to the same extent as when fed on balanced diet with or without milk. The indications were that parasites in rats required something more than provided by an exclusive milk diet.

Weights of all animals were recorded twice a week. There was a progressive increase and no appreciable difference was seen in weights of the experimental and control groups.

DISCUSSION.

Factors inimical to growth of parasites in susceptible hosts can be (*a*) deficiency of nutritional requirements of parasites, (*b*) immunity and (*c*) therapeutic agents affecting parasites specifically. The results of the present investigation seemed to indicate that some factor or factors were deficient in milk diet for normal growth of *P. berghei* in rats. This is in accordance with the views expressed in a special editorial of the *British Medical Journal* (1952). Parasites were never totally absent in any of the animals on milk diet, but were less in numbers as compared to those on other diets with or without milk. Further, as stated above, the course of parasitæmia was appreciably different in two groups of animals treated identically with the exception of pre-conditioning treatment before inoculation which in one case was on milk and the other on a milkless diet. In the former, the peak as well as the average daily parasitæmia were appreciably lower than in the latter. The cause of such difference was probably a deficiency in the milk diet given for 17 days in one case, and only for 10 days in the other. Such a conclusion seemed justifiable as the indices of parasitæmia were higher in the groups fed on a milkless diet and balanced diet inclusive of milk as compared to those on an exclusive milk diet.

As the first experiment was in progress, Maegraith, Deegan and Jones (*loc. cit.*) announced that milk diet had a suppressive effect on the course of *P. berghei* infection in albino rats. They had used cow's milk, human milk, reconstituted Australian dried milk, and Ostermilk, and found that parasitæmia was almost totally suppressed in animals fed on any one of the types of milk as compared to normal diet, and concluded that milk contained something that inhibited or restricted the development of parasites. Their results were similar to the effects of starvation of rats reported by Ramakrishnan (*loc. cit.*).

*Colony diet differed from control diet No. 1 in that the Klim powder was substituted by skimmed milk powder.

The special editorial of the *British Medical Journal* referred to above also drew attention to the fact that milk was deficient in biotin and whether that could explain the restricted multiplication of parasites in the experiments of Maegraith *et al.* (*loc. cit.*). From results of experiments carried out on biotin deficient rats, it appeared that the deficiency did not cause any restriction on the multiplication of *P. berghei*. These results will be communicated at a later date.

At the time of writing this paper Dr. Harper of May and Baker Ltd., Dagenham, London, visited the Institute. While discussing the results with him, he pointed out that milk proteins were devoid of chromo-proteins as compared to meat and raised the question whether this could be the factor responsible for the observed results. It is an attractive hypothesis and would appear to be borne out by the results of experiments on rats on a meat diet (to be reported later) in which the parasite density was considerably higher than in the controls.

SUMMARY.

The course of *P. berghei* infection in rats on an exclusive milk diet was milder than in those on milkless diet as well as diet which included milk. It has been concluded that such an effect was most probably due to some deficiency in milk.

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Indian Journal of Malariology, 7, 1, *March* 1953.

STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

*X. A critical analysis of experimental mosquito transmission.

BY

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(March 15, 1953.)

INTRODUCTION.

A CONSIDERABLE number of vertebrates, nineteen of the order *Rodentia* and one of *Chiroptera*, have been found to be susceptible to blood-induced infections of *P. berghei* (Satya Prakash, Krishnaswami and Ramakrishnan, 1952). Attempts at experimental infection of mosquitoes, however, have not been equally successful. Vincke and Lips (1948) were not able to infect *A. gambiæ*, *A. coustani*, *A. maculipalpis*, and *A. funestus*, which were fed on infected mice. Raffaele and Baldi (1950) fed *Ædes ægypti* and *C. pipiens* on infected mice without success. Infection of the salivary gland in a single specimen of *A. stephensi* (type) out of a lot fed on an infected mouse was reported by Ramakrishnan and Satya Prakash (1950). Rodhain and Vincke (1951) described a mild gut infection in one out of four *A. maculipennis* (*atroparvus*) fed on infected *Thamnomys* but none fed on infected cotton rats. Unequivocal infection of mosquitoes in the laboratory was successfully

*The investigation was partly financed from a grant by the Indian Council of Medical Research.

accomplished by Yeoli and Wall (1951) who found oöcysts and sporozoites in *A. stephensi* as well as *A. quadrimaculatus* and *A. maculipennis* (*atroparvus*), fed on infected hamster.

In view of the above, it was found necessary to examine in detail the known factors influencing insect transmission, namely, the parasite and its gametocytes, the vertebrate and the invertebrate hosts and the environmental conditions in reference to the last. This paper presents a critical analysis of the available data.

MATERIAL AND METHODS.

Rodents.—A few albino mice and rats were used from the colonies maintained at the Institute, and the rest were purchased from other laboratories*. The squirrels were trapped locally, and specimens of *R. rattus* were obtained from the local municipal authorities. Four hamsters were received from the Liverpool School of Tropical Medicine by the kind courtesy of Professors Davey and Gordon, while the fifth was obtained through the courtesy of the Director, Pasteur Institute, Coonoor. All animals were maintained in laboratory on the standard diet given on an *ad lib.* basis.

Mosquitoes.—*A. stephensi* (type) and *Ædes ægypti* were available from the colonies maintained at the Institute, while *A. fluviatilis* specimens were obtained from the colony at the Field Station of the South India Branch at Mettupalayam. Adults of *A. splendidus* and *A. jamesi* were bred from larvae caught in and around Gadalur (South India), while the other species were bred out of larvæ caught around Delhi.

Parasite.—Two strains of the parasite (designated as Strains I and II) were used in the experiments. Strain I was obtained by the courtesy of Professor Emile Brumpt, Paris, in 1950, and has been maintained at the Institute by serial blood passage in albino rats. Strain II arrived in May, 1952, through the courtesy of Brigadier J. S. K. Boyd of the Burroughs Welcome Research Laboratories, London, and has since been maintained in albino rats in a manner similar to the Strain I. The second strain had been established bysporozoites obtained in nature from *A. dureni* in the Belgian Congo.

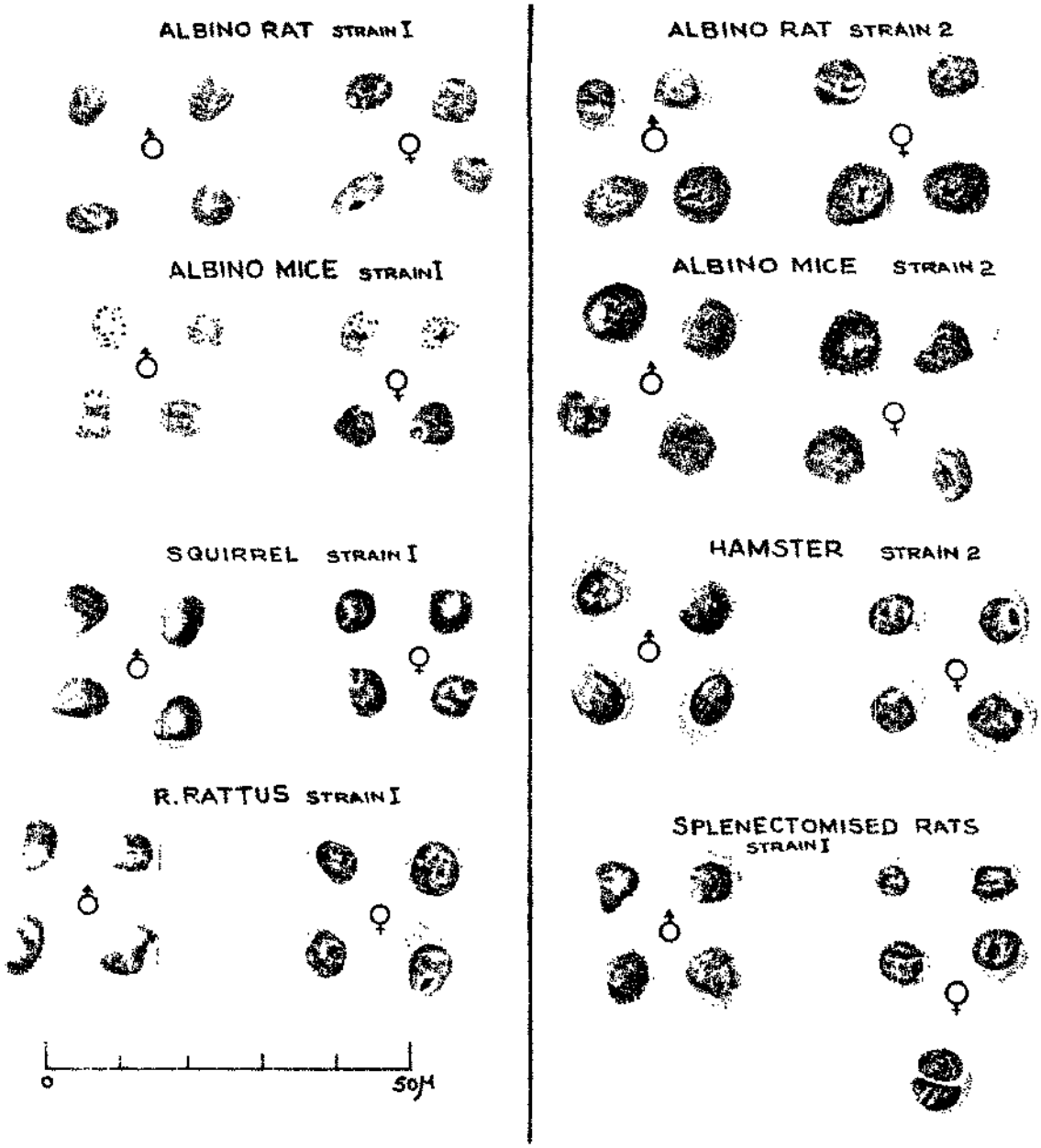
The dose of infection was invariably 1×10^6 parasitized erythrocytes except for the first few passages when it was 8×10^5 parasitized erythrocytes. The inoculum in every case was obtained from the tail blood during the rising phase of acute parasitæmia and injected into the recipient intraperitoneally. At the time of reporting, Strains I and II had undergone one hundred forty-five and forty-five serial passages respectively in albino rats. It was only possible to passage the latter twice in hamsters due to the limited number of animals available.

Blood examination.—Blood was obtained by pricking the tip of the tail after cleansing it with methylated spirit and allowing it to dry. Thin smears were made, air dried, fixed in methyl alcohol and stained by the rapid method of Jaswant Singh and Bhattacharji (1944). Asexual and mature sexual forms of parasites were counted against 10,000 erythrocytes.

Mosquito feeding.—Ten to fifteen mosquitoes, starved overnight, were enclosed in a tube (about one inch in diameter and two inches long) the open ends of which

*Haffkine Institute, Bombay; Indian Veterinary Research Institute, Izatnagar.

PLATE I.


ALBINO RAT STRAIN I
ALBINO RAT STRAIN 2
ALBINO MICE STRAIN I
ALBINO MICE STRAIN 2
SQUIRREL STRAIN I
HAMSTER STRAIN 2
R. RATTUS STRAIN I
SPLENECTOMISED RATS
STRAIN I
0
50μ

were covered by pieces of netting. One end of the tube was applied to a shaven part of the body of the rodent held with a cloth wrapped over it. In the case of *R. rattus* and a few squirrels the animals were anæsthetized by Nembutal (15 mg. per 20 gm. body weight) given intraperitoneally and left till recovery in a cage containing mosquitoes starved overnight. Fully fed mosquitoes were separated in a second cage and stored in the insectary under standard conditions till they were dissected for detection of gut and gland infections.

The temperature and humidity conditions of the insectary were roughly adjustable by improvised methods. The temperature during the experiments in 1950, 1951, and 1952 at Delhi varied between 75 and 85°F., and the relative humidity was between 65 and 75 per cent. In Mettupalayam, the insectary temperature varied between 77 and 87°F. and relative humidity was not measured but was found to be satisfactory as judged by the survival of mosquitoes and the consistently positive results obtained concurrently in the transmission of *P. gallinaceum* through *Ædes ægypti*.

CYTOLOGICAL CHARACTERISTICS OF GAMETOCYTES

The morphology and staining reactions of *P. berghei* gametocytes in mice, rats, and squirrels were described by Ramakrishnan and Satya Prakash (1950). It seems desirable to give a full description of the gametocytes that were encountered in the present investigations.

When stained with J. S. B. or Geimsa stains, gametocytes were of characteristic appearance. Macrogametocytes appeared spherical with a rose coloured chromatin-nucleus surrounded by cytoplasm which stained a deeper blue than that of asexual forms. The chromatin was dense and compact. Such cells were found in both polychromatophilic and mature erythrocytes. Scattered pigment granules were seen when in the latter.

The microgametocytes were also spherical, with the chromatin loosely arranged. The cytoplasm was blue, less dense than in the macrogametocytes in Strain I. In Strain II, till the tenth passage in rats, cytoplasm of the microgametocyte appeared pinkish, or biscuit coloured and differentiation between the cytoplasm and the chromatin was difficult. They gave a similar appearance to those seen in a hamster slide received through the courtesy of Professor Garnham. But after the tenth passage they started looking exactly like those of Strain I. When at a later date, Strain II was passaged into hamsters, after the second passage a few microgametocytes looked as they did before the tenth passage in rats.

In all animals, the micro- and macro-gametocytes did not occupy the entire erythrocytes; only three-fourths of the cells were filled by the parasites. Almost invariably a halo was present around the chromatin mass in both. Camera lucida drawings of gametocytes in the different animals are shown in Plate I.

The question of what constitutes a mature gametocyte has confronted many workers. The generally accepted criterion for maturity is scattering of the pigment (Gambrell, 1937). From time to time difficulty in differentiating forms, that look like immature gametocytes, from large schizonts, has been recorded by

many workers (Taliaferro, 1925; Huff, 1927; Shah, 1934). These forms must be pre-gametocytes for when an asexual form approaches their size the nucleus should have divided. In the present investigations, however, such doubtful forms were counted as asexual, and only mature gametocytes were classified as sexual forms.

FIRST APPEARANCE OF GAMETOCYTES.

Satya Prakash *et al.* (*loc. cit.*) came to the conclusion that parasitized erythrocytes introduced into the peritoneal cavity, found their way intact into the peripheral blood. This being the case, one can expect to see gametocytes in the circulation of the inoculated animal on the very first day of patency, depending of course on the gametocyte density in donor's blood.

Gametocytes were seen in peripheral blood on an average of 2 days in rats (Strain I), 3-4 days in Strain II and 4·4 days in mice (Strain I) after the first day of patent parasitæmia. Individual variations were to be expected as it is well known that gametocyte production is better in some hosts than in others although factors responsible for the difference are not known.

RATIO OF GAMETOCYTES TO ASEXUAL PARASITES AND THEIR RELATIONSHIP.

Table I indicates the daily percentage prevalence of mature gametocytes to total parasitæmia in mice, rats and hamsters. It was clear that the gametocyte density followed closely that of asexual parasites.

Acquired immunity of varying degrees in animals appeared to affect density of gametocytes which seemed to disappear from the peripheral blood along with the asexual parasites.

SEX RATIO OF GAMETOCYTES.

The ratio of macro- to micro-gametocytes on different days in mice, rats, and hamsters is depicted in Table II. With few exceptions females were more in number and the relative prevalence of males varied from a third to half the number of females.

PERIODICITY OF GAMETOCYTES.

P. berghei was found to complete its erythrocytic schizogony in 24 hours (Ramakrishnan and Satya Prakash, *loc. cit.*). In four mice infected with Strain I and five rats with Strain II, the number of mature gametocytes in a population of 200 parasites were counted in smears taken every four hours through fourteen days. Twenty-four hour counts were totalled up and are shown in Charts 1 and 2 as actual numbers as well as percentages. The percentage curve of gametocytes in mice showed a rythymic rise on the second, sixth and tenth days respectively. In rats on the other hand a similar rise was seen on the first and the twelfth day respectively. It would seem that mature gametocytes appeared in the peripheral blood in waves at intervals of about 3 to 4 days during primary parasitæmia. Gametocytes were not patent in peripheral blood when the asexual parasites became sub-patent during the chronic phase of infection in rats.

TABLE I.

Average percentage of gametocytes to total parasites in mice, rats and hamsters inoculated with Strains I and II of P. berghei.

Animal and parasitic strain.	Days from infection.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mice (Strain I)	7·3(18)	7·6(17)	7·0(17)	6·6(14)	5·7(14)	8·0(13)	6·2(13)	5·0(13)	5·5(12)	6·3(12)	5·9(12)	4·2(8)	5·0(5)	5·1(5)
Rats (Strain I)	15·1(3)	5·3(6)	5·9(4)	8·3(8)	4·3(4)	4·1(2)	3·7(4)	5·8(4)	5·3(3)	9·0(3)	6·9(3)	7·9(4)	3·6(1)	5·8(1)
Rats (Strain II)	4·7(5)	35·8(5)	1·9(5)	1·8(5)	1·3(5)	3·1(5)	4·4(5)	4·6(5)	4(5)	1·2(5)	5·7(5)	8·9(5)	4(4)	...
*Hamster	32·8(2)	9·2(2)	9·7(2)	6·8(2)	6(2)	6·1(2)	2·5(2)	4(2)	2·7(2)	4·2(2)	3·8(2)	2·1(2)	3·6(2)	4·3(2)
†Hamster	6·3(2)	14·3(2)	19(2)	14(2)	4·5(2)	7·6(2)	7·4(2)	4·3(2)	7·5(2)	5·6(2)	5·2(2)	3·8(2)	2(2)	2·7(2)

Note.—Figures in brackets show the number of animals under observation.
*Strain II passaged from rat to hamster.
†Strain II passaged from hamster to hamster.

TABLE II.

Percentage prevalence of micro- and macro-gametocytes in the different hosts from the first to fourteenth day of infection.

Animal and parasitic strain.	Sex	Day following infection													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Mice (Strain I)	Males	28·1	31·9	35·4	34·3	33	45·2	34·8	33·2	30	37·0	38·9	...	...	...
	Females	71·9	68·1	64·6	65·7	67	54·8	65·2	66·8	70	63	61·1	...	...	...
Rats (Strain I)	Males	37·4	33·3	48·2	42·9	51·9	33·3	43·2	35·5	46·1	38·5	29	33	...	...
	Females	62·6	66·7	51·8	57·1	48·1	66·7	56·8	64·5	53·9	61·5	71	67	...	...
Rats (Strain II)	Males	20·0	18·1	34·8	27·2	19·5	33·3	25·0	40·0	21·4	100·0	57	25	...	...
	Females	80	81·9	65·2	72·8	86·5	66·7	75	60·0	78·6	...	43	75	...	...
*Hamster	Males	...	10·5	11·5	19·3	28.3	25·5	32	34·5	29·5	31	31·4	33·3	31·2	37
	Females	...	89·5	88·5	80·7	71·7	74·5	68	65·5	70·5	69	68·6	66·7	68·8	63
†Hamster	Males	...	36	38·2	37·2	36	35·5	36	39	36·2	33	34·5	33	33	46·5
	Females	...	64	61·8	62·8	64	64·5	64	61	63·8	67	65·5	67	65	53·5

*Strain II passaged from rat to hamster.
†Strain II passaged from hamster to hamster.

CHART 1.

P. berghei GAMETOCYTE PERIODICITY IN ALBINO RATS

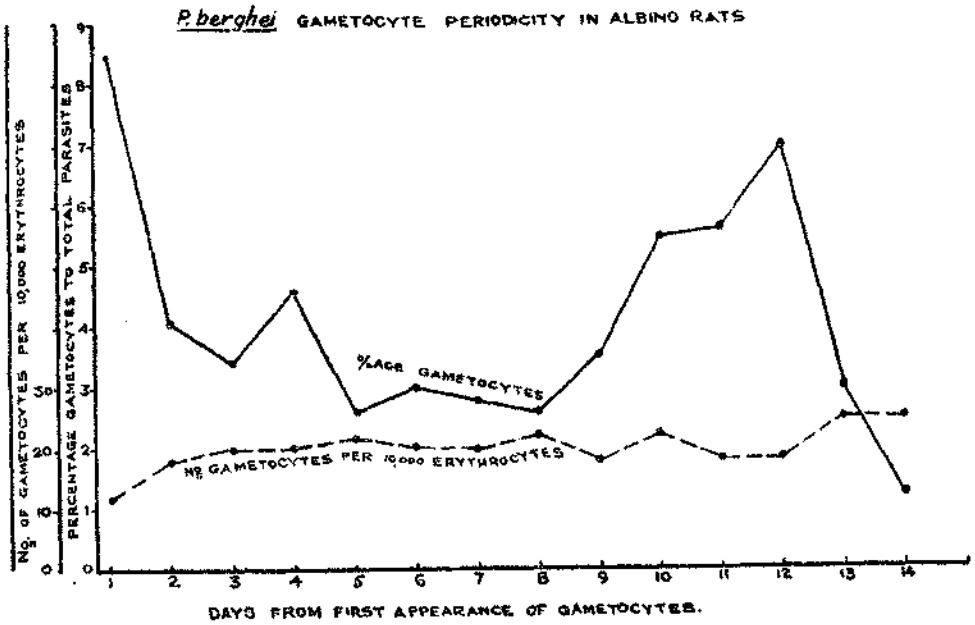
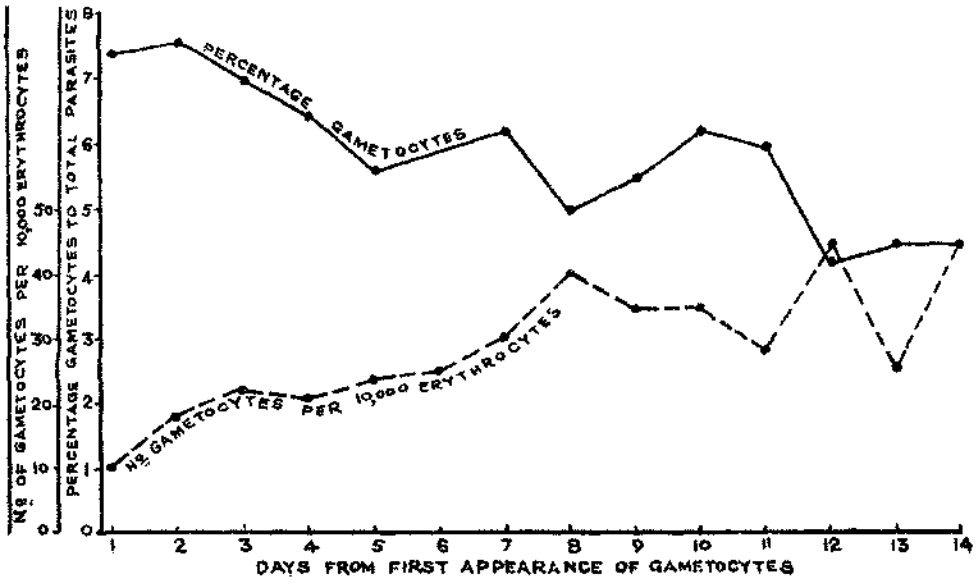


CHART 2.

P. berghei GAMETOCYTE PERIODICITY IN ALBINO MICE



EFFECT OF SERIAL PASSAGE ON GAMETOCYTES.

At the time of writing, as already stated, Strain I had undergone one hundred and forty-five and Strain II forty-five serial passages in albino rats. Gametocytes of Strain I showed no change whatsoever, either in morphology, staining reactions or their numbers. Those of Strain II, however, showed certain changes in respect of the males as described earlier.

EXFLAGELLATION STUDIES.

Thin blood films from infected mice, rats, squirrels and hamsters kept in a moist chamber at room temperature in summer at Delhi for intervals of 5 to 60 minutes, and later dried, fixed and stained, failed to reveal any exflagellation of microgametocytes. Such observations were repeated several times.

Smears of gut contents of mosquitoes fed on gametocyte carriers were examined at intervals of 5 minutes during the first half hour and then at half hourly intervals through 24 hour periods. Neither exflagellation of microgametocytes, nor zygotes or oökinetes could be observed.

PARASITIC RELAPSE.

Parasitic relapses were shown to occur in about fifty per cent of rats with chronic infection (Ramakrishnan *et al.*, 1951). It was also shown that such relapses were transient, rarely lasting for more than 3 days. Careful examination of blood films of six rats during relapses failed to reveal gametocytes.

THE VERTEBRATE AND INVERTEBRATE HOSTS.

As seen from the previous section, both Strains I and II of *P. berghei* produced gametocytes in all the four hosts namely, mice, rats, squirrels and hamsters. Ten species of Anopheles, *Culex bitæniorynchus* and *Ædes ægypti* were fed on different gametocyte carriers. Gametocyte counts at the time of feeding are indicated in the Tables against each animal. With the exception of one specimen of *A. stephensi* (type) fed on infected albino mouse, which showed sporozoites in its salivary glands, all the other attempts to infect mosquitoes were unsuccessful.

Tables II to V and VI to VIII depict details of mosquitoes dissected after a single feed on one of four different types of hosts infected with either Strain I or II of *P. berghei*.

On the basis of observations by James (1926) that mosquitoes which at first seemed refractory to infection could become infected by repeated feeding on suitable patients, some were fed twice and others thrice on gametocyte carriers of Strain I before dissection. The details are given in Table IX.

TABLE III.

Analysis of dissection of mosquitoes after a single feed on albino rats with gametocytes of Strain I.

Mosquito species.	Number fed.	Interval in days between feed and dissection.	Number dissected for gut and gland.	Gametocytes per 10,000 erythrocytes.
Ædes ægypti ...	288	6 to 19	59	19–38
A. annularis ...	2475	5 to 21	1164	4–56
A. stephensi (*mysorensis*)	2422	5 to 22	947	3–42
A. stephensi (type) ...	1324	5 to 20	353	3–46
A. subpictus ...	99	8 to 13	17	7–24
A. culicifacies ...	116	4 to 9	12	10
A. splendidus ...	20	11 to 20	6	11–17
A. hyrcanus ...	5	6	4	22–27
A. jamesi ...	52	8 to 19	9	9–18
A. fluviatilis ...	30	7 to 8	15	22–31

TABLE IV.

Analysis of dissection of mosquitoes after a single feed on albino mice with gametocytes of Strain I.

Mosquito species.	Number fed.	Interval in days between feed and dissection.	Number dissected for gut and glands.	Gametocytes per 10,000 erythrocytes.
Ædes ægypti ...	4	7	1	9–96
A. annularis ...	79	6 to 13	38	12–28
A. stephensi (*mysorensis*)	1278	5 to 22	515	6–160
A. stephensi (type)	335	4 to 19	60*	18–71
A. hyrcanus ...	2	3 to 4	2	22–27

*Sporozoites found in the glands of one specimen.

TABLE V.

Analysis of mosquitoes dissected after a single feed on R. rattus *with gametocytes of Strain I.*

Mosquito species.	Gametocytes per 10,000 erythrocytes.	Number fed.	Number dissected.	Interval in days between feed and dissection.
A. annularis ...	16 to 59	520	241	3 to 23
A. stephensi (*mysorensis*)	3 to 28	532	210	3 to 21
A. stephensi (type) ...	6 to 34	282	76	7 to 21
A. subpictus ...	1 to 25	387	33	3 to 13
A. pulcherrimus ...	Not counted.	1	1	14

TABLE VI.

Analysis of mosquitoes dissected after a single feed on squirrels with gametocytes of P. berghei *Strain I.*

Mosquito species.	Number fed.	Number dissected.	Interval between feed and dissection in days.	Gametocytes per 10,000 erythrocytes.
A. annularis ...	81	67	5 to 8	11 to 24
A. stephensi (type) ...	29	14	7 to 15	1 to 24

TABLE VII.

Analysis of mosquitoes dissected after a single feed on albino mice with gametocytes of Strain II.

Mosquito species.	Number fed.	Number dissected.	Interval in days between feed and dissection.	Gametocytes per 10,000 erythrocytes.
A. stephensi (type) ...	13	1	9	Not counted.

TABLE VIII.

Analysis of mosquitoes dissected after a single feed on albino rats with gametocytes of Strain II.

Mosquito species.	Number fed.	Number dissected.	Interval in days between feed and dissection.	Gametocytes per 10,000 erythrocytes.
A. stephensi (*mysoriensis*)	92	33	5 to 14	8 to 28
A. stephensi (type) ...	979	243	7 to 16	Not counted.
Aëdes ægypti ...	893	181	6 to 18	Not counted.
A. annularis ...	316	85	2 to 15	Not counted.

TABLE IX.

Analysis of mosquitoes dissected after a single feed on hamsters with gametocytes of Strain II.

Mosquito species.	Number fed.	Number dissected.	Interval in days between feed and dissection.	Gamtocytes per 10,000 erythrocytes.
A. annularis ...	94	76	5 to 14	Not counted.
A. fluviatilis ...	775	305	2 to 22	18 to 147
A. stephensi (type) ...	2743	1379	2 to 22	18 to 147
C. hitæniorhynchus ...	3	3	9 to 11	44
Aëdes ægypti ...	4	4	9 to 11	44

Although gametocytes were numerically present in blood on the day the mosquitoes were fed, it was considered possible that they were not sufficiently mature to progress in their development. In the absence of any test for maturity of gametocytes the only possibility was to feed mosquitoes on consecutive days on the same gametocyte carrier, on the chance of some gametocytes being mature on any one day of their patency. Details of such feeding are given in Tables X and XI.

TABLE X.

Analysis of mosquitoes dissected after multiple feeds on rodents with gametocytes of Strain I.

Mosquito species.	NUMBER DISSECTED AFTER.		Rodent.	Interval in days between dissection and last feed.	Gametocytes per 10,000 erythrocytes.
	Two feeds.	Three feeds.			
A. annularis	142	29	Albino rat	5 to 21	4 to 56
	4	Nil	*Rattus rattus*	23	16 to 59
A. stephensi (type)	107	25	Albino rat	20	3 to 46
	10	Nil	*R. rattus*	21	6 to 34
A. stephensi (*mysorensis*)	9	1	Albino rat	22	3 to 42
A. jamesi ...	7	Nil	Albino rat	19	18
A. splendidus	3	Nil	Albino rat	20	17

TABLE XI.

Analysis of mosquitoes dissected after multiple feeds on consecutive days on albino rats with Strain I from the day of inoculation.

Mosquito species.	Number of days consecutive feed on the same rat.	Number fed.	Number dissected.	Interval in days between dissection and last feed.
A. annularis	6	100	15	2 to 5
	9	212	72	4 to 14
A. stephensi (type)	6	354	48	15 to 16
	6	279	54	15 to 16
	4	183	17	13 to 16
	9	70	62	7 to 9

DISCUSSION.

For a parasite that was first discovered in its sporozoite stages occurring naturally in *A. dureni* (Vincke and Lips, 1948) attempts at experimental infection of mosquitoes with *P. berghei* have offered considerable difficulties. The data presented in this paper cover most factors known to influence plasmodial infection

in mosquitoes. Each one of the factors will be discussed below in order to examine the reasons, if any, for the failures and account for the successful infection of *A. stephensi*, *A. quadrimaculatus* and *A. maculipennis* (*atroparvus*) by Yoeli and Wall (1951).

THE PARASITE AND GAMETOCYTE.

It would appear that gametocytes as determined by their morphology and cytology were produced by both strains of *P. berghei* in mice, rats, squirrels and hamsters, but were not infective to a variety of anopheline species and *Culex bitæniorynchus* and *Ædes ægypti*. The number of gametocytes and the ratio between sexes were not inadequate for infection of insects.

After repeated failures to infect mosquitoes with gametocytes of Strain I, it was considered that serial passage may have modified them in some way and a freshly isolated sporozoite-induced Strain II was obtained. As pointed out earlier, gametocytes of this strain also were found incapable of infecting mosquitoes. As stated earlier microgametocytes of Strain II when received, stained pinkish or biscuit coloured and the differentiation between chromatin and cytoplasm was slight. As already mentioned, such a picture of microgametocytes was identical to that found in the type slide of an infected hamster, obtained from the London School of Tropical Medicine. After the tenth passage in rats they looked like those of Strain I and again resumed their original characters after the second hamster passage.

Vincke and Lips (*loc. cit.*) reported exflagellation of microgametocytes in gut contents of some mosquitoes fed on infected mice. Coradetti and Verolini (1951) observed exflagellation *in vitro* of microgametocytes situated in mature erythrocytes after 15 to 20 minutes in a moist chamber. In the present series, exflagellation of male gametocytes of either strain from any one of the five vertebrate hosts could not be observed either after exposure to moist chamber, or smears of gut contents of mosquitoes soon after their feed.

It appeared that gametocytes of *P. berghei* present in mice, rats, squirrels and hamsters were for some unknown reasons functionally deficient and were incapable of infecting the several species of mosquitoes used during the investigation.

THE INVERTEBRATE HOST.

Of the three Anopheles species that have been successfully infected with *P. berghei* in the laboratory, namely, *A. stephensi*, *A. quadrimaculatus* and *A. maculipennis* (*atroparvus*) (Yoeli and Wall *loc. cit.*), at least one (*A. stephensi*) was employed extensively in the investigations under report. Both the known varieties of this mosquito, type and *mysorensis*, were fed and dissected in large numbers, with negative results except the single specimen of *A. stephensi* (type) which was positive. As *A. stephensi* was included in the series, it was justifiable to conclude that negative results of the investigation were not due to unsuitability of mosquitoes but to some other factor.

Yoeli and Wall (*loc. cit.*) showed that the extrinsic incubation period was seven days in mosquitoes stored at 78·8 to 80·6°F. The temperature and humidity

conditions in the insectary under which insects were stored after feed were found to be suitable for the survival of a good number of the mosquitoes for two and three weeks longer than the known extrinsic incubation period.

Jaswant Singh, Ray and Nair (1949 : 1950) reported positive infections of *P. knowlesi* in *A. stephensi* and *A. annularis* stored in the same insectary under identical conditions. It appeared, therefore, that environmental conditions in which the mosquitoes were stored, were not responsible for negative results.

THE VERTEBRATE HOST.

The report of successful infection of *A. stephensi*, *A. maculipennis* (*atroparvus*) and *A. quadrimaculatus* fed on infected hamster by Yoeli and Wall (*loc. cit.*) who also observed that attempts to infect *A. stephensi* from infected rats were unsuccessful, indicated the important part played by vertebrate hosts in such experiments. Such a phenomenon is not entirely unknown as Huff (1948) showed that gametocytes of *P. relictum* in pigeons were not infective to *C. pipiens* while the same strain of parasites when inoculated into canaries, infected these mosquitoes. Reference has already been made to failures to infect mosquitoes from mice and cotton rat by Vincke and Lips (*loc. cit.*), Raffaele and Baldi (*loc. cit.*) and Rodhain and Vincke (*loc. cit.*).

With the above background in view, hamsters were obtained. Attempts to infect mosquitoes from these hosts infected with the more recently isolated Strain II were also unsuccessful. The number of hamster passages of the strain were limited to two due to non-availability of more animals.

It would appear justifiable to conclude that the gametocytes of *P. berghei* were in some way modified in the mice, rats and squirrels and therefore, were not infective to mosquitoes. The modification did not appear to be transient and caused only by the erythrocytes or the plasma of the animal as two serial passages in hamsters did not render them infective to mosquitoes. It is not possible to forecast whether successive passages into a large number of hamsters would render the strain infective to mosquitoes. It would be highly interesting if such proved to be the case.

SUMMARY.

The conditions under which attempts were made to infect a variety of mosquitoes with *P. berghei* from infected mice, rats squirrels and hamsters are described. The attempts were unsuccessful except, in the case of a single specimen of *A. stephensi* (type) fed on an infected mouse.

Each of the known factors influencing successful infection of mosquitoes, namely, the parasite and its gametocytes, the vertebrate and the invertebrate hosts and the temperature and humidity conditions in relation to the mosquitoes, are fully discussed.

It is concluded that gametocytes of both sexes were present in mice, rats and squirrels but were in some way inhibited from infecting mosquitoes. The

strain was passaged through hamsters twice and gametocytes in them also failed to infect mosquitoes. The question is posed whether serial passage through a large number of hamsters will revive the gametocytes functionally.

ACKNOWLEDGEMENT.

The authors are grateful to Mr. A. David, Assistant Research Worker, and Mr. Bua Dass, Laboratory Assistant, for their help in the care and dissection of mosquitoes, and to Mr. S. K. Ochaney, the Artist at the Institute for his accurate drawings on plate.

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Indian Journal of Malariology, **7**, 1, *March* 1953.

NOTES ON KALA-AZAR AND ITS CONTROL IN ISWARGANJ THANA, MYMENSINGH DISTRICT, EAST BENGAL.

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(May 18, 1953.)

INTRODUCTION.

THE present investigation was carried out in the course of a malaria control demonstration campaign in East Pakistan (Bengal) by a World Health Organization/United Nations International Children Emergency Fund Team assisting the Pakistan Government. Although the survey and control measures were primarily meant for malaria control, advantage has been taken of some facilities offered by the local Ministry of Health in order to make some observations on Kala-Azar incidence and its eventual control by spraying D.D.T. residual as was employed for malaria control.

Insufficient time, means and personnel which could be detailed for observations on kala-azar, as well as completion of the antimalaria project, did not permit for the collection of statistically significant observations. For the same reason it was not possible to confirm the interruption of kala-azar transmission after D.D.T. spraying, suggested by some of the findings.

It is nevertheless believed that the present observations may, as a whole, contribute to some extent to the knowledge of the problem in this area.

*Whose participation in this research is limited to the classification of *Phlebotomus* collected.

Two aspects of the kala-azar problem in Iswargunj Thana are dealt with :—

1. A study on the diffusion of kala-azar and its epidemiology;
2. Some results of D.D.T. residual spraying.

Programme of the research included :—

(*a*) A preliminary survey in a definite area, including spleen and blood test (aldehyde test) of children below 15 years.
Collection of *Phlebotomus*.

(*b*) Spraying with D.D.T. residual of part of the area.

(*c*) A follow-up survey including :

Spleen and blood test of the children already examined during the preliminary survey.

Search for *Phlebotomus*.

1. *Description of the area.*—Iswarganj Thana, part of the district of Mymensingh, lies in the central part of the plains of Bengal at 24° 50′ latitude north and 90° 50′ longitude east. The area is intersected by innumerable water courses originating from the Brahmaputra River. The altitude is under 100 feet; the sub-soil water level varies from 3 to 10 feet according to seasons. The vegetation is luxurious. The land is mostly made of silt. Population density is very high : 1,250 per sq. mile; the people living mostly in bamboo huts, usually built over a low platform of pressed earth and arranged in groups of 3 to 4 huts per family, each hut being meant for a different purpose (stable, sleeping room, store, visitors' room). The walls of the houses are mostly made out of bamboo and plastered with mud mixed with cow-dung. Replastering is performed by the inhabitants usually once a year at the end of the rainy season, *i.e.*, during November. A small percentage of houses (roughly less than 10 per cent) have their walls made of corrugated iron sheets, bricks or cement. There are a few villages with grouped houses, most of the compounds being evenly scattered in the cultivated land. Mean monthly temperatures vary from 64°F. in January to 82°F. in August. Rainfall occurring mostly during monsoons (May-September) averages 91·39 inches per year. Relative humidity at 8 a.m. varies from a monthly average of 92 per cent (September) to 79 per cent (April).

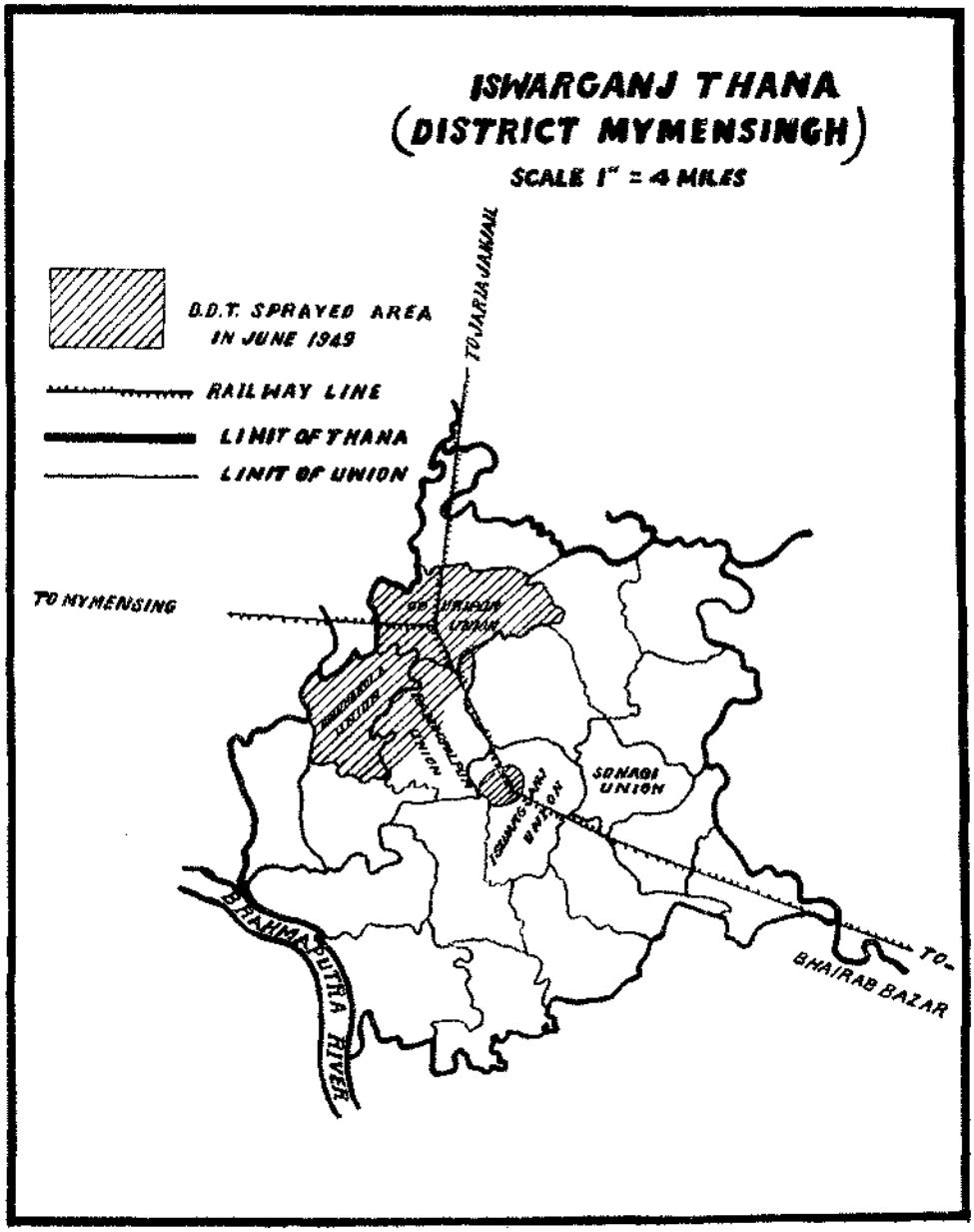
The area of observation included the Union Boards of Iswarganj, Dowhakola, Ramgopalpur and Sohagi, with a total surface of 42 sq. miles and a total population of 45,000 (Map I). Malaria is predominant in this area, spleen index among children being 55 per cent and parasite index 11 per cent in the month of June.

2. *Method of collection of data.*—The diagnosis of kala-azar cases has been based upon : (1) Results of serum tests; (2) Enlargement of spleen.

Among the various serum tests all depending on the increase of the euglobulin fraction, and particularly the most used tests of Napier (aldehyde) and Chopra (antimony), the authors have chosen one based on the aldehyde. Although the antimony test has the advantage of giving a positive result earlier in the disease, it is liable to give (according to Napier and others) a false positive reaction in cases with a large spleen not due to kala-azar, which would have been a disadvantage in the area under observation which is hyperendemic for malaria. The

incubation period of kala-azar is considered to be 2-4 months, which means that a total of 5-9 months elapses from the moment when the infection has occurred to the period when the test gives a full positive result. The two surveys performed in the area under observation were made at an interval of 7-10 months.

MAP I.



In the original Napier's method, one or two drops of commercial formalin are added to 1 c.c. of clear serum. In a strongly positive result the serum becomes solid and completely opaque, like the white of a hard-boiled egg, within a few minutes; if it becomes completely opaque within 24 hours, the result is still positive. Doubtful results are expressed by solidification of the serum with various degrees of cloudiness. In a negative result the serum remains crystal clear, although it may solidify. A comparison of aldehyde test result with splenic enlargement is necessary according to Napier, to judge of doubtful serum results, according to the following table.

Size of spleen. According to Hackett (1944)	Aldehyde test reading.		
	Positive.	Doubtful.	Negative.
4–5	Kala-azar	Doubtful	Not Kala-azar
3	Kala-azar	Probably Kala-azar	Not Kala-azar
2	Kala-azar	Kala-azar	Doubtful
1	Kala-azar	Kala-azar	Doubtful

Instead of the original Napier's test, the authors have used the modification described by Raghavan (1949) which is performed in capillary tubes and does not require drawing the blood from a vein. This excellent and simple method allowed the authors to perform several thousand tests in a milieu which would otherwise have made the research almost impossible. Raghavan (*loc. cit.*) suggests also that eosin be mixed with formalin or urea-stibamine as it affords a better chance of diffusing the reagent through the plasma. The changes in colour of eosin makes the reading easier. We have used Raghavan's capillary tube method without eosin, using commercial formalin 40 per cent as a reagent.

Capillary tubes 1 m.m. in diameter and 9 c.m. long are employed for this method. A small amount of 2 per cent potassium citrate is introduced by capillarity into the tube to about 1/4 of its length. Then a small amount of blood exuded from a pricked finger or ear lobe is added to the tube by capillarity to about 2/3 of the length of the tube. The capillary tube is then stuck vertically on a base of plasticine and the blood cells are allowed to sediment. After the plasma has well separated (after about 2 or 3 hours) the lower portion of the capillary tube containing deposits of cells and plug of plasticine is broken and discarded; the portion containing the column of fluid is brought into contact with 40 per cent formalin or urea-stibamine and the liquid allowed to enter by capillarity into the tube to reach together with the fluid a total length of about 3/4 of the tube. The positive K. A. sera give a leucogel reaction in varying periods of time from a few minutes to 3 hours. According to Raghavan (*loc. cit.*) a number of comparisons between the capillary tube method using aldehyde and the original Napier's method gave almost identical results. Some comparisons made by the authors confirmed this statement. Occasional checks with normal sera were also made.

Spleens were classified into 5 classes according to Hackett (1944). All the cases which fell in the categories situated below the line drawn on the table of interpretation of the results given above, were considered positive for kala-azar. The survey was performed by house to house visits in the selected area and the following data were collected : age of the patient, size of the spleen, result of kala-azar test, result of blood examination for malaria, occurrence of other kala-azar infections in the same family, occurrence of mixed malaria and kala-azar infections. Individual kala-azar cards were issued for each person examined irrespective of whether he had given a positive or negative kala-azar test in order that he might be traced again for control in the subsequent survey. Blood from adults of over 15 years who showed clinical symptoms of kala-azar were also tested. Each individual (adult or child) who gave a positive kala-azar test, was given a course of treatment with urea-stibamine.

Phlebotomus were collected once every week from suitable catching stations selected beforehand. Each catch lasted half an hour. Collections were made inside the houses by means of aspirator tubes. Identification of *phlebotomus* could not be made locally on account of lack of literature. The species were identified only a few months later.

The first survey lasted from January to June, 1950, and as many children below 15 years as possible were examined in the area.

The second survey lasted from October, 1950, till January, 1951, and the various villages were surveyed in the same order as in the first survey. During this survey all children who could be re-traced and who had given a negative kala-azar test during the first survey were re-examined and re-tested for kala-azar ; the purpose of this second survey was to find out whether any of the children who were negative in the first survey had become positive in the meantime. Regular fortnightly catches of sandflies were carried on from the beginning till the end of the observation period.

For purposes of DDT spraying operations the area under observation was divided into two sections : One included the Union of Dowhakola and part of Iswarganj and Ramgopalpur unions where all the inside surfaces of every structure were first sprayed for malaria control purposes, in June 1949 with D.D.T. at the rate of 2 gm./sq. meter. This area had a surface of approximately 17 sq. miles and a population of 19,000 inhabitants. The other section of the area including the Union of Sohagi and part of the unions of Iswarganj and Ramgopalpur was left unsprayed as a check area and was eventually sprayed for the first time in May-June, 1950, with the same dosage of D.D.T. The two gm. D.D.T. per sq. meter sprayed on the walls maintained its lethal effect for over 14 months irrespective of the nature of the surfaces existing in the area, on which it had been sprayed as proved by means of biological tests conducted on anophelines.

RESULTS.

(*a*) *Spleen and blood tests.*—During the first survey which lasted from January to end of July, 1950, 6,108 children below 15 years were examined in 82 villages, 2,718 children were found with enlarged spleen (44·5 per cent spleen rate), average enlarged spleen being 1·9. 510 children gave a positive aldyhyde test, making

a total of 8·35 per cent of the children in the area, positive for kala-azar. The average enlarged spleen of the kala-azar test positive children was 2·4. In 61 out of 149 families, in which positive kala-azar cases were found, there were more than one kala-azar patient in the same family (41 per cent of the families had more than one person infected). The following table indicates the distribution of positive kala-azar cases for age groups out of 276 cases :—

Age.	Number positive for kala-azar.	Age.	Number positive for kala-azar.	Age.	Number positive for kala-azar
15	34	10	19	5	32
14	19	9	30	4	12
13	21	8	31	3	14
12	32	7	11	2	3
11	6	6	11	1	1

The youngest kala-azar patient found was a baby 16 months old.

The following table shows the coincidence of malaria and kala-azar in the same person :—

Patients with positive kala-azar test examined for malaria		123
Positive for malaria also		10
Percentage of mixed malaria and kala-azar infections		8·1 per cent
Species of malaria found	7	*P. falciparum*
	1	*P. vivax*
	2	*P. malariæ*

Eight of these with both infections were below 15 years. The other two were respectively 25 and 40 years of age.

Since the general malaria survey of the same area had given a malaria parasite rate of 11·0 per cent among the children, it is reasonable to infer that there does not appear to exist any relevant incompatibility for malaria and kala-azar infections in same individuals.

The second survey was started in October, 1950, and continued till January, 1951. 3,052 children that had been kala-azar negative at the time of the first survey, were retraced and retested. Of these, 899 were living in the area sprayed since 1949 and out of them only one (0·11 per cent) gave a positive kala-azar test, but the boy gave a history of having been outside the sprayed area for two months (May and June). 2,153 were living in the area sprayed for the first time only in May-June, 1950, and out of them 40 (1·85 per cent) gave a kala-azar positive test. This suggests that kala-azar transmission had occurred to a certain extent since the beginning of 1950 in the area that had been sprayed for the first time in May-June, 1950, but it had not occurred—or only to a very limited extent—in the area sprayed since 1949.

Catches of *Phlebotomus* gave the following results :—

Month 1950.	Unsprayed stations.*		D.D.T. sprayed stations.†	
	Number captured.	Instances in which *Phlebotomus* were found.	Number captured.	Instances in which *Phlebotomus* were found.
Mar.	72	15	...	...
Apr.	129	19	...	...
May	135	13	...	...
Jun.	8	2	...	...
Jul.	29	5	...	...
Aug.	9	4	4‡	1‡
Sept.	20	6	...	...
Oct.	14	4	...	...
Nov.	53	14	...	...
Dec.	25	4	...	...

* Sixteen catching stations searched for half an hour once a week located outside the sprayed area.

† Five catching stations searched for half an hour once a week located in the area sprayed since June, 1949. These stations were positive for *Phlebotomus* before that date with an index comparable to that of the stations in the check area, but on account of the irregular visits performed for only 2 months before that date, the results obtained are not given here in detail. The spraying was performed in June, 1949, and in May-June, 1950. It may be added that besides the fixed catching stations, all other premises in the sprayed area which were regularly being searched for mosquitoes once every week on account of a contemporary malaria control programme (25 premises) were never positive for sandflies during this period.

‡ Found in one house at Kataria (Dowhakola) on August 7, 1950. The house had been completely rebuilt with new bamboo walls (unsprayed) two months before.

This table shows a considerable increase in the *Phlebotomus* population of the area left unsprayed in the pre-monsoon months (April-May) and a minor increase after the monsoon (November). *Phlebotomus* population seem to show its lowest ebb during the monsoon (June-September). This is in accordance with Napier's finding. Further, the table shows that no *Phlebotomus* have been found in the premises sprayed with D.D.T., with the single exception noted above.

Unfortunately, lack of technical literature on the spot prevented the authors from making an immediate diagnosis of species of the *Phlebotomus* captured, and the local climatic conditions spoiled a large number of the specimens caught. By the time a proper identification could be made of species, only 134 specimens were left in good condition.

The specimens examined belong to the following species :—

	♂	♀
Phlebotomus argentipes, Annandale and Brunetti, 1908	1	0
Phlebotomus (*Prophlebotomus*) *squamipleuris* var. *indicus*, Theodor, 1951	8	34
Phlebotomus (*Prophlebotomus*) *africanus* var. *asiaticus*, Theodor, 1933	12	48
Phlebotomus (*Prophlebotomus*) *shortii*, Adler and Theodor, 1937	16	15
	37	97

Total specimens = 134

The only important fact which arises from this observation is the scarcity of specimens of *P. argentipes* found in this highly endemic area (only one male, *i.e.*, 0·75 per cent of the total specimens examined). No definite conclusions can be drawn from this insufficient observation, but the fact is nevertheless worth noting.

SUMMARY.

A kala-azar survey in an area of East Bengal (Iswarganj Thana) of 6,108 children below 15 years by means of formaldehyde test in capillary tubes showed 8·35 per cent positive.

Notes on seasonal incidence and prevalence of the species of sandflies are reported as well as some observations regarding the effect of D.D.T. spraying on kala-azar transmission.

ACKNOWLEDGEMENTS.

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The following are some of the abbreviations in use :—

Amer. J. Hyg.
Ann. Trop. Med. Parasit.
Brit. Med. J.
Bull. Ent. Res.
Bull. Soc. Path. Exot.
C. R. Soc. Biol.
Geneesk. Tijd. Ned.-Ind.
Ind. J. Mal.
Ind. J. Med. Res.
J. Mal. Inst. Ind.
Jl. R. A. M. C.
J. Trop. Med. Hyg.
Meded. Volk. Ned.-Ind.
Rec. Mal. Surv. Ind.
Rev. Appl. Ent.
Riv. Malariol.
Trans. Roy. Soc. Trop. Med. Hyg.
Trop. Dis. Bull.
U.S.A. Pub. Hlth. Rpts.

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STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

XI. Hæmatology of Blood-induced Infections in Albino Rats.*

BY

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SOME aspects of hæmatology of *P. berghei* infections in albino mice were studied by Galliard and Lapierre (1950*a*). Similar studies were reported in respect of albino rats by Coradetti and Verolini (1951), who confined themselves to changes in erythrocytes. The present study deals in detail with the hæmatology of malaria in albino rats during untreated acute and chronic infections and parasitological relapses. The investigations reported here were carried out during 1951 and 1952.

MATERIAL AND METHODS.

A few of the albino rats were available from the colony at the Institute and the rest were purchased from other sources.† The animals used in the studies were full grown adults 15 to 20 weeks old. They were all fed once a day on a standard diet and provided with water in bottles.

The strain of parasite was the same as reported previously (Ramakrishnan and Satya Prakash, 1950) maintained in albino rats by serial blood passage. The dose of infection in each case was 80,000 parasites inoculated intraperitoneally.

Blood was drawn from the donor by pricking the tip of the tail after cleansing it with methylated spirit and allowing it to dry. Undue pressure on the tail was

*The investigation was partly financed by a grant from the Indian Council of Medical Research.

†Haffkine Institute, Bombay, and the Indian Veterinary Research Institute, Izatnagar.

avoided in order that the samples of blood may be uniform. The drawing of blood for the different counts was carried out between 9 and 10 a.m., before the animals were fed, in order to avoid variations due to tides of the different cells in relation to food and different times of the day (Taliaferro and Kluver, 1940).

The total erythrocyte and leucocyte counts were made by standard methods using a Spencer " Bright Line " hæmocytometer. Final figure for each sample represented the average of counts made in two counting chambers. For differential erythrocyte and leucocyte counts, air dried thin films were made and stained by the rapid method of Jaswant Singh and Bhattacharji (1944). Parasites were enumerated in dry smears against 10,000 erythrocytes inclusive of the polychromatophilic cells.

To stain reticulocytes, a drop of one per cent solution of brilliant cresyl blue in normal saline was mixed with a drop of blood for half to one minute and then a smear made (Kolmer and Boerner, 1945). This was air dried, fixed with methyl alcohol, and counterstained with J.S.B. stain (*loc. cit.*).

DEFINITIONS.

The designation mature erythrocytes, was given to the circular non-nucleated acidophilic corpuscles in the sense they were mature forms. Polychromatophilic erythrocytes stained basophil and were larger in diameter than the above, and were also non-nucleated. The term erythrocytes includes both the above.

The neutrophil leucocytes were similar to the one seen in human blood. There were some forms which looked like immature neutrophil leucocytes (La Cour, 1944) and had annular or ring shaped neuclei with thickenings at different parts and these were counted as neutrophil leucocytes. The large and small lymphocytes were counted together. The eosinophils and the basophils were characteristic as in human blood.

NORMAL HÆMATOLOGY.

The normal blood picture was determined on sixty different occasions and the average of counts are shown in Table I.

TABLE I.

Normal hæmatology of adult albino rats.

Sex	Number of observations.	Erthrocytes in millions per c.mm.	Polychromatophilic cells per 10,000 mature erythrocytes.	Leucocytes per c.mm.	DIFFERENTIAL LEUCOCYTE COUNT PER CENT.				
					Neutrophil.	Lymphocytes.	Monocytes.	Eosinophil.	Basophil.
Males	30	6·33	259	11,735	47	41	9	2	1
Females	30	6·48	265	12,374	48	40	9	2	<1
Average for both sexes ...		6·40	262	12,055	48	41	9	2	<1

In the normal rats, the number of erythrocytes were found to vary between six and seven millions and the leucocytes between eleven and twelve thousands per c.mm. Two to three per cent of the erythrocytes were found to be polychromatophilic cells. The neutrophils and the lymphocytes were about equal in number while the monocytes formed nine per cent of the total. These figures were at variance with the averages reproduced below for both sexes for Wistar strain of rats (Farris and Griffith, 1949).

Erythrocytes, millions per c.mm.	Leucocytes per c.mm.	Differential leucocyte count per cent.				
		Neutrophils.	Lymphocytes.	Monocytes.	Eosinophils.	Basophils.
9·35	9,000	20	78	< 1	2	< 1

Explanation for the difference between the normal counts of local rats and standards for the Wistar strain is not known. It is possible that climatic conditions may play a part. Another factor responsible may be that the local strains were not infection-free as was found in a separate experiment, when large numbers of them after splenectomy showed infections of *Bartonellosis*.

HÆMATOLOGY OF ACUTE MALARIA.

Parasites were patent in the peripheral blood for about 20 days during the acute infection. Fifty-two rats were inoculated and, hæmatological as well as parasite counts, were made on them on different days following infection. The average counts are presented in Table II.

Table II.

Hæmatology in acute malaria.

Days of patency.	Number of observations.	Parasitised cells per 10,000 erythrocytes.	Erythrocytes in millions per c.mm.	Polychromatophils in 10,000 erythrocytes.	Leucocytes per c.mm.	Differential leucocyte count per cent.				
						Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
1 to 5 ...	14	268	5·17	456	12,900	51	36	9	3	1
6 to 10 ...	18	438	4·44	542	14,900	48	40	9	2	1
11 to 15 ...	15	310	4·11	899	13,900	48	40	9	2	1
16 to 19 ...	5	184	4·88	639	12,300	50	40	8	2	...
Average ...	52	331	4·58	631	13,281	49	39	9	2	1

It was found that as the infection progressed the total erythrocytes were reduced in number, although there was a relative increase in the polychromatophilic cells. Further, the increase in the latter was maintained after the peak of the infection which occurred during the six to ten day period of patency. Throughout the acute infection the total leucocyte count showed a slight increase mainly due to increased neutrophils. The lymphocytes were slightly decreased while the monocytes, eosinophils and basophils remained unchanged.

HÆMATOLOGY IN LATENCY.

A group of fifteen animals which developed chronic infections without any treatment was selected for hæmatological observation during different periods of latency and the results are presented in Table III.

TABLE III.

Hæmatology in latent malaria.

Period of latency in days.	Number of observations.	Erythrocytes in millions per c.mm.	Polychromatophilic cells per 10,000 RBC.	Leucocytes per c.mm.	Differential leucocyte count per cent.				
					Neutrophil.	Lymphocyte.	Monocyte.	Eosinophil.	Basophil.
1 to 10* ...	16	6·57	279	11,788	49	39	9	2	1
63 to 73 ...	4	6·62	122	7,120	42	43	13	<2	<1
86 to 93 ...	3	6·60	124	6,587	35	47	15	2	<1
103 to 118 ...	3	6·76	223	6,773	37	50	12	<1	<1
148 ...	1	6·84	212	6,160	31	52	14	3	...
225 ...	1	6·25	232	9,600	40	45	14	1	...
233 ...	1	7·34	210	9,660	31	55	13	1	...
Average of 13 observations excluding the first 16 ...		6·69	168	7,229	37	47	13	2	1

*Initial latency.

Soon after the infection became subpatent, the erythrocyte count increased approaching normal and the proportion of polychromatophilic cells fell as compared to the acute infection phase. There appeared to be a progressive leucopenia upto twenty-one weeks. The neutrophils showed progressive decrease while the lymphocytes increased in numbers.

HÆMATOLOGY DURING RELAPSE.

Counts were made in six animals which showed parasitological relapse. The average counts are shown in Table IV.

TABLE IV.

Hæmatology in relapse.

Number of observations.	Polychromatic cells per 10,000 erythrocytes.	Erythrocytes in millions per c.mm.	Number of parasitized cells per 10,000 erythrocytes.	Leucocytes per c.mm.	Differential leucocyte count per cent.				
					Neutrophils.	Lymphocytes.	Monocytes.	Eosinophils.	Basophils.
6	163	5·58	5·3	9,297	42	45	10	2	< 1

The blood picture in relapse was in general agreement with that during latency. The erythrocyte count, however, was depressed due to a renewed destruction of the cells. The leucopenia of latency continued during the relapse but the neutrophils were relatively more in number, than during latency. The monocyte count was depressed almost to that of normal and acutely infected animals.

Relative proportion of polychromatophilic cells to normal erythrocytes.—It was apparent that the polychromatophilic cells increased during the acute infection and soon became normal in number in initial latency. In advanced latency, however, the cells were below normal in numbers and again increased during relapse. The data are summarized in Table V.

TABLE V.

Average count of polychromatophilic cells per ten thousand erythrocytes in normal and infected animals.

Normal.	Stage of infection.			
	Acute.	*Early latency.	Advanced latency.	Relapse
262	631	279	168	163

*Within 20 days of sub-patency of primary parasitæmia.

Reticulocytes.—To determine the proportion of reticulocytes among the polychromatophilic cells, duplicate smears were made from each of twelve rats with early infection and 107† normal animals. One set was stained by the special

†Taken from Table I as polychromatophilic counts were not made for 107 observations.

method for reticulocytes and the other by the J. S. B. method, both techniques referred to earlier. The results are summarized below.

TABLE VI.

Relative proportion of reticulocytes to polychromatophils in normal and infected rats.

Group of animals.	Number of observations.	Average number of polychromatophilic cells per 10,000 RBC.	Average number of reticulocytes per 10,000 RBC.	Percentage of reticulocytes to polychromatophils.
Normal ...	107	262*	203	77
Acute infection	52	631	489†	67·5†
Relapse ...	6	163	120	74

*Taken from Table I as polychromatophilic counts were not made for 107 observations.

†Estimated from counts made on 11 animals.

It was evident that the reticulocyte count increased during infection. About seventy per cent of the polychromatophilic cells were found to be reticulocytes. The remaining thirty per cent were transitional cells in which the dissolution of the nucleus was complete, but still stained basophilic, and had not yet become mature erythrocytes.

Observations were made in twenty-one animals to determine the relative infestation of polychromatophilic and mature erythrocytes. The results are summarized in Table VII.

TABLE VII.

Relative infestation of mature and immature red cells by P. berghei *in albino rats.*

Days after inoculation.	1	2	3	4	5	6	7	8	9	10
Number of observations		2	5	3	3	2	3	1	1	1
	Count per 10,000 erythrocytes.									
Average number of cells parasitized ...		231	198	199	245	366	408	168	136	666
Average number of mature erythrocytes parasitized ...		71	37	31	46	131	64	26	32	128
Average number of polychromatophilic cells parasitized ...		160	161	168	199	235	344	142	104	478
Percentage parasitized polychromatophils to total number of cells parasitized ...		61	86	84	81	64	84	85	76	79

DISCUSSION.

Special features of the investigation were that the animals were of approximately the same age, the dose and route of inoculation identical in every case. Procedures adopted for the different counts were uniform and the time for drawing blood for the observations was kept constant. The handling of the animals was minimum in order to avoid as far as possible emotional disturbances which are known to influence the blood picture (Taliaferro and Kluver, *loc. cit.*).

Thus, most factors known to produce variations in the blood picture were eliminated as far as possible. But as mentioned earlier, it was found in the course of other investigations that most of the albino rats from the different laboratories in India were all infected with *Bartonella Sp.* In this respect also, the conditions of experimentation were more or less constant as it was almost certain that every animal carried that infection.

The extensive literature on the hæmatology of simian and human malaria was reviewed by Taliaferro and Kluver (*loc. cit.*), Maegraith (1948) and Kitchen (1941 : 1949). With regard to erythrocytes in monkey as well as man infected with malaria, the findings have been consistent in that a large destruction of the cells occurred in the disease giving rise to anæmia of a microcytic type with its attendant stimulation of hæmopoiesis resulting in increased numbers of immature cells in circulation. The degree of anæmia was greater during the period when parasitæmia was patent and less so during subpatent infections.

Albino rats infected with *P. berghei* showed the same sequence of changes in erythrocytes as monkey and man infected with their respective malaria parasites. The erythrocyte count of 6·4 million per c.mm. in normal animal fell to 4·6 million during acute infection and rose again to 6·7 million during latency. During a relapse, however, the count fell once more to 5·6 million (Tables I, III and IV). Proportionately with the destruction of erythrocytes there was an increase in the number of polychromatophilic cells about seventy per cent of which were reticulocytes (Tables V and VI). The progressive anæmia showed, as expected, different degrees of anisocytosis and a certain proportion of nucleated erythrocytes.

The results in Table VII confirmed the preference of *P. berghei* to invest immature erythrocytes as previously reported by Ramakrishnan and Satya Prakash (*loc. cit.*) and Baldi (1950) and confirmed by Coradetti and Verolini (1951). Pigment was visible only in mature schizonts in polychromatophilic cells and most stages of parasites in mature erythrocytes. There were three possible explanations for this finding. One was that there is no hæmoglobin in the polychromatophilic cells about seventy per cent of which were reticulocytes. This was not in accordance with known teaching (Samson Wright, 1948) that hæmoglobin is to be found in reticulocytes. A second was that hæmoglobin may be quantitatively present in reticulocytes, but somewhat different in quality and therefore not metabolised by the parasite (Coradetti and Verolini, *loc. cit.*). A third was that the hæmoglobin content of the reticulocyte was as in mature cells, but the majority of parasites found in reticulocytes corresponded to the exo-erythrocytic stage of development as in *P. elongatum* (Huff and Bloom, 1935). This possibility was considered by Ramakrishnan *et al.* (1951) and by Garnham (1951).

With regard to the leucocyte picture, opinions are divided and there have been observations to show leucopenia to be a characteristic of uncomplicated malaria as well as others which showed the opposite or a leucocytosis during patent infection (Taliaferro and Kluver, *loc. cit.*; Maegraith, *loc. cit.*, and Kitchen, *loc. cit.*). In the present investigations, a mild leucocytosis was observed in the rats throughout the period of patent parasitæmia of acute infection (Tables I and II). This continued in initial latency (Table III), that is, within the first ten days of sub-patency after acute infection. This observation was in agreement with the findings of Galliard and Lapierre (1950*b*) who also found a pronounced leucocytosis during the patent period in rats. After this period, however, there was a leucopenia, which persisted during relapse although to a less extent than during latency (Table IV).

One of the most uniform findings in literature is that the three types of leucocytes influenced by malaria are neutrophils, lymphocytes and monocytes. Opinions are varied, however, in regard to the type and degree of changes in the three types of cells. The eosinophils and basophils do not seem to be affected by uncomplicated malaria. Findings in the present investigations were in agreement with the general conclusion.

In the reviews mentioned above, the leucocytosis or leucopenia during the acute phase has been attributed to changes in neutrophil numbers. The mild leucocytosis found in rats during the acute phase and early latency was also found to be due to a relative increase in neutrophils. (Tables II and III).

The most consistently affected single leucocyte in malaria is the monocyte. Taliaferro and Kluver (*loc. cit.*) found in simian malaria that increase in monocytes persisted throughout the patent period but disappeared during latency. A similar increase in monocytes was observed in acute human (*P. falciparum*) infections (Bianchi, 1940, quoted by Maegraith, 1948), and in acute simian infections by Krishnan, Lal and Napier (1932) and Malamos (1934). In *P. berghei* infections of rats, on the other hand, the monocytes did not show any increase throughout the period of acute parasitæmia as well as early latency (Tables III and IV). Similar findings were reported by Galliard and Lapierre (1950*a*) in respect of albino mice infected with *P. berghei*. In established latency, however, the monocytes showed a definite increase (Table IV).

The present investigation showed lymphocytes were relatively reduced during the acute phase and early latency (Tables II and III). In established latency, however, there was a relative increase of lymphocytes which persisted through the relapse period. In human malaria such an increase of lymphocytes was found to occur only during the apyrexial intervals of an attack and Stephens and Christophers (1908) considered it a diagnostic sign of malaria. Bianchi (*loc. cit.*) also observed a lymphocytosis in acute *falciparum* malaria. Taliaferro and Kluver (*loc. cit.*) described an irregular increase in lymphocytes both during patent and latent infections in simian malaria. The finding that lymphocytosis occurred in *berghei* malaria during latency *pari pasu* with monocytosis is in accordance with the classical observations of Taliaferro and Mulligan (1937) on the intimate relationship between the two types of cells.

Thus, the leucocyte picture of albino rats with *P. berghei* infection seemed to be characterised by a mild leucocytosis in acute infection and early latency contributed by a relative increase of neutrophils. In established latency and relapse the picture was one of leucopenia accompanied by a relative increase in lymphocytes and monocytes.

SUMMARY.

Certain hæmatological changes in the course of blood-induced *P. berghei* infections in albino rats have been reported.

A fall in the erythrocyte count during the primary parasitæmia—with a relative increase in polychromatophils—was followed by a recovery during the latent phase. A similar anæmia was recorded during relapse.

The parasites showed a selective invasion of the immature erythrocytes.

A mild leucocytosis was observed during the acute stage and early latency. In advanced latency and relapse the blood showed leucopenia with a relative increase in monocytes and lymphocytes.

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STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

XII. Attempts to estimate *in vivo* the acquired immunity in Albino rats.*

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"It is impossible to understand the present position of immunology with its mixture of established fact, half knowledge, hopeful guessing and frank bewilderment without an adequate grasp of the difficulties involved in measuring immunity reactions in the living animal, and in assessing the significance of such measurements when they have been obtained" (Topley and Wilson, 1947).

The degree of enlargement of the spleen provides an indirect method to assess the immunity quantitatively in malarious individuals as well as community. Homologous superinfection accompanied by negative parasitological results, has been widely used to demonstrate the existence of acquired immunity in experimental malaria. An extension of the same procedure with predetermined doses of superinfection can be expected to provide a measure of acquired immunity. The possibility was pointed out by Fabiani and Vargues (1951).

The object of the present investigations was to examine the extent to which progressively increasing challenge doses of homologous superinfection would provide a quantitative measurement of immunity acquired by albino rats† as a result of untreated blood-induced infections of *P. berghei*. In addition, two strains‡ of

*The investigation was partly financed by a grant from the Indian Council of Medical Research.

†Most of the rats were from the colony at the Institute and a few were purchased from Haffkine Institute, Bombay, and the Central Drug Research Institute, Lucknow. Majority of them were 15 to 20 weeks old while a few 30 to 42 weeks old. Both sexes were represented. The animals were maintained on a standard diet given once a day on an *ad lib* basis.

‡The first strain was received in 1950 by courtesy of Prof. E. Brumpt of Paris, and the second in 1952 by courtesy of Brigadier J. S. K. Boyd of the Burroughs Wellcome Laboratories, London. The latter was established from sporozoites found in naturally infected *A. dureni* in Belgian Congo.

P. berghei maintained separately at the Institute were compared immunologically to determine if they differed from one another.

METHODS.

The parasitæmia was estimated against 10,000 erythrocytes in thin blood films stained by the J.S.B. technique. Blood was drawn from the donor into a tuberculin syringe containing a known amount of 2 per cent citrate-saline. The contents of the syringe were so diluted that each 0·1 c.c. contained one million parasites which was the primary dose in every case. Test doses to challenge the immunity were multiples of the primary dose and were administered between the 1st and 13th day of latency.* All inoculations were given by the intraperitoneal route.

Stained thin blood films from each animal were examined daily and when positive, the parasites were enumerated. Slides were declared negative when no parasite was encountered in 100 oil immersion fields. Blood smears of immune animals were examined for 10 to 15 days following superinfection.

RESULTS.

Of the thirty-five animals superinfected with doses upto 200 times the primary dose (Table I), twelve showed a few circulating parasites in the peripheral blood for 1-2 days in 7 rats and 3-4 days in the rest. The transient nature of the patent parasitæmia appeared to be due to the presence of a few of the re-inoculated parasites. When the challenge dose was relatively high (two thousand million parasites), the immune recipients showed on the day following superinfection, approximately one per cent cell infection which fell abruptly and the blood smears were negative in 2-4 days (Table III). Non-immune animals receiving similar large doses, developed progressive high parasitæmia and succumbed within six days.

TABLE I.

Details of homologous superinfections of P. berghei.

Day of superinfection and sub-patency following primary parasitæmia.	Challenge dose in million parasites.	Number of animals.	Strain of *P. berghei*.	Number positive and duration.
5 and 7 ...	1	2	I	1 positive for 2 days.
1, 6 and 7 ...	2	3	I	Nil.
6 and 10 ...	3	3	I	2 positive for 2 days.
5, 6 and 7 ...	4	3	I	Nil.
5 and 8 ...	20	2	I	Nil.
5 and 9 ...	40	2	I	Nil.
6 ...	100	1	I	Nil.
6, 7, 9 and 13 ...	200	5	I	2 positive for 2 and 4 days.
5, 6 and 9 ...	100	4	II	2 positive for 2 days.
6 and 7 ...	150	2	II	1 positive for 1 day.
6, 7 and 8 ...	200	4	II	Nil.
6, 11 and 14 ...	1,000	4	II	All positive for 3 days.

*Sub-patency following primary parasitæmia.

TABLE II.

Results of highest challenge dose.

Group of rats.	Challenge dose in million parasites.	Number of animals.	PARASITE COUNT /10,000 RBC ON DAYS FOLLOWING SUPERINFECTION.					
			1	2	3	4	5	6
Latent (6th to 15th day) ...	2,000	2	81	42	3	0	0	0
Normal (Control)	2,000	2	110	541	436	608	710	1,623

In order to examine whether the two strains mentioned earlier differed antigenically, the immunity acquired to one was challenged by the other (Table III). It appeared that the two strains received at different times from different laboratories were immunologically identical.

TABLE III.

Results of "heterologous" superinfections.

Primary infection.	Number of animals.	Day of superinfection.	Challenge infection.	Challenge dose in million parasites.	Results.
Strain I ...	5	22, 24, 28, 29, 31	Strain II	1	All negative.
Strain II ...	5	31, 37, 39, 40, 41	Strain I	1	All negative.

DISCUSSION.

It appeared that a quantitative estimation of individual immunity *in vivo* using viable antigen, though theoretically conceivable, was not practically possible. An interesting observation of an increase in immunity quantitatively of a herd was furnished by Ciuca *et al.* (1934) who analysed their results of malaria therapy of 1,198 patients and concluded that successive homologous superinfection progressively increased the number of persons showing immunity to further challenge doses.

Acquired immunity in malaria consists of two factors, namely, the humorol and cellular (Taliaferro and Taliaferro, 1929 : Taliaferro and Cannon, 1930). In the living animal both the components are dynamic and progressive, as long as the antigenic stimulation lasts. Although the dose of primary infection was one million parasitized erythrocytes in every case, it has to be remembered that the

parasites were viable and multiplied in enormous numbers during the course of the disease, till they became subpatent and the antigenic stimulus was in reality many times that afforded by the million parasites originally inoculated. Actual day to day counts were made in a small sample of infected rats, and the number of parasites invading the total volume of blood [reckoned at 6·7 c.c. per 100 gm. weight (Farris and Griffith, 1949) and the erythrocite count at 4·5 millions per c.mm. during the acute infection as recorded by Ramakrishnan *et al.* (1953)] was calculated to be at least 18×10^{10} parasites, about 180 times larger than the highest challenging dose tried. It was thus seen that challenge doses even 1,000 times the dose of primary infection, did not give rise to patent parasitæmia which could be considered as resultant infection.

SUMMARY.

Attempts at quantitative estimation of immunity acquired by albino rats during an untreated primary infection with *P. berghei* by superinfection with graded doses of the homologous strain proved unsuccessful.

Two strains of *P. berghei* maintained at the Institute were found to be immunologically identical.

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HOST PREDILECTION OF *A. FLUVIATILIS* IN TERAI REGION OF UTTAR PRADESH, INDIA.

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THE vectorial status of *A. fluviatilis* has long been recognized in the foot-hills of peninsular India, while till recently all attempts to incriminate it as a vector in the foot-hills of Uttar Pradesh in the north of India have been unsuccessful. From the results of precipitin tests and dissections of *A. fluviatilis* carried out in different parts of India, Covell (1939 : 1944) postulated the probable existence of more than one biological race of the species. The anthropophilic index of *A. fluviatilis* from the U.P. Terai was 1·8 and 1·4 per cent in 1938 and 1939 respectively, while it was found to be much higher by the present authors in 1949-52. The possible explanations for such a change in host preference of the mosquito and how far precipitin tests may be used for determining the existence of biological races in any area are considered in this paper.

MATERIAL AND METHODS.

During 1949-52, seven hundred and four samples of blood meals from *A. fluviatilis* were forwarded to the Institute for precipitin tests by Dr. P. C. Issaris, the Leader of the World Health Organization Malaria Control Demonstration Team and Mr. V. Ramakrishna, the Entomologist of the parallel team, both working in the Uttar Pradesh Terai. These were stored in a dry condition in a refrigerator till they were subjected to tests.

Samples of blood meals were tested only against human antisera prepared at the Malaria Institute. Adult Rhode Island red and white leghorn fowls were immunised with human plasma (Jaswant Singh *et al.*, 1952) obtained from the local blood bank. A single dose of 3 c.c. of the plasma was administered intravenously and on the tenth day the bird was bled, and the serum tested against the

antigen and titrated for potency. Only sera which showed specificity for human antigen and with titres 1 in 2,000 were used in the tests. The technique employed for the precipitin tests was the one described by Rice and Barber (1935) and reactions were read at the end of 20 minutes of antigen antibody contact at room temperatures in Delhi.

ANALYSIS OF RESULTS.

Of the 704 samples tested, 310 or 47 per cent gave positive reactions against anti-human sera. The blood meals were classified according to the place of capture of mosquitoes and the analysis of the results are given below :—

TABLE I.

Results of precipitin tests on blood meals of A. fluviatilis *from the Terai region of Uttar Pradesh with anti-human serum.*

Year.	PLACE OF CAPTURE.							
	HUMAN DWELLINGS.		CATTLESHED.		OUTDOOR.		UNCLASSIFIED.	
	Number tested.	Per cent positive.	Number tested.	Per cent positive.	Number tested.	Per cent positive.	Number tested.	Per cent positive.
1949 ...	13	69	17	58	...	...	...	...
1950 ...	5	40	295	44	...	...	...	...
1951 ...	86	56	37	51	38	45	...	...
1952 ...	45	24	46	22	4	25	118	47
Totals	149	47	395	42	42	43	118	47

The results were re-analysed (Table II) to determine whether there were any seasonal changes in anthropophilic index of *A. fluviatilis* in the Terai region.

TABLE II.

Anthropophilic index of A. fluviatilis *in the Uttar Pradesh Terai according to cold, hot and wet seasons.*

Season.	Number tested.	Per cent positive.
November to February (cold)	264	38
March to June (hot)	410	50
July to October (wet)	30	17
TOTAL	704	47

In the small sample collected during the rainy season, unfavourable for the breeding of *A. fluviatilis*, the anthropophilic index was the lowest. It was highest in the hot season during which maximum transmission was possible, and low during the cold season. No general conclusion was possible in the absence of larger samples.

DISCUSSION.

Analysis of blood meals recorded in this paper is at great variance with those reported earlier. The anthropophilic index of *A. fluviatilis* in the Terai during 1949-52 is 47 per cent and the sporozoite rate 1·6 per cent (Srivastava and Chakrabarti, 1952). The comparative figures are as shown in Table III.

TABLE III.

Anthropophilic index and infection rates in A. fluviatilis *in the Uttar Pradesh Terai.*

Period.	Anthropophilic index.	Infection rate in glands.
1938* ...	1·8	0
1949–52 ...	47	1·6

*Covell (*loc. cit.*)

This marked change in the anthropophilic index of *A. fluviatilis* of the Uttar Pradesh Terai in the course of about 10 years requires explanation. In the past, the region was highly malarious, densely wooded with little human population. Since 1949, however, great changes have taken place in the area. Forests have been cleared, large areas drained, and reclaimed for extensive agricultural operations. Besides, large scale indoor residual D.D.T. spraying operations have been in force since 1947 and several thousands of people have been colonized in the area (Srivsatava, 1950).

While it is difficult to assess how the various individual factors affect the degree of contact between *A. fluviatilis* and man in the Uttar Pradesh Terai, in general it can be stated that the changes in the environment have resulted in marked increase in the *fluviatilis*-man contact. The large scale clearance of forests and undergrowth must have considerably reduced the game population. Although real urbanisation has not yet occurred in the region, it is nevertheless true that the human population has considerably increased. Greater opportunities exist now for a very much higher degree of mosquito-man contact, than during the pre-colonisation period.

Precipitin tests of mosquito blood meals have certainly made it possible to determine with some exactness the degree of contact between mosquito and man in any given area. It has not been possible to prove whether the degree of contact is due to deliberate preference on the part of the mosquito or due to relative availability of different types of blood meal. As is well-known in experimental

studies on malaria transmission, it is possible, with few exceptions, to make mosquitoes of different species feed on almost any type of host.

Changes in the environment like, improvement in agricultural practice and improved stabling of cattle, have altered the association of malaria vectors with man and brought about a natural recession of autochtonous malaria in many parts of northern Europe (Boyd, 1949). A reversal of this phenomenon is also well-known, namely, an increase of human malaria by anophelines predominantly zoophilic turning to man and getting infected from immigrant population during the war, when cattle was destroyed. A similar change of vector tropism of anophelines in Bohemia at the end of the last war re-introduced malaria for the first time for over a hundred years. (Burke-Gaffney, 1952).

Viswanathan (1950) observed that, to the extent to which the indices had computed, very high anthropophilic indices in *A. fluviatilis* were associated with very high infection rates and *vice versa*. He did not find any significant variations in the morphological characteristics of the vector and non-vector "types" of *A. fluviatilis* in Bombay State during any stage of their development and considered that the two races of this species ought to be determined by the variation in their biological behaviour rather than morphological characteristics.

Can differences in the results of precipitin tests alone form the basis of differentiation of biological races in a species complex? Such differences in the *maculipennis* complex were later found to have a taxonomical basis for their differentiation in so far as the study of their egg types were concerned (Falleroni, quoted by Hackett, 1934). As already stated no such differences have been made out in the egg, larvæ, pupæ or adult stages of the anthropophilic and zoophilic types of *A. fluviatilis* (Rao, 1937 ; Viswanathan, *loc. cit.*).

Indeed, apart from the non-existence of taxonomical differences in specimens of *A. fluviatilis* occurring in different parts of India, the larvæ of this species are indistinguishable from those of a closely allied species, *A. minimus*. The differences in the adults of the two species are such that slight variations in the palpal bandings may confuse the identification. Puri (1952), who has devoted considerable attention to the systematics of the two species has an open mind regarding the possibility of a gradation of taxonomical changes between the two that a close study may reveal the merging of both into one, each being a variety of the same species.

Feeding habits of mosquitoes are influenced by a variety of factors. As pointed out by Thomson (1951), they may be influenced by the type of construction of houses and cattlesheds in which collections are made and by the relative proportion of man and animals. Afridi *et al.* (1939) in their work on *A. culicifacies* at Delhi, whose findings are reproduced below, found that a larger proportion of *A. culicifacies* had fed on human beings when relatively larger numbers of the latter were available.

Ratio of men to cattle.	Human positives, per cent.
13 : 1	3·2
9 : 1	1·9
1·5 : 1	0·9

In so far as mosquitoes are concerned, the present knowledge indicates that they are facultative in their preference of hosts which appears to vary with the availability of particular types of blood meals. On the other hand a true zoopholism appears to exist for the Tse-tse flies (*G. morsitans*) in Africa as shown by ample evidence that it becomes extinct in the absence of game (Carmichæl, 1952). Even in this case of true zoophilism, the fly can, at least occasionally, bite man and infect him with *T. rhodesiense* when he went into *morsitans* areas which contained much game (Fairbairn quoted by Carmichæl, 1952).

Bates (1949) reviewed the situation and pointed out that it may be possible to establish strains with particular biting habits by selective breeding from a mixed stock, thus indicating that there may be a genetic factor involved in the food preferences. The possibility has to be considered for *A. fluviatilis* species complex in any area being a heterogenous group consisting of a mixture of strains, some preferring to feed on man and others on animals. Due to changes in the environment and relative availability of one or the other type of blood meal it is conceivable that only strains for which the suitable blood meal exists, are able to inbreed and multiply while the others become extinct in the area.

It was found (Table I) that 42 of the blood meals tested were from *A. fluviatilis* captured in outdoor resting places and 43 per cent of them proved to be human blood. This is the first record of precipitin tests carried out on blood meals of specimens of the species resting outdoors. If it is remembered that to collect satisfactory samples of blood from mosquitoes for precipitin tests, the specimens captured for the purpose must be freshly fed ones, then it follows that the specimens under consideration must have left indoors immediately or at least very soon after their meal.

It is concluded that while precipitin tests of mosquito blood meals are extremely useful in the study of epidemiology of malaria, and indicate accurately the degree of man-mosquito contact under a given set of conditions, it is not in itself a method of determining any fixed predilection for hosts. Nor are results of precipitin tests unsupported by taxonomical or other criteria sufficient to discriminate races within species.

SUMMARY.

1. Results of precipitin reactions of blood meals of *A. fluviatilis* in the U.P. Terai during 1949-52 are presented.

2. The anthropophilic index of *A. fluviatilis* in this area has shown a considerable increase over that observed a decade ago.

3. The factors likely to have resulted in this change in food preferences are discussed.

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SOME OBSERVATIONS ON EXPERIMENTAL INFECTIONS IN DOMESTIC PIGEONS (*COLUMBA LIVIA* GMELIN) WITH *P. RELICTUM*.

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(March 30, 1953.)

JASWANT SINGH *et al.* (1951) reported that none out of 214 domestic pigeons examined, harboured any natural infection of *P. relictum*. Wenyon (1926) stated that *P. praecox* (*P. relictum*) most usually occurred in small and larger birds like the sparrow and pigeon. Other worker who observed natural infection of *P. relictum* in pigeon was Coatney (1938).

With a view to find out if *P. relictum* occurring in house sparrows in Delhi and its environs would infect the pigeons from the same area, laboratory experiments were undertaken and the results are set out in this paper.

MATERIALS AND METHODS.

P. relictum was obtained from house sparrows locally trapped. The pigeons were purchased from a bird market in Delhi. In one instance a young pigeon which was reared in a screened cage in the laboratory, was also used.

Blood smears of pigeons were examined daily for a week prior to experiments and then at 2 days intervals after they had been inoculated both by (*a*) blood transfer, and (*b*) by sporozoite infection.

For blood-induced infections, parasitized blood was obtained mostly from the wing vein of infected sparrows and in a few cases where large quantities were required, the birds were sacrificed. The dosage of inoculum varied in different birds and it was calculated in millions of parasites per kg. of body weight. Inoculations were given into the wing veins.

Sporozoites of *P. relictum* were obtained by feeding laboratory bred *Culex fatigans* on infected sparrows. The salivary glands were dissected in normal saline

8-10 days after feeding and sporozoites were collected into a syringe and injected into the wing veins of pigeons. In one instance, 13 *Culex fatigans* with sporozoites of *P. relictum* were made to feed on a normal pigeon.

Controls were kept by inoculating house sparrows with *P. relictum* and later feeding *Culex fatigans* on those birds when they became positive.

RESULTS.

Blood-induced infection.—Eight pigeons were used for this experiment. The results are tabulated in Table I. Of twenty one sparrows inoculated as controls, twelve became positive. Seventy per cent of the eighty *Culex fatigans* fed on them showed sporozoites in glands when dissected after an adequate interval.

TABLE I.

Serial number of pigeon used.	Dosage in million parasites per kg. body weight.	Observation period in days.	Number positive.
1.	11	89	Nil
2.	11·75	138	,,
3.	12·5	122	,,
*4.	27	62	,,
5.	50	70	,,
6.	72	122	,,
7.	73	70	,,
8.	200	53	,,

*This bird was reared in the laboratory in a screened cage.

From the above it was clear that the pigeons did not take the infection in spite of massive doses of parasites inoculated.

Sporozoite-induced infection.—Five pigeons were used for sporozoite-induced infection. The results are set out in Table II.

TABLE II.

Serial number of pigeons used.	Total number of pair of positive glands injected.†	Observation period in days.	Number positive.
9	19	30	Nil
10	12	30	,,
11	12	30	,,
12	12	30	,,
13	18	30	,,
14 (infective mosquitoes by natural bites.)	13‡	38	,,

†After crushing.

‡13 mosquitoes fed on pigeon No. 14. Presence of sporozoites in their glands was confirmed by dissection after feeding.

It is evident from the Table that no patent blood infection resulted in pigeons infected with sporozoites.

Post-mortem was carried out on five pigeons and their organs and tissues have been preserved for further studies.

DISCUSSION.

Wolfson (1937) successfully infected pigeons with *P. cathmerium* isolated from a wood thrush and suggested that pigeons could be used as experimental birds. With a strain of *P. relictum* isolated from mourning dove, Coatney (*loc. cit.*) successfully infected the pigeon and he too felt that pigeons were better birds for experimental studies as they were highly susceptible to *P. relictum*. But from the studies reported here it is clear that there is not the slightest evidence to show that local pigeons are susceptible to the strain of *P. relictum* of Delhi house sparrows. Redmond (1944) also failed to infect pigeons by inoculations with sporozoites of *P. relictum* obtained from mosquitoes fed on canaries. It could be suspected that the pigeons inoculated with sporozoites and infective blood of *P. relictum* may have been immune due to prior natural infection. It is justifiable to eliminate this possibility in view of the fact that as stated previously not one of 214 pigeons examined during five years revealed *P. relictum* in its blood. It is also to be noted that one of the birds used was young and was reared in a screened cage. This was also negative to inoculation. The use of pigeons in Delhi as experimental hosts for laboratory studies with *P. relictum* is not possible on account of their apparent natural immunity to this strain of plasmodium.

SUMMARY.

House pigeons were found to be refractory to both blood-induced and sporozoites-induced infections of *P. relictum* of house sparrows.

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SCREENING OF ANTIMALARIALS AGAINST *P. GALLINACEUM* IN CHICKS*

PART II.

BY

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IN an earlier paper Jaswant Singh, Basu and Ray (1952) described the technique for assay of antimalarials. They had also established the minimum effective dose (M.E.D.) and quinine equivalent of some of the more well-known antimalarials like mepacrine, proguanil, amodiaquin and chloroquine.

In the present paper the authors report their observations on the effect of pyrimethamine on blood-induced infections of *P. gallinaceum* in chicks.

MATERIALS AND METHODS.

Avian host.—Sixty, 7-days old chicks were used for study of M.E.D. of pyrimethamine.

The chicks were obtained from fertilized eggs hatched and reared in the laboratories of this Institute.

Plasmodium.—The strain of *P. gallinaceum* maintained in these laboratories for years was used for these investigations. The dose of inoculum, $0{\cdot}5 \times 10^6$

*This investigation was carried out under the scheme "Screening of antimalarial drugs" which is financed by the Council of Scientific and Industrial Research.

erythrocytic parasities, per gm. of the body weight of the chick was injected intravenously through the right jugular vein.

Antimalarial.—Pyrimethamine was received through the courtesy of Messrs. Welcome Laboratories, London, in bottles of 500 tablets, each containing 25 mg. base. The dose was calculated in milligramme per 50 gm. of body weight.

Weighing, numbering and administration of the drug was carried out in a manner similar to that described previously by Jaswant Singh, Basu and Ray (*loc. cit.*). The first dose was administered on the day of inoculation (0 day). Two such doses were administered daily on the following 3 days. Thus a total of 7 such doses was administered.

Activity.—A dosage schedule was considered to be effective if the average 4th day count of the treated group did not exceed 25 per cent of that of the comparison group (Wiselogle, 1946). The minimum dose which effected such deceleration in the course of parasitæmia was the M.E.D. of the drug.

RESULTS.

From Table I, it would be observed that Class II effect was produced with a dose of 0·1 mg. Although there was some reduction in doses of 0·001 and 0·0005, parasitæmia was over 25 per cent of the comparison group. However, a dose of 0·0015 brought a reduction below the required level and hence this was established as the M.E.D. for this drug.

TABLE I.

Parasitæmia on the 4th day in the comparison group and those under different schedules of pyrimethamine.

Schedule number.	Dose in mg./50 gm.	Number of chicks.	Average 4th day count per 10,000.	Comparison with control group (per cent).	Remarks.
I.	0·1	4	0	0·00	
II.	0·05	6	1·6	0·04	
III.	0·01	5	2·5	0·07	
IV.	0·005	6	5	0·14	
V.	0·003	5	26	0·77	
VI.	0·0015	5	140	4·1	M.E.D.
VII.	0·001	5	898	26·7	
VIII.	0·0005	6	1238	36·8	
IX.	0·0003	5	1345	40·2	
X.	0·0001	5	2333	66·4	
Comparison group		8	3360		

DISCUSSION.

Against simian malaria, pyrimethamine was reported to be more effective than proguanil. Schmidt and Genther (1953) had observed that it is 30 times more effective than proguanil against *P. cynomolgi*, whereas against a strain of *P. knowlesi* it was found to be only 4 times more potent (Jaswant Singh *et al.*, 1951). According to Falco *et al.* (1951), pyrimethamine was found to be 200 times more active in *P. berghei* than proguanil whereas Jaswant Singh, Krishnaswami *et al.* (1952) observed that the drug showed powerful action in as small a dose as 10^{-6} mg. per 20 gm. body weight.

During the present investigation, the minimum effective dose for Class I effect was established as 0·0015 mg./50 gm. The M.E.D. for quinine being 1·6 mg./50 gm. chick, the quinine equivalent of pyrimethamine is in the region of 1,066 (1·6/0·0015). Further when it is compared to proguanil (M.E.D., 0·1 mg.), pyrimethamine appears to be 66 times (0·1/0·0015) more effective even though there is somewhat structural similarity between the two compounds. These results appear to be similar to those reported by Falco *et al.*, (*loc. cit.*) who had observed that by comparison pyrimethamine is 60 times more effective than proguanil.

SUMMARY.

Sixty chicks were infected each with a dose of $0·5 \times 10^6$ parasitised cells from a fowl infected with *P. gallinaceum*.

Fifty-two chicks were placed under various treatment schedules with pyrimethamine, and 8 were kept for comparison.

The minimum effective dose was determined as 0·0015 mg./50 gm. From this the Q.E. was observed to be 1,066 and it was found to be 66 times more powerful than proguanil.

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AVIAN PLASMODIUM IN INDIAN BIRDS.

Part I.

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THE findings of a plasmodium resembling *P. polare* in partridges has recently been recorded and detailed studies are in progress (Ray *et al.* 1953). This species of partridge (*Francolinus pondicerianus interpositus* Hartert) locally known as '*Titar*' (Hindi) is widely distributed in many parts of India, and live in thickets and shrubs in fields and jungles. During the winter months these wild caught birds are sold in the markets of Delhi as edible poultry.

In one of such birds, another plasmodium was detected recently. No evidence of any mixed infection could be detected in blood smears taken every 4 hours for 4 or 5 days.

The present report records the morphological character and the duration of schizogony of the parasite.

Morphology.—All stages of asexual forms and gametocytes were detectable in the erythrocytes as shown in Plate II.

Compared to other avian parasites like *P. gallinaceum* or *P. relictum* maintained in these laboratories, the asexual forms of the parasite appeared to be small and the size varied from 1 to 3 μ. The largest of the gametocytes was 7·8 μ.

Trophozoites.—The early form is usually round or oval. The nucleus is large. The cytoplasm contains a prominent vacuole. As the parasite develops, it is slightly amoeboid and assumes oval or quadrangular shape. The nucleus becomes larger and appears to be out of proportion to the rest of the parasite. The vacoule is still visible. The pigment is detectable at this stage and contains usually two granules.

CHART 1

DIFFERENTIAL PARASITE COUNT IN P.ROUXI

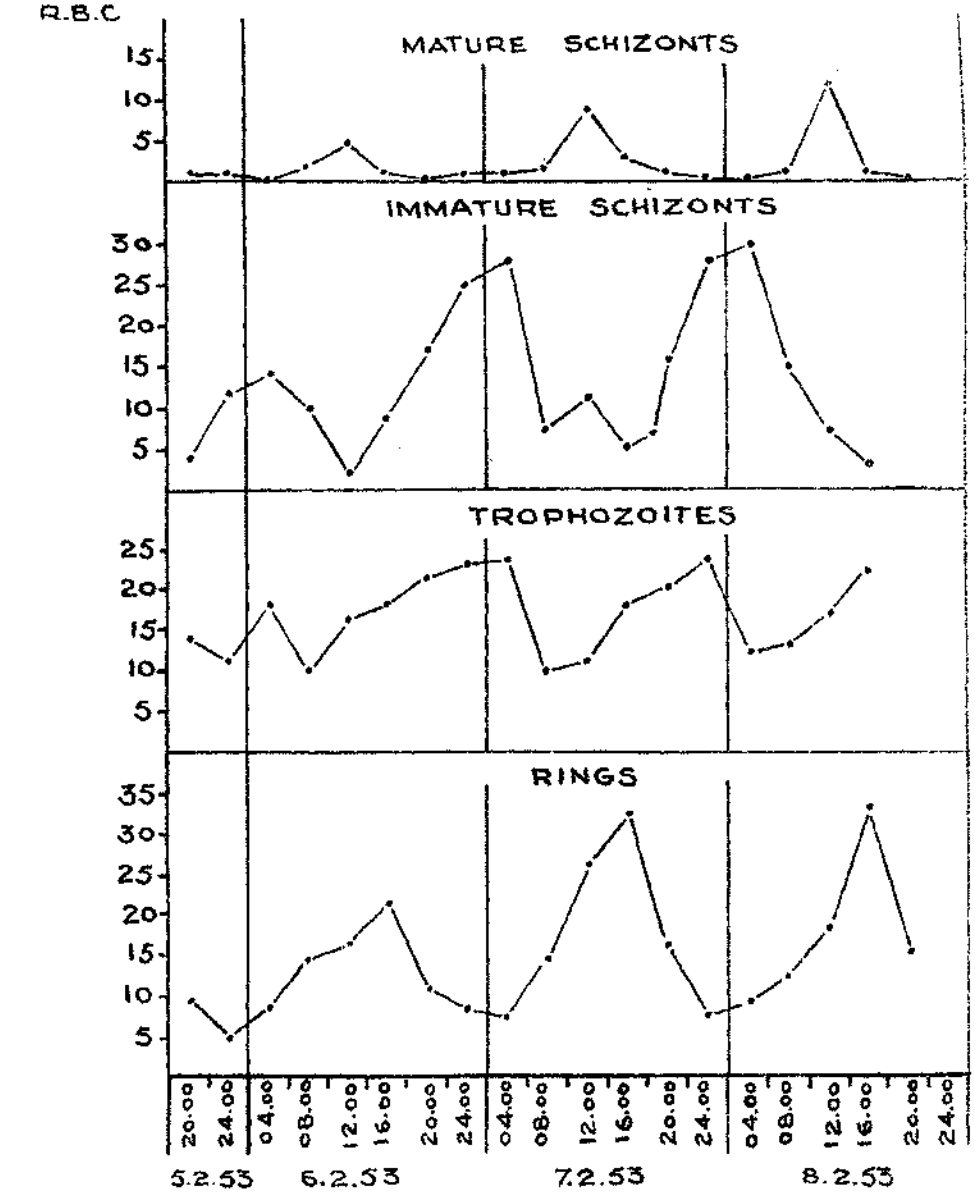
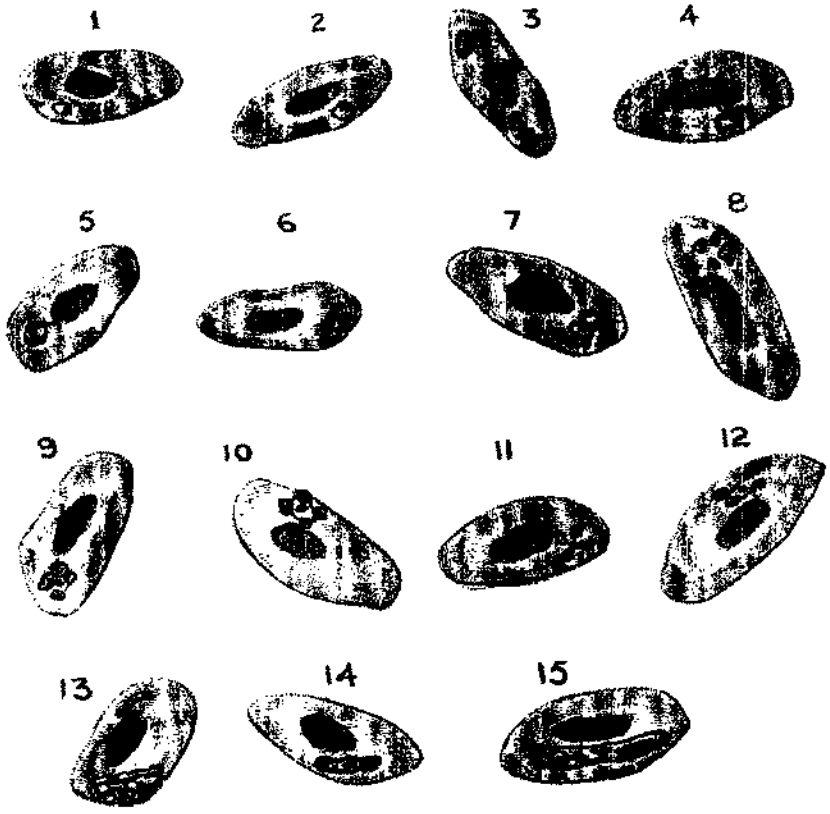


PLATE II.



GAMETOCYTES & ASEXUAL FORMS OF *P. falciparum*.

The chromatin divides fairly early before the parasite can grow bigger. At first it is divided into two and lies diagnoally opposite, usually connected with a strand of chromatin (Plate II, Figures 5 and 6). At about this period, two dark pigment granules, one large and one small, became prominent.

The two chromatin bodies divide further, each into two, thus forming a total of 4 divisions. Quite often these are connected by strands of chromatin (Plate II, Figures 9-12). The merozoites take certain positions and this is responsible for the various shapes the parasites assume.

No further division has been observed although over hundreds of mature schizonts have been examined. The number of merozoites in mature schizonts appears to be constantly four.

At this stage, a clump (usually round) of dark pigment is present either in the periphery (Plate II, figures 7 and 8) or in the centre (Plate II, Figures 9-12).

Gametocytes.—Gametocytes are usually elongated though early forms may be oblong in shape. The longer ones run parallel to the whole length of the nucleus. The cytoplasm is abundant and sometimes appears to be irregular or ragged. The chromatin is prominent. The pigment is granuler in character and is more numerous than in the asexual forms. The granules may be round, rod like or irregular. As a rule they are dark in colour.

Host cell.—The size or shape of the cell is never altered. Rarely the early trophozoites touch the nucleus of the erythrocyte. As a rule the older and divided forms move towards one end (Polar) of the nucleus which is not displaced at any stage.

Periodicity.—To determine the periodic cycle, blood smears were taken every 4 hours and the principle followed was on a line similar to that adopted by Mulligan (1935). Four stages of the parasites were considered during differential parasite count. These were rings, growing trophozoites, immature and mature schizonts.

The stages of parasites observed during the various hours are shown in Chart 1. It will be observed that the maximum number of mature schizonts and rings were detectable at 12·00 noon and at 4·00 p.m. respectively every day, though it may be noted that any of the 4 stages could be detected during most part of the day and night.

DISCUSSION.

From the elongate character of the gametocytes, non-displacement of the host cell nuclei, and the nature of the schizont as described in the identification table by Manwell (1938), Giovannola (1939) and Hewitt (1940), the present species appear to resemble *P. rouxi*, first described by Sergent *et al.* (1931). Further from the morphological characters, the most important of which is the number of merozoites in a mature schizont being constantly four, and the type of pigments, the parasite seems identical to that described by Giovannola (*loc. cit.*).

From the differential parasite count it is clearly evident that the length of the schizogony cycle is of 24 hours though the synchronicity is low as was first observed by Wolfson (1936) in respect of *P. rouxi.*

Prior to the discovery of the plasmodium resembling *P. polare* (Ray *et al.*, *loc. cit.*) and the present species, no reference is available of any plasmodial infection in the common Indian partridges. Hence the findings of two different species in these birds have thrown open further scope of work in this line.

SUMMARY.

An avian species of plasmodium was found in a common Indian partridge. The parasite showed the following characteristics :—

(i) Small in size.

(ii) Mature schizonts contain always 4 merozoites.

(iii) During division of chromatin, the parasite is found near one pole.

(iv) Pigments in the trophozoites are nearly always two while in mature schizonts it forms into one dark round mass.

(v) Gametocytes are elongated.

(vi) Periodic cycle of asexual schizogony is of 24 hours.

In view of the above, the parasite is established as *P. rouxi.*

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NOTES ON THE ANOPHELINE FAUNA OF A HILL TRACT IN MYSORE STATE, INDIA.*

BY

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INTRODUCTION.

In the course of studies on malaria and anopheline mosquitoes in the western hills of Mysore State, India, field investigators had the opportunity to record a body of information pertaining not merely to malaria vectors but to the anopheline fauna as a whole. This knowledge was gained through standard methods of population sampling as practised by malariologists, namely: the dipping of larvæ from breeding places and the collection of imagines from daytime resting places in human dwellings, "mixed" dwellings and cattlesheds.

The data thus accumulated are of some importance from a number of points of view. In some instances the abundance of a non-vectorial species of Anopheles may serve as a rough guide to the potential presence of an undetected vector, owing to known similarity of larval or adult habitats and behaviour of the two species. In other cases a highly prevalent although non-vectorial species of Anopheles may serve as a splendid indicator of the residual toxicity of DDT in sprayed structures. In both these cases, a knowledge of the annual cycle of abundance of such so-called "unimportant" anophelines is needed if one wishes to use the collateral evidence which their presence provides.

Under the impression that knowledge of all mosquitoes is useful, no matter how trivial some of the species appear to be when human disease is the topic of discussion, one presents the following summaries of anopheline annual population cycles as displayed during a one-year period of observation in Mysore.

MATERIALS AND METHODS.

Locale.—The ensuing narrative is concerned with a remarkable terrain, since variations in rainfall—and consequently in flora and fauna—occur on an

*The work on which this paper is based was performed with the support and under the auspices of the Division of Medicine and Public Health of The Rockefeller Foundation in co-operation with the Bureau of Malariology of the Mysore State Health Department.

unusually sharp gradient in the region here considered. Mysore State occupies the centre of South India. The larger part of the State consists of the southernmost extension of the Deccan Plateau, an extremely ancient geological formation, three thousand feet above the sea. The State is cradled in a horseshoe of hills—the Eastern Ghats, the foothills of the Nilgiris to the south, and the Western Ghats. Only in its northern frontier is Mysore continuous with the great central plateau of India.

The Western hills, or ghats, rise to about 4,500 feet, and although their slopes are at least fifty miles removed from the Arabian Sea, they present the first obstruction encountered by rain-laden clouds of the annual southwest monsoon. During June, July and August these clouds behave as if pricked by the peaks of the ghats. But just as a ruptured container of liquid is soon rid of its contents, so the monsoon clouds, driven north-eastward, appear shortly to have been drained of their burden. Measurements of annual rainfall in the Western Ghats of Mysore begin with figures such as two hundred thirty inches, but decrease at the average rate of ten inches per mile as one progresses from west to east. Thus a region that is virtually drenched each summer may be no more than twenty-five miles removed from another region where ascending dust clouds mingle with soaring serpent-eagles even during the height of the southwest monsoon.

The hilly regions of excessive rainfall, known as the Malnad, are reputed to be highly malarious, and extensive depopulation during the past thirty years is attributed by the remaining inhabitants to voluntary migration to drier areas to escape the fevers. The wettest zones are consequently lightly populated. Cardamom, a spicy herb grown in shady forests, is the chief—and the most valuable—crop ; a small amount of tea is produced ; timber of good size is also important. In the narrow valleys rice is raised annually with dependence on the southwest monsoon for the required irrigation.

East of the ridge marking the divide between watersheds of the Arabian Sea and the Bay of Bengal, in areas with annual rainfall of 80 to 100 inches, the human population is considerably denser and cultivation of the land is correspondingly more intensive. Cardamom, coffee, pepper and some citrus fruits are commercially raised in partially cleared forest, while the broader and less precipitous valleys afford greater opportunities for rice cultivation for local consumption. Water for irrigation in this instance is provided principally by surface run-off in the southwest and northeast monsoon seasons, although only irregular and minor additions in September-December may be credited to the northeast monsoon. During the first half of each year most of the area is dry, and since there are very few " tanks " for impounding run-off waters, the baked paddy fields remain fissured and idle.

Ten miles or so east of this area of intermediate rainfall, annual precipitation has declined to sixty inches or less. The land now supports extensive areas of " scrub jungle ", which the imported *Lantana* has frequently overrun in dense thickets. Crops depending on forest shade are less extensively grown, and virtually the entire economy is based on rice cultivation, supplemented by dry cereal crops such as *ragi* and *jola*. For the latter reason, and also in view of the scant rainfall, tanks are almost a necessity. Thus it is sometimes possible to observe a thriving

tank-fed series of ricefields here, at a time when fields in the contiguous intermediate area, more abundantly endowed with annual rains, paradoxically lie fallow for want of moisture.

Selection of study areas.—Owing to the alleged intensity of Malnad malaria, it was decided to establish a Malaria Investigation Centre in the heart of one such area. The town of Sakleshpur, in Hassan District, was chosen for this purpose. This town lies in the intermediate rainfall zone and affords easy access by motor roads to the other two zones. In order to gain information as to the nature of the epidemiology of Malnad malaria in typical undisturbed situations, three groups of small villages were chosen for study after preliminary malaria surveys had been made.

The first group of villages, somewhat less than 3,000 feet in elevation, was situated about ten miles west of Sakleshpur and 4 miles southwest of the ridge marking the watershed. Average rainfall for the previous ten years was 223 inches annually. The second group of villages, about 3,000 feet in elevation, 20 miles south of Sakleshpur and 10 miles northeast of the ridge, was fairly typical of the intermediate zone. The ten-year annual rainfall average was 83·19 inches. The third group of villages, 3,000 feet in elevation, 20 miles northeast of Sakleshpur, although only 16 miles northeast of the ridge marking division of the watersheds, had an annual average rainfall over the past ten years of 48·23 inches.

Anopheline surveys.—Beginning in March 1951, routine collections of larval and adult anophelines were carried out in the three study areas on a weekly basis. Identifications of specimens were made at the Malaria Investigation Centre on the day of collection. During the course of malaria investigations there was some variation in the number and quality of personnel engaged in entomological work, but records were reduced to uniformity in so far as possible by expressing anopheline prevalence in terms of the numbers collected per man-hour. In each village of each area it was attempted to establish three fixed catching stations for adult mosquitoes comprising a human dwelling, a mixed dwelling and a cattle-shed. A " mixed " dwelling was defined as a structure which housed both people and domestic animals at night with at the most an incomplete partition separating the two populations. Mosquitoes caught in either part of such a dwelling could be considered as having been attracted by either class of occupants, regardless of the sites of capture within the structure.

According to local customs of villagers in the different areas, mixed dwellings were found in some regions and not in others. In the latter case only human dwellings and cattlesheds were designated as fixed mosquito catching stations. The construction of all types of structure was in general similar throughout the Sakleshpur area, being typified outstandingly by primitive use of crude materials in response to evident poverty. In one or two villages a pretentious house with carved supporting timbers and a corrugated tile roof was to be found, but in general the architecture consisted of walls made of mud, or of sticks, split bamboos or palm thatch, and roofs made with rice straw thatch or country tile. Uneven earthen floors, slicked down with liquid cow dung, were likewise the conventional mode. Housekeeping was usually light ; ancient cobwebs hanging from walls and lofts afforded favoured sites for anopheline roosting.

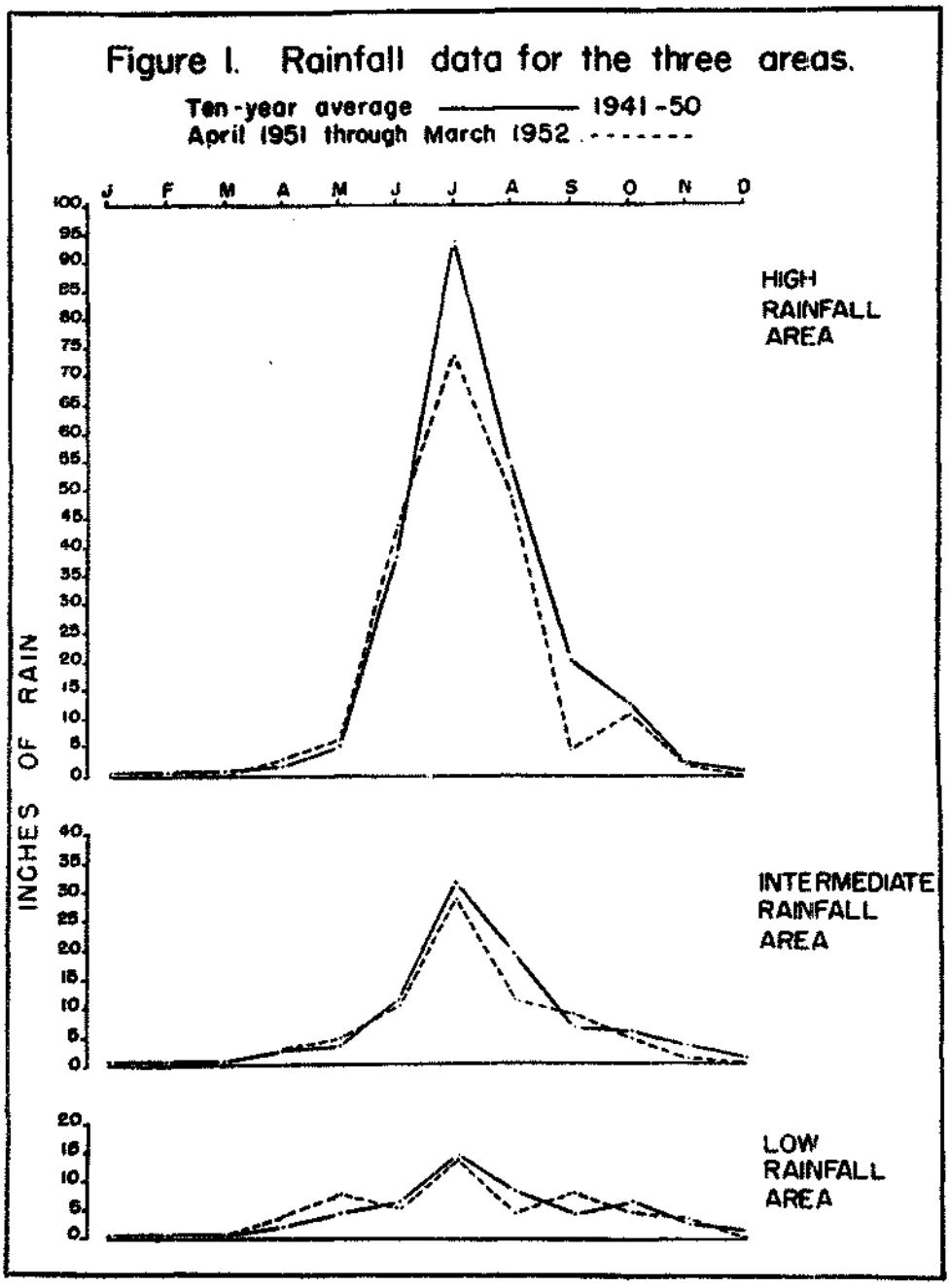


Figure I. Rainfall data for the three areas.
Ten-year average ——— 1941-50
April 1951 through March 1952 - - - - - - -
J F M A M J J A S O N D
INCHES OF RAIN
HIGH RAINFALL AREA
INTERMEDIATE RAINFALL AREA
LOW RAINFALL AREA

Daytime collections of mosquitoes were made invariably between the hours of 10·00 and 12·00. Individual collectors routinely canvassed the interior of structures with flash-light and aspirator tube, the time allotted being half an hour or an hour per structure according to its size. The structures were usually untenanted at this time, but it was determined that night inhabitants included human beings, cattle, chickens, occasional water buffaloes, dogs, cats and sometimes sheep or goats. Pigs, if present, were kept in separate enclosures.

Collections of adult anophelines from daytime outdoor resting places were not made. Nor were artificial collecting means such as animal traps, window traps, light traps, keg shelters or the like employed. A few night collections were made and will be discussed later.

There is undoubtedly some weighting of larval records on the side of stream-breeding species, for the larva collectors were explicitly instructed to concentrate their efforts on running water. This was because the investigators were especially interested in learning the distribution and abundance of the alleged chief malaria vector in the Malnad, *Anopheles fluviatilis*. However, since it was desired also to obtain information about all other indigenous anophelines, the larva collectors were told to spend part of their time collecting from tanks, paddy fields and domestic water collections—in fact, not to neglect any body of water whatsoever. Hence the anopheline larval survey is comprehensive as regards the incidence of species within the area, even though it may lack something of a quantitative character.

The one-year period covered in this study extends from the middle of March, 1951, to the same date in 1952. This seemed to be the best time to begin and end a year owing to the fact that the rains had not yet begun, while surface water had become scarce. Thus many anopheline populations were approaching their lowest point for the year.

The actual rainfall in the three areas during this study period, as compared with the ten-year average, is shown in Figure 1. It will be noted that the year was fairly normal in this respect, although there was a slight deficiency of rain throughout the region. Temperature and humidity data are not available.

RESULTS.

Species encountered in the study area.—The twenty-two species encountered in the several areas may be most conveniently presented in the form of three lists (Table I).*

Granting that the collection of adult mosquitoes from man-made structures must be an unfair test of the real prevalence of anopheline populations, it is nevertheless of significance in any consideration of bionomics. Tables II, III and IV give a rough idea of the monthly incidence of species thus obtained in the three areas. In the case of less common species, the absolute numbers taken are probably more significant than the per man-hour figures. But in the case of abundant species, per man-hour statements of density are the more revealing, for variations took place from month to month not only in the number of collectors employed but also in the length of time they spent individually at their work.

* *Anopheles annandabi* var. *interruptus* Puri has since been found once in the intermediate rainfall area.

TABLE I.

Total numbers of adult and larval anophelines collected in the three areas during one year. (Western Mysore State).

Species.	High rainfall.		Medium rainfall.		Low rainfall.	
	Adults.	Larvæ.	Adults.	Larvæ.	Adults.	Larvæ.
(Man-hours)	182·5	175·0	160·5	172·0	221·5	230·0
Aconitus ...	3	2	9	44	44	21
Aitkeni	2	251	0	206	1	3
Annularis ...	65	11	33	7	302	30
Barbirostris ...	51	1,150	16	1,036	28	861
Culicifacies ...	19	7	303	11	249	15
Fluviatilis ...	31	16	24	51	81	261
Hyrcanus var. *nigerrimus*	13	923	5	786	13	879
Insulæflorum ...	0	5	0	2	0	2
Jamesi	27	172	28	165	60	199
Jeyporiensis ...	2,108	304	1,250	497	250	1,258
Jeyporiensis var. *candidiensis* ...	2	0	0	0	1	0
Karwari ...	2	19	5	3	7	3
Leucosphyrus ...	1	2	0	3	0	9
Maculatus ...	1	3	0	0	1	3
Majidi	0	3	0	4	0	0
Pallidus ...	38	11	1,835	76	6,947	162
Philippinensis ...	0	0	0	172	0	6
Splendidus ...	0	5	4	10	5	38
Subpictus ...	125	16	106	23	200	10
Tessellatus ...	9	2	14	1	43	11
Tuckhudi ...	0	0	0	0	2	0
Vagus	148	22	135	25	125	8
Varuna ...	1	1	3	5	0	6
TOTALS ...	2,646	3,125	3,770	3,127	8,359	3,785

Total adults	...	14,775
Total larvæ	...	10,037
Total records	...	24,812

TABLE II.

Monthly total (T) and per man-hour (MH) incidences of mosquitoes obtained by daytime collection in human dwellings and cattlesheds in the high rainfall area (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Aconitus ...	T			2								1	
	MH			0·154								0·062	
Aitkeni ...	T				2								
	MH				0·200								
Annularis ...	T	7	7	33	5	1			1	5		1	2
	MH	0·350	0·424	2·538	0·500	0·134			0·054	0·294		0·062	0·126
Barbirostris	T	1	1	1					2	23	20	3	
	MH	0·050	0·060	0·078					0·108	1·352	1·020	0·188	
Culicifacies ...	T			1	5	4	4	2			1	2	
	MH			0·078	0·500	0·534	0·400	0·108			0·052	0·126	
Fluviatilis ...	T	8	10	12	3								
	MH	0·400	0·606	0·924	0·300								
Hyrcanus ...	T	1								3	6	1	2
	MH	0·050								0·176	0·308	0·062	0·126
Jamesi ...	T	5	3	1			2		2		2	4	8
	MH	0·250	0·182	0·078			0·200		0·108		0·102	0·250	0·500
Jeyporiensis	T	178	413	235	24	41	53	82	48	183	245	481	143
	MH	8·900	25·030	18·078	2·400	5·466	5·300	4·432	2·596	10·882	12·564	25·062	8·938
Karwari ...	T							1	1				
	MH							0·054	0·054				
Leucosphyrus	T								1				
	MH								0·054				
Maculatus ...	T	1											
	MH	0·050											
Pallidus ...	T	3		6		1	1		2	3	3	11	8
	MH	0·150		0·462		0·134	0·100		0·108	0·176	0·154	0·688	0·500
Subpictus ...	T	1	54	28	22	2	3	2		4	4	5	
	MH	0·050	3·272	2·154	2·200	0·267	0·300	0·108		0·236	0·204	0·312	
Tessellatus ...	T	2										2	5
	MH	0·100										0·126	0·312
Vagus ...	T	6	7	22	21	20	16	20	1	4	3	19	9
	MH	0·300	0·424	1·690	2·210	2·667	1·600	1·082	0·054	0·236	0·154	1·188	0·562
Varuna ...	T			1									
	MH			0·078									

Table III.

Monthly total (T) and per man-hour (MH) incidences of mosquitoes obtained by daytime collection in human dwellings and cattlesheds in the intermediate rainfall area. (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
Aconitus ...	T	5		1							1	1	1
	MH	0·278		0·082							0·058	0·072	0·072
Annularis ...	T	11	8		7	3			1	1			2
	MH	0·616	0·552		0·700	0·300			0·062	0·064			0·142
Barbirostris	T								1	5	7	3	
	MH								0·062	0·322	0·412	0·214	
Culicifacies ...	T	24	73	66	63	56	8	2	1			8	2
	MH	1·334	5·028	5·500	6·300	5·600	1·066	0·166	0·062			0·572	0·142
Fluviatilis ...	T	5	6	5	7	1							
	MH	0·278	0·414	0·416	0·700	0·100							
Hyrcanus ...	T	1										2	2
	MH	0·056										0·142	0·142
Jamesi ...	T	8	5	1			1	3				2	8
	MH	0·444	0·344	0·082			0·134	0·250				0·142	0·572
Jeyporiensis	T	144	86	41	5	7	19	55	108	168	234	252	131
	MH	8·000	5·932	3·416	0·500	0·700	2·534	4·584	6·750	10·838	13·784	18·000	9·358
Karwari ...	T							2	1	2			
	MH							0·166	0·062	0·130			
Pallidus ...	T	56	20	6	5	7	5	42	58	378	430	593	235
	MH	3·112	1·380	0·500	0·500	0·700	0·667	3·500	3·626	24·388	25·294	42·356	16·756
Splendidus ...	T	2			1								1
	MH	0·112			0·100								0.072
Subpictus ...	T	1	47	12	2	5	4	8	4	9	6	6	2
	MH	0·056	3·242	1·000	0·200	0·500	0·534	0·667	0·250	0·580	0·352	0·428	0·142
Tessellatus ...	T			1		1	1	4	1		1	3	2
	MH			0·082		0·100	0·134	0·334	0·062		0·058	0·214	0·142
Vagus ...	T	3	9	5	16	24	15	18	5	10	10	16	4
	MH	0·166	0·620	0·416	1·600	2·400	2·000	1·500	0·312	0·646	0·582	1·142	0·286
Varuna ...	T	1	1										1
	MH	0·056	0·068										0·072

TABLE IV.

Monthly total (T) and per man-hour (MH) incidences of mosquitoes obtained by daytime collection in human dwellings and cattlesheds in the low rainfall area. (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Aconitus ...	T	13	8	7			1	3	2		3	4	3
	MH	0·456	0·364	0·424			0·100	0·162	0·112		0·120	0·182	0·126
Aitkeni ...	T				1								
	MH				0·100								
Annularis ...	T	37	110	104	5		1	3	3	5		21	13
	MH	1·298	5·000	6·304	0·500		0·100	0·162	0·166	0·256		0·954	0·542
Barbirostris	T			1					3	1	18	5	
	MH			0·060					0·166	0·052	0·720	0·228	
Culicifacies	T	5	19	15	50	15	65	67	9	2		1	1
	MH	0·176	0·864	0·910	5·000	2·000	6·500	3·622	0·500	0·102		0·046	0·042
Fluviatilis ...	T	16	20	9	2	1	4	2				3	24
	MH	0·562	0·910	0·546	0·200	0·134	0·400	0·108				0·136	1·000
Hyrcanus ...	T	1									8	4	
	MH	0·036									0·320	0·182	
Jamesi ...	T	5	5	7		1	2	13	8	1		11	7
	MH	0·176	0·228	0·424		0·134	0·200	0·702	0·444	0·052		0·500	0·292
Jeyporiensis	T	21	42	37	4	2	2	13	6	18	48	30	27
	MH	0·736	1·910	2·242	0·400	0·267	0·200	0·702	0·334	0·924	1·920	1·364	1·124
Karwari ...	T							2	3		2		
	MH							0·108	0·166		0·080		
Maculatus ...	T												1
	MH												0·042
Pallidus ...	T	289	337	389	356	280	242	476	610	1361	1215	1072	320
	MH	10·140	15·318	23·334	35·600	37·334	24·200	25·730	33·838	69·794	48·600	48·728	13·334
Splendidus ...	T			1	3		1						
	MH			0·060	0·300		0·100						
Subpictus ...	T	2	104	37	22	3	5		3	5	6	9	4
	MH	0·070	4·728	2·242	2·200	0·400	0·500		0·166	0·256	0·240	0·410	0·166
Tessellatus ...	T	1	2	1			6	14	4	2	4	6	3
	MH	0·036	0·090	0·060			0·600	0·756	0·222	0·102	0·160	0·272	0·126
Turkhudi ...	T			2									
	MH			0·122									
Vagus ...	T	5	6	11	5	7	24	23	9	8	11	10	6
	MH	0·176	0·272	0·667	0·500	0·934	2·400	1·244	0·500	0·410	0·440	0·454	0·250

These tables present a monthly breakdown of daytime adult captures in the three areas, while Figure 2 presents some of the same data in visual form. Larval collections made simultaneously are grouped for convenient perusal in Tables V, VI and VII, and also charted in Figure 2. The ratios of all larvæ to adults collected during the year, on a per man-hour basis, have been calculated : these are listed in Table VIII and shown graphically in Figure 3. The latter Figure, embodying much of what has already been presented in this paper, will bear particular discussion against its background.

A preliminary word must be interpolated here to validate the larva-adult ratios used in this study. It is often true that observed larva-adult ratios on a given day are not indicative of the true relationship between these fractions of a species population, since today's adults are yesterday's larvæ, where as today's larvæ will become tomorrow's adults. Owing to the tendency toward geometric rates of increase or decrease of a species in response to climatic, reproductive and other forces, a correlation between adult and larval populations must usually be made in reference to an elapsed unit of time rather than to an instant. But in the present case the larva-adult ratios were derived from the summation of per man-hour rates of capture for a twelve-month period ; this should theoretically, and without much doubt actually, serve to eliminate discrepancies that might be observed in casual studies confined to brief periods of time. It therefore makes no difference whether species X was abundant as a larva in June although rare as an adult, or whether such a relationship was reversed one or two months later. The ratios give overall information as to the likelihood of capturing larvæ and adults of species X in the three areas during a year of study.

Since the most common form of malaria survey includes collection of anophelines, adult and sometimes larval, on a daytime basis, this paper will be confined to consideration of the data thus far presented. It gives certain information on annual cycles of anopheline species in the Sakleshpur region, but only in so far as the methods employed have revealed them. It is hoped to enhance the interpretation of present results in a future paper dealing with night collections and other methods of entomological investigation.

DISCUSSION.

Before proceeding to discussions of the cycles of individual species, it will be necessary to consider the implications of observed adult and larval densities in the case of daytime collections made simultaneously.

Adults.—Adult occurrences in human dwellings, mixed dwellings and cattle-sheds presuppose that a mosquito has entered on the previous night to feed and rest, or has entered at dawn merely to rest. Naturally those mosquitoes that entered for whatever purpose at night and then vacated the premises will not be included in the sort of census that is considered here. Failure to record a species at all, or in significant numbers, within these shelters does not automatically prove that it has not frequented such shelters or is not commonly present in the local environment.

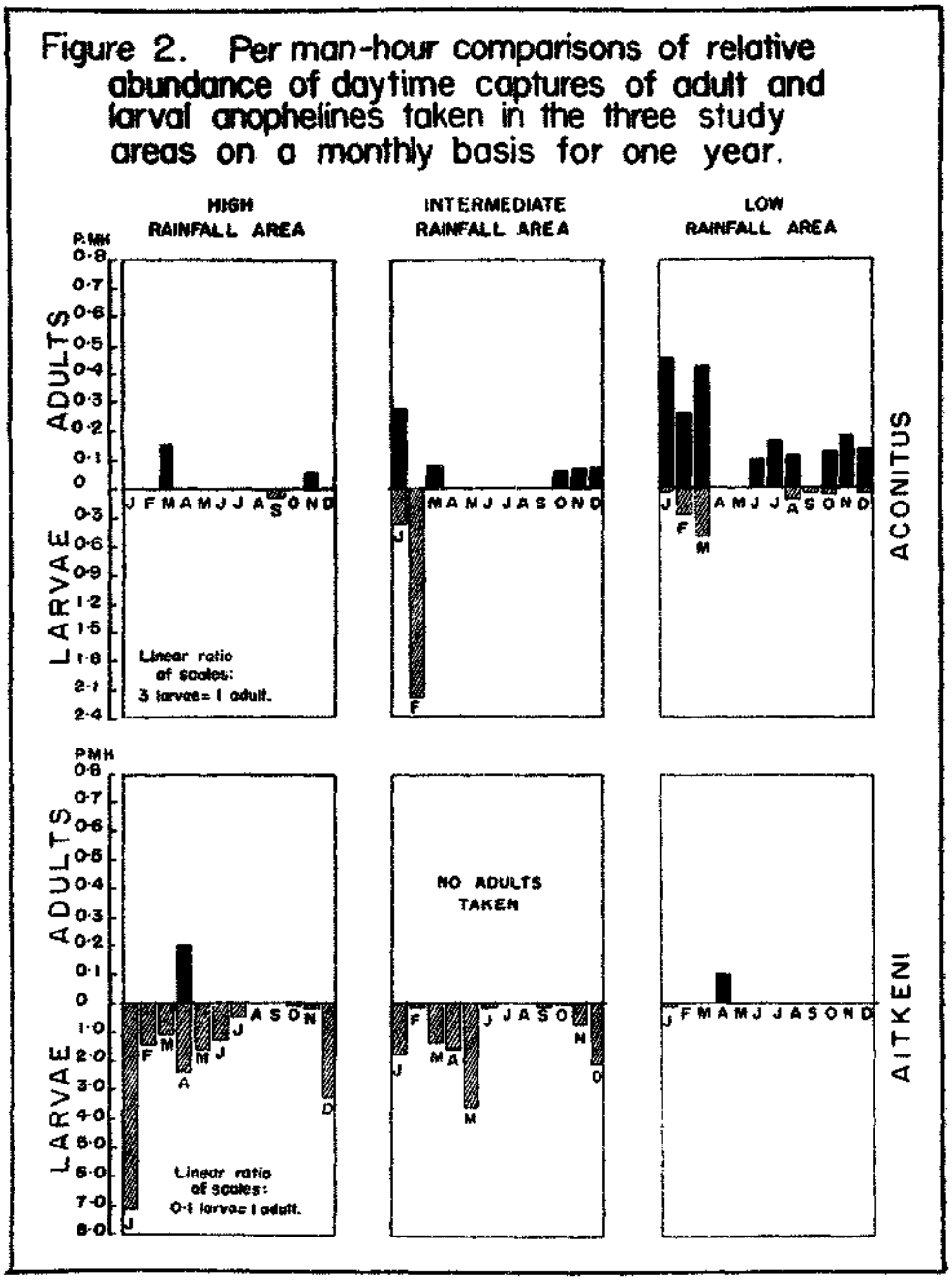


Figure 2. Per man-hour comparisons of relative abundance of daytime captures of adult and larval anophelines taken in the three study areas on a monthly basis for one year.

Figure 2, Continued.

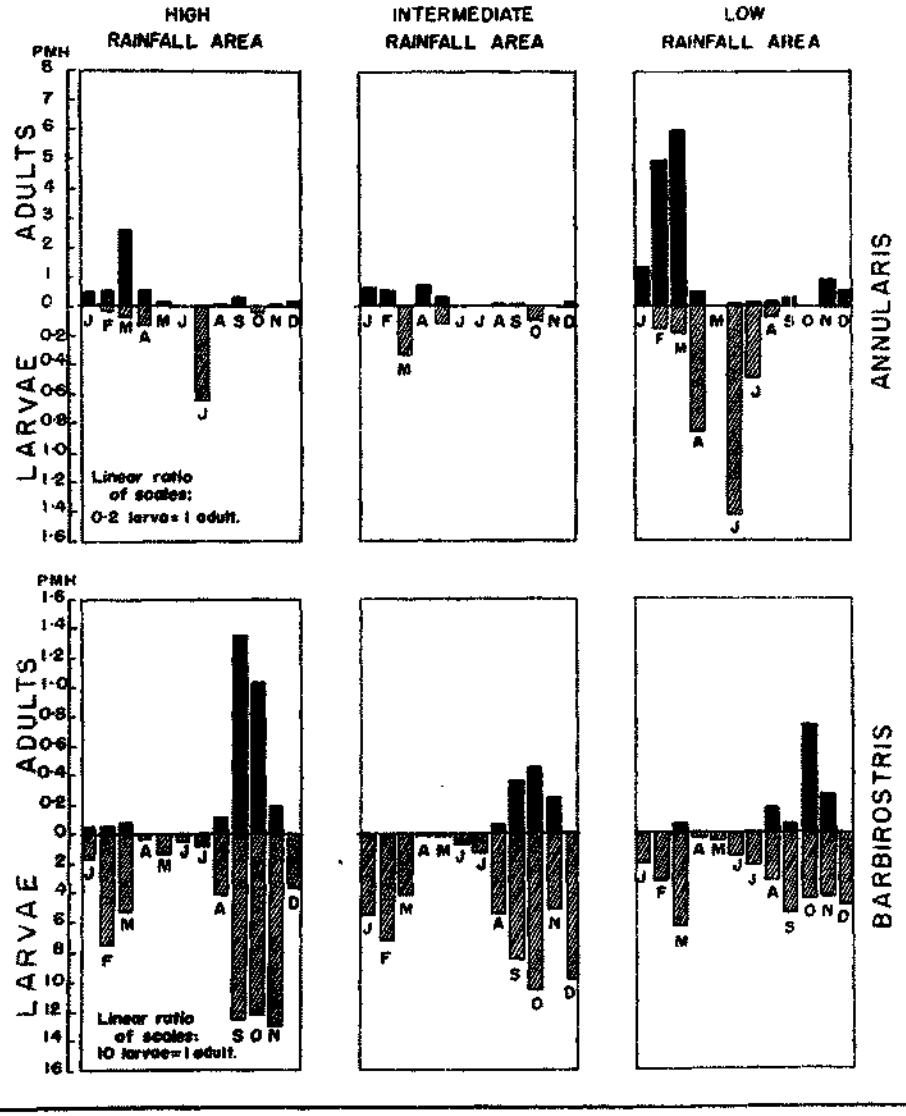
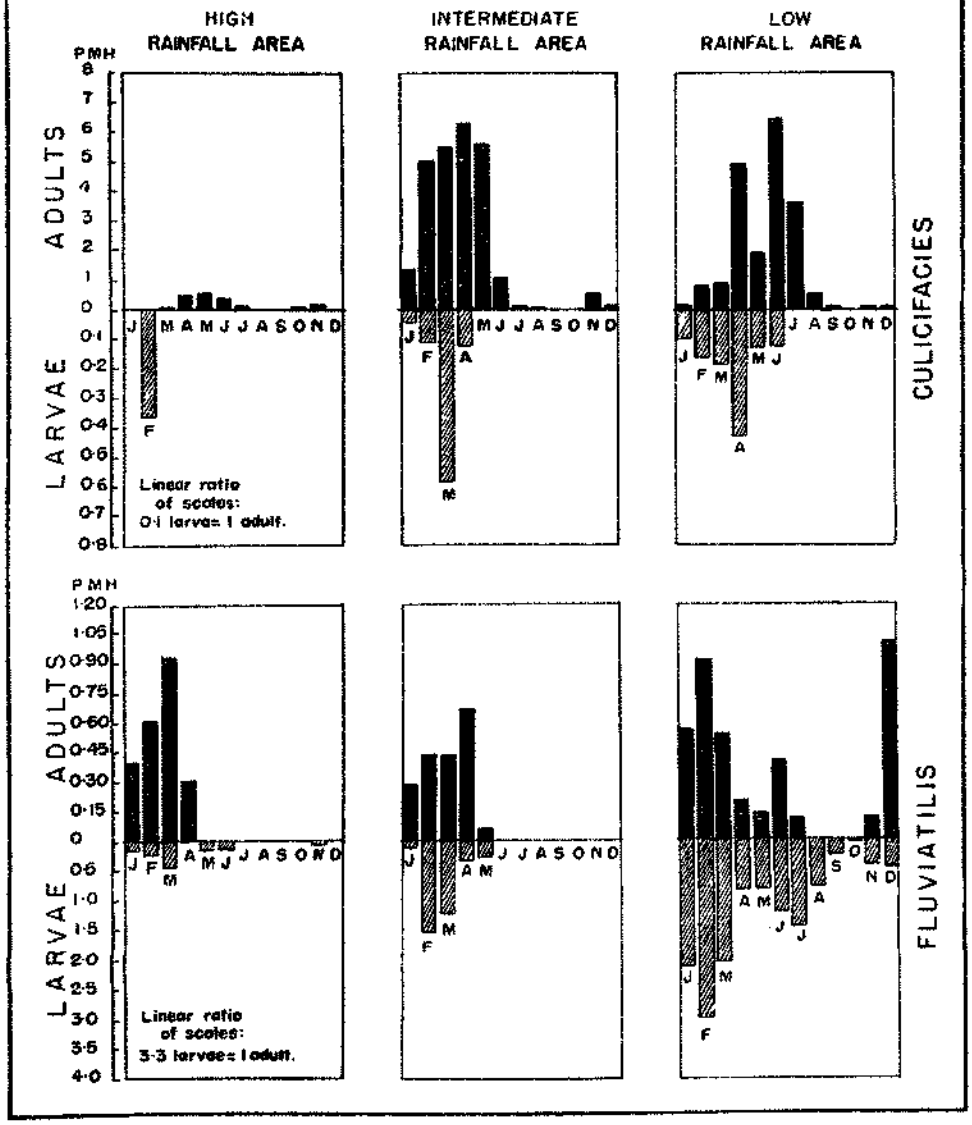


Figure 2, Continued.



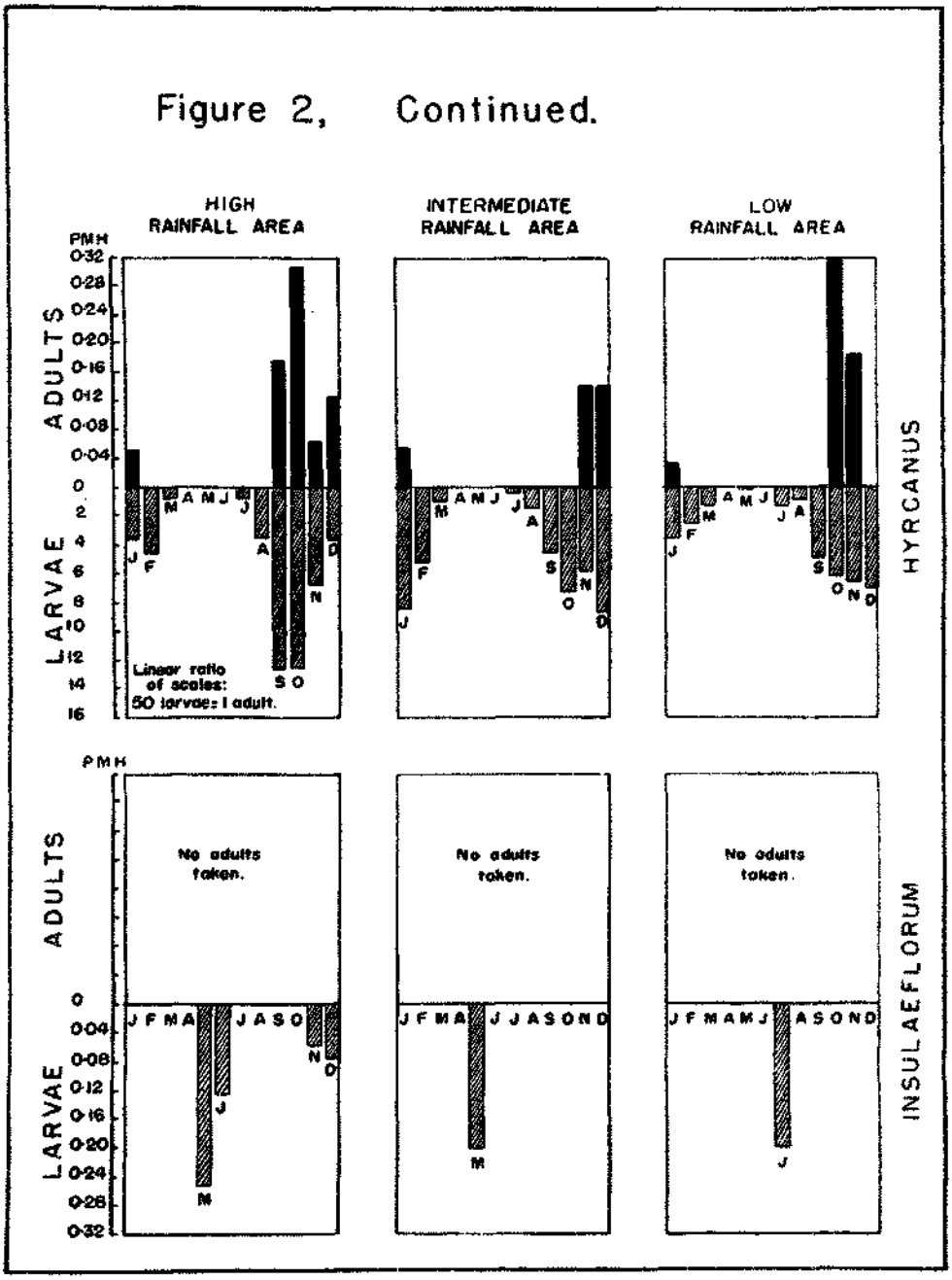
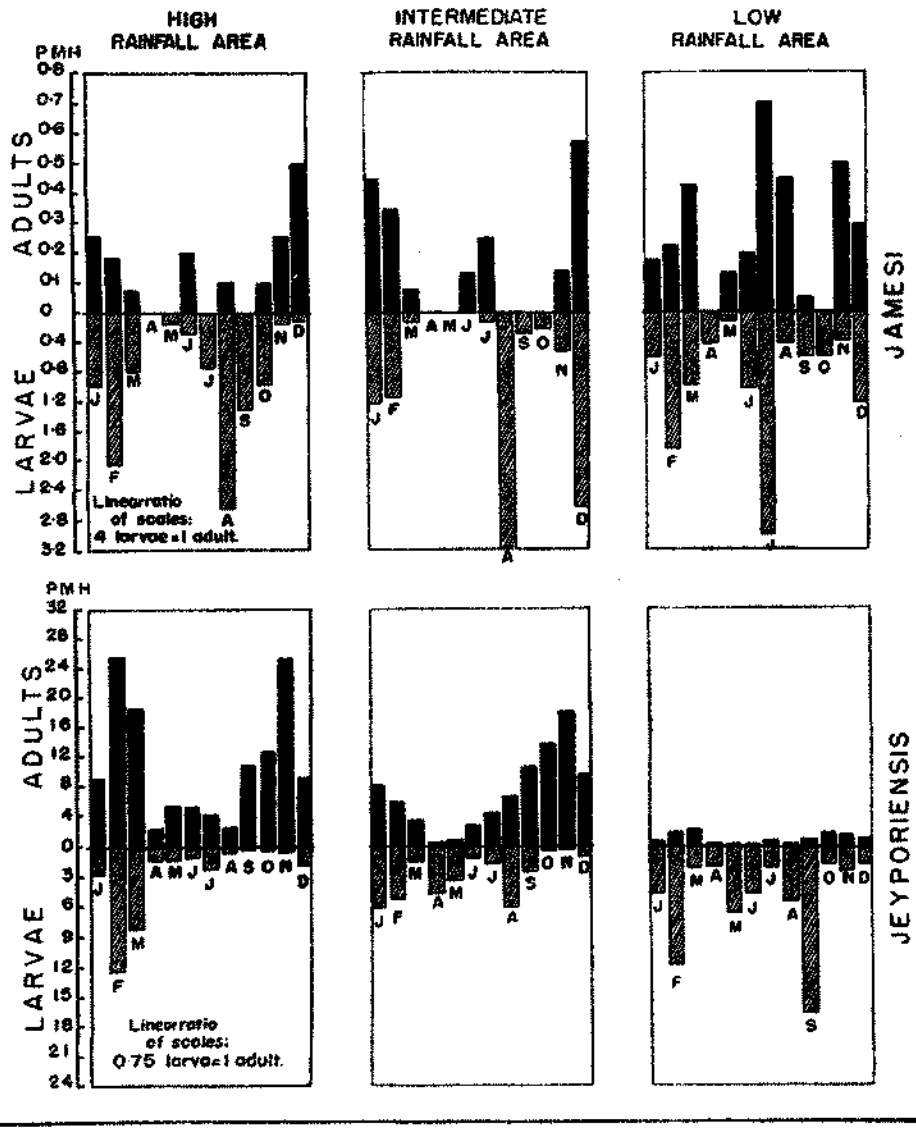


Figure 2, Continued.
HIGH RAINFALL AREA
INTERMEDIATE RAINFALL AREA
LOW RAINFALL AREA
PMH
ADULTS
LARVAE
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
0
2
4
6
8
10
12
14
16
Linear ratio of scales: 50 larvae: 1 adult.
HYRCANUS
No adults taken.
J F M A M J J A S O N D
INSULAEFLORUM

Figure 2, Continued.



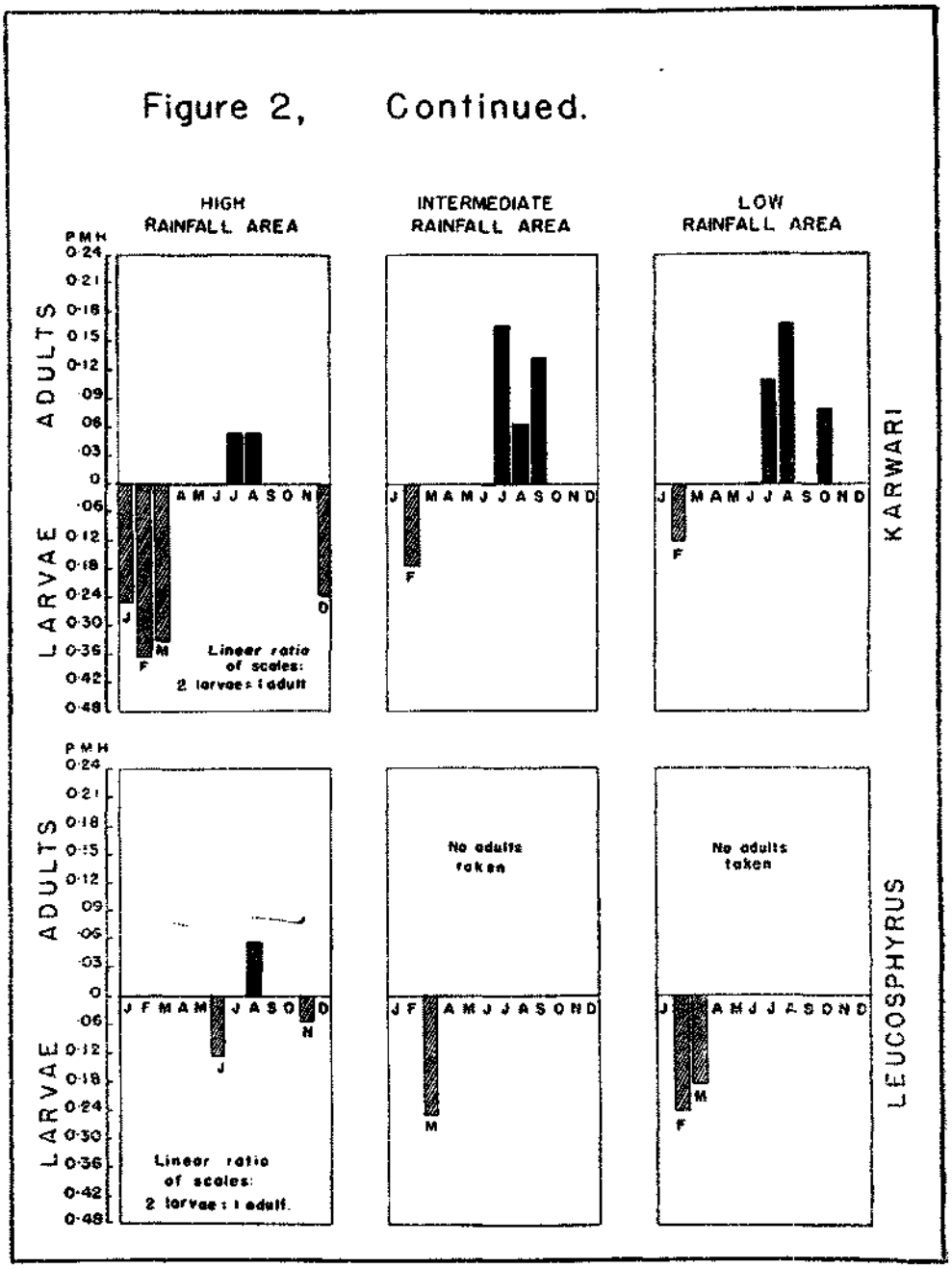


Figure 2, Continued.
HIGH RAINFALL AREA
INTERMEDIATE RAINFALL AREA
LOW RAINFALL AREA
PMH
ADULTS
LARVAE
KARWARI
LEUCOSPHYRUS
Linear ratio of scales: 2 larvae : 1 adult
No adults taken

Figure 2, Continued.

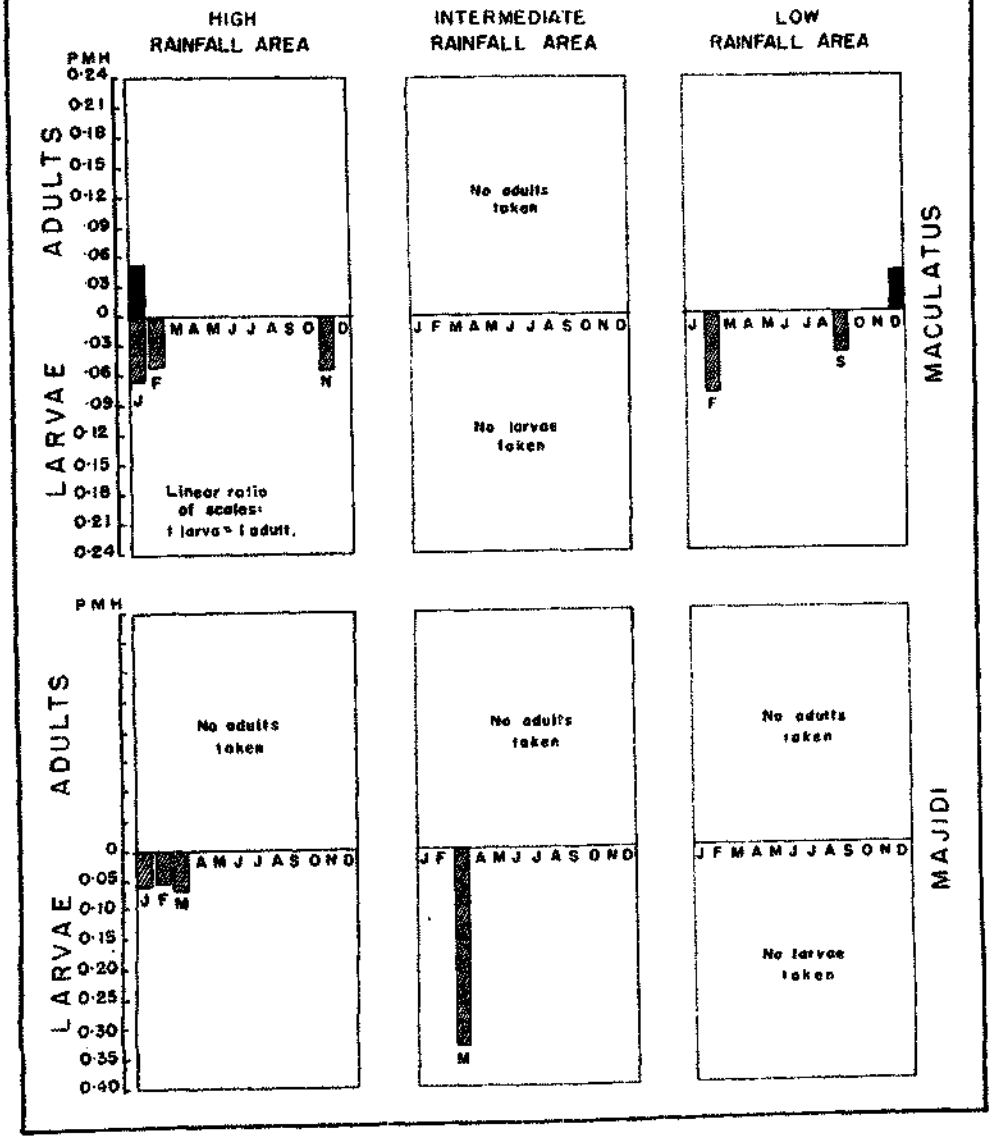
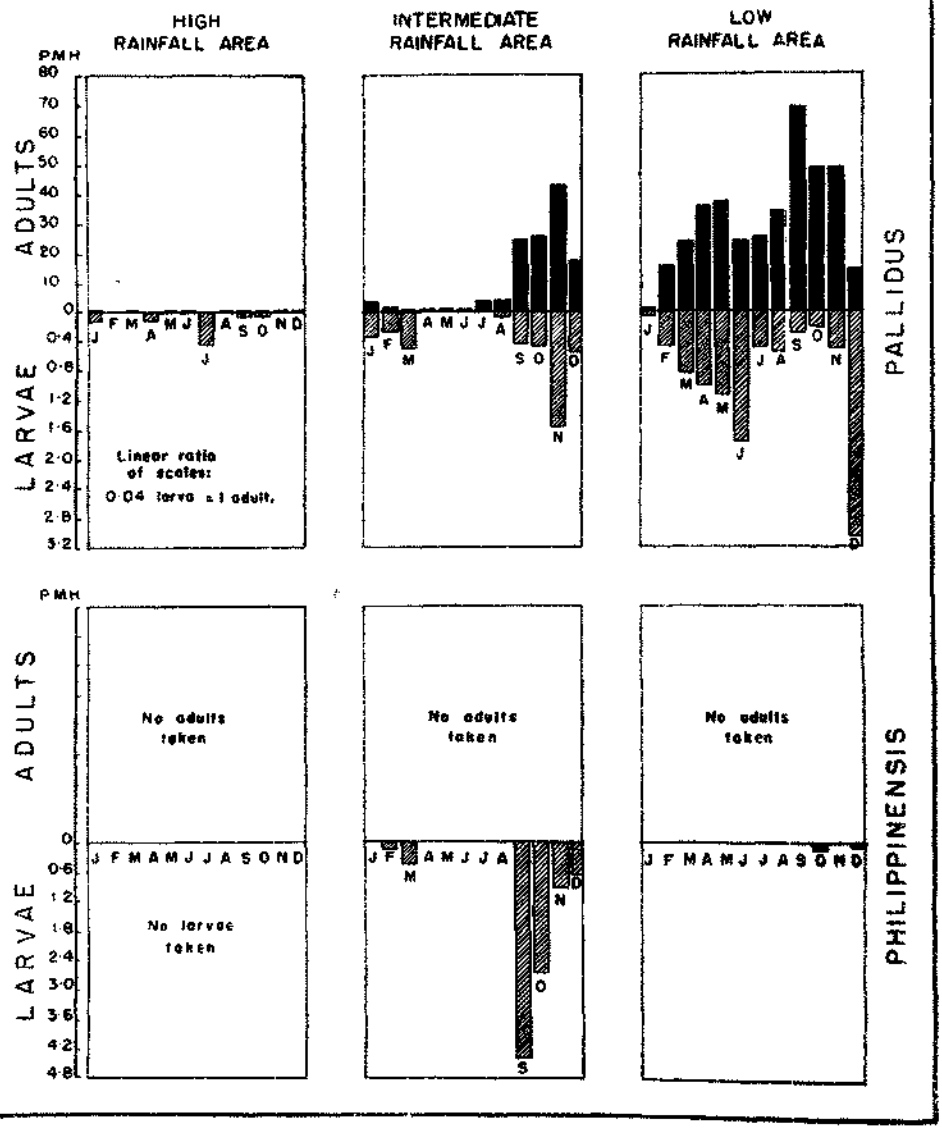


Figure 2, Continued.



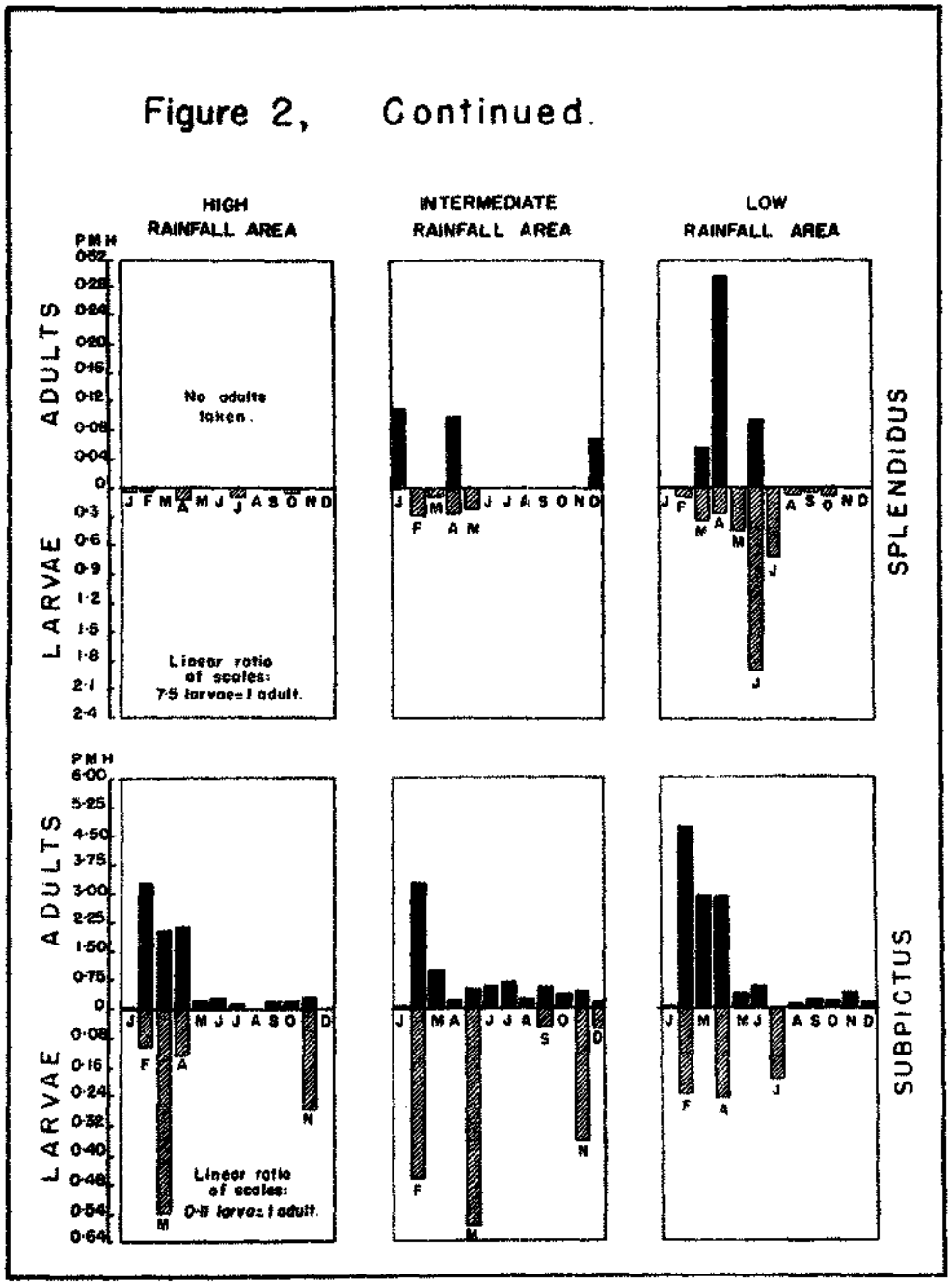


Figure 2, Continued.
HIGH RAINFALL AREA
INTERMEDIATE RAINFALL AREA
LOW RAINFALL AREA
PMH
ADULTS
LARVAE
No adults taken.
Linear ratio of scales: 7·5 larvae=1 adult.
SPLENDIDUS
Linear ratio of scales: 0·1 larvae=1 adult.
SUBPICTUS

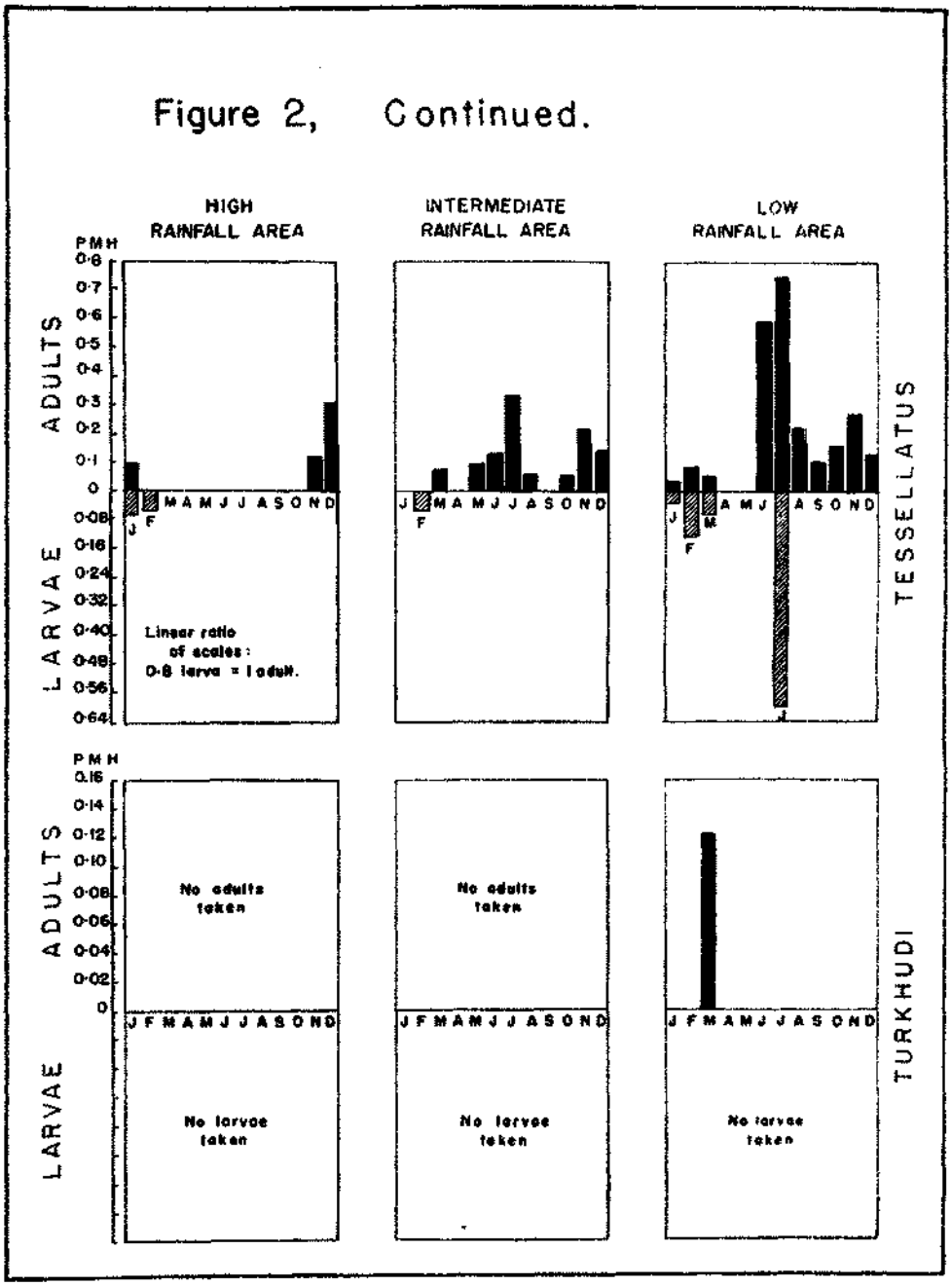


Figure 2, Continued.
HIGH RAINFALL AREA
INTERMEDIATE RAINFALL AREA
LOW RAINFALL AREA
TESSELLATUS
TURKHUDI
ADULTS
LARVAE
PMH
J F M A M J J A S O N D
Linear ratio of scales: 0·8 larva = 1 adult.
No adults taken
No larvae taken

Figure 2, Continued.

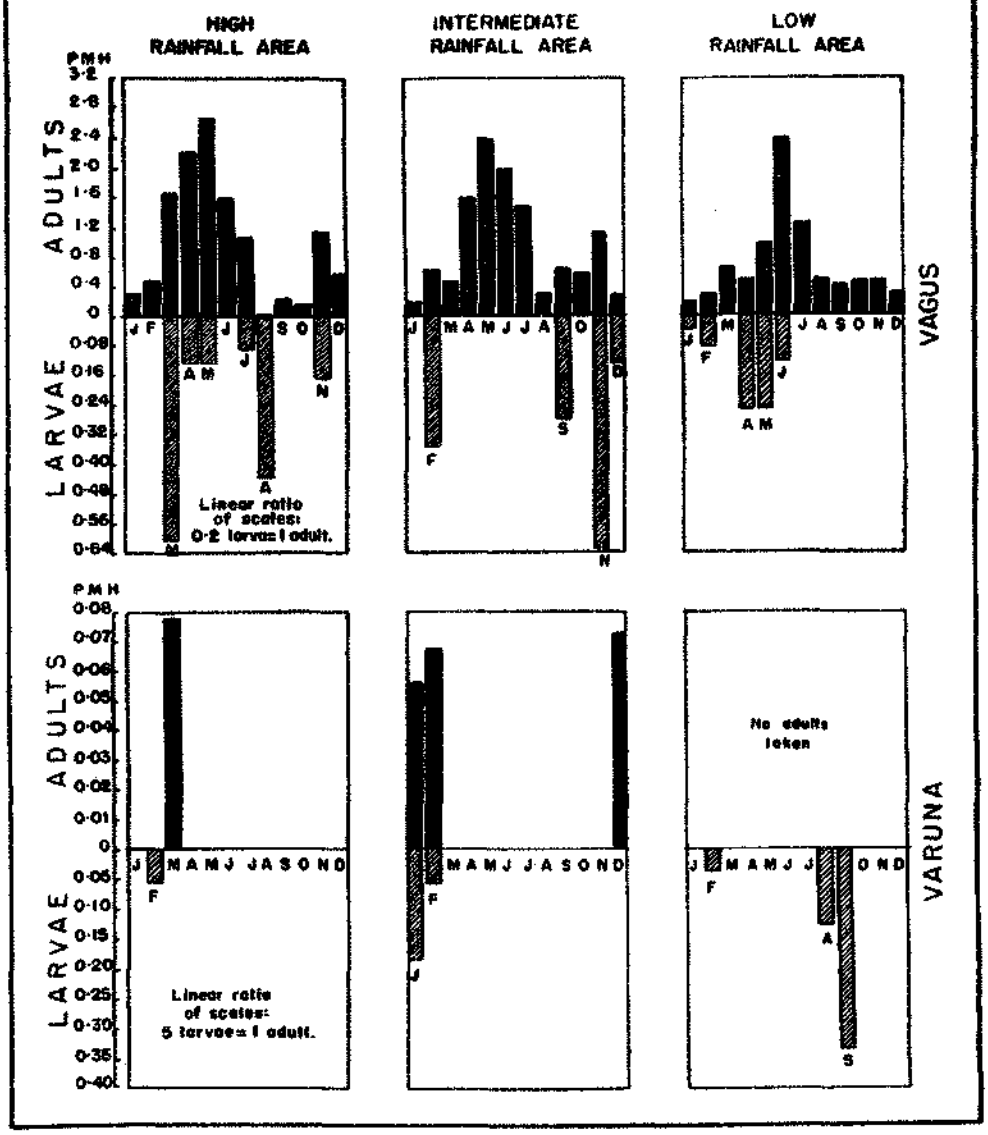


Table V.

Monthly total (T) and per man-hour (MH) incidences of larvæ collected in the high rainfall area. (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Aconitus ...	T									2			
	MH									0·100			
Aitkeni ...	T	115	27	16	19	13	10	5			1	3	42
	MH	7·118	1·421	1·067	2·375	1·625	1·250	0·455			0·043	0·167	3·238
Annularis ...	T		1	1	1			7			1		
	MH		0·053	0·067	0·125			0·636			0·043		
Barbirostris	T	25	145	78	1	9	3	10	65	253	278	233	50
	MH	1·563	7·632	5·200	0·125	1·125	0·375	0·909	4·063	12·650	12·087	12·944	3·846
Culicifacies ...	T		7										
	MH		0·368										
Fluviatilis ...	T	2	4	7		1	1					1	
	MH	0·125	0·211	0·467		0·125	0·125					0·056	
Hyrcanus ...	T	58	84	11				9	53	254	288	119	47
	MH	3·625	4·421	0·733				0·818	3·313	12·700	12·522	6·611	3·615
Insulæflorum	T					2	1					1	1
	MH					0·250	0·125					0·056	0·077
Jamesi ...	T	15	39	12		1	2	8	42	26	22	3	2
	MH	0·938	2·053	0·800		0·125	0·250	0·727	2·625	1·300	0·957	0·167	0·154
Jeyporiensis	T	43	231	122	10	10	8	24	8	5	6	13	24
	MH	2·688	12·158	8·133	1·250	1·250	1·000	2·182	0·500	0·250	0·261	0·722	1·846
Karwari ...	T	4	7	5									3
	MH	0·250	0·368	0·333									0·231
Leucosphyrus	T						1					1	
	MH						0·125					0·056	
Maculatus ...	T	1	1									1	
	MH	0·063	0·053									0·056	
Majidi ...	T	1	1	1									
	MH	0·063	0·053	0·067									
Pallidus ...	T	2			1			5		1	2		
	MH	0·125			0·125			0·455		0·050	0·087		
Splendidus ...	T	1	1		1			1			1		
	MH	0·063	0·053		0·125			0·091			0·043		
Subpictus ...	T		2	8	1							5	
	MH		0·105	0·533	0·125							0·279	
Tessellatus ...	T	1	1										
	MH	0·063	0·053										
Vagus ...	T			9	1	1		1	7			3	
	MH			0·600	0·125	0·125		0·091	0·438			0·167	
Varuna ...	T		1										
	MH		0·053										

TABLE VI.

Monthly total (T) and per man-hour (MH) incidences of larvæ collected in intermediate rainfall area. (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Aconitus ...	T	7	37										
	MH	0·364	2·176										
Aitkeni ...	T	40	19	17	13	35	1			2	1	14	34
	MH	1·818	0·118	1·417	1.625	3·500	0·167			0·111	0·048	0·737	2·125
Annularis ...	T			4		1					2		
	MH			0·333		0·100					0·095		
Barbirostris	T	125	124	51	2	2	5	8	89	151	223	97	159
	MH	5·682	7·294	4·250	0·250	0·200	0·833	1·333	5·563	8·389	10·619	5·105	9·935
Culicifacies ...	T	1	2	7	1								
	MH	0·045	0·118	0.583	0·125								
Fluviatilis ..	T	3	27	15	3	3							
	MH	0·136	1·588	1·250	0·375	0·300							
Hyrcanus ...	T	182	87	11				2	23	79	152	112	138
	MH	8·273	5·118	0·925				0·333	1·438	4·389	7·238	5·895	8·625
Insulæflorum	T					2							
	MH					0·200							
Jamesi ...	T	27	19	2				1	51	5	5	10	45
	MH	1·227	1·118	0·167				0·167	3·188	0·278	0·238	0·526	2·613
Jeyporiensis	T	134	89	20	36	33	6	11	97	47	3	5	16
	MH	6·091	5·235	1·667	4·500	3·300	1·000	1·833	6·061	2·611	0·333	0·263	1·000
Karwari ..	T		3										
	MH		0·176										
Leucosphyrus	T			3									
	MH			0·250									
Majidi ...	T			4									
	MH			0·333									
Pallidus ...	T	7	5	6					1	8	10	30	9
	MH	0·364	0·294	0·500					0·061	0·444	0·476	1·579	0·563
Philippinensis	T		2	5						79	57	18	11
	MH		0·118	0·417						4·389	2·714	0·947	0·688
Splendidus ...	T		5	1	2	2							
	MH		0·294	0·083	0·250	0·200							
Subpictus ...	T		8			6				1		7	1
	MH		0·470			0·600				0·056		0·368	0·063
Tessellatus ...	T		1										
	MH		0·059										
Vagus ...	T		6							5		12	2
	MH		0·353							0·278		0·632	0·125
Varuna ...	T	4	1										
	MH	0·182	0·059										

Table VII.

Monthly total (T) and per man-hour (MH) incidences of larvæ collected in the low rainfall area. (Western Mysore State).

		Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
Aconitus ...	T	1	7	8					2	1	2		1
	MH	0·033	0·280	0·500					0·125	0·042	0·063		0·056
Aitkeni ...	T	3											
	MH	0·100											
Annularis ...	T		4	3	7		11	5	1				
	MH		0·160	0·188	0·875		1·425	0·500	0·064				
Barbirostris	T	62	82	101	3	4	12	21	52	132	144	159	89
	MH	2·067	3·280	6·313	0·425	0·500	1·500	2·100	3·250	5·500	4·500	4·453	4·944
Culicifacies ...	T	3	4	3	3	1	1						
	MH	0·100	0·160	0·188	0·425	0·125	0·125						
Fluviatilis ...	T	64	75	33	7	7	10	15	13	7	1	16	13
	MH	2·133	3·000	2·064	0·875	0·875	1·250	1·500	0·813	0·292	0·031	0·457	0·724
Hyrcanus ...	T	106	61	20		1		13	14	118	196	225	125
	MH	3·533	2·440	1·250		0·125		1·300	0·875	4·917	6·125	6·429	6·944
Insulæflorum	T							2					
	MH							0·200					
Jamesi ...	T	18	46	15	3	1	8	30	7	15	20	14	22
	MH	0·600	1·840	0·938	0·425	0·125	1·000	3·000	0·438	0·625	0·625	0·400	1·222
Jeyporiensis	T	141	297	31	15	55	39	20	88	403	54	81	34
	MH	4·700	11·880	1·938	1·875	6·875	4·875	2·000	5·500	16·792	1·688	2·314	1·889
Karwari ...	T		3										
	MH		0·120										
Leucosphyrus	T		6	3									
	MH		0·240	0·188									
Maculatus ...	T		2							1			
	MH		0·080							0·042			
Pallidus ...	T	2	12	15	8	9	14	5	9	8	7	18	55
	MH	0·067	0·480	0·938	1·000	1·125	1·750	0·500	0·563	0·333	0·219	0·514	3·056
Philippinensis	T										4		2
	MH										0·125		0·111
Splendidus ...	T		2	5	2	3	15	7	1	1	2		
	MH		0·080	0·313	0·250	0·425	1·875	0·700	0·064	0·042	0·063		
Subpictus ...	T		6		2			2					
	MH		0·240		0·250			0·200					
Tessellatus ...	T	1	3	1				6					
	MH	0·033	0·120	0·064				0·600					
Vagus ...	T	1	2		2	2	1						
	MH	0·033	0·080		0·250	0·250	0·125						
Varuna ...	T		1						2	3			
	MH		0·040						0·125	0·333			

Figure 3. Ratios of annual per man-hour rates of capture of anopheline larvae to adults in areas of high, intermediate and low rainfall.

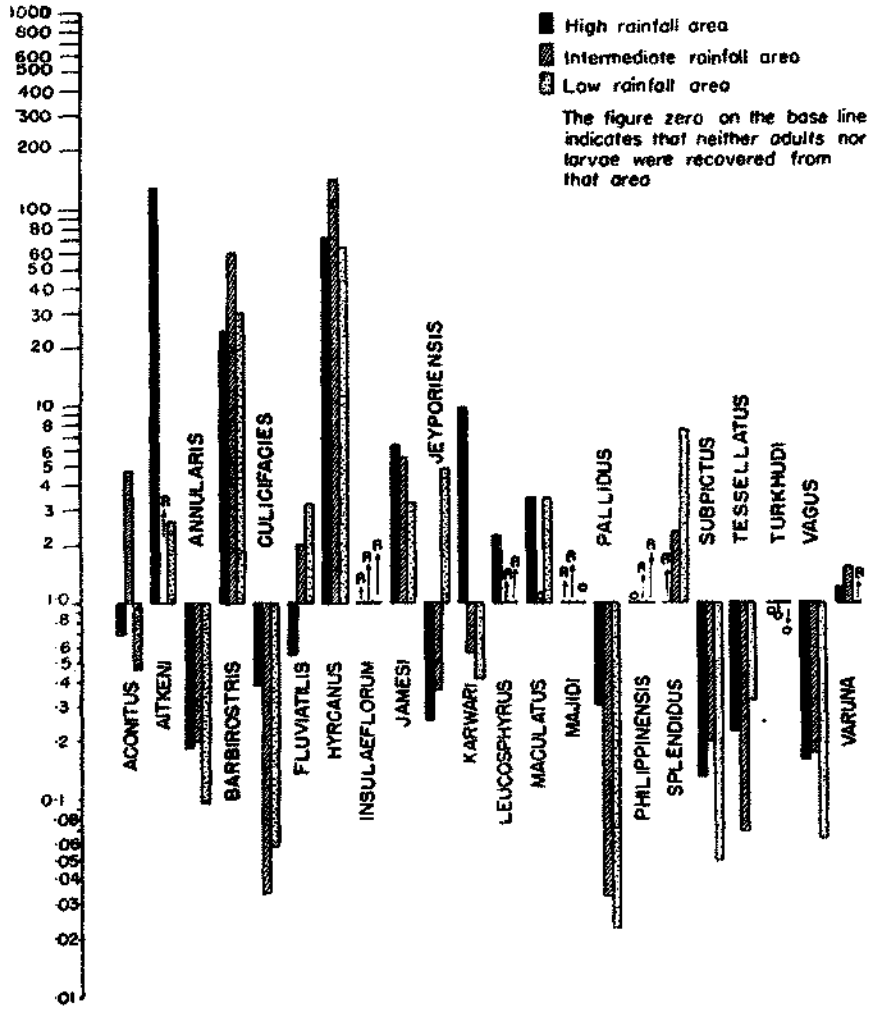


TABLE VIII.

Ratios of per man-hour larval to adult captures. Daytime collections made simultaneously. Data for one year (Western Mysore State).

Anopheline species.	Rainfall area.		
	High.	Intermediate.	Low.
Aconitus ...	0·69 : 1	4·6 : 1	0·46 : 1
Aitkeni ...	130 : 1	∞ : 1	2·6 : 1
Annularis ...	0·18 : 1	0·20 : 1	0·095 : 1
Barbirostris ...	24 : 1	60 : 1	30 : 1
Culicifacies ...	0·38 : 1	0·034 : 1	0·058 : 1
Fluviatilis ...	0·54 : 1	2·0 : 1	3·2 : 1
Hyrcanus ...	74 : 1	147 : 1	65 : 1
Insulæflorum ...	∞ : 1	∞ : 1	∞ : 1
Jamesi ...	6·4 : 1	5·5 : 1	3·2 : 1
Jeyporiensis ...	0·25 : 1	0·36 : 1	4·8 : 1
Karwari ...	9·8 : 1	0·55 : 1	0·41 : 1
Leucosphyrus ...	2·2 : 1	∞ : 1	∞ : 1
Maculatus ...	3·4 : 1	...	3·4 : 1
Majidi ...	∞ : 1	∞ : 1	...
Pallidus ...	0·30 : 1	0·033 : 1	0·022 : 1
Philippinensis ...	...	∞ : 1	∞ : 1
Splendidus ...	∞ : 1	2·3 : 1	7·6 : 1
Subpictus ...	0·13 : 1	0·20 : 1	0·048 : 1
Tessellatus ...	0·22 : 1	0·068 : 1	0·32 : 1
Turkhudi ...	...	...	0 : 1
Vagus ...	0·16 : 1	0·17 : 1	0·062 : 1
Varuna ...	1·2 : 1	1·5 : 1	∞ : 1

Larvæ.—Larvæ are not likely to shift from one habitat to another unless mechanically propelled by a flood. During such conditions, however, gravid mosquitoes may oviposit in unusual locations. Thus the routine dipping for larvæ in established collecting areas may at times give reliable information as to species prevalence but at other times only a confusing or negative indication. This consideration has been especially worrisome in the high rainfall area,—so

provocative indeed that much time has been spent—fruitlessly—in attempting to learn how some anopheline species that appear annually in that area survive the period of apparent (or real) absence during the south-west monsoon. This problem is discussed in another paper that is under preparation (Malaria Investigation Centre : Report on studies on *A. Fluviatilis*).

Any consideration of adult and larval anopheline captures in the boundaries of the present study must therefore be guarded by a realization of the limitations placed upon interpretation of data in each case. The following comments, on a species basis, are presented with these limitations in view.

One of the principal anomalies to arise in this study (and that must have arisen repeatedly in anopheline surveys elsewhere) is the one in which overall adult census figures consistently exceed larval enumerations. It does not require a Malthusian analysis to elucidate the principle that larvæ must always be more numerous than adults on an annual basis. In instances when adults are found in excessive numbers over a long time period, the only conclusions to be drawn are that larval populations are not being efficiently canvassed and/or that adult populations are being concentrated in collecting stations owing to their predilection for the hosts in those stations or to the attraction of the stations themselves as daytime resting places. Bates (1949) has given due stress to this limitation of collecting methods. A further inference is that adults may be living longer and hence accumulating in domestic shelters (*see* below).

This problem is of paramount significance. The conventional methods for making entomological malaria surveys, while time-tested and by no means brought into question by the present study, are fraught with the very errors that are brought out in the following paragraphs. A species-to-species analysis, and area-to-area analysis, in the regions within a few miles of Sakleshpur disclose that daytime collections of anopheline larvæ and adults frequently exhibit disparities that are contrary to expectation. Since ordinary entomological malaria surveys must be brief, it would seem that their chief value must be in revealing merely the presence and general distribution of established vectors ; the role of such vectors in a given region requires vast further study for elucidation.

Comparisons of adult and larval densities on the basis of per man-hour collections serve the purpose of disclosing the readiness with which the two forms may be found. But in addition to the fact that one stage may be more numerous than the other in their respective habitats, it is evident that the per man-hour comparison is itself mathematically invalid. For in the case of adults a visual method of collection is used, not feasible for larval dipping. Were it possible readily to see larvæ in their habitats, collecting would become at once far more efficient, for the entomologist could concentrate his efforts in the most densely populated portions of each habitat, including submerged regions in the cases of those larvæ in which surface-avoidance is an outstanding trait. The very fact that comparisons of adult and larval densities by these imperfect means reveal differences among various species, or among various geographic ranges of a given species, confronts the entomologist with proof of the invalidity of his collecting methods, while at the same time provides the malariologist with information that he can utilize

because the imperfect methods are part of his standard practice. The anachronism must stand until field collecting procedures are improved.

Adult longevity.—The following thoughts may also be expressed in this regard. During a static period so far as production of larvæ of an anopheline species is concerned, when methods of collecting larvæ and adults of that species remain constant, there may still be a conceivable variation of larva-adult ratios in response to climatic variations. This is in those cases when adult longevity is influenced by changes in temperature and/or humidity. The larva-adult ratio, based on per man-hour collections of both larvæ and adults, will show a higher proportion of larvæ if adult life is short ; this, superficially viewed, is what one should expect owing to a presumed fixed rate of mortality of larvæ, pupæ, emerging imagines and flying insects.

But when physical conditions favour the survival of adults, there may be a large building up of the mosquito population, despite a concomitant mortality rate. Since the fourth instar larva exists in this form for only about two days, but adult mosquitoes may live for more than twenty days, the larva-adult ratio may come to a figure that appears to contradict the known laws of nature. Hypothetical examples may be given as follows.

Let it be arbitrarily supposed that there is normally a mortality rate of twenty per cent per day at the stages in the life cycle of an anopheline species embracing fourth instar larvæ, pupæ and imagines, an assumption not out of keeping with the finding of Russell and Rao (1942) that the death rate of *A. culicifacies* in an outdoor cage was fifty per cent every two days. Then, if 1,000 fourth instar larvæ of this species are found today, it will be assumed that half of them would ordinarily pupate tomorrow and the other half on the day after tomorrow. In the meantime, enough third instar larvæ will moult to keep the fourth instar population constant at 1,000 from day to day. The pupal period is assumed to be two days, and adult life will continue about three weeks in those that survive that long. Thus the fate of a given batch of 1,000 fourth instar larvæ will be as follows :—

Day.	Stages present.		Numbers.		
1	4th Instar larvæ,	old	500		
	4th Instar larvæ,	new	500	Total	1,000
2	4th Instar larvæ,	old	400		
	Pupæ,	new	400	Total	800
3	Pupæ,	old	320		
	Pupæ,	new	320	Total	640
4	Adults,	new	256		
	Pupæ,	old	256	Total	512
5	Adults				410
6	,,				322
7	,,				258

Day.	Stages present.	Numbers.
8	Adults	207
9	,,	166
10	,,	133
11	,,	106
12	,,	85
13	,,	68
14	,,	54
15	,,	43
16	,,	34
17	,,	27
18	,,	22
19	,,	18
20	,,	14
21	,,	11
22	,,	9

Thus by the eighteenth day following the first emergence of mosquitoes, we are down to one per cent of the original batch of 1,000 larvæ. This is approximately in accord with current beliefs related to the natural longevity of mosquitoes.

However it is now necessary to remember that the following mosquito population will be in existence, despite the fact that only 1,000 fourth instar larvæ have been present on any given day. The original batch will have 9 survivors ; the following day's batch will have 11 survivors, and so on. The total number of adult mosquitoes on hand will be about 2,500 and the ratio of larvæ and pupæ to adults is therefore 0·4 : 1, a figure not out of keeping with some of the observed results at Sakleshpur, for example in the instance of *Anopheles jeyporiensis* in the intermediate rainfall area.

But let us now assume a set of conditions in which larval and pupal mortalities remain constant at twenty per cent, there still being 1,000 fourth instar larvæ present daily, but in which adult mortality suddenly rises to fifty per cent. On the fourth and subsequent days we shall have :—

Day.	Stages present.		Numbers.		
4	Adults,	new	180		
	Pupæ,	old	256	Total	436
5	Adults				218
6	,,				109
7	,,				55
8	,,				28
9	,,				14
10	,,				7

Thus it takes only seven days following the first emergence of adult mosquitoes for the original batch of larvæ to be reduced to one per cent. The total adult anopheline population on a given day, moreover, is only about 600, so that a reversal of the larva-adult ratio takes place, in this case being 1·67 : 1. With an even more greatly accelerated adult mortality rate in *A. jeyporiensis* the larva-adult ratio found in the low rainfall area, 4·8 : 1, could theoretically be achieved.

It is interesting to remember that in these two examples there has been no suggestion of a decline in the anopheline population, seasonally or otherwise : in both cases it is demonstrated how two populations might exist in a stable condition, although with different larva-adult ratios. (In the first case, with greater adult longevity, the restriction of fourth instar larval populations to 1,000 must be attributed to limitation of suitable breeding habitats or some other theoretical brake on the early larval stages.)

In practice this concept may be modified by the numbers of batches of eggs laid by females of different longevities, by different resting habits in response to seasonal variations in humidity, etc. Larva-adult ratios will be correspondingly modified.

A high larva-adult ratio may therefore sometimes be an indication of shortened adult longevity. In such cases the finding would be of significance to the transmission of pathogens requiring appreciable extrinsic incubation periods in mosquitoes. With respect to malaria, it might mean that a vector was able to take only one blood meal. The vector could therefore survive as a species, but it would be unable to accomplish the maturation and dissemination of plasmodial sporozoites.

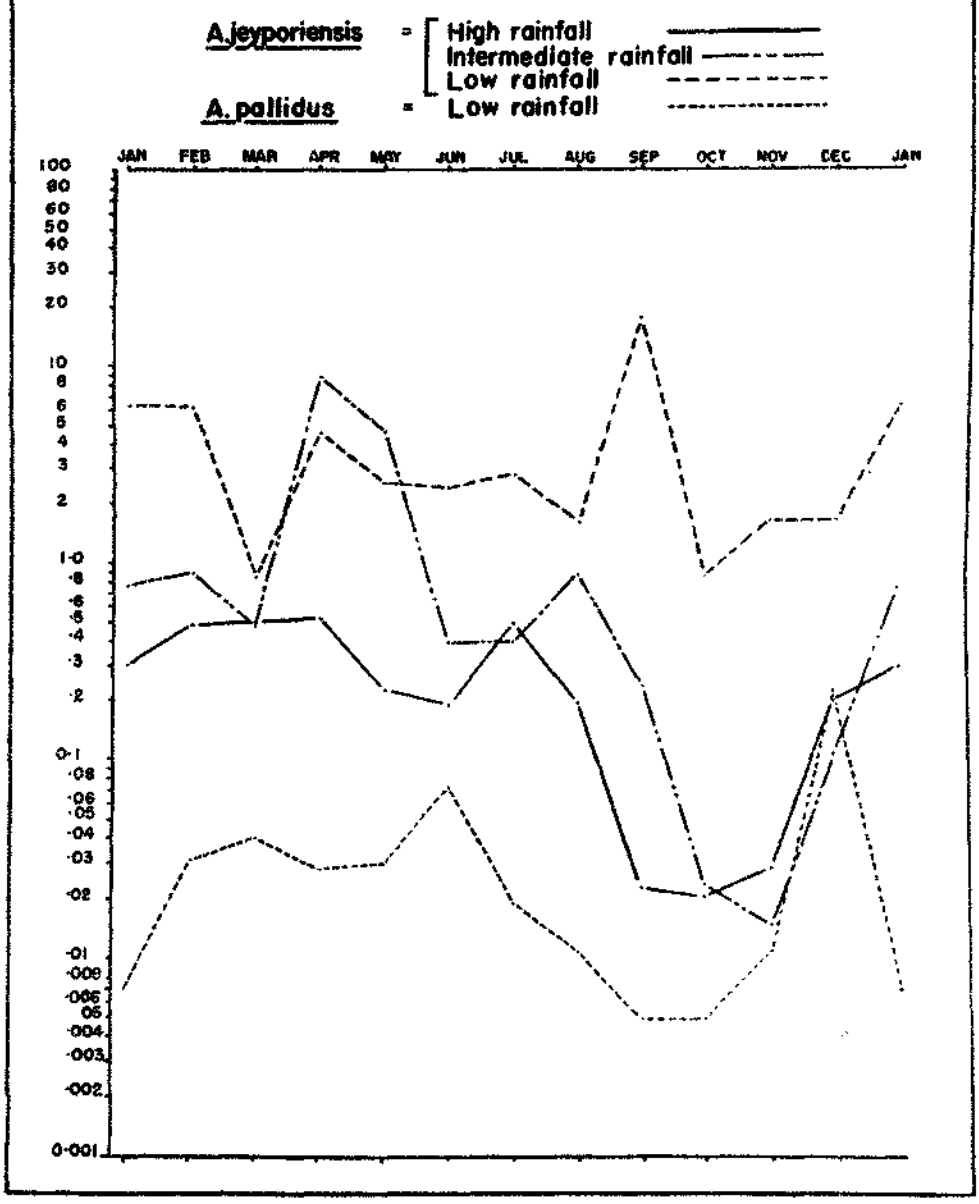
Finally, since larva-adult ratios, as already mentioned, reflect many diverse environmental factors besides climate (*i.e.*, adult and larval habitats and behaviour), it may be suggested that the greatest use of this figure is in the study of individual

species over periods of time. For example under conditions which appear to be constant, a rise in the larva-adult ratio after the spraying of a village with D.D.T. would suggest shortened adult longevity. A subsequent lowering of the ratio would suggest loss of residual potency of the insecticide. In the interests of economy, it might be more expedient to gauge respraying programmes on the possibilities for transmission than on the bald fact that infiltration of anophelines had been detected in the sprayed area. Studies could probably be devised locally to reveal a critical larva-adult ratio related to adult longevity compatible with malaria transmission. A saving might thereby be effected in either the number of rounds of spraying per year or the quantity of deposit adopted for adequate malaria control. Other methods of judging age of mosquitoes caught in the field are of doubtful validity in many cases, and in each instance require the services of a highly skilled technician, so that periodic determination of larva-adult ratios might in some situations provide approximate information that would be otherwise unavailable. Such studies could be made most fruitfully with the most abundant local house-invading anopheline species, regardless of whether it was the local vector or not, for they would give immediate and direct information as to the current status of the toxicity of the residual insecticidal deposit.

An attempt may now be made to test some of these theories against the actual data. If we select a species that occupies all three Sakleshpur areas in reasonable abundance, or select two species occupying the same area in reasonable abundance, we may first tabulate their monthly larva-adult ratios through the year (Table IX) or plot these values on a graph (Figure 4) and then see whether the resulting information is in accord with the known bionomics of the species concerned. For this purpose *Anopheles jeyporiensis* and *A. pallidus* will be utilized, the former in all the Sakleshpur areas and the latter in the low rainfall area. The reasons for this choice are twofold. First, the species were both found to occur in sufficient abundance in the areas mentioned to lend themselves to study on a monthly basis. Secondly, the adults were attracted to the collecting stations and remained within them during the daytime in good numbers, so that a fairly valid notion of adult abundance was obtained. Christophers (1933) states of *A. jeyporiensis* that it is "commonly taken in houses and cattlesheds", while *A. pallidus* exhibits similar behaviour but with special attraction to stables and cowsheds. As for larvæ, those of *A. jeyporiensis* were also taken in significant numbers in the three areas, but only few *A. pallidus* larvæ were encountered even in the stronghold of this species in the dry region. This scarcity was only apparent since breeding areas in ricefields and channels afforded opportunity for wide dissemination of the larval population. Since habitats of this species were extensively searched, the data on larvæ, although numerically small, probably reveal comparative population levels.

Anopheles jeyporiensis is found by such analysis to exhibit sharp difference in the three areas. Its larva-adult ratio is lowest, generally speaking, in the high rainfall area, reaching its minimum after the southwest monsoon. At this time there is plenty of water for breeding. Although the rains have ceased, the region is still wet ; humidity is relatively high and temperatures are moderate. Later in the autumn and through the winter and spring, breeding areas dry up, the

Figure 4. Monthly larva-adult ratios of two anopheline species



temperature rises and atmospheric humidity progressively decreases. Concomitantly the larva-adult ratio rises.

TABLE IX.

Monthly larva-adult ratios of two anopheline species. (Western Mysore State.)

Month.	*Anopheles jeyporiensis.*			*Anopheles pallidus.*
	Rainfall area.			Low rainfall area.
	High.	Intermediate.	Low.	
January ...	0·302	0·761	6·386	0·007
February ...	0·486	0·883	6·217	0·031
March ...	0·500	0·488	0·864	0·040
April ...	0·541	9·000	4·688	0·028
May ...	0·229	4·714	2·575	0·030
June ...	0·189	0·395	2·438	0·072
July ...	0·492	0·400	2·849	0·019
August ...	0·193	0·898	1·647	0·011
September ...	0·023	0·241	18·065	0·005
October ...	0·021	0·024	0·879	0·005
November ...	0·029	0·015	1·696	0·011
December ...	0·207	0·107	1·681	0·229

In the area of intermediate rainfall there is a similar reaction of the larva-adult ratio to the monsoon, although it is somewhat delayed, since full monsoon effects are felt earlier west of the Divide than in the intermediate rainfall area. The phenomenon of desiccation is more marked here than to the westward, being especially pronounced in the hot months of April and May, when the larva-adult ratio attains a high peak.

The area of low rainfall experiences the lowest humidity and the highest temperatures of these three regions, with fewer extreme variations during the course of a year. Larva-adult ratios are correspondingly higher and more uniform than in the two more western regions. The peak in September, 1951, was associated with the termination of the southwest monsoon.

Christophers (1933) indicates that *A. jeyporiensis* is generally distributed over the east and south of the Indian peninsula, but adds that it is common in the Jeypore Hills at 2,000-3,000 feet and on the Nilgiri Plateau at 6,000 feet. This

would suggest that the species is adapted, although not restricted, to areas of moderate or high rainfall.

The conclusion may therefore be drawn that the seasonal variations of larva-adult ratio of *A. jeyporiensis* within the three Sakleshpur areas, as well as the contrast observed among the three areas, result from variations in adult longevity as controlled by atmospheric humidity, adult life being longest at maximum saturation.

If we now compare larva-adult ratios of *A. jeyporiensis* with those of *A. pallidus* in the low rainfall area (Table IX and Figure 4), we find an interesting contrast. *Anopheles pallidus* seems to behave in an opposite manner to *A. jeyporiensis*, reaching a minimum ratio when *A. jeyporiensis* is at its maximum and more or less fluctuating in reciprocal fashion throughout the year.

Christophers (1933) says of *A. pallidus* that it has a wide distribution but makes special mention of its relationship to ricefields in Central India. It may therefore be presumed to be adapted to plain regions, which are notoriously hot and dry. Adult longevity in this species may therefore be less dependent on the humidity factor than in the case of *A. jeyporiensis*.

A direct corroboration of these findings can be derived from a glance at Table I in which it is seen that *A. jeyporiensis* adults were taken abundantly in the high rainfall area but in decreasing numbers in the progressively drier areas. The ratios are roughly 8 : 5 : 1. *Anopheles pallidus*, however, was captured with opposite frequency, its ratios being about 1 : 46 : 174.

But larval catches did not exhibit such wide differences. In the case of *A. jeyporiensis* they may be reduced to the approximate ratio of 2 : 2 : 5. For *A. pallidus* the larval ratio is 1 : 7 : 15. Thus it would appear that the aquatic environment of non-stream-breeding species such as these is inclined to be somewhat constant, whereas it is in the adult state that mosquitoes have most difficulty in adjusting themselves to varying external physical conditions.

In conclusion, in the two examples given, there appears to be evidence that larva-adult ratios are positively related to the longevity of adult mosquitoes. When future collections provide sufficiently high numbers of adults and larvæ of other species, similar analyses will be attempted in order to learn whether this manipulation of data gives valid information in all cases.

Adult and larval population curves.—According to accepted notions respecting mosquito populations, a large output of larvæ ordinarily precedes a large output of adults by a period of one to three weeks. If we plot the monthly per man-hour captures of *A. jeyporiensis* larvæ and adults in the high rainfall area on a graph (Figure 5), we find that this is not invariably the case. In fact there are two peaks of adult abundance, one of which is accompanied by a larval peak, and the other of which is not. Referring to Figure 4 and to the discussion of larva-adult ratios, we can say that the peak in February-March, when both stages were caught in abundance, occurred at a time of short adult longevity owing to the dry season. Although larvæ were common, they could not push adult prevalence to a higher figure because the adults died at almost the same rate as that at which larvæ were being produced.

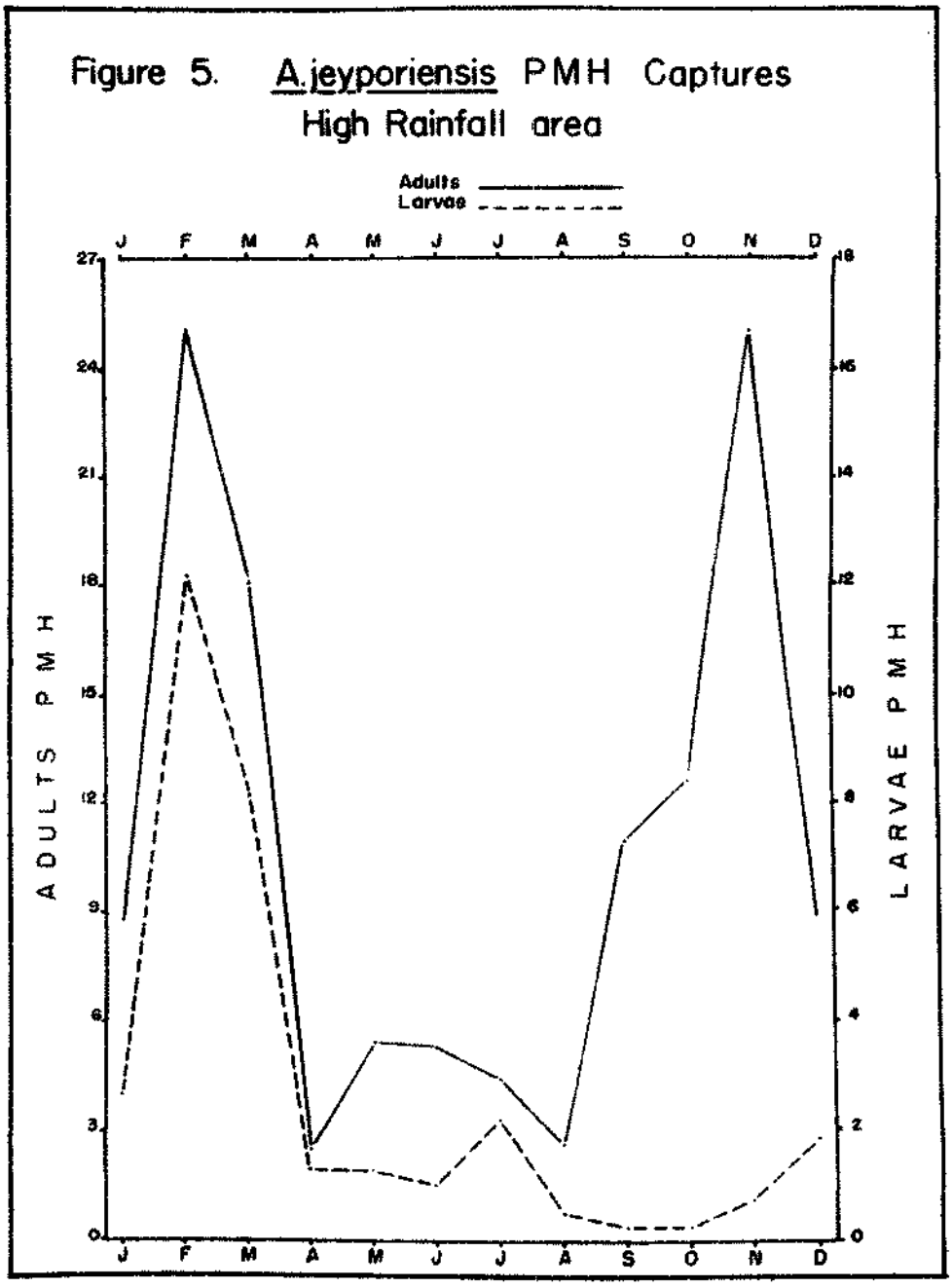


Figure 5. A.jeyporiensis PMH Captures
High Rainfall area
Adults
Larvae
J F M A M J J A S O N D
ADULTS PMH
LARVAE PMH
0 3 6 9 12 15 18 21 24 27
0 2 4 6 8 10 12 14 16 18

But in the case of the adult peak which reached its height in November, the lowest larval population of the year was nevertheless able to produce a second peak in adult abundance. At this season the weather was cool and damp, and adult longevity must have been at its maximum.

Figure 6 presents comparable information about *A. jeyporiensis* in the intermediate rainfall area. Adult abundance exhibits an almost perfectly smooth annual curve in relation to two seasons, the hot dry months of April and May, and the cool weather following the southwest monsoon and extending into the light northeast monsoon. Larval densities are marked by three peaks, none of which affects the smooth adult curve. As in the case of *A. jeyporiensis* in the high rainfall area, one larval peak coincides with a period of minimum adult population, although in this case it is in the dry period during April-May. Likewise in October and November the adult peak of the year is accompanied by the year's lowest larval densities. In this case, however, the adult peak has been preceded by a larval peak. Since these two maxima are separated by three months, they are not likely to be related in an immediate biological fashion, that is, the November mosquitoes are not solely August's metamorphosed larvæ. It appears more likely that the larval peak in August is only incidental, and that the adult peak in November represents principally a lengthened span of life of more recent imagines that are emerging from the current sparse larval population.

Figure 7, showing adult and larval per man-hour captures of *A. jeyporiensis* in the low rainfall area, repeats only some of the foregoing observations. A larval and adult peak in February, followed by a drop first in larvae and then in adults in the two succeeding months, has the appearance of a conventional wave of mosquitoes following a period of larval abundance. However, the subsequent larval peak during the hot month of May is unable to produce a comparable increase in the adult population. But the adult peak in October is again related to an antecedent peak of larval prevalence. Since it has already been suggested that this region is not optimal for *A. jeyporiensis*, there being the shortest adult longevity here, it is probably to be expected that adult peaks must be more closely related to larval abundance here than in situations more favourable for the prolongation of adult life.

Turning now to *Anopheles pallidus* in the low rainfall area, we find (Figure 8) that there is first of all a baffling situation resulting from the extreme disparity between per man-hour captures of adults and larvæ. It is nevertheless possible to observe that during the first five months of the year there is a rough parallel in the fluctuation of the two populations, but after this the trends are in opposite directions. Since this region has been assumed in the foregoing discussion to be favourable for *A. pallidus*, it is now not unexpected to observe that a low larval density from August through November is able to support the major adult peak of the year. Since this is the very time when *A. jeyporiensis* attains a peak in the same area only by virtue of maximal larval abundance, the contrast between the adaptation of these two species to a region of low rainfall is again emphasized.

As for the assumption that *A. pallidus* is adapted to less humid conditions than *A. jeyporiensis*, the evidence is not as clear in Figure 8 as it is in the depiction of larva-adult ratios in Figure 4. It is to be observed that there is a minor adult

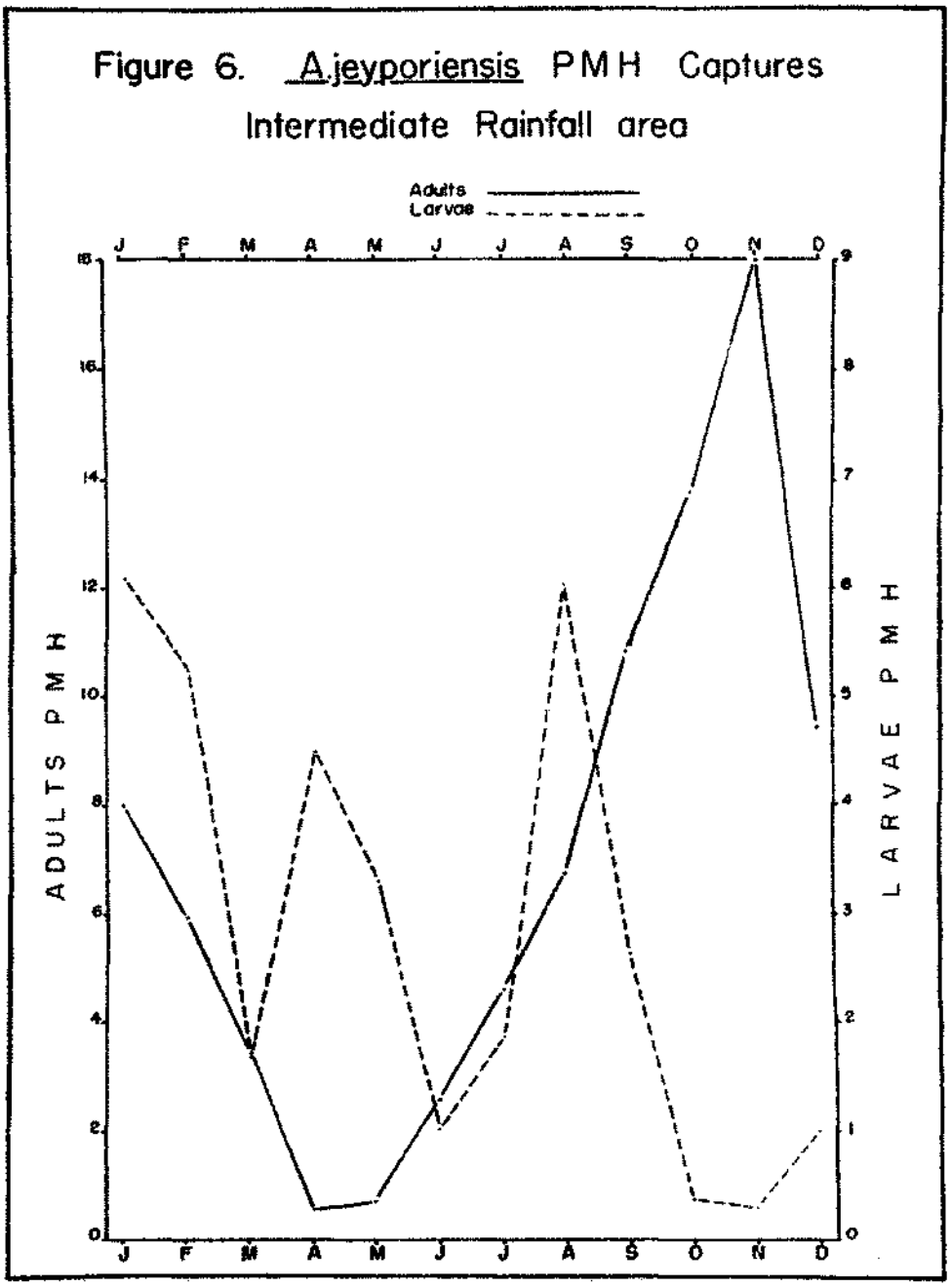


Figure 6. A.jeyporiensis PMH Captures
Intermediate Rainfall area
Adults
Larvae
J F M A M J J A S O N D
ADULTS PMH
LARVAE PMH
0 2 4 6 8 10 12 14 16 18
0 1 2 3 4 5 6 7 8 9

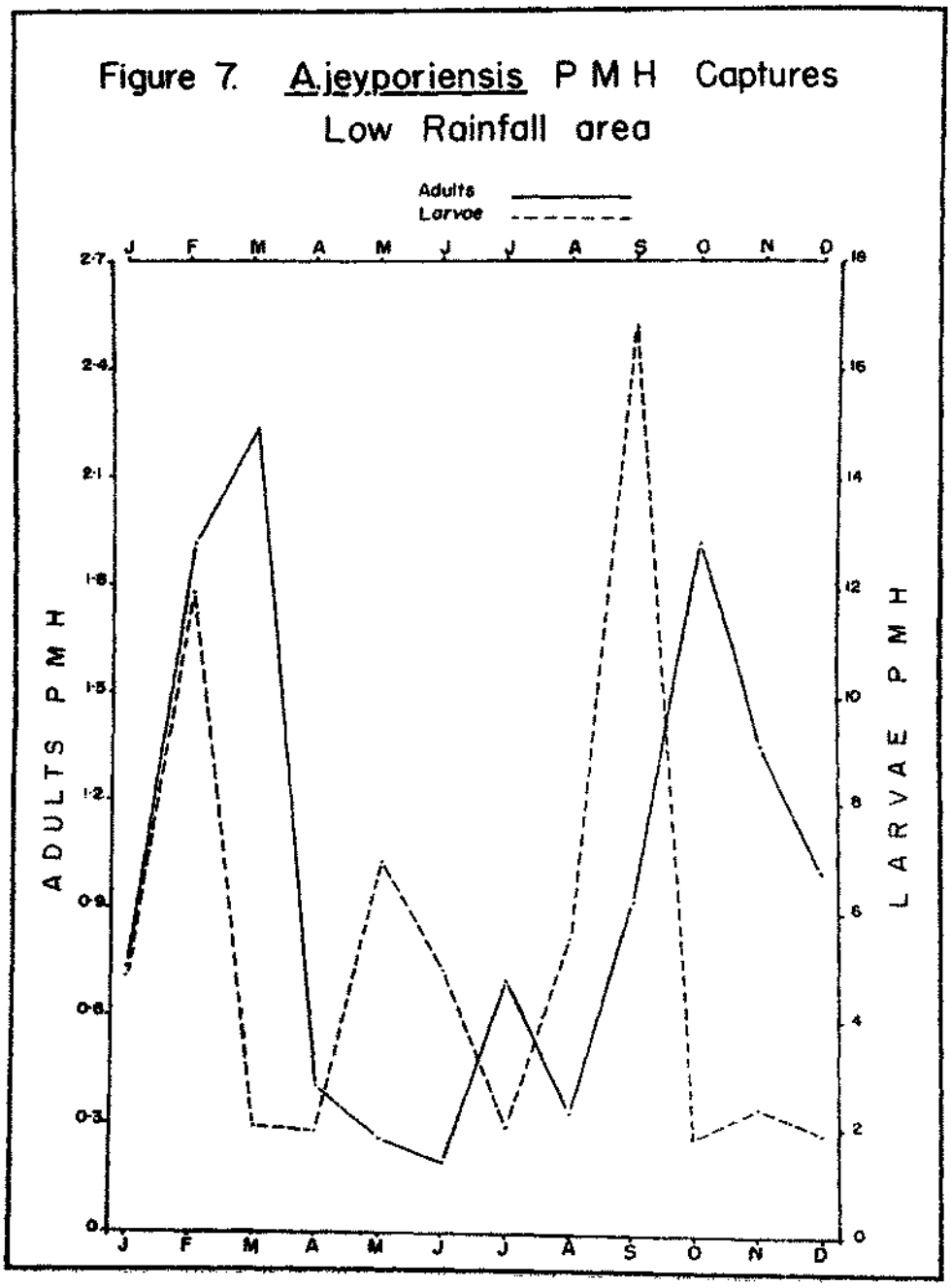


Figure 7. A.jeyporiensis P M H Captures
Low Rainfall area
Adults
Larvae
J F M A M J J A S O N D
ADULTS P M H
LARVAE P M H
0
0·3
0·6
0·9
1·2
1·5
1·8
2·1
2·4
2·7
2
4
6
8
10
12
14
16
18

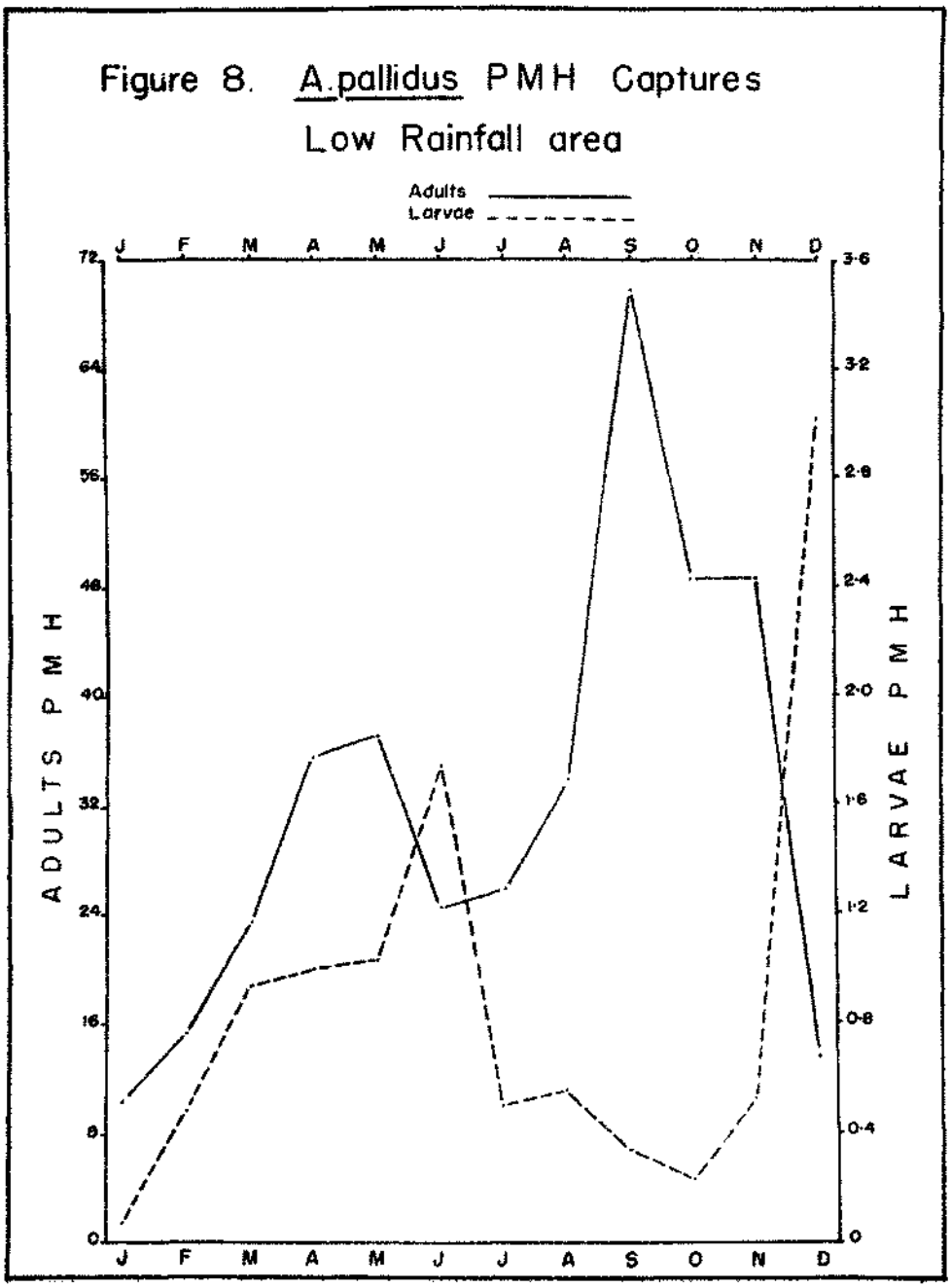


Figure 8. A.pallidus PMH Captures
Low Rainfall area
Adults
Larvae
J F M A M J J A S O N D
72
64
56
48
40
32
24
16
8
0
ADULTS P M H
3·6
3·2
2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
LARVAE P M H
J F M A M J J A S O N D

peak during the hot weather of April and May, a season when adult *A. jeyporiensis* populations are depressed to their lowest levels in each of the three Sakleshpur regions. The highest peak of *A. pallidus* adults, however, occurs in the post-south-west monsoon period, more or less in coincidence with peaks of *A. jeyporiensis* adults in the same area and in the two other areas. Therefore *A. pallidus* may be adapted to dry climates in the sense that it is able to withstand them more successfully than *A. jeyporiensis*, even though increased humidity favours longevity of *A. pallidus* just as in the case of *A. jeyporiensis*. Such a result cannot lead to any confusion, for it must be remembered that all mosquitoes are essentially similar in their reactions to heat and humidity, being universally killed at the extremes of either environmental factor, but merely reacting in slightly different ways at various points between these extremes.

In the hot, dry months of April and May, when several anopheline species may be detected as thriving larval populations, but adult catches in man-made structures are small, it has just been suggested that adult longevity is shortened. Another supposition should be considered, namely, that during this season the adults have changed their usual daytime resting places. The interior of cattlesheds become hot by midday, even though humidity may not decline correspondingly owing to the saturation of the earthen floor with animal urine. Is it possible that mosquitoes would then fly out in search of more hospitable daytime shelters ?

Only a tentative answer can yet be given to this question. Preliminary results of night collections of mosquitoes in the Sakleshpur villages indicate little numerical difference between night and day densities of those anopheline species which habitually rest in cattlesheds during daytime. Hence the small numbers of mosquitoes collected by day in the hot season may be presumed to reflect a not imperfect image of adult densities at that time. Therefore the idea of shortened adult longevity may still be entertained.

An advance sample of such data, gathered in 1952, may be presented in the case of *A. jeyporiensis* in the intermediate rainfall area. Table X and Figure 9 show the per man-hour catches of this species effected by : (1) daytime hand-catching at the designated stations in the villages of this area ; (2) night hand-catching in the same structures, plus additional hand-catches from animals tethered immediately outside the structures ; and (3) the conventional weekly larval survey.

It is seen at once that daytime adult and larval catches follow closely the pattern exhibited by this species during the hot April-May period in 1951 (Figure 6), i.e., there is larval preponderance, again suggesting abbreviated adult longevity.

Night catches of *A. jeyporiensis* within this period, and indeed in the periods preceding and following, duplicate the daytime adult curve to a remarkable degree, not only in pattern but in the absolute numerical sense. There can be no doubt that these figures refer to a homogeneous adult population, rather than to segregated fractions with different behaviours. It appears, therefore, that at least in this case the daytime catch within man-made structures continued to be a valid census method during the hot period of 1952. The uniform one-month leftward shift of the curve pertaining to night catches, while worthy of future

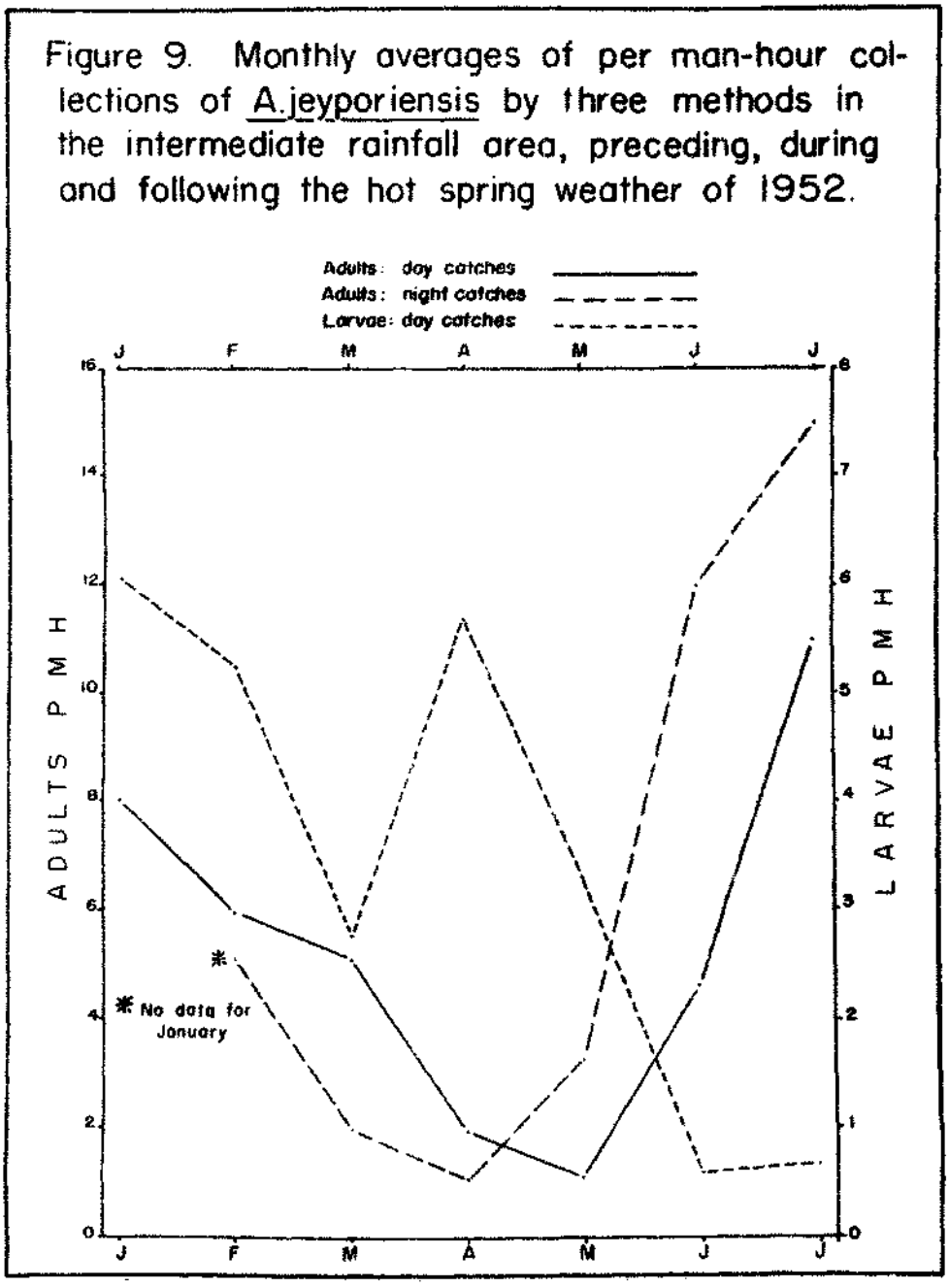


Figure 9. Monthly averages of per man-hour collections of A.jeyporiensis by three methods in the intermediate rainfall area, preceding, during and following the hot spring weather of 1952.
Adults: day catches
Adults: night catches
Larvae: day catches
J F M A M J J
ADULTS P M H
LARVAE P M H
0 2 4 6 8 10 12 14 16
0 1 2 3 4 5 6 7 8
* No data for January

study, is not germane to present considerations, since a longer period of time is being examined.

TABLE X.

Monthly averages of per man-hour collections of A. jeyporiensis *by three methods in the intermediate rainfall area, preceding, during and following the hot spring weather of* 1952 *(Western Mysore State).*

Month.	Adults per-man-hour.		Larvæ per man-hour.
	Daytime hand catches.	Night-time hand catches.	
January ...	8·000	...	6·041
February ...	5·932	5·042	5·235
March ...	5·043	1·962	2·750
April	1·875	1·030	5·667
May	1·059	3·244	3·273
June	4·348	12·000	0·583
July	11·000	15·600	0·667

It remains then only to question whether the apparent larval preponderance during the hot spring period is real. Since water collections are then drying up, larvæ may be concentrated within a relatively few habitats and therefore be collected in enhanced numbers. This argument can be met rather easily by pointing out that the even greater peak of *A. jeyporiensis* larval collections in August 1951 coincided with a period of abundant larval habitats, when the larvæ might well be expected to be so dispersed as to appear scarce. A study of larval catches in the other areas (Figures 5 and 7) reveals similar evidence that larval peaks and troughs cannot be consistently related to the current extent of suitable aquatic habitats. No sufficient reason can be advanced, therefore, to regard the larval census as unsatisfactory in the case of this easily-collected species.

In summary it may be ventured as a hypothesis that not all adult mosquito populations in the Sakleshpur area necessarily exhibit annual population cycles comparable to those observed in temperate zones. Adult peaks in abundance are based not on sudden explosive larva production, but on the existence of conditions favourable for adult survival. The lowest annual larval densities are capable of producing—or sustaining—the highest annual adult populations. Likewise a low adult density is capable of maintaining high larval populations.

These findings lead to two further observations : (1) The reproductive capacity of individual mosquitoes is sufficient, even in times of adult scarcity, to ensure species survival. (2) Larval mortality must be rather low, since small populations can nevertheless sustain high adult densities. The latter observation is a further

indication of the tropical character of the ecology of Sakleshpur mosquitoes, for there is suggestive evidence from other sources that predation may be of a lower order in the tropics than in temperate regions, *i.e.*, in certain studies on birds and mammals. A high survival rate in Sakleshpur mosquito larvæ, plus the fact that adult longevity is determined chiefly by climate, leads to the speculation that tropical anopheline mosquitoes in this part of India are not unduly troubled by predators at any stage of their life cycle. This is not to deny that larvivorous fish and aquatic insects take their toll of mosquito larvæ, but it does predicate that these enemies play only a negligible part in the regulation of Sakleshpur mosquito populations.

Influence of rainfall gradient.—While the rainfall gradient may have a deciding influence on the frequency with which anopheline larvæ or adults are recovered, numerous instances can be selected from the foregoing data in which the incidence of captures seems to have some other basis. Of the twenty-two species of Anopheles recorded from the Sakleshpur area, only thirteen were taken as both larvæ and adults from all three study areas. In the high rainfall area four of the thirteen species exhibited the highest larva-adult ratios encountered ; three species exhibited the lowest ratios ; and six species exhibited intermediate ratios. In the intermediate rainfall area the numbers of species exhibiting such maximum, minimum and intermediate ratios were six, two and five respectively, while in the low rainfall area the corresponding figures were three, eight and two (Table XI). There is no discernible trend in these results.

TABLE XI.

Distribution of maximum, intermediate and minimum per man-hour larva-adult ratios of thirteen anopheline species which occurred as both larvæ and adults in all three areas, excluding data from Table VIII involving infinity and zero (Western Mysore State).

Anopheline species.	Rainfall area.								
	High.			Intermediate.			Low.		
	Larva-adult ratio level.			Larva-adult ratio level.			Larva-adult ratio level.		
	Max.	Int.	Min.	Max.	Int.	Min.	Max.	Int.	Min.
Aconitus		×		×					×
Annularis		×		×					×
Barbirostris			×	×				×	
Culicifacies	×					×		×	
Fluviatilis			×		×		×		
Hyrcanus		×		×					×
Jamesi ...	×				×				×
Jeyporiensis			×		×		×		
Karwari	×				×				×
Pallidus	×				×				×
Subpictus		×		×					×
Tessellatus ...		×				×	×		
Vagus		×		×					×
TOTAL ...	4	6	3	6	5	2	3	2	8

Considering the species from the standpoint of their occurrence in all three areas as larvæ or adults, but not necessarily as both, it is found that six species consistently appeared predominantly as adults and eight species as larvæ, while four species were inconsistent in this respect (Table XII). This division appears to display some degree of rationality ; but the interpretation to be placed upon it cannot be one involving rainfall but must be concerned with larval habitats and adult behaviours.

TABLE XII.

Anopheline species in which the larva-adult ratio was above or below 1 : 1 in all three areas (excluding zero) (Western Mysore State).

Preponderance of adults in all areas.	Preponderance of larvæ in all areas.	Adult or larval (preponderance mixed in the areas).
Annularis	*Aitkeni*	*Aconitus*
Culicifacies	*Barbirostris*	*Fluviatilis*
Pallidus	*Hyrcanus*	*Jeyporiensis*
Subpictus	*Insulæflorum*	*Karwari*
Tessellatus	*Jamesi*	
Vagus	*Leucosphyrus*	
	Splendidus	
	Varuna	
6 species.	8 species.	4 species.

One of the remarkable findings of this analysis is that not more of the species displayed a regular decline or increase in larva-adult ratios correlated with the rainfall gradient, and moreover that there were three species that showed an increase in the ratio with increasing rainfall while an equal number showed a decrease (Table XIII). Of the remaining species for which sufficient data for analysis were available, no less than ten were inconsistent from this standpoint.

Since there is little virtue in the tabulation of data without their interpretation, one is tempted to essay a few remarks on these fundamentally unsatisfying results. Of course there is even less virtue in guesses that cannot be verified. But it would seem inescapable that the three Sakleshpur areas must be regarded as small macrocosms or large microcosms in themselves. Rainfall exerts its influence to be sure, but apart from that force there are local conditions that modify findings profoundly. This conclusion is not to be regarded with astonishment, however, for the region, with its twenty-two species of Anopheles, is obviously typical of its removal only thirteen degrees from the equator, and Darwin has long since clarified the relationship between multiplicity of inches and abundance of species

TABLE XIII.

Anopheline species showing regular decline or increase in larva-adult ratios in correlation with the rainfall gradient (Western Mysore State).

Increase of larva-adult ratio with increasing rain.	Decrease of larva-adult ratio with increasing rain.	Inconsistent species.
Jamesi	*Fluviatilis*	*Aconitus*
Karwari	*Jeyporiensis*	*Aitkeni*
Pallidus	*Varuna*	*Annularis*
		Barbirostris
		Culicifacies
		Hyrcanus
		Splendidus
		Subpictus
		Tessellatus
		Vagus
3 species.	3 species.	10 species.

in such tropical latitudes. With only small increments of migration by populations of the tropics, the sedentary majorities are prone to make strong local adaptations to conditions within limited environments. This question is taken up in the case of *A. fluviatilis* and the distribution of malaria in another paper (Malaria Investigation Centre : Report on studies on *A. fluviatilis*).

It has long been suspected that various anopheline species populations in India may comprise sibling species such as are exhibited by the *Anopheles maculipennis* complex in Europe. Studies designed to differentiate these cryptic species in India have been successful in very few cases and failures in others. It has then been postulated that distinct species may nevertheless exist as biological variants despite their morphological homogeniety. The present study may lend support to the latter contention in a more hair-splitting degree than has previously been considered. There may well be populations of given species of Anopheles in South India, living within a few miles of one another, but so far removed by ecological barriers as to live essentially different lives and to approach mutual reproductive isolation.

Comments on rare species.—Some anopheline species were taken so infrequently in the Sakleshpur region that their status cannot be analyzed on the basis of numerical records. Their very scarcity in routine collections is of interest, however, for one wonders whether they are really rare or whether the collecting methods

are at fault in these cases. If it is a matter of collecting methods, then one would like to know what techniques would disclose the species in their true abundance. If it is a matter of actual rarity, then one is curious to know how a species can exist from year to year in such small numbers. Quite probably some of the Sakleshpur records are concerned with one type of rarity and some with the other, that is with both apparent and real rarity.

A rare species in a general region might be expected to appear in collections in a sporadic manner in both time and space. Each small subdivision of the region might be populated by the species in some years but not in others. There might be continual extinction of isolated populations and continual reinvasion of formerly populated territories. The region as a whole, then, would be the scene of constant shifting of small population groups, so that a map showing the local distribution of the species would have to be equipped with a battery of small electric lights which blinked on and off constantly in an irregular way.

A species is rare throughout its range in some cases, resulting in a situation that may be most difficult to understand. More often rarity results because of lack of suitable environment in a given location, the species being abundant somewhere else. In the case of mosquitoes the needed environmental factors include those that may influence requirements at any or all stages of the annual cycle. Thus some of the Sakleshpur records demonstrate that high rainfall may be necessary to a species, which is therefore rare in the low rainfall area, and vice versa. But when a species is rare in all three areas, the riddle becomes more obscure. The lack of intraspecific competition that may be presumed in cases of rarity would suggest that there is no barrier to an increase of the species population. Possibly there is difficulty in finding mates, or perhaps swarms of males, if required for mating, cannot congregate in sufficient density to attract females. It must not be forgotten that rarity, as used in the present context, refers only to population densities as determined by entomological collections. In some cases a species may be more abundant than is suspected, and apparent rarity may then be at variance with other known facts about the species.

In any event it appears that the rare species continue to survive in the general area. Anopheline surveys were conducted for two decades at Mudigere, some twenty miles northwest of Sakleshpur, and the twenty-two species of Anopheles discussed in this paper were taken without exception also in the Mudigere region (Sweet and Rao, 1933). Preliminary results of continued work at Sakleshpur during 1952 indicate that the rare species encountered in 1951 will again all be collected, not merely in the general area but in the same village groups as before. Thus there appears to be a highly potent force uniting a rare species to its environment, and the distributional map with its flashing lights may be an inexact figure : the lights may burn constantly, or only infrequently blink here or there. This, in fact, accords better with the concept of sedentary populations in the tropics.

Comments on individual species.—While the tables and figures included in this paper give information as to the distribution and abundance of twenty-two anopheline species in the Sakleshpur region, a few comments should be made to expand the bare data. This will now be done briefly in the cases of those species which have not already been more thoroughly discussed.

1. *Anopheles aconitus* Dönitz.—While this species must be classified as rare in the high rainfall area, its virtual absence is inexplicable. Christophers (1933) records it as widespread in the Oriental Region, while Puri (1941) lists it from all the subdivisions of the Indian peninsula. Christophers adds that it " normally occurs at moderate altitudes ; recorded by Mangkoewinoto in Java at 2,800 feet ". It would thus appear to be adaptable over a wide range of habitats.

The reputed readiness with which *A. aconitus* enters man-made structures and feeds on animals or man, and the lack of specialized larval habitats, probably account for the fact that adults and larvæ were taken in fairly comparable numbers in the intermediate and low rainfall areas.

The chief evidence for an annual cycle in the populations of this species is found in the absence of both larvæ and adults from collections in all three areas during the hot season in April and May. It would thus appear likely that adults are sensitive to high temperatures and low humidity.

2. *Anopheles aitkeni* James.—The sporadic capture of adults is in accord with the statement by Christophers (1933) that *A. aitkeni* " is a wild and shy species, not frequenting houses or cattlesheds ". The more profuse larval captures reflect, at least in part, the special thoroughness with which streams were canvassed for larvæ.

This species also has a wide distribution in the Orient, but it favours hill regions, being " very abundant in the Nilgiris at 6,000 feet ", while at Shillong it occurs " especially toward the end of the cold season " (Christophers, 1933). The capture of most of the Sakleshpur larvæ in the area of high rainfall coincides with the first quotation, while the concentration of captures during December and January suggests that the population of *A. aitkeni* has an annual cycle in this area that is also correlated with cold weather.

3. *Anopheles annularis* van der Wulp.—The larva-adult ratio of this species in the three Sakleshpur areas is of the " *pallidus* " type as in the foregoing discussion. This probably is a reflection of the wide variety of habitats in which larvæ will develop freely, as listed in Christophers (1933). It may also represent a concentration of the adult population in man-made structures, for the same author cites many references to the strong attraction of *A. annularis* toward cattle.

Like *Anopheles pallidus*, furthermore, this species exhibits a predilection for the low rainfall area. The peak of its annual adult population cycle falls earlier in the year, however, occupying the late winter months preceding the hot weather of April and May. Dense larval populations in the latter period are not accompanied by comparable numbers of adults, so that longevity of the imagines must then be drastically abbreviated.

The maximum seasonal prevalence of *A. annularis* does not coincide with the period of rice cultivation in any of the Sakleshpur areas. This finding is parallel to that of Chang, Watson and Chow (1950) in southern Formosa, although they found a peak of abundance of the species in autumn rather than in spring.

4. *Anopheles barbirostris* van der Wulp.—Regarding *A. barbirostris*, Christophers (1933) states that " in the Indian area it is not a common house species ".

Preliminary results of recent night hand-catching and window-trapping experiments at a cattleshed in the low rainfall area of Sakleshpur indicate that *A. barbirostris* enters to feed and then leaves the shelter. In three selected tests, 33 mosquitoes of this species were caught in the window-trap at night, two were taken in night hand-collections, but none was encountered in day collections. The 33 specimens constituted 7·25 per cent of the total anopheline catch in the trap. However, during the one-year study period covered in this paper, the incidence of this species in daytime collections was only 0·7 per cent of all the anophelines caught. These findings suggest that Christophers' remarks probably apply to daytime collections of mosquitoes.

The high larva-adult ratio is in accord with a low rate of daytime resting indoors. On the other hand there are some special reasons why larvæ were collected in such abundance. The breeding places are easily accessible to larva collectors, and despite instructions to concentrate on stream collecting, the collectors were often tempted to dip in the stagnant margins of a conveniently located tank or other collection of still water. This was not only easier but more likely to result in greater collections so that the employee could make a good showing. Furthermore *A. barbirostris* larvæ are large and conspicuous, so that they were not likely to be overlooked.

There is some concentration of records of this species in the high rainfall area, but it is not marked. Annual peaks of adult populations are not readily surmised from the data, but the evidence in the case of larvæ would suggest that the species flourishes in greatest abundance during the latter six months of the year with a peak in September-October. *Anopheles barbirostris* would thus fall into the category of mosquitoes reacting unfavourably to heat and low humidity.

5. *Anopheles culicifacies* Giles.—Christophers (1933) characterizes this anopheline as being " ordinarily a plains species ". Its scarcity in the high rainfall (Malnad) area of Sakleshpur may be attributed to this trait. It is probable also that suitable larval habitats are less frequent in hilly terrain, for there is less opportunity for the collection of quiet pools or slowly moving bodies of fresh water in such well-drained areas.

The larva-adult ratio is again of the "*pallidus*" type, and widely disseminated larvæ and zoophilic adults are again the explanations of the phenomenon. However, we observe in the case of *A. culicifacies* a distinctly different pattern in the annual cycle of adult populations. The hot period in April-May, the bugaboo of so many other anopheline species, seems to leave adult *A. culicifacies* populations not only untouched but actually enhanced. Peak levels in the intermediate rainfall area occur from February through May, and about a month later than this in the low rainfall area. Seasonal larval abundance is somewhat better correlated with the status of adult populations than in most of the foregoing species, so that a more characteristic " temperate zone " curve is exhibited.

Adult longevity is not demonstrably affected during hot weather. The chief hazard to be faced annually by this species would appear to be a serious decimation concomitant with the southwest monsoon, possibly as the result of

the flooding out of larval habitats. Whatever its cause, the depletion is so great that adult populations are not appreciably restored until early in the following year.

In the absence of actual field readings of temperature and humidity, it is impossible to compare the foregoing suggestive evidence regarding longevity of *A. culicifacies* in the Sakleshpur region with the experimental results obtained by Rajindar Pal (1943) and by Russell and Rao (1942). But there does appear to be a discrepancy between the Sakleshpur experience and the report of the latter investigators, who found the shortest survival time of this species in an outdoor cage to be related to high temperature and low humidity. Possibly their results would have been more in accord with those presented here if they had kept their cage in an occupied cattleshed, especially since *A. culicifacies* characteristically rests in such shelters during the day.

Furthermore in none of the Sakleshpur areas can *A. culicifacies* prevalence, adult or larval, be correlated with the annual practices of rice cultivation, since the major incidence of the species occurs in the months when paddy fields are not only fallow but also devoid of standing water. This phenomenon is probably related to the fact that irrigation does not exist on a large-scale basis such as in some of the extensive river and canal schemes elsewhere in India. For example Russell and Rao (1941) observed a parallel between rice cultivation and the abundance of *A. culicifacies* in south-eastern Madras, a region where irrigation was widespread for eight months of the year. The lowest adult densities were seen during the remaining four months, "from April to early June", when most of the habitats were dry. It would seem that conditions at Sakleshpur are totally different and that the single annual rice crop dependent on the southwest monsoon is too small a factor to influence the bionomics of the species in this region.

6. *Anopheles fluviatilis* James.—The status of this species in the Sakleshpur areas will be discussed at length in another paper (Malaria Investigation Centre : Report on *A. fluviatilis*). Suffice it to say here that it displays irregular larva-adult ratios in the different areas, but that in no case is there a wide departure from parity. However, since larvæ of this species were searched for most intensively, this ratio should probably be lowered to put the species in proper contrast with its sister anophelines.

It is in the case of *A. fluviatilis* especially that the question of daytime resting places must be raised in connection with adult collections made exclusively in man-made structures. This factor will not be discussed here except to point out that adult densities may be considerably greater than indicated by the data under review in this paper.

The correlation between seasonal larval densities and adult populations is good in the intermediate and low rainfall areas. Total annual abundance was greatest in the low rainfall area, which is contrary to the generally accepted notion, well expressed by Christophers (1933), that *A. fluviatilis* is distributed "especially in foothill areas and hilly or rocky tracts".

The annual peak of adult populations, in the area as a whole, is from the first of the year through at least March. The April-May hot spell is attended by decreased but persisting adult catches ; hence, if these catches reflect truly the

relative abundance of the species, some ability of the adults to withstand desiccation is apparent. The most striking feature of the annual cycle of the species in all its stages in the high and intermediate rainfall areas is its virtual disappearance following the onset of the southwest monsoon and its continued absence through the remainder of the year. This phenomenon has been recorded also at Mudigere (Sweet and Rao, 1933), and may be attributed, at least during the monsoon, to the flushing of stream habitats. Where such flooding is less extensive, as in the low rainfall area, *A. fluviatilis* populations may be detected, although in lowered densities, during the monsoon and post-monsoon periods.

7. *Anopheles hyrcanus* var. *nigerrimus* Giles.—The larva-adult ratio of this species is similar to that of *A. barbirostris*, owing to the infrequency with which adults are collected in man-made structures and, again, to the distribution of the conspicuous larvæ in easily accessible habitats.

Seasonal prevalence of adults and larvæ is also closely similar to that of *A. barbirostris*, and correlation of larval with adult populations during periods of abundance is generally possible. The number of adults collected is too small to permit a statement as to their reactions to heat and dry weather. There is a remarkable absence of adults from February through August in all three areas, although larvæ are scarce or absent only from April through June. Adult and larval peaks appear to occur in general from September through December, with their maximum in October-November. However, these peaks are seen somewhat earlier in the high rainfall area than in drier terrain. Since rice cultivation also is begun sooner in the wettest region, there is an indication that in Sakleshpur *A. hyrcanus* also "breeds especially in association with rice-fields" (Christophers, 1933). Indeed many of the larvæ were obtained from this habitat.

8. *Anopheles insulæflorum* Swellengrebel and Swellengrebel de Graaf.—This is a mysterious species in that no adults were recognized, although it is possible that females might have been mistaken for *A. aitkeni*. A further peculiarity lies in the fact that larvæ, although taken in all three areas, were found almost exclusively in May, June and July. Puri (1941) notes that larvæ of *A. insulæflorum* and *A. aitkeni* are both found in "shady pools along forest streams". In the Sakleshpur areas this description is apt in the case of *A. aitkeni*, but *A. insulæflorum* larvæ were usually taken along the edges of streams more in the open, often not far from larval habitats of *A. fluviatilis* and *A. varuna*.

The feeding behaviour and annual population cycle of *A. insulæflorum* remain unknown, although the detection of this species in all three study areas, with their diverse climates, suggests that this may be a widespread mosquito.

9. *Anopheles jamesi* Theobald.—The breeding of this species in "tanks and seepage pools with grass growing along the edges", as expressed by Puri (1941), was confirmed in the Sakleshpur areas. Christophers (1933) is able to cite only one reference to adult behaviour, *viz.*, "Found in houses and stables (Ramsay; Sweet)". The preponderance of larval over adult captures suggests that *A. jamesi* either enters houses infrequently or else does not utilize structures as daytime shelters. This impression is sustained by results of window-trapping and night hand-catching. In the preliminary experiment already mentioned, 39 *A. jamesi*

were taken in the trap at night, 56 by the night hand-collectors, but only four by the daytime hand-collectors.

Annual prevalence of larval and adult populations of this species is marked by two seasonal peaks as shown in Figure 2. The months of April and May are accompanied by a decline of both segments of the population. The southwest monsoon period is marked by increased prevalence of larvæ, although a corresponding adult population develops only in the low rainfall area. September and October are the time of a second general decline of the species, but after this there follows a five-month period of steady prevalence.

The annual cycles are remarkably similar to one another in the three Sakleshpur areas. It would seem that *A. jamesi* must be well adapted to the region. Similarity of the three cycles would suggest that local populations exist, each of which has adjusted its inherent specific traits to the annual climatic conditions that prevail in the given areas.

10. *Anopheles jeyporiensis* James.—The type form of this species has already been discussed. The finding of *A. jeyporiensis* var. *candidiensis* Koidzumi, 1924, in the high and low rainfall areas of Sakleshpur is of importance even though only three specimens were recognized. Where such a variety is outnumbered by the typical form to the extent exhibited here, one is tempted to suspect that the variety is not in any sense a race, but only an uncommon recessive genetic combination. It then becomes interesting to read that " all specimens from eastern India, as distinct from the Peninsular area, showed the palpal markings as described for the variety ", while specimens from South India resembled the type form (Christophers, 1933). Here we seem to have an instance in which the genetic potentialities for evolution within a species are being expressed in one region but not in a contiguous region, and in which the evolutionary change is based not on mutation but on mendelian selection. This sort of evolution would not lead rapidly to reproductive isolation and might, in fact, eventually be nullified by chance merging of the typical and variant populations.

It is likely that the term, " variety ", as now used by students of mosquitoes, covers a number of categories, ranging from a simple uncommon recessive genetic character to an almost complete biologic departure from the main stock, *i.e.*, the threshold of identity as a new species.

11. *Anopheles karwari* James.—The rarity of *A. karwari* in the Sakleshpur areas has no demonstrable basis. This is a wide ranging species in the Oriental Region, with South India as part of its stated range (Christophers, 1933). Puri (1941) indicates that larvæ are found in " seepage pools and small fresh water springs with vegetation along the edges ", a statement that has been verified in the present studies. There is no lack of such habitats in the Sakleshpur area. Christophers (1933) reports that adults have been found in houses and cowsheds and will feed on man.

Why *A. karwari* should then exhibit such erratic patterns of prevalence (Figure 2) is a puzzle. That it was found as both larvæ and adults in all three areas indicates that it is adapted to all types of the local climatic conditions. But adults and larvæ were not found in the same seasons. In all three areas adults

appeared in July and August, tapering off through September in the intermediate rainfall area and through October in the low rainfall area. Larvæ, on the contrary, were found only in February in the latter two areas, while in the high rainfall area they occurred from December through March.

The larva-adult ratios were inconsistent in the three areas, but total numbers of both stages collected were so small that this is not significant. It may be questioned whether *A. karwari* is as domestic in the Sakleshpur region as one is led to assume from Christophers' sketchy comments, for if it were, it would probably be more abundant here. One suspects that the species may have some other specialized requirement which is not satisfied in this part of India.

12. *Anopheles leucosphyrus* Dönitz.—The classification of this mosquito as a wild jungle species (Christophers, 1933) is applicable at Sakleshpur. The paucity of records renders a discussion of annual cycles impossible. Its capture in all three areas is significant from the standpoint of distribution and adaptability to diverse climates, but its rarity precludes the possibility of malarial vectorship as displayed by the species in Borneo (unless revised collecting methods at Sakleshpur alter the present belief in its scarcity there). Possibly different adult collecting methods would reveal it as more abundant than here shown, but this is not likely since the larval survey should in that case have made such a disclosure.

13. *Anopheles maculatus* Theobald.—Although *A. maculatus* is reputed to have a wide distribution, it occurs in peninsular India "notably in the Nilgiris" and is "not commonly found in the great alluvial plains" (Christophers, 1933). Hence its rarity in the Sakleshpur region may indicate that the areas studied are not sufficiently montane in character. The two adult records, and five of the six larval records, occurred in the four relatively cool months from November through February. Although *A. maculatus* was not collected in the intermediate rainfall area, its presence in the two others indicates that it is a generally distributed but sparse mosquito in the region.

14. *Anopheles majidi* McCombie Young and Majid.—Sakleshpur is in the known limited range of this species in southwest India, but *A. majidi* must nevertheless be classified as one of the rarest species in the region studied. The absence of adults in collections suggests that it may not enter or remain in man-made structures. Since only seven larvæ were found during an entire year, however, it may be dangerous to draw such an inference. All the larvæ were taken from January through March, three in the high rainfall area, four in the intermediate area and none in the low rainfall area. Hence *A. majidi* may be characteristic of cool and more humid regions.

15. *Anopheles pallidus* Theobald.—Populations of this species have already been contrasted with those of *A. jeyporiensis* in the low rainfall area (Figures 4, 7 and 8). It was proposed in the accompanying discussion that *A. pallidus* adults are able to survive hot dry periods with a measure of considerable efficiency.

It is therefore of interest to note the sharp gradient in the reduction of annual *A. pallidus* populations as one progresses to areas of higher rainfall (Figure 2). In the intermediate rainfall area there is a peak of adults and larvæ that builds up and declines from September to January, coincident with the single rice crop dependent on gradual run-off of surface and seepage water after the southwest

monsoon. In the high rainfall area, run-off is so rapid that rice is cultivated during the monsoon itself, when all mosquito breeding is drastically limited by the rapid movement of all waters. Hence no favourable time occurs during the year for *A. pallidus* larvæ in the heart of the Ghats.

There is a minor indication that conditions are also less favourable for adults of *A. pallidus* in areas of higher rainfall than in drier regions, for a rise in the larva-adult ratios toward moister zones is seen, indicating progressively shorter adult longevity (Figure 3). If this is significant, it means that *A. pallidus* is not merely able to tolerate decreased humidities but is positively adapted to some degree of that condition. Such an adaptation would indeed fit it for life in the Indian plains.

16. *Anopheles philippinensis* Ludlow.—Although adults of *A. philippinensis* have been found " in houses, cattlesheds and stables " (Christophers, 1933), only larvæ were encountered during the Sakleshpur studies. The ones in the intermediate rainfall area were taken abundantly from a small tank close to houses and cattlesheds, so that the absence of adults in collecting stations in this village signifies their reluctance to enter or remain in such shelters. Possibly the species feeds on pigs or other animals that are not carefully housed at night. In line with what has been said about larva-adult ratios in relation to adult longevity, the absence of adults of this species in collections may also reflect a highly abbreviated span of imaginal existence, although if this were the only factor involved, one would expect to have recovered at least one or two adults from shelters.

The seasonal peak of *A. philippinensis* larvæ in this village in September, with a decline through the next three months, corresponded with gradual depletion and stagnation of water in the small tank.

The absence of *A. philippinensis* from the high rainfall area is probably related to the lack of suitable collections of still water for tenure by larvæ.

Larvæ were too rare in the low rainfall area to give an indication of the local status of the species, although they were found at a time contemporaneous with larval prevalence in the intermediate rainfall area. Since records of *A. philippinensis* are rather scattered in India and the Oriental Region, it is possible that this mosquito is distributed patchily in response to the need for some peculiar environmental requirements that exist in suitable combination only here and there.

17. *Anopheles splendidus* Koidzumi.—The classification of this species as " a northerly Oriental form " and its distribution from " extreme northwest (India) to Upper and Lower Burma and South India " (Christophers, 1933) would indicate adaptation in at least part of its range to arid or semi-arid climates. The prevalence of *A. splendidus* in greatest concentration in the low rainfall area of the Sakleshpur region, plus its maximum abundance there during the hottest season of the year, tend to confirm such an impression. Christophers (1933) states that adults are " found, usually in small numbers, in houses, outhouses, cowsheds, etc.". In the brief window-trapping experiment already alluded to, there was an indication that this species normally utilizes outdoor resting places, for ten mosquitoes were found in the trap, two were taken by the night hand-collectors, but none by the day hand-collectors. Thus Christophers' " small numbers " in

shelters, and the acutal paucity of adult specimens in the year's study at Sakleshpur, may be at least partially accounted for.

The rather sparse collections of *A. splendidus* in the whole area, as well as its decline along a gradient of changing climate, are characteristic of a species at or near the edge of its range. In this case the inference is that *A. splendidus* should be found more commonly in the adjacent plains, unless its entire hold on South India is only tenuous.

18. *Anopheles subpictus* Grassi.—The geographical and seasonal distribution of *A. subpictus* in the Sakleshpur region is rather uniform, as may be seen in Figure 2. Larva-adult ratios are low in all three areas, no doubt owing to the wide dispersal of larvæ in almost all sorts of habitats and the readiness with which adults enter houses and cattlesheds (Christophers, 1933). In northern Indiath is species is uncommon before the monsoon, whereas in South India it is said to occur "more or less throughout the year" (Christophers, 1933). While the latter statement is technically correct when applied to the Sakleshpur region, the data indicate that there is a late winter and early spring peak of abundance, with a maximum in February, in all three areas and that adult populations are sharply reduced during the rest of the year.

Some ability of *A. subpictus* adults to withstand lowered humidity can be observed in the high and low rainfall areas during April, but populations decline in all the areas during the ensuing hot month. This species must be regarded as an adaptable one, considering its wide range ; its adjustment to divergent climates at Sakleshpur is therefore not remarkable.

19. *Anopheles tessellatus* Theobald.—Christophers (1933) is of the opinion that *A. tessellatus* is a more or less domestic species, although he cites one investigator, Stookes, who " seldom found it in houses, even though larvæ were abundant ". The low larva-adult ratios in all three Sakleshpur areas tend to support Christophers' view. But window-trapping results in the low rainfall area of Sakleshpur tend to confirm Stookes' observation : 153 specimens were taken in the trap, 29 by the night hand-collectors, and only five by the day hand-collectors.

The wide range of this species through India and the Orient indicates its adaptability to various conditions, but a rather strong predilection for the low rainfall area of Sakleshpur is nevertheless shown. Here *A. tessellatus* achieves peak adult abundance during the southwest monsoon, prevailing at lower levels thereafter and disappearing in the hot pre-monsoon months of April and May. A secondary adult peak occurs in November, possibly this time in relation to the northeast monsoon.

In the intermediate rainfall area *A. tessellatus* likewise exhibits an adult peak during the southwest monsoon, although this is of smaller magnitude. The secondary November peak is discernible here also. In the high rainfall area the summer peak is altogether wanting, if one may judge this on the small numbers of specimens collected ; only the later annual up-swing is represented.

This type of yearly and distributional cycle is unique among the twenty-two anopheline species of the Sakleshpur region. Other species that are most prevalent in the low rainfall area experience peaks of adult population at times

of the year when the benefits—if such they are—of a dry climate may be more obviously utilized. It appears contradictory for a species to adapt itself to the rainy season of a dry area when rain is to be had in greater profusion in adjacent terrain. Nevertheless a suggestive paragraph in Chang, Watson and Chow (1950) may point to the explanation of this behaviour. In southern Formosa, these workers also noted peaks of prevalence of *A. tessellatus* during the rainy season, apparently because of the requirement of pools by larvæ. When the rains stopped, the pools dried up and adult density diminished. In the Sakleshpur areas, pools develop and are maintained better in rolling lands on the fringe of the monsoon than among the drenched hills. Christophers (1933), however, lists numerous alternative larval habitats, so this may not be the whole story. A need for studies of the ecology of *A. tessellatus* populations is apparent.

Larval habitats listed by Christophers (1933) and Puri (1941) are varied. In the Sakleshpur area larvæ were found so inconsistently that it is not possible to generalize as to their habitats. Therefore no conclusion as to adult longevity can be drawn from larva-adult ratios. The seasonal fluctuation of adult populations nevertheless indicates relatively low tolerance for heat and dryness.

20. *Anopheles turkhudi* Liston.—The collection of only two adult specimens within an entire year reflects the uncommon status of this species at the southern limit of its range. Christophers (1933) implies that it is characteristic of, although not restricted to, plains regions. Hence its occurrence in the low rainfall area at the beginning of the hot season is logical. However, as already mentioned, *A. turkhudi* has been recorded at Mudigere (Sweet and Rao, 1933) ; this is an area of higher rainfall. Since adults are said to be caught in houses (Christophers, 1933), the scanty records from Sakleshpur probably reflect true rarity of the species. The failure to detect larvæ in the region is further confirmation of such status.

This is an outstanding example of the puzzle presented by a rare species. It will be of interest to learn whether *A. turkhudi* continues in the future to present itself in the form of isolated specimens, and if so, why.

21. *Anopheles vagus* Dönitz.—This mosquito, like *A. subpictus*, has a wide geographical range in India and the Orient, as well as a wide variety of larval habitats. Like *A. subpictus*, it also seems well adapted to the three Sakleshpur areas. But in a study of Figure 2 it is possible to conclude that *A. vagus* is the better adapted of the two species, particularly as annual rainfall increases. This is apparently a result of the ability of *A. vagus* to withstand the hot weather of April and May, when its adult populations actually reach their peak in the two more western areas despite the dry weather at that time. In the low rainfall area the species has greater difficulty, for desiccation is more extreme, but it is still able to maintain respectable adult densities compared with many other local anophelines.

Adult annual population cycles in the three areas present an almost uniform appearance. Minor differences consist of two variations in the low rainfall area where the summer peak is reached a month later than in the moisture zones and where a secondary peak in November fails to appear.

Larvæ were collected in only small numbers. While their occurrence seemed at times to be correlated with adult prevalence, at other times the reverse association obtained. The only statement that can be made concerning larva-adult ratios is that they reflect, again as in the case of *A. subpictus*, the diversity of niches occupied by larvæ and the frequency with which adults enter man-made structures.

22. *Anopheles varuna* Iyengar.—The species is rather puzzling because of its apparently discontinuous prevalence in the Sakleshpur region. Obviously the data are incomplete. Its occurrence in all three areas indicates a general distribution, but the records themselves suggest either scarcity or only localized abundance. Further work is required to establish the status of *A. varuna* in Sakleshpur.

Other species.—It is likely that other anopheline species will be recorded from Sakleshpur, since *A. minimus* and *A. stephensi* are known from Mudigere, while *A. annandalei* was found recently in the north-western part of Mysore State. The Western Ghats at their highest elevations in Mysore have not been surveyed for anopheline mosquitoes. Presumably such species as *A. gigas* and possibly *A. lindesayi* exist there, and any of these might eventually appear as stragglers in Sakleshpur, particularly in the high rainfall area. Such events would only add to the list of rare species of this region. The invasion of hitherto unrecorded species from the plains, however, might lead to successful colonization of the low rainfall area.

As facilities for travel and the number of travellers in India increase over the next few decades, the transport of mosquitoes over long distances may become commonplace. Mechanical transference of this sort could involve not only the appearance of new species in a region but also the introduction of new strains of already indigenous species. The concept of sedentary tropical populations, existing as reproductively isolated groups, would then become obsolete. Perhaps the first indication of the admixture of genes from such new strains would be a departure from established annual population cycles. A formerly rare species might be enabled to become common, or a species that was formerly not anthropophilic might assume a form of behaviour leading it to become a malaria vector. The need for foreknowledge of the bionomics of currently rare or non-vectorial anophelines can be stressed on this theoretical basis in addition to the more practical reasons mentioned here and there in the preceding pages.

SUMMARY.

1. The anopheline fauna in a hill region of western Mysore State, India, was surveyed from the middle of March, 1951, to the same date in 1952. Three areas near Sakleshpur were chosen for study because of the sharp rainfall gradient along which they were situated.

2. Entomological methods employed were the conventional ones utilized in malaria reconnaissance, namely the daytime collection of mosquitoes from houses and cattlesheds in villages and the recovery of larvæ from adjacent aquatic habitats. Records of both types of captures were reduced in so far as possible

to a per man-hour basis. Although this was an admittedly rough way of collecting and manipulating data, the uniformity of field methods and mathematical treatment resulted in the possibility of making a number of revealing correlations.

3. Twenty-two species of Anopheles were encountered in the region. Analyses of the annual cycle of each species were made from the standpoints of: larval prevalence and habitats; adult prevalence and daytime resting places; ratio of larval to adult abundance on annual and seasonal bases; relation of the species to intensity of rainfall; relation of the species to the dry season; and significance of the combined findings in relation to the status of the species in the region and in its several climatic subdivisions.

4. The bulk of the findings was presented in the form of suggestions, since there were no experimental data beyond rainfall readings and the date and place of collection of individual larval and adult mosquitoes. Numerous patterns in the findings recurred sufficiently constantly to lend the suggestions considerable interest.

5. The larva-to-adult ratio of per man-hour captures, when reduced to monthly averages and plotted against the year's climatic cycle, suggested that some anopheline species are characterized by adults that have greater ability to withstand desiccation in hot weather than those of other species. This ratio was found to be applicable to various species with known predilection respectively for both dry and moist climates.

6. The use of the larva-adult ratio as an indicator of longevity of adult anopheline populations in areas sprayed with residual insecticides was advocated.

7. The generally non-migratory characteristics of tropical populations were thought to be exhibited by some of the anopheline species encountered. This attribute was reflected in the lack of a wave of larval abundance as the invariable precursor of a comparable eruption of adults. The larval population remaining essentially constant, a wave of adults was then considered to result from a change in environmental conditions favouring increased adult longevity.

8. The implications of sedentary tropical anopheline populations in relation to the evolution of local genetic strains was discussed. The significance of rare species was also considered.

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RECENT ADVANCES IN SYNTHETIC ANTIMALARIALS 1948-1952.*

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(June 30, 1953.)

CONTENTS.

Recent advances in different groups have been arranged in the order of (i) introduction, (ii) chemical studies and relationship of their chemical structure to biological activity, and (iii) studies on drug metabolism and other physico-chemical observations.

INTRODUCTION.

PAMAQUINE (plasmoquine) (III), the first synthetic remedy for malaria was discovered in 1926 and was followed by mepacrine (atebrin) (II) in 1931. Till 1939, the tempo of work on synthetic antimalarials was leisurely and even mepacrine and pamaquin had only received limited trials. During the World War II,

*Revised to include publications available up to June 1953.

the German workers had pushed their work on quinolines to resochin (X) (chloroquine) and sontochin, and both these drugs were being clinically tried. During the same period American workers examined about 14,000 compounds, out of which nearly hundred drugs, derived from various chemical groups, were tested against human malarias (Wiselogle, 1946 ; Bami *et al.*, 1947*a*). This was apart from their extensive pharmacological, parasitological and biological investigations on various aspects of malaria chemotherapy. These studies revealed the true potentialities of mepacrine, confirmed the utility of pamaquin as a curative drug, and established chloroquine as a suppressive antimalarial of high potency. Amongst drugs similar to pamaquin, pentaquin and isopentaquin proved equally active and relatively less toxic. The British investigators studied nearly 1,700 compounds belonging to hitherto unexplored fields of pyrimidines, guanidines and biguanides. Their extensive studies till 1947 resulted in the discovery of M3349 and paludrine (proguanil) (XII) (Curd and Rose, 1946 ; Bami *et al.*, 1947*a* ; Rose, 1951 ; Davey, 1951).

This short account of work does not adequately summarise the total chemical and biological data obtained but most of the work indicated above has been published and well reviewed apart from the fact that new drugs discovered till 1947, have been clinically evaluated and either accepted or rejected according to their merits (Coatney and Cooper, 1948 ; Coggeshall and Craige, 1949 ; Field, 1949 ; Cooper, 1949 ; Fairley, 1949 ; Jaswant Singh, 1949 ; 1950 ; Davey, 1951 ; Findlay, 1951 and Coatney *et al.*, 1953). The object of the present paper is to review the latest developments in the field of antimalarials from 1948 to 1952 from chemical, physico-chemical and certain biochemical aspects.

The new entrants to the field of synthetic antimalarials during the period under review are camaquin (XI), primaquin (VI), daraprim (pyrimethamine) (XVI) and lapinone (M2350) (XVIII). Camaquin, a drug structurally similar to chloroquin (X) has been confirmed as a potent suppressive antimalarial while primaquin has proved to be highly curative with very low toxicity when compared to other 8-aminoquinolines, like pamaquin, pentaquin and isopentaquin (Edgecomb *et al.*, 1950). Daraprim, a new 2 : 4- diaminopyrimidine derivative (XVI), is being introduced as a suppressive with low toxicity but it is too early to give a final opinion about its utlimate utility for the treatment of human malaria (Hitchings *et al.*, 1952 ; Jaswant Singh, Ray, Basu and Misra, 1952 and Jaswant Singh, Ray, Basu and Nair, 1952). Extensive chemical and biological investigations on naphthoquinones by Fieser, Berliner *et al.*, (1948) resulted in the discovery of lapinone (M2350) (XVIII) which has not utlimately competed so well with other well-known antimalarials (Fawaz and Haddad, 1951). Latest development in the field of natural drugs is the discovery of a highly active alkaloid from a common garden plant hydrengea (Baker, Schaub, McEvoy and Williams, 1952) whose chemical structure is identical with that of febrifugine (I), an alkaloid from *Dichora febrifuga* Lour (Koepfli, 1950 ; Baker, McEvoy, *et al.*, 1953*a*). Chemical and pharmacological investigation on chemical structures allied to paludrine, chloroquine, daraprim, mepacrine, lapinone and febrifugine have not offered a compound so far which could compete with the parent drug at clinical level. However, during these studies many interesting observations have been made regarding the relationship of biological activity with chemical structure.

The synthesis of new antimalarial remedies has been more or less empirical, in spite of the fact that existing knowledge regarding relationship of chemical structure to activity was of considerable help. It was thought, therefore, that a better understanding with regard to mechanism of drug action and route and manner by which they are degraded *in vivo*, could be helpful to the synthetic chemist in his quest for better antimalarials. In this connection degradative studies with pamaquin have established at least one of the possible routes of its breakdown *in vivo* (Josephson, Taylor *et al.*, 1951 ; Josephson, Greenberg, *et al.*, 1951), while similar studies with paludrine have offered highly active triazine compounds (XIV) (Carrington *et al.*, 1951 ; Crounse, 1951 ; Crowther and Levi, 1953). Synthesis of pentaquin (IV) labelled with radio-active isotope N^{15} at different positions (Elderfield *et al.*, 1953 ; Blatt and Gross, 1953) has been accomplished and degradative studies with radio-active materials have yielded interesting results (Elderfield and Smith, 1953). It may be mentioned that p-bromo analogue of paludrine (bromoguanide) labelled with radio-active bromine has also been used in certain absorption and excretion studies by Crowther and Levi (1953). *Naphthoquinones*, acridines, and *4-aminoquinolines* have also been similarly studied with varying degree of success.

Study of physico-chemical properties of various antimalarials in relation to their biological response usually gave inconclusive results (Gage, 1949*a* : 1949*b* : 1949*c* ; Hammick and Mason, 1950*a* : 1950*b*).

The progress in the fields of tissue culture of plasmodia (Hawking, 1951) and metabolism of malaria parasites (Gieman, 1948 ; Fulton, 1951) has been well reviewed. These two aspects could also give clues for the synthesis of potential antimalarials based upon chemical structures of the nutritional requirements of the parasites but the progress in this direction has been slight. Discovery of a rodent malaria parasite *P. berghei* (Vincke and Lips, 1948) aroused considerable interest regarding its use as a test parasite for new potential antimalarials (Goodwin, 1949 ; Thurston, 1950). Results obtained with *P. berghei* usually vary from those obtained with other strains but its utility for rapid screening and confirmation of the data obtained with other plasmodia is now well established. Recent discovery of pre-erythrocytic stages of various human and experimental malaria parasites has been a notable achievement which has greatly helped to understand the chemotherapy of malaria from this background (Shortt, 1950).

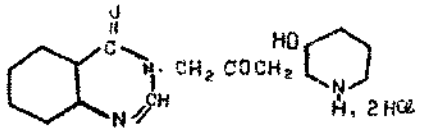
The problem of drug resistance in malaria chemotherapy has increasingly come into prominence. Although it has been easy to produce drug-resistant strains of various plasmodia in the laboratory, acquiring of resistance to well-known drugs by human parasites in the field has neither been easy nor frequent (Bishop, 1951). Another interesting aspect of resistance has been the discovery of cross-resistance between various drugs such as paludrine and daraprim to a particular malaria parasite which could be explained on the basis of structural similarities and hence similar mode of action of drugs involved. A relieving feature of the phenomena of resistance and cross resistance is that paludrine and daraprim resistant strains, which are comparatively easily produced, can be treated with drugs of acridine and quinoline groups satisfactorily (Jaswant Singh, Ray, Basu and Nair, 1952 ; Schmidt and Genther, 1953).

Considering the amount of work done during the last five years it could be said that degradation studies on various antimalarials still remain to be completed and deserve vigorous tackling. As regards synthetic investigations, pyrimidines, triazines, biguanides and quinolines can be fruitfully further investigated in the light of what has already been achieved.

ALKALOIDS AND RELATED COMPOUNDS.

In spite of greater emphasis on synthetic antimalarials, cinchona alkaloids and alkaloids derived from various other vegetable sources have been successfully investigated during the period under review. Alkaloids obtained from an evergreen Chinese shrub " Chang Shan " (*Dichroa febrifuga* Lour ; indigenous to South Western China and India) have been extensively investigated and recently febrifugine (I) (Koepfli, 1950), the active principal of this plant, has been found to be chemically identical with the alkaloid obtained from common garden plant hydrengea (Baker, Schaub, McEvoy and Williams, 1952 ; Baker, McEvoy *et al.*, 1953*a*). This alkaloid (I) has shown high antimalarial activity but low chemotherapeutic index, which limits its usefulness (Hewitt *et al.*, 1952). For instance, febrifugine is hundred times as active as quinine but probably three hundred times as toxic, when tested against *P. cynomolgi*, while in the case of *P. vivax* even low dosages were too toxic to be of any practical value (Coatney *et al.*, 1950). Total alkaloids as obtained from cinchona tree have been found to be equal or superior to quinine while percentage of quinine in this mixture did not appear to determine the activity as various alkaloids mutually acted as synergists (Baranger and Filer, 1948). This finding is of special interest to under-developed countries growing cinchona who can produce much larger amounts of " total cinchona alkaloids " for their needs as against pure quinine.

Chemical studies.—Chinese investigators obtained three isomeric alkaloids from *D. febrifuga* Lour, which they tentatively called alpha, beta and gamma dichroines (Jang *et al.*, 1948 ; Chou *et al.*, 1948 ; Yang, 1951). Gamma-dichroine was found to be highly effective against avian malarias (Henderson *et al.*, 1949). Similarly Kuehl *et al.* (1948) after examining six hundred plants, obtained two antimalarial alkaloids, I and II, from *D. febrifuga*. Further detailed examination of the same herb by Koepfli *et al.* (1949) resulted in the discovery of two interconvertible alkaloids febrifugine and isofebrifugine, the former displaying good antimalarial activity. Koepfli *et al.* (1949) attempted to reconcile all the above findings by different workers and concluded that isofebrifugine, Kuehl's alkaloid I and alpha-dichroine were identical, while beta and gamma-dichroine and Kuehl's alkaloid II may correspond to febrifugine, which is dimorphic in character. There was also a general agreement in all the above cases as regards a molecular formula of $C_{16}H_{19}O_3N_3$ for these alkaloids and a 4-quinazolone structure substituted at 3-nitrogen (Jang *et al.*, 1948 ; Chou *et al.*, 1948 ; Koepfli *et al.*, 1949). Finally Koepfli (1950) assigned configuration (I) to febrifugine and gamma-dichroine which has been recently confirmed by Baker, McEvoy *et al.* (1953*b*).



FEBRIFUGINE (I)
(ALKALOID FROM HYDRENGEA)
3-[beta-keto-gama-(3-hydroxy-2-piperidyl) propyl]-4-quinazolone.

Display of high activity in the case of (I) prompted examination of other plants. Eventually, hydrengea, a common garden plant, offered a crystalline alkaloid which resembled febrifugine as regards its chemical and biological properties (Ablondi *et al.*, 1952). This fact was further confirmed by degradative studies (Hutchings *et al.*, 1952) and in order to determine the chemical structure of this alkaloid a comprehensive study of the functional derivatives of 4-quinazolones with 3-substituted side chains was undertaken (Baker, Querry, Kadish and Williams, 1952*a* ; Baker Querry, Schaub and Williams, 1952). Out of eighty such functional derivatives studied, only 3-[-beta-keto-gamma-(2-piperidyl) propyl]-4-quinozolone was found to be one hundredth as active as hydrengea alkaloid and equal to quinine activity (Baker, Querry, Schaub and Williams, 1952 ; Hewitt *et al.*, 1952). Further extension of the same studies (Baker and co-workers, 1952) finally resulted in the synthesis of hydrengea alkaloid (I) which is 3-[beta-keto-gamma-(3-hydroxy-2-piperidyl)propyl]-4-quinozolone having *cis* configuration in the piperidine ring (Baker, Schaub, McEvoy and Williams, 1952 ; Baker, McEvoy *et al.*, 1953*a*). Mediocre chemotherapeutic index in case of (I) (Hewitt *et al.*, 1952) prompted the study of its analogues where the benzene ring in the parent structure (I) carried one or more halogen, alkyl, nitro or alkoxy substituents (Baker, Schaub, Joseph *et al.*, 1952*b* ; 1952*c* ; Baker, Joseph, Schaub *et al.*, 1952). The effect of such substitution and alterations on the biological activity of the molecule has been summarized below (Hewitt *et al.*, 1952) :—

(a) Substituents in position 5 of structure (I) increased the antimalarial activity while substituents in position 6 gave compounds with lower chemotherapeutic index (Baker, Schaub, Joseph *et al.*, 1952*c*).

(b) Extensive investigations regarding substituents at position 5 revealed that 5-flouro derivative was as good as chloro analogue although their replacement by iodo group resulted in a compound of lower activity. Amongst 5-alkyl chains, the activity was inversely proportional to the length of alkyl chain. As regards chemotherapeutic index, 5-trifluoromethyl analogue of (I) was the best although the quinine co-efficient came down from 100 to 35 (Baker, Schaub, Joseph *et al.*, 1952*c*).

(c) Substituents in positions 7 or 8 gave compounds which had lower activity or chemotherapeutic index or both. While 6- or 7-substituted derivatives of (I) were inactive (Baker, Schaub, Joseph *et al.*, 1952*c*).

(d) Similarly (5, 8) (7, 8) (5, 7) or (6, 7) disubstituted derivatives of (I) having two identical substituents were found to be less active. However, 3, 5, 6-trisubstituted derivatives showed ani ncrease in activity by 30 per cent (Baker, Schaub, Joseph *et al.*, 1952*b*).

(e) 5-chloro-6-methyl analogue of (I) was only as active as its 5-chloro analogue while certain dichloro substituted compounds though more active have shown lower chemotherapeutic index (Baker, Joseph, Schaub *et al.*, 1952).

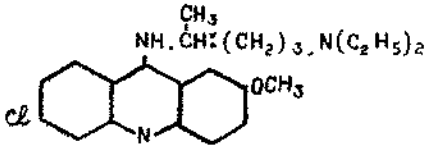
(f) Hydrengea alkaloid (I) can be considered as a 5, 6-substituted-4-pyrimidone derivative and consequently quinazolone moiety of (I) has been replaced by various 5-aryl-4-pyrimidones. The resulting compounds, however, had very low quinine co-efficient (Baker, Schaub, Joseph, *et al.*, 1953). Similarly a thiophene isoster of (I) was also studied (Baker, Joseph, Schaub *et al.*, 1953).

In order to study the effect of replacement of quinoline ring of quinine by isoquinoline, 3-isoquinolyl-2-quinuclidyl ketone has been reported but the biological data was not given (Clemo and Popli, 1951). Alkaloids from *Leonotis nepetaefolia* have been investigated and only the seeds displayed some antimalarial activity (Asenjo *et al.*, 1948).

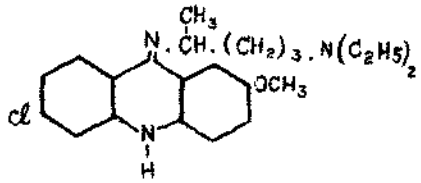
Biological studies and physico chemical observations.—Quinine is degraded in the system to a 2-hydroxy derivative which was positively inferior to quinine as regards specific antimalarial activity (Marshall and Rogers, 1948) and in order to block this mode of detoxication, various 2-alkylamino-quinolines were prepared but no test report is available (Luthy *et al.*, 1949).

ACRIDINES.

Mepacrine (atebrin) (II), the well-known antimalarial of this family has not been surpassed in activity by any other acridine derivative built on its pattern. Studies have been conducted on the degradative metabolism of acridines but the results were inconclusive, while attempts to correlate biological activity with physico-chemical properties have also met with little success.



MEPACRINE (II)



MEPACRINE (Resonance hybrid) (IIa)

[2-Methoxy-6-chloro-9-(4-diethylamino-1-methylbutylamino) acridine.]

Chemical studies.—A number of active 2-methoxy-6-chloro-9-dialkylamino-alkylamino-acridines have been reported (Eli Lilly & Co., 1948*a* ; Perrine and Sargent, 1949) while various 9-alkyl- (or aryl-) -amino-acridines having different substituents in the two benzenoid rings were usually found to be devoid of activity (Grigorovskii and Terenteva, 1947 ; Kshatriya and Nargund, 1948 ; Parke Davis & Co., 1948 ; Singh and Singh, 1948 ; Singh and Ahmed, 1949 ; Kshatriya *et al.*, 1950 ; Tamemasa, 1951). Out of 5- and 8-chloro isomers of (II), the latter

was found to be less active than the parent drug (Dauben, 1948). Replacement of the mepacrine side chain with a 4-diethylaminocyclohexylamino group offered a compound which was only slightly inferior to mepacrine when tested against human malaria with no unpleasant side effect or skin coloration (Asano *et al.*, 1950). Certain 2-methoxy-6-chloro-9-(diethylamino-methylphenylene-alkylamino) acridines have been found to possess high activity (Stavrovskaya, 1951) while 1-(1, 1, 3, 3-tetrahydromethyl) butyl-4-methoxy-9-(dialkylaminoalkylamino)-acridines and their 6-chloro analogues were all inactive when tested against *P. lophuræ* (Niederl and Hundert, 1950). Certain 3- and/or 6-chloro-2-nitro-9-dialkylaminoalkylamino-acridines (Shah and Nargund, 1951) and various salts of atebrin (Eli Lilly & Co., 1948*b* ; Steck *et al.*, 1952) have also been reported.

The activity of certain pyridoacridines, prompted the study of a number of 8-chloro-benzoacridine derivatives having a mepacrine side chain at position 5-. Their antimalarials' screening tests revealed that, position to which the extra ring is attached to the acridine group, is of importance while the nature of the ring itself is of little consequence (Dobson *et al.*, 1948). Similarly certain 4-(dialkylaminoalkyl-amino)-p-phenanthrolines, relative to above mentioned pyridoacridines, displayed some antimalarial activity (Douglas and Kermack, 1949). Certain open models of mepacrine viz. 4-dialkylaminoalkylamino-3-phenylquinolines and their 6-chloro analogues have been prepared as 3-phenylquinoline was previously found to be active (Adam and Hey, 1950).

Out of certain amino-carbinols derived from dihydroacridines, x-(2-diethylamino-1-hydroxyethyl)-10-acetyl-9 : 10-dihydroacridine gave best results against *P. gallinaceum* while its dialkyl analogues also displayed feeble antimalarial activity (Sargent and Small, 1948*b*). However, Linnell and Sharp (1950) were unable to condense an alkylamino chain with 4 : 9-dichloro-1 : 2 : 3 : 4-tetrahydroacridine.

Metabolic studies and physico-chemical data.—The study of basic excretion products of mepacrine (II), by spectrographic methods revealed that diphenylamine derivatives, and acridanes are likely possibilities (King *et al.*, 1946). However, relatively low stability of acridanes made their presence in the urine or urine extract very improbable, more so when the acid extraction of urine could not be applied to acridanes as they are acid insoluble. Further spectrographic evidence has completely ruled out acridanes as possible metabolic products but compounds of the type, 5-chloro-4-methoxy-diphenylamine-2-carboxylic acid are very likely to be the breakdown products of mepacrine (II) (Tarnoky, 1950).

Similarly, study of absorption and distribution of 9-aminoacridines similar to atebrin revealed that a neutral acridone (without the side chain) has been produced but in this case also no conclusive proof of its existence was available (McChesney and McAuliff, 1949).

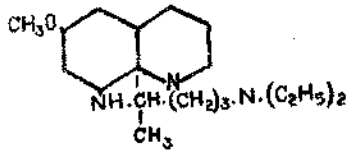
9-amino-acridines were found to have the greatest proton accepting strength (basicity) which is due to the resonance of the donor (NH_2) group. The resonance hybrid in such a case is a benzenoid-quinonoid structure (II*a*) for whose existence additional experimental evidence has been provided by Irvin and Irvin (1950). Schonhofer (1942) had previously attributed the activity of mepacrine to such a quinonoid structure (II*a*). Mepacrine has also been resolved into its optically

active isomers with d-4 : 6 : 4′ : 6′-tetranitrodiphenic acid (Brown and Hammick, 1948).

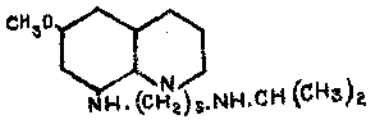
No definite correlation between antimalarial activity and either basicity or reduction potential could be observed in the case of mepacrine and its analogues. Lipoid partition co-efficients, surface activities and protein affinities of 9-amino acridines also failed to give any definite relationship between antimalarial activity and physico-chemial properties or chemical structure (Hammick and Mason, 1950*b*). These results could be justified on the ground that a drug must traverse the cellulor tissues of the host as well as that of the parasite before reaching the site of action where the factors governing the enzyme inhibition may be different from those made available outside (Hammick and Mason, 1950*a*). 9-aminoacridines have been found to inhibit the oxidation of diamines by diamine-oxidase and study on these lines has also failed to give a suitable relationship between chemical configuration and biological activity (Mason, 1950). The distribution of acridine isomers between two solvents by counter-current technique, revealed that the impurities have a different partition ratio than the pure compound (Craig *et al.*, 1948). This technique has been used with advantage in various metabolic studies on antimalarials (Titus *et al.*, 1948 ; Crounse, 1951).

8-SUBSTITUTED QUINOLINES.

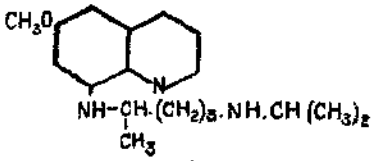
Curative properties as displayed by 8-aminoquinoline drugs with or without the simultaneous administration of suppressive antimalarials, is the most outstanding feature of this group. Pamaquin (III), pentaquin (IV), isopentaquine (V) and primaquin (VI) are the outstanding members of this series which differ from each other in the nature of terminal alkylamino chain attached to 6-methoxy-8-aminoquinoline group.



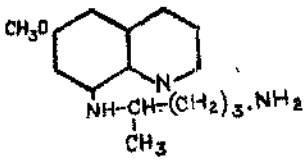
PAMAQUIN (PLASMOQUIN) (III)
6-methoxy-8(4-diethylamino-1-methylbutylamino)-quinoline.



PENTAQUIN (IV)
6-methoxy-8 (5-isopropylamino-amylamino)-quinoline.



ISOPENTAQUIN (V)
6-methoxy-8-(4-isopropylamino-1-methylbutylamino)-quinoline.



PRIMAQUIN (VI)
6-methoxy-8-(4-amino-1-methyl-butylamino)-quinoline.

Pentaquin is about half as toxic as pamaquin and twice as active, while isopentaquin is about half as toxic as pentaquin and equally active (Alving, 1948). Primaquin (VI) first studied by Alving (1948), has recently proved to be most

active and considerably less toxic than the rest of the drugs of this series. Its chemotherapeutic index is 10 when compared to that of pamaquin and isopentaquin whichare 1 and 2·5 respectively. Unlike III, IV and V primaquin maintained a high prophylactic and curative ratio when given in safe therapeutic doses without the simultaneous administration of any suppressive antimalarial like paludrine, quinine (Edgecomb *et al.*, 1950). The curative properties of these compounds (or perhaps their metabolites) are due to their action on late exo-erythrocytic stages of malaria parasites. This action was further potentiated by the simultaneous presence of suppressive antimalarials, which otherwise have no action on the exo-erythrocytic forms. Toxic manifestations in case of these drugs are a serious handicap for their unsupervised general use. Primaquin appears to be more promising in this respect but it is too early to give a definite opinion. Studies with pamaquin have revealed a possible mode of its degradation (Josephson, Greenberg *et al.*, 1951) while similar studies with radio active pentaquine have also been of considerable interest (Elderfield and Smith, 1953).

Chemical studies.—Commercial pamaquin was found to contain an isomer which was due to the contamination of 1-diethylamino-4-bromo-pentane with its 3-bromoisomer during the course of its manufacture. Considering this difficulty, reductive amination reaction involving 6-methoxy-8-amino-quinoline and 1-diethylamino-pentanon-4 was successfully tried as an easier method for the manufacture of pure pamaquin (Elderfield *et al.*, 1948 ; Barber *et al.*, 1948). Elderfield and Ressler (1950) have verified the chemical structure of pamaquin and plasmocid (6-methoxy-8-(3-diethylaminopropylamino)-quinoline) by amine degradation method, wherein the side chain is cleaved by oxidation and later chemically identified. Newer methods for the synthesis of pentaquin (IV) have also been investigated by Green (1951).

Synthesis of a number of 6-methoxy-8-aminoquinolines allied to pamaquin and having primary or secondary alkyl-amino side chains, has been reported (Cope *et al.*, 1949). Similarly in continuation of their work on pentaquin, Drake *et al.*, (1949) studied a number of analogues compounds but none was found to be less toxic than pentaquin itself. Antimalarial properties of 1, 2, 3, 4-tetrahydropamaquin, 1, 2, 3, 4-tetrahydropentaquin and 8-(3-amino-propyl-amino)-6-methoxy-1, 2, 3, 4-tetrahydroquinoline were compared with those of their simple quinoline isomers and tetrahydro derivatives were 2 to 4 times as active although the toxicity was also generally higher (Gray and Hill, 1949).

5 : 6-dimethoxy-6-dialkylamino alkylamino-quinolines (Elderfield and Head, 1949 ; Tatsuoka *et al.*, 1949*a*) as well as 5, 6-alkylenedioxy-8-dialkylamino alkylamino quinolines have been reported, out of which the latter group has shown good activity against avian malaria (Lott *et al.*, 1948). Similarly a number of compounds based upon pamaquin structure such as 6-methoxy (or hydroxy)-8-(dialkylamino alkylamino)-4-methyl-quinolines (Campbell, 1950) ; 6-methoxy-5-amino-8-(dialkylaminoalkylacetamido)-quinolines (Tatsuoka *et al.*, 1950) ; 6-methoxy-8-[(2-hydroxy-3-disubstituted amino) propylamino] quinolines (Eli Lilly, 1948*c*) ; 6-hydroxy analogue of pamaquin (Steck and Boemhe, 1952) and 8-(2 : 5-dimethyl-1-pyrryl) quinolines (Sen *et al.*, 1953) have been prepared as potential antimalarials.

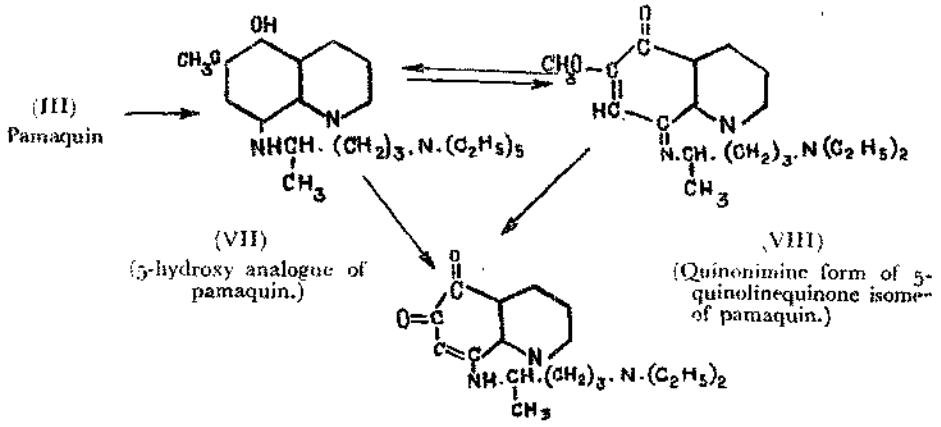
2-p-chloroaniline-8-beta-diethylaminoethylamino-quinoline and other analogous compounds, as extension of the previous work on similar pyrimidines, have been investigated but none of them had any significant antimalarial activity (Bennett *et al.*, 1949).

Study of 8-quinolyl-alpha-dialkylaminoalkyl carbinols has not yielded promising results (McCoubery and Webster, 1948*b*); Topchiev and Bekhli, 1948) while quinolyl-biguanides and quinolyl guanidines have been discussed under biguanides (pp. 199-200). Sulphuric, 3 : 5-dinitrobenzoic and 8-hydroxy-5-quinoline sulphonic acid salts of various 6-methoxy-8-dialkylamino-alkylamino-quinolines have been investigated (Tatsuoka *et al.*, 1949*b*), while 6-methoxy-8-aminoquinoline was also diazotised and coupled with stovarsol and the resulting dye was devoid of activity (Giral, 1948).

Metabolic studies.—There appeared to be no correlation between plasma concentration of pamaquin and its antimalarial activity (Berliner *et al.*, 1948) while pamaquin and its homologues had very little activity *in vitro* although they were highly active *in vivo* (Gieman, 1948; Greenberg, Taylor and Josephson, 1951). These findings were indicative of the fact that pamaquin might be exerting its therapeutic action through metabolic degradation product(s). The toxic effects of pamaquin include production of methemoglobin and occurrence of acute hemolytic anemia but no such reactions were observed *in vitro* unless the concentration was far greater than that obtainable in plasma with therapeutic doses. Possibly, the toxicity was also exerted through a metabolic product (Brodie and Udenfriend, 1950). Pamaquin and other 8-aminoquinolines were rapidly degraded in the system (Zubrod *et al.*, 1948 ; Hughes and Schmidt, 1950) and considering the above facts extensive investigations have been undertaken to elucidate the pathway of their metabolism.

Hughes and Schmidt (1950) studied the metabolism of pamaquin (III), isopentaquin (V), pentaquin (IV), primaquin (VI) and certain 2-methoxy-8-dialkyl-aminoalkylamino-quinolines in monkeys and it was found that each of them is degraded to a varying extent, to an alkli soluble, ethylene-dichloride insoluble acid derivative. However, the chemical nature of these products remained obscure.

Brodie and Udenfriend (1950) investigated the alkaline degradation products of pamaquin and 6-methoxy-8 (3-diethylamino-propylamino)-quinoline and in each case, two metabolites were obtained. One of them was an unstable compound which could produce methemoglobin and lyse erythrocytes *in vitro* while the other was a stable fluorescent compound. Considering the high *in vitro* activity of 6-hydroxy-8-amino-quinolines and 5 : 6-dihydroxy-8-aminoquinolines (Greenberg, Taylor and Josephson, 1951) it was argued that pamaquin was converted into a 5-hydroxy-derivative (VII) which then acted as a methemoglobin former through reversible conversion to quinonimine (VIII) (Blanchchard and Schmidt, 1946 ; Brodie and Udenfriend, 1950). However none of these products could be actually chemically identified.



PAMAQUIN METABOLITE (IX)
8-(4-diethylamino-1-methylbutylamino)-5, 6-quinolinequinone

Josephson, Taylor, *et al.* (1951) observed that concentrates obtained from blood, tissues and droppings of the chickens treated with pamaquin, possessed antimalarial activity *in vitro*, which could not be accounted for on the basis of pamaquin present in these concentrates. Previous unfinished observations (Brodie and Udenfriend, 1950 ; Hughes and Schmidt, 1950) and the above findings led to an extensive examination of this problem. Eventually a metabolite of pamaquin was obtained (Josephson, Taylor *et al.*, 1951) which differed from those obtained previously, but was closely similar to a quinolinequinone obtained by ultra-violet irradiation of pamaquin (Elderfield and Werble, 1950), as regards ultraviolet spectrum, inability to couple with diazotised sufanilic acid, antimalarial activity *in vitro* and methemoglobin forming properties. The structure of the irradiated product and that of the new metabolite was found to be identical *viz.* 8-(diethylamino-1-methylbutyl- amino)-5, 6-quinolinequinone (IX) and this compound was 16 times as active as pamaquin *in vitro* (Josephson, Greenberg, Taylor and Bami, 1951).

Identification of a pamaquin metabolite (IX) established one point in the route of pamaquin degradation. Taking an analogy from the 4-aminoquinolines (Titus *et al.*, 1948) and pentaquin degradation (Elderfield and Smith, 1953), the terminal alkyl chain cleavage could give rise to other similar quinolinequinones, none of which has however been identified so far. On the other hand pamaquin (III) when converted into (IX), must have passed through two intermediate stages, namely (VII) and (VIII) (Josephson, Greenberg, Taylor and Bami, 1951). These findings experimentallys ubstantiate Schonhofer's 1942 hypothesist hat 6-methoxy -8- aminoquinolines are degraded through hydroxyquinolines to quinolinequinones. It would be interesting to record that metabolite (IX) failed to show antimalarial activity *in vivo* in spite of its high *in vitro* activity (Schmidt, 1951).

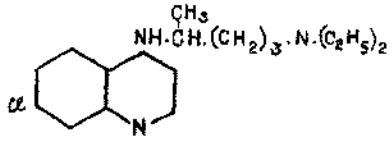
Drake and Pratt (1951) studied synthetically a few quinones and hydroquinones related to pentaquin (IV) and in this case also, quinonoid product like (IX) having a pentaquin side chain instead, was found to be highly active *in vitro* when compared to pentaquin (Greenberg, Taylor and Josephson, 1951). These results

further confirmed the above findings, that position 5- of 6-methoxy-8-aminoquinolines was most vulnerable to oxidative degradation.

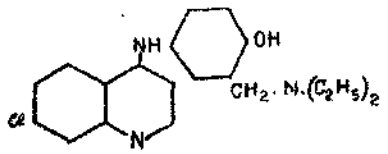
Considering that 8-aminoquinolines labelled with radio active isotopes could be studied with advantage as regards degradation metabolism, Elderfield *et al.*, (1953) synthesised pentaquin (IV) labelled in quinoline ring with N^{15}. Similarly pentaquin with labelled nitrogen in the terminal amino group of the alkyl chain as well as pentaquin having radio active nitrogen at the 8-amino group were also synthesised (Blatt and Gross, 1953). *In vivo* degradative studies with the last two compounds revealed that the alkyl-amino side chain was easily and completely removed in the system, subsequently de-aminated and its components excreted with the normal body metabolites. This work has been of considerable interest but no definite metabolite of pentaquin could be isolated (Elderfield and Smith, 1953).

4-SUBSTITUTED QUINOLINES.

Amongst 4-substituted quinolines, chloroquine (resochin) (X) and camoquin (cam-aqi) (XI) are the two outstanding suppressive antimalarials. Chloroquine has received exhaustive clinical trials as a suppressant while camoquin which was discovered in 1948 (Burckhalter *et al.*, 1948) is fast coming into prominence as a prophylactic and suppressive antimalarial (Jaswant Singh, 1950 ; Davey, 1951 ; Bertanga, 1951 ; Hitchings *et al.*, 1952 ; Jaswant Singh *et al.*, 1953). Major chemical work has centred round synthesis and examination of closely allied 4-amino-quinoline structures but in no case, a better antimalarial could be found. Metabolic studies on these drugs involving demethylation of alkyl-amino chain have been of considerable interst (Titus *et al.*, 1948).



CHLOROQUINE (RESOCHIN) (X)
7-chloro-4-(4-diethylamino-1-methyl-butylamino)-quinoline.



CAMOQUIN (CAM-AQI) (XI)
7-chloro-4-(3-diethylaminomethyl-4-hydroxy-anilino) quinoline.

Chemical studies.—Hydroxychloroquine, 7-chloro-4-(4-(N-ethyl-N-β-hydroxy-ethylamino)-1-methylbutyl-aminoquinoline, was found to be 3 to 7 times more active than mepacrine when tested against *P. lophuræ* (Surrey and Hammer, 1950) while single dose therapy was likely to be effective against an acute attack of *P. falciparum* (Longhlin *et al.*, 1952). Replacement of the terminal alkyl radical of chloroquine with a pyrrolidyl group yielded a compound whose activity and toxicity were analogous to that of chloroquine (X) when tested against *P. gallinaceum* (Reitsema and Hunter, 1949). Similarly introduction of aryl groups between the two amino radicals of the chloroquine alkyl side chain also offered active compounds (Sterling Drug Co., 1950). Various 4-dialkyl-amino-alkylamino-quinoline derivatives having 2-(2-furyl) (Andrisano and Modena, 1950) ; 5 : 8-, 5 : 6-

and 6 : 8-dichloro (Surrey, 1951), 6-hydroxy (Eli Lilly and Co., 1948*c*) and 2-p-chlorophenyl (Bachman *et al.*, 1950) substituents in the quinoline ring have been reported. While chemical data on 4-(4-diethylamino-1-methylbutylamino) quinolines having poly alkyl and/or halogen substituents in the quinoline ring have been reported (Steck *et al.*, 1948), their activities have been listed previously (Wiselogle, 1946). A study of a number of chloroquine type of compounds has revealed that 7-chloro radical is optimal for activity while introduction of 3-methyl group (this compound is known as sontochin or nivaquin) lowered activity and 3 : 8 dimethyl group completely destroyed the activity (Gray and Hill, 1949). 3-nitro and 3-amino analogues of chloroquine were also found to be less active than the parent drug (Surrey and Cutler, 1951). Various methods for the manufacture of chloroquine have been worked out and recently an improved synthesis has been offered by Johnson and Buell (1952).

The hydroxy group in the benzene ring of camoquin (XI) has been replaced by fluoro and methoxy radicals and the resulting fluoro analogue was considerably less active than the parent drug (Sveinbjornsson and Vanderwerf, 1951). However, the methoxy analogue of (XI) showed high antimalarial activity which could probably be due to its demethylation *in vivo* into the active parent drug (Burckhalter, 1949). Chandran *et al.* (1951) reported 4-(7-chloro-8-amino-4-quinolylamino)-alpha-diethylamino-*o*-cresol which displayed some suppressive action in experimental malaria. Methods for the preparation of camoquin have also been investigated (Burckhalter *et al.*, 1948 : 1949 : 1950).

A number of other 4-aminoquinoline derivatives namely 2-arylamino-4-dialkylamino-alkylamino quinolines (Curd, Landquist *et al.*, 1950) ; 4-alkylamino-3 : 8- dimethylquinolines (Eichinger and Struckwisch, 1949) ; 2-furyl-6-methoxy-4-(4-diethylamino-1-methyl-butylamino) quinoline (Andrisano, 1950) ; 4-(3-diethylamino-propylamino) 2- and/or 3-methyl-quinolines, their 6-methoxy and 7-chloro analogues (Wiselogle, 1946 ; Wheeler *et al.*, 1949 ; Landquist, 1951) ; 4-(dialkylaminoalkylamino) quinoline-(4 : 3-2 : 3)-quinolines (Kermack and Storey, 1951) ; 2- or 4-(dialkylaminoalkyl thio-butylamino)-quinolines (Gilman and Plunkett, 1949) ; and 4-phenyl-2-(4-diethylamino-1-methylbutylamino)quinolines (Reynolds and Hauser, 1950) have been reported as potential antimalarials. Certain pyrido-(4-3-b) quinolines have been found to be devoid of activity (Bachman and Barker, 1949).

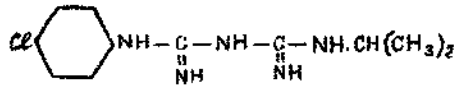
King and Wright (1948) have investigated alpha-(aminomethyl)-2-phenyl-6 : 7-dimethyl-4-quinolinemethanol and its NN'-dibutyl, diamyl- and dihexyl-analogues. They were all found to be superior to quinine when tested against avian malaria but the experimental evidence did not prove that riboflavin antagonism is the mechanism of their action. Similarly 2-aryl-alpha-dialkylamino-methyl-4-quinoline methanols with halogen substitutions in the quinoline ring (Becker, 1949 ; Lutz *et al.*, 1950) and beta-diketone analogues of 4-quinoline methanol (Shivers and Hauser, 1948) were also studied. Considering the biological activity of 4-hydroxy-quinoline, 3-alyl-4-hydroxy-2-methyl-quinoline and hundred other closely allied compounds were examined for antimalarial activity. This led to the discovery of endochin (3-heptyl-4-hydroxy-7-methoxy-2-methyl quinoline), which though very active against avain malaria failed to give satisfactory results in clinical trials (Salzer *et al.*, 1948).

Metabolic studies and physico-chemical observations.—Chloroquine (X), 4-(3-diethylamino-1-hydroxy-propyl-amino)-7-chloro-quinoline (SN 8137) and 4-(3-diethylaminopropyl-amino)-7-chloro-quinoline (SN 9584) were studied for their degradative metabolism in human beings. By using counter-current method of purification (Craig *et al.*, 1948), in each of the above cases, a corresponding demethylated product having one terminal methyl group removed, was obtained (Titus *et al.*, 1948). These findings are apparently not in confirmity with those obtained in the case of pamaquin (Josephson, Greenberg, Taylor and Bami, 1951; Greenberg, Josephson, Bami and Taylor 1951) and pentaquin (Elderfield and Smith, 1953). Ultraviolet irradiation of 4-amino-7-chloroquinoline derivatives and study of their breakdown products did not reveal new products (Price *et al.*, 1948).

A number of 4-dialkylaminoalkylamino quinolines as well as their 7-chloro analogues have been studied as regards their absorption spectra and degree of ionisation. It was concluded that essential pre-requisite for activity in these cases consisted of an amino-nitrogen para to the protonised heterocyclic nitrogen atom, both of which share the cationic charge. This hypothesis was only applicable to mepacrine, aminopyridines and aminopyrimidines (Gage, 1949*a*), and supported the previous suggestions put forward by Schonhofer (1942). Basic alkylamino side chain in the above cases had its own effect on electron distribution while the terminal nitrogen displays its conductophoric properties separately but both of these were essential for activity and could not be considered separately. Notable exceptions to this view are quinine, quinoline carbinols and pamaquin which are not ionised at the physiological pH while the first two do not have nitrogen atom opposite the ring nitrogen. Chloroquine (X) has been resolved into its optically active form which showed significantly different antimalarial activity against avian malaria (Riegel and Sherwood, 1949).

BIGUANIDES, GUANIDINES, ETC.

Simplicity of chemical structure coupled with high antimalarial activity and low toxicity of biguanide derivatives, has prompted numerous investigations on substituted biguanides. The well-known member of this class *viz.* paludrine (proguanil) (XII) has been thoroughly investigated at clinical level (Cooper, 1949; Jaswant Singh, 1950; Davey, 1951; Findlay, 1951) and has established itself as a routine prophylactic and suppressive with low toxicity. When compared to mepacrine, chloroquine and camoquin, paludrine is rather slow acting like daraprim. Development of resistance to paludrine both in the laboratory and field is a serious drawback but luckily paludrine resistant strains of malaria are susceptible to other antimalarials like mepacrine, chloroquine, etc. (Bishop, 1951; Findlay, 1951; Jaswant Singh, Ray, Basu and Nair, 1952; Schmidt and Genther, 1953).



PALUDRINE (PROGUANIL) (XII)
N¹-p-chlorophenyl-N⁵-isopropyl-biguanide.

Extensive chemical and bio-chemical investigations on nearly fourteen types of biguanides allied to paludrine, have not so far yielded a compound better than the parent drug (XII). However, bromoguanide (p-bromo analogue of paludrine) (Jaswant Singh *et al.*, 1949 : 1950) and 3 : 4-dichlorophenyl isomer of plaudrine (Curd, Davey *et al.*, 1950 ; Chaudhuri *et al.*, 1951 : 1952) did show high antimalarial activity but they were both more toxic than paludrine. Search for metabolites of paludrine and final isolation of an active metabolite *in vivo* (Carrington *et al.*, 1951 ; Crowther and Levi, 1953) has been of great interest. Recently bromoguanide labelled with radio active bromine has also been prepared and used for excretion studies (Crowther and Levi, 1953).

Chemical studies.—Using four basic routes for the synthesis of biguanide derivatives (Rose, 1951), numerous N^1-aryl-N^5-alkyl (dialkyl or cycloalkyl) biguanides including plaudrine have been reported by a number of workers (Fernandes and Ganapathi, 1948 ; Curd, Hendry *et al.*, 1948 ; Ashworth *et al.*, 1949 ; Bami and Guha, 1949*a* ; Hart and Vanderwerf, 1949 ; Birtwell *et al.*, 1949 ; Curd and Rose, 1949 ; Crowther, Curd, Davey *et al.*, 1951 ; Crowther, Curd and Rose, 1951 ; Guha and Guha, 1952*a* : 1952*c*). Majority of these compounds have shown some activity and from this data the following relationship between their chemical structure and biological activities has been drawn :—

(a) Introduction of any substituent into one or both ortho positions of the phenyl ring detsroyed activity (Crowther, Curd and Rose 1951 ; Rose, 1951).

(b) Introduction of halogen at the meta position resulted in active compounds (Bami *et al.*, 1949; Jaswant Singh *et al.*, 1949 ; Crowther *et al.*, 1949).

(c) Replacement of para-chloro radical of (XII) with other halogen atoms such as bromo, iodo and fluoro gave active compounds with comparatively higher toxicity (Ainley *et al.*, 1949 ; Bami *et al.*, 1949 ; Jaswant Singh *et al.*, 1949 : 1950) *e.g.* p-bromo isomer of paludrine (bromoguanide) was as active but more toxic than paludrine (Jaswant Singh *et al.*, 1949 : 1950).

(d) Amongst dihalogen analogues of paludrine, 3 : 5 dichloro isomer was slightly active while a similar bromo derivative was as active as paludrine. However, 3 : 4-dichloro analogue of the parent drug was 3 to 10 times as active and considerably more toxic (Curd, Davey *et al.*, 1950 ; Crowther, Curd, Davey *et al.*, 1951).

(e) N^1-3 : 4 : 5-trichlorophenyl-N^5-alkyl (or dialkyl)-biguanides have shown good antimalarial activity generally but they are also more toxic (Crowther, Curd, Davey *et al.*, 1951).

(f) Isopropyl group at the terminal nitrogen always offered compounds with maximum activity in any given series. Propyl isomers are a little less active while the methyl group in place of isopropyl radical completely destroyed activity. In short, increase or decrease in the length of isopropyl alkyl chain, always resulted in the decrease of biological activity (Rose, 1951).

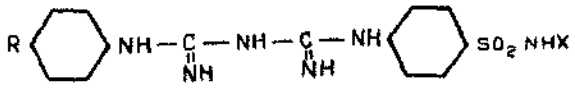
(g) N^1-aryl group is essential for activity and if an additional aryl group is introduced, the activity is greatly lost. For instances N^1 : N^2-diaryl-N^5-alkyl-biguanides (Ashworth *et al.*, 1949 ; American Cynamide Co., 1950) as well as N^1-6-hydroxy-8-quinolyl-N^5-isopropyl-biguanide (Bami, 1953*b*) were all inactive.

(h) Introduction of additional alkyl group to the N^1-aryl-N^5-alkyl-biguanides also gave compounds of low antimalarial activity. N^1-aryl-N^2 : N^5-dialkyl biguanides (Crowther, Curd, Richardson and Rose 1948 ; Crowther, Curd and Rose, 1951) N^1-aryl-N^2 : N^4 : N^5-trialkyl-biguanides and N^1-aryl-N^4 : N^5-dialkyl biguanides (Ashworth *et al.*, 1949 and Crowther, Curd and Rose, 1951) were either devoid of activity or it was of a very low order.

(i) Shifting of N^5-alkyl group to N^3 position, as in the case of N^1-aryl-N^3-biguanides, offered compounds with moderate activity (Bekhli *et al.*, 1947 ; Ashworth *et al.*, 1949), while N^1 : N^2 : N^5-trialkyl-biguanides were completely inactive, (American Cyanamide Co., 1950).

(j) Any alteration of the basic biguanide molecule (*i.e.* replacement of one or more nitrogens with sulphur or oxygen atoms) in any manner results in complete loss of activity (Birtwell, 1949 ; Curd, Davey and Richardson, 1949 ; Curd, Davey, Richardson and Ashworth, 1949).

Gupta and Guha (1949) and Guha and Guha (1952*b*) made various unsuccessful attempts to synthesise substituted polyguanides, but under certain conditions, the formation of melamines could only be detected (Gupta and Guha 1949). Various N^1-benzyl-N^5-aryl or alkyl biguanides have also been reported, as potential antimalarials (Tendick and Burckhalter, 1950 ; Funke and Kornmann, 1947).



R=H, Cl, Br, CH_3O etc.
X=H, Heterocyclic

SULPHABIGUANIDES (XIII)
N^1-aryl-N^5-(p-sulphonamido)phenyl-biguanides

A number of arylalkyl-biguanides have been discussed previously but the introduction of therapeutically active chemical structures into the basic biguanide molecule was also considered of interest. Consequently Bami *et al.* (1947*b* : 1948*b*) commenced studies on a number of sulphabiguanides of the type (XIII) wherein a number of well-known sulpha drugs have been linked to any arylbiguanide chain. Sulpha-drugs studied in this manner were : sulphanilamide, sulphathiazole, sulphadiazine (Bami *et al.*, 1947*b*), sulphamerazine, sulphamethazine (Bami *et al.*, 1948*b*), sulphapyridine (Bami, 1950), sulphapyrazine (Gupta, 1952), sulphabenzamide (Gupta and Guha, 1950*a*) and 3 : 5-dibromo sulphanilanilide (Gupta, 1952). Sulpha biguanides derived from metachloridine (SN. 11437) were also studied in a similar manner (Bami *et al.*, 1948*a*). Amongst all these

compounds the activity was generally low whenever present (Jaswant Singh *et al.*, 1949 ; Bami *et al.*, 1949) which could be attributed to the absence of terminal isopropyl group, which has invariably been associated with high antimalarial activity (Rose, 1951). Consequently a number of N^1-p-sulphonamidophenyl-N^5-isopropyl biguanides (N^1-isopropyl analogues of XIII) have been synthesised and some of them have displayed good antimalarial activity against *P. gallinaceum* (Srinivas *et al.*, 1953 ; Bami, 1953*a*). Nagy (1948) had also patented certain similar type of compounds but biological testing data is not available. Certain N^1-arylsulphonyl-N^5-alkyl (or aryl) biguanides (Funke and Kornmann, 1947 ; Bami 1953*a*), N^1-aryl-N^5-phenylarsenic acid biguanides (Roy and Guha, 1950*b*) and 4-4-di (N^1-arylbiguanido) diphenyl sulphides and sulphones (Roy *et al.*, 1950) have also been reported but the first mentioned were devoid of activity (Bami, 1953*a*).

High antimalarial activity as displayed by quinoline group of drugs stimulated researches in the field of quinoline biguanides. A number of N^1-aryl biguanides carrying a 8-chloro-5-quinolyl (Gupta and Guha, 1948*a*), 8-quinolyl (Gupta *et al.*, 1948) ; 7-chloro-8-quinolyl (Gupta, 1952 ; Sen, Riachaudhari and Basu, 1952) ; 5 : 6-dimethoxy-8-quinolyl (Sen, Ray and Basu, 1952*a*) ; 5-chloro-8-quinolyl (Gupta, 1952) and 6-methoxy-8-quinolyl (Basu, 1952) groups at the N^5-position have been reported. Out of these N^1-aryl-N^5-(5 : 6-dimethoxy-8-quinolyl) biguanides were both active and toxic (Sen, Ray and Basu, 1952*a*) while N^1-p-anisyl-N^5-(6-methoxy-8-quinolyl) biguanide showed encouraging activity against experimental infections (Basu, 1952).

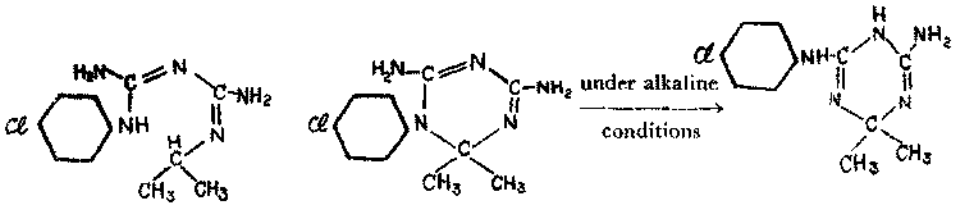
Other heterocyclic systems studied in case of N^1-aryl-N^5-heterocyclic-biguanide were : 5-acridyl (Gupta and Guha, 1950*b*) ; 2-chloro-7-methoxy-5-acridyl (Gupta and Guha, 1951) ; 2-pyridyl (Roy and Guha, 1950*a*) ; 6-substituted-2-benzothiazolyl (Guha and Guha, 1952*d*) ; and 2-thiazolyl (Bami and Guha, 1949*b*). Acridyl biguanides were devoid of antimalarial properties (Sirsi *et al.*, 1950) and so were N^1-p-chlorophenyl-N^5-2-thiazolyl biguanide (Bami *et al.*, 1949) and N^1-aryl-N^5-benzothiazolyl biguanides (Guha and Guha, 1952*d*). Tetra- and tri-arylbiguanide substituted derivatives of 3-phenylchelidonic acid and 3-phenylchelidamic acid respectively also displayed only limited suppressive action against *P. gallinaceum* (Neelakantan *et al.*, 1952*b*).

Paludrine was found to be de-aminated into N-p-chlorophenyl-N'-isopropyl-guanylurea by long standing in hydrochloric acid. Study of similar N-aryl-N'-alkyl (or $N'N'$-diaklyl) guanylureas showed them to be only moderately active against erythrocytes of *P. gallinaceum* and completely inactive against the exo-erythrocytic forms. While isomeric N'-isopropyl-N-p-chlorophenyl guanylurea, 1-p-chlorophenyl-5-alkyl (or dialkyl) biurets (Curd, Davey and Richardson, 1949) ; and certain 1-aryl-5-alkyl-isodithio-biurets (Curd, Davey, Richardson and Ashworth, 1949) were found to be devoid of activity. Replacement of the terminal guanidine group of paludrine with s-isopropylisothiourea or an amidine group also resulted in complete loss of activity (Birtwell, 1949).

Various N^1:N^3-aryl (and/or) alkyl guanidines (Gupta and Guha, 1948*b*) ; sulphuryl-bis-guanidines (Sur *et al.*, 1952) ; N^1 : N^3-bis (p-sulphonamido phenyl) guanidines (Guha *et al.*, 1953) ; N^1-aryl-N^3-(7-chloro-8-quinolyl) guanidines (Sen,

Raichaudhuri and Basu, 1952) ; N^1-aryl-N^3-p-phenylarsonic acid-guanidines (Guha *et al.*, 1952) and N^1-aryl-N^3-(6-methoxy-8-quinolyl) guanidines (Chandran *et al.*, 1952) have been synthesised as potential antimalarials and the last two groups failed to reveal any activity. Toxicity of 8-aminoquinolines is influenced by the type of alkylamino chain attached and this led to the synthesis of N^1-(5-(6-methoxy-8-quinolylamino)-amyl) gaunidine (DR. 15526) and other related compounds. Although DR. 15526 was sixteen times less toxic than paludrine, it failed to show any curative action against *P. cynomolgi* and *P. vivax* (Drake and Garman, 1949). Certain 2-guanidino-quinazolines (Theiling and Mckee, 1952) and 2-guanidino-benziminazoles, embodying the paludrine skeleton have been synthesised but the later type was devoid of activity (King *et al.*, 1948). Srinivas *et al.*, (1952) studied a number of substituted hydrazodicarbonamidines as potential antimalarials.

Studies on degradation and physico-chemical data.—Hawking and Perry (1948) suggested that paludrine itself is not active but is converted into an active metabolite *in vivo*. Fraser and Kermack (1951) tried to explain this by the assumption that paludrine combined with a normal metabolite of the body, such as, aceto-acetic acid (an intermediate product of fatty acid metabolism). However, condensation products of proguanil with acetoacetic ester and acetic anhydride were either inactive or only slightly active. Chase *et al.* (1951) strongly suggested the possibility of a cyclic structure for paludrine metabolite due to the polyfunctional nature of the biguanide chain and the structural resemblence between paludrine (XIIa) and highly active 2 : 4-diamino-pyrimidines, such as daraprim (XVI) (Falco, Goodwin *et al.*, 1951). Birtwell (1952) suggested that oxidation being a common mode of detoxication, paludrine may be transformed into (XIV) which has still greater structural resemblence with daraprim (XVI). Meanwhile from the urine of the monkeys receiving paludrine, Crounse (1951) was able to isolate, p-chlorophenyl biguanide and an inactive triazine, *viz.* 2-amino-4-p-chloroanilino-6 : 6-dimethyl-1 : 3 : 5-triazine (XV). It was suggested that compound (XIV) could also be formed but under their experimental conditions it could not be isolated (Crouse, 1951). It may be added here that inactive triazine (XV) was previously synthesised (Birtwell *et al.*, 1948 ; Birtwell, 1952) and found to be inactive.



PALUDRINE (XIIa)

PALUDRINE METABOLITE (XIV)
2:4-diamino-1-p-chlorophenyl-1:6-dihydro-6:6-dimethyl-1:3:5-triazine

INACTIVE TRIANZINE (XV)
2-p-chloroanilino-4-amino-6:6-dimethyl-1:3:5-triazine

Carrington *et al.* (1951) and Crowther and Levi (1953) were able to isolate the active paludrine metabolite (XIV) as well as the inactive compound

isolated by Crounse (1951) from rabbit fæces and human urine. It was shown that (XIV) was readily converted into its inactive isomer (XV) which in turn was hydrolysed to p-chlorophenyl-biguanide. Crounse (1951) was thus only able to isolate two inactive products due to the labile nature of (XIV). Paludrine metabolite (XIV) was ten times more active than paludrine when tested against *P. gallinaceum* (Carrington *et al.*, 1951) while six times as active as quinine, twice as active as mepacrine or paludrine and half as active as pamaquin when tested against *P. lophuræ* (Modest *et al.*, 1952). A 3 : 4-dichloro analogue of (XIV) has been isolated as a metabolite of N^1-3 : 4-dichlorophenyl-N^5-isopropyl biguanide while radio-active bromo analogue of paludrine has been used in excretion and absorption studies (Crowther and Levi, 1953). A p-bromo analogue of (XIV) has also been isolated as a metabolic product of bromoguanide (Bami, 1953*b*).

Schmidt *et al.* (1952) have observed recently that paludrine is 2-4 times as active as its metabolite (XIV) when tested against *P. cynomolgi* in monkeys. These findings were not in confirmity with the previous screening results in avian malaria and perhaps inherent differences in susceptibilities of the two strains or differences in the physiological disposition of the metabolite were responsible for this disparity. However, considering that P. *cynomolgi* is a better indicator for clinical usefulness of a drug, metabolite (XIV) may not ultimately prove to be better than paludrine. Naturally more work would be needed to settle this point, but these results do explain partly the failures encountered by Crounse (1951) when *P. cynomolgi* alone was being used for screening the paludrine metabolites. It has also been observed that paludrine and its metabolite (XIV) were both feebly active *in vitro* against *P. gallinaceum* (Taylor *et al.*, 1952) which is specially surprising in case of (XIV) which is supposed to be an active breakdown product of paludrine. Is it that high *in vitro* antimalarial activity of the serum obtained from experimental animals receiving paludrine (Hawking and Perry, 1948) was not due to the presence of (XIV) but some other factors ?

Paludrine and sulphadiazine potentiate each other's antimalarial action, although their toxicity has also been additive (Greenberg *et al.*, 1948 ; Greenberg, 1949*a* : 1949*b*). Similarly cross resistence between sulpha drugs and paludrine has been noted in the case of *P. gallinaceum* (Bishop and McConnachie, 1950). These findings cannot be explained on the basis of the structural similarity or a similar mode of action, because unlike paludrine, the antimalarial action of sulpha drugs can be inhibited by para-amino benzoic acid. A satisfactory explanation of this observation is not yet forthcoming (Bishop and McConnachie, 1951). Studying the effect of paludrine on the respiration of *P. gallinaceum in vitro*, Srinivasan and De (1951) observed that unlike mepacrine, paludrine did not bring about the inhibition of respiration by affecting the flavo-proteins like d-amino-acid oxidase.

A number of arylalkyl biguanides, aryl-guanidines and amidines (as biguanide fragments) were studied for their pK values but this physical data could not be satisfactorily correlated with anitmalarial activity. Out of the three possible configurations of the biguanide ions, the one responsible for activity could not be singled out (Gage, 1949*b*). Recent physico-chemical data and extensive investigations on substituted biguanides, have prompted the rejection of earlier hypothesis concerning the relationship of activity with chemical structure, which was based

upon potential tautomerism within the active biguanide molecule (Curd and Rose, 1946). The same question has now been considered in the light of properties of the amidine group based upon resonance energies. According to this, all active molecules must have an amidine or a vinyloguous structure, associated with either aromatic or alkyl or both groups and so influenced by relevant substituents that at physiological hydrogen ion concentration, they are largely present as the amidinium ion (Gage 1949*a* : 1949*b* : 1949*c*). A chlorophenyl residue, associated but not necessarily in conjugation with an amidine or extended amidine system and in a structure that provides the necessary cationic functions, would give more often than not, active antimalarials. The intensity of action and toxicity, however, cannot be predicted by this rule (Rose, 1951) and it also cannot explain the cause of activity in the case of quinine, pamaquin and quinoline-carbinols.

TRIAZINES AND TRIAZOLES.

Numerous substituted triazines had been investigated previously (Wiselogle, 1946 ; Curd *et al.*, 1947) but activity whenever present was feeble. The discovery of triazines as degradation products of paludrine (for details see page 200) stimulated interest in this field. A number of active triazines allied to (XIV) have been prepared and found highly active against avian infections. Close similarity of this class of compounds (XIV) to highly active biguanides (XII*a*) and 2 : 4-diamino pyrimidines (XVI) warrants further chemical and biological investigations.

Chemical studies.—A 3 : 4-dichloro analogue of (XIV) *viz.*, 2: 4-diamino-6 : 6-dimethyl-1 : 6-dihydro-1-(3 : 4-dichlorophenyl)-1 : 3 : 5-triazine was hundred times as active as paludrine against *P. gallinaceum* (Carrington *et al.*, 1951). Against *P. cynomolgi*, this compound was even less active than paludrine, although more active than (XIV) (Schmidt *et al.*, 1952). Recently it has also been obtained as an *in vivo* metabolite of 3 : 4-dichloro analogue of paludrine (Crowther and Levi, 1953).

1-p-bromophenyl-2 : 4-diamino-1 : 6-dihydro-6 : 6-dimethyl-1 : 3 : 5-triazine was found to be 256 times as active as quinine and twice as active as (XIV) when screened against *P. gallinaceum* while its 6 : 6-diethyl isomer was completely inactive (Bami, 1953*b*). Study of p-ethoxyphenyl, phenyl, p-methoxy-phenyl and 2 : 4-dichlorophenyl analogues of triazine (XIV) revealed that the first two compounds are devoid of activity (Modest *et al.*, 1952) while the remaining two are only as active as quinine when tested against avian malaria (Bami, 1953*b*). Basu *et al.*, (1952) have reported a p-sulphonamidophenyl analogue of the active triazine (XIV) but the biological testing results are not indicated. In case of all the above triazines, treatment with alkali easily converted them into respective 4-amino-2-anilino-6 : 6-dialkyl-1 : 3 : 5-triazines which were invariably devoid of activity (Crounse, 1951 ; Carrington *et al.*, 1951 ; Basu and Sen, 1952 ; Crowther and Levi, 1953 ; Bami, 1953*b*). Birtwell *et al.* (1948) had also previously synthesised the inactive type of triazines (XV).

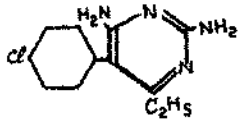
Cuthberston and Moffatt (1948) prepared a number of 2-(6-methoxy-8-quinolylamino)-4- amino-6-chloro/or amino-1 : 3 : 5 triazines, 2-(diethylamino-1-

methylbutylamino)-4- amino-6-chloro/or methoxy 1 : 3 : 5 triazines and 2-(p-chloroanilino)-4-iso-propylamino-6-chloro-(methyl or unsubstituted)-1 : 3 : 5-triazines. Out of these, only 2-(p-chloroanilino)-4-isopropylamino-1 : 3 : 5-triazines showed some activity. Similarly a possible metabolite of paludrine *viz.*, 2-p-chloroanilino-6-methyl-4-isopropylamino-1 : 3 : 5-triazine was devoid of anti-malaria activity (Fraser and Kermack, 1951).

A number of 3 : 5-diamino-1-aryl-1 : 2 : 4-triazoles were studied as anti-malarials but the activity was lacking in all the cases (Thurston and Walker, 1952). Similarly 3-p-chloroanilino-5-isopropyl-1 : 2 : 4-triazole and other analogous compounds, although embodying the paludrine skeleton, were found to be inactive (Curd, Davey, Richardson and Ashworth, 1949).

PYRIMIDINES.

Antimalarials of the pyrimidines series were introduced as precursors of paludrine (Curd and Rose, 1946) and further variations in this group have resulted in the discovery of daraprim (pyrimethamine) (XVI) which is a 2 : 4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine (Falco, Goodwin *et al.*, 1951 ; Russell and Hitchings, 1951). This drug has been found to be highly active against avian, simian and rodent malarias (Falco, Goodwin *et al.*, 1951 ; Jaswant Singh *et al.*, 1951 ; Hitchings *et al.*, 1952 ; Jaswant Singh, Ray, Basu and Nair, 1952 ; Schmidt and Genther, 1953) and clinical trials with daraprim so far have proved it to be a good suppressant with low toxicity (Hitchings *et al.*, 1952 ; Jaswant Singh, Ray, Basu and Nair, 1952 ; Jaswant Singh *et al.*, 1953). It is similar to paludrine as regards slowness of its action, low toxicity and acquiring of resistance by various strains of malaria (Rollo, 1952 ; Jaswant Singh, Ray, Basu and Nair, 1952 ; Hitchings *et al.*, 1952 ; Schmidt and Genther, 1953). Cross resistance between daraprim and proguanil in the case of human and experimental malarias shows a similarity of mode of action of these two drugs (Schmidt and Genther, 1953 ; Jaswant Singh *et al.*, 1951 ; Jaswant Singh, Ray, Basu and Nair, 1952 ; Robertson *et al.*, 1952 ; Hitchings *et al.*, 1952).



DARAPRIM (PYRIMETHAMINE) (XVI)
2:4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine

Chemical studies.—Paludrine and 2 : 4-diamino-5-p-chlorophenoxy-pyrimidine were not only structurally similar but also powerful antagonists of pteroylglutamic acid in cultures of *Lacto bacillus* casei (Falco *et al.*, 1949). Considering the high potency of paludrine, it was thought that the above 2 : 4-diaminopyrimidine might also display antimalarial activity. This was found to be so in the above case (Falco *et al.*, 1949) as well as in the case of its 6-methyl homologue (Goodwin, 1949). However, the antagonism between these pyrimidines and pteroylglutamic

acid was not proportional to the antimalarial activity (Falco *et al.*, 1949). These results encouraged further studies and consequently a number of 2 : 4-diamino-5-aryloxyl-6-methyl (or 6-unsubstituted)-pyrimidines (Falco, Russell and Hitchings, 1951) ; 2 : 4-diamino-5-benzyl-6-alkyl (or 6-unsubstituted)-pyrimidines (Falco, DuBreil and Hitchings, 1951) ; 2 : 4-diamino-5-aryl-6-alkyl (or aryl)-pyrimidines (Russell and Hitchings, 1951 ; Chase *et al.*, 1951) and 2 : 4 : 6-triamino-5-alkyl (benzyl or aryl) pyrimidines (Russell and Hitchings, 1952) were synthesised. Many compounds of the above series showed high antimalarial activity against *P. gallinaceum* in chicks and *P. berghei* in mice and the general relationship of chemical structure to the biological activity could be summarised as follows :

(a) 2 : 4-diamino group was essential for good activity in this class of compounds and substitution of either or both of the amino groups resulted in considerable loss of activity.

(b) 2 : 4 : 6-triamino-5-substituted pyrimidines were only slightly active (Russell and Hitchings, 1952).

(c) 2 : 4-diamino-6-alkyl pyrimidines with 5-phenyl substituents were the most active followed by 5-benzyl and 5-phenoxy isomers respectively.

(d) Electro attracting halogen and nitro groups at para position of the 5-phenyl ring yielded active compounds while similar substituents at ortho and meta position gave compounds of much lesser potency.

(e) p-bromo-phenyl and p-fluoro-phenyl analogues of daraprim (XVI) were less active than the parent drug.

(f) 5-(3 : 4-dichlorophenyl)-6-alkyl-2 : 4-diamino-pyrimidines were equally active though more toxic than their corresponding 5-p-chlorophenyl analogues (Hitchings *et al.*, 1952) (*cf* : 3 : 4-dichloro-phenyl analogue of paludrine).

(g) Alkyl group at position 6- is also essential for good activity and in the 5-aryl series (Russell and Hitchings, 1951), peak activity was obtained with ethyl group. Longer alkyl chains at 6-position offered compounds with low activity.

Curd and Rose (1946) had previously reported a number of active 2-arylguanidino-4-dialkylamino aklylamino-6-methyl-pyrimidines and further variations in the nature of different substituents did not yield better results (Cliffe *et al.*, 1948). Changing of the guanidino group in the above type of compounds, into ureido or thioureido group did not give significantly active compounds (Ashworth *et al.*, 1948). Various 4-arylguanidino-2-dialkylaminoalkylamino-6-methyl-pyrimidines and 4-arylguanidino-6-dialkylaminoalkylamino-2-methyl-pyrimidines displayed activity in contrast to their inactive anilino isomers, which was probably due to the introduction of a guanidino linkage between the aryl and the alkyl group in the former two types of compounds (Crowther, Curd and Rose, 1948). However, when the aryl group in the above active pyrimidines was replaced by 6-quinolyl (Gulland and Macey, 1949) or 6-methoxy-8-quinolyl radicals, the resulting compounds were inactive (Sen, Ray and Basu, 1952*a*). Number of other pyrimidines such as 4-dialkylamino alkylamino (or pyridylamino)-2-arylamino (or 2'-pyridylamino)-6-alkyl pyrimidines (Curd, Graham and Rose, 1948 ; Moffatt,

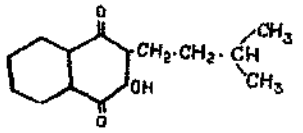
1950) ; 4 : 6-bis:(dialkyl-aminoalkylamino)-2- anilino-pyrimidines (Moffatt, 1950); 4 - diethylamino - propylamino - 2 - p - methylsulphonylphenyl-6 - methyl - pyrimidine (Forrest and Walker, 1948) and 6'-beta-diethylaminoethylamino-4 ; 4' : 6-trimethyl-2 : 2'-dipyrimidylamine (Curd, Graham and Rose, 1948) were investigated but the antimalarial activity if present, was very feeble.

Biological investigations and physico-chemical observations.—Schmidt *et al.* (1953) have extensively studied the absorption, distribution, degradation, excretion and toxicity of daraprim (XVI) and it has been found to be half as toxic as paludrine and one third of chloroquine toxicity. Daraprim was excreted as a non-basic closely allied metabolite whose true chemical nature has not been revealed (Hitchings *et al.*, 1952 ; Schmidt *et al.*, 1953). Furthermore, *in vitro* inactivity of daraprim also indicated its possible degradation in the system prior to its exerting the therapeutic action (Taylor *et al.*, 1952). Daraprim is remotely structurally related to folinic acid and has been found to be antagonist of folicfolinic acid family of vitamins (Hitchings *et al.*, 1952). Greenberg and Richeson (1950) observed that like paludrine, 2 : 4-diamino-5-phenoxy-pyrimidines potentiated the action of sulphadiazine on *P. gallinaceum* which made it likely that these were acting by involving the same metabolic system probably those mediated by pteroylglutamic acid. In spite of the fact that antagonism to pteroylglutamic acid and antimalarial activity in the case of pyrimidines and paludrine did not run parallel, they may have a similar mode of action due to similarities of chemical structures and biological responses (Jaswant Singh *et al.*, 1951 ; Jaswant Singh, Ray, Basu and Nair, 1952 ; Hitchings *et al.*, 1952 ; Schmidt and Genther, 1953).

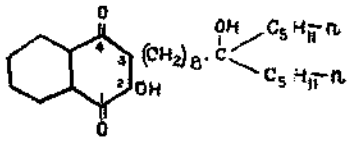
Physico-chemical measurements like, ultraviolet spectra and electrolytic dissociation constants, in the case of number of pyrimidine derivatives, have failed to offer a satisfactory correlation between chemical structure and biological activity. It is argued that heterocyclic nucleus and the side chain must be ionised at the physiological pH for the specific antimalarial activity to be exhibited (Gage, 1949*c*; Rose, 1951). However, biological activity is more closely related to the distribution of charge on the molecule than to its basicity *per se* (Hitchings *et al.*, 1952).

NAPHTHOQUINONES.

In 1943, several hundred random samples, when put to antimalarial screening, revealed that simple structure like hydrolapachol (XVII) and related quinones were active. This was of particular significance because (XVII), unlike all the rest of antimalarials, did not contain either nitrogen or sulphur. These findings prompted extensive chemical and biological investigations on substituted naphthoquinones under Dr. Fieser at Harvard and Dr. Leffler at Abbott Laboratories, which resulted in the evolution of lapinone (M2350) (XVIII) (3-(8'-hydroxy-8-diamyl-methyloctyl)-2-hydroxy-1 : 4-naphthoquinone (Fieser, Berliner *et al.*, 1948 ; Fawaz and Fieser, 1950). Lapinone is the most active member of this group and has shown some promise in clinical trials (Wiselogle, 1946 ; Fawaz and Haddad, 1951). Extremely poor absorption of this drug by the oral route, makes it necessary that it should be given intravenously, which is a serious drawback.



HYDROLAPACHOL (XVII)
3-isoamyl-2-hydroxy-1:4-naphthoquinone.



LAPINONE (M 2350) (XVIII)
3-(8'-hydroxy-8-diamyl-methyloctyl)-2-hydroxy-1:4-naphthoquinone.

Chemical studies.—Chemical investigations mostly centred around modification of the 3-alkyl chain of hydrolapachol (XVII). Nearly three hundred derivatives of the type 3-substituted-2-hydroxy-1 : 4-naphthoquinone were synthesised, mostly by peroxide alkylation of hydroxy naphthoquinones and by Hooker oxidation reaction in certain cases (Fieser and Fieser, 1948). While 1 : 4-naphthoquinones themselves have been prepared by an improved diene synthesis using butadiene and benzoquinones (Fieser, 1948*a*). Some of the substitutions tried at 3-position were alkyl, isoalkyl, cycloalkylalkyl, 4-cyclohexycyclo-hexyl, cycloalkyl, arylalkyl, aryl, and alkyl chains containing nitrogen or halogen (Fieser, Berliner *et al.*, 1948 ; Zaugg *et al.*, 1948). A number of similar naphthoquinones with substitution in the benzenoid ring as well as replacement of 2-hydroxy group with chloro, amino, groups, etc., were also prepared and tested (Fieser, Berliner *et al.*, 1948). Cram (1949) further studied derivatives similar to lapinone, especially with regard to presence of oxygen in the 3-alkyl side chain while methods for the separation of naphthoquinones have also been worked out (Fieser, 1948*c*). Some important relationship between biological activity and chemical structure in this class of compounds (XVIII) are summarised below (Fieser and Richardson, 1948).

(a) In the case of normal, iso and branched alkyl chains at position 3- of 2-hydroxy-1 : 4- naphthoquinone the activity against *P. lophurae* increased upto C_9 and then fell off.

(b) 3-alkyl chains containing one or two alicyclic rings gave peak activities at C_{10}-C_{11} and C_{12}-C_{13} respectively, while aryl substituents produced a still greater shift.

(c) Presence of substituted aminomethyl groups (Leffler and Hathaway, 1948), aryl (or alkyl)-mercaptoalkyl groups (Moser and Paulshock, 1950) and aryloxyalkyl groups (Paulshock and Moser, 1950) at position 3- of 2-hydroxy-1 : 4-naphthoquinone, offered compounds with low antimalarial activity.

(d) The quinonoid 2-hydroxyl group seemed indispensable as its replacement by any other group invariably resulted in the loss of activity (Fieser, Berliner *et al.*, 1948).

(e) Substitution in the benzenoid ring of naphthoquinone mostly destroyed activity (Fieser, Berliner *et al.*, 1948).

(f) Presence of a halogen, double bond, nitrogen or oxygen in the 3-alkyl chains up to C_{12} completely destroyed the activity, but C_{16}-C_{21} side

chains containing a tertiary hydroxyl group gave highly potent drugs like (XVIII).

Metabolic, biochemical and physico-chemical studies. - Nearly twenty 2-hydroxy-3-alkyl-1 : 4-naphthoquinones were studied for their degradative metabolism *in vivo* both in man and animals and it was observed that a rapid oxidation of 3-alkyl chain occurred with the production of hydrophilic compounds. These degradative products were isolated and characterised as secondary or tertiary alcohols or carboxylic acids (Fieser, Chang *et al.*, 1948). Naphthoquinones having a hydrocarbon side chain terminating in a ring, on degradation gave weekly active products hydroxylated in the ring (Fieser, Heymann and Seligman, 1948). Similar metabolism studied with lapinone (XVIII) have shown that it was considerably more resistant to degradation, hence so very active. Metabolic studies were usually conducted with blood and urine samples but in certain cases, it was found that this procedure was not applicable as very little of the drug was excreted in the urine. Consequently the rate and extent of degradation was measured by using *in vitro* respiration inhibition technique (Fieser, Heymann and Seligman, 1948; Heymann and Fieser, 1948*a*).

Metabolic oxidation involving the side chain and not the nucleus can be synthetically reproduced using chromic anhydride. In this case also, the products were quinone acids, ketones and alcohols (Fieser, 1948*b*). Ettlinger (1950) has also been able to show a photocatalysed autoxidation in ether in the case of these compounds.

Naphthoquinones were not only active against blood-induced avain malaria but also against three sporozoite induced avain infections (Wiselogle, 1946; Clarke and Theiler, 1948). Walker and Richardson (1948) found that 3-beta-decalyl(cis)-propyl-2-hydroxy-1 : 4-napthoquinone (M2279) potentiated the curative action of pamaquin. Most of the naphthoquinones were tested by *in vitro* screening procedure based upon inhibition of respiration of parasitized red blood cells drawn from a duck infected with *P. lophurae* (Heymann and Fieser, 1948*a*; Fieser and Heymann, 1948; Fieser, Heymann and Seligman, 1948). Compounds with peak potency were mostly antagonised in decreasing order by human, monkey and duck plasma proteins and most likely these drugs acted by combining with a respiratory enzyme which resulted in the deactivation of the latter (Fieser and Heymann, 1948). The extent to which these drugs were susceptible to protein antagonism (as measured by the depression of the inhibitory action of the naphthoquinones on the respiration of parasitized cells), varied with the type of serum and drug involved (Heymann and Fieser, 1948*a*). Similarly no correlation could be established between inhibition of respiration of succinate oxidase and antimalarial activity *in vivo* (Heymann and Fieser, 1948*b*).

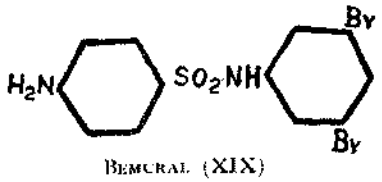
Extensive studies of distribution characteristics of naphthoquinones revealed that a balance between lipophilic and hydrophilic characteristics was required for optimum activity in any given series of hydroxynaphthoquinones (Fieser, Ettlinger and Fawaz, 1948). It has also been observed that these drugs rapidly get oxidised into hydrophilic compounds of low potency, hence for optimum activity it was necessary that the 3-alkyl side chain should carry such substituents which made it resistant to degradation, as well as offered suitable balance between lipophilic and

hydrophilic properties. In the case of lapinone (XVIII), introdution of a hydroxyl group to C_9-alkyl chain, blocked the metabolic degradation and further lengthening of the alkyl side chain counterbalanced the hydrophilic properties conferred by this hydroxyl group (Fieser, Heymann and Seligman, 1948).

SULPHONAMIDES AND SULPHONES.

It has been well recognised that antimalarial activity of sulpha drugs is due to their antibacterial properties. Previously sulpha drugs have shown only limited promise against human malarias (Wiselogle, 1946 ; Findlay, 1949 : 1951), although their action against avian and simian malarias has continued to play an important rôle in the development of new antimalarials and better understanding of the existing ones. During the period under review, no outstanding advance has been made in this field although problems of cross resistance and potentiation of antimalarial action between sulpha drugs and other antimalarials, have been of considerable interest.

Chemical studies.—A number of 4-amino-3'-5'-disubstituted -benzene sulphanilides have been studied, out of which Bemural (XIX) (4-amino-3'-5'-dibromo-benzene-sulphanilide) has shown optimum activity (Behmisch, 1948 ; Wiselogle, 1946). In the above type of compounds, 4-amino group was essential for activity while replacement of halogens by other radicals always decreased the activity (Behmisch, 1948). Bemural (XIX) and sulphaguanidine were diazotised and coupled with N-(4-diethylamino-1-mtehylbutyl) aniline and the resulting products showed some activity against simian malaria (Tani *et al.*, 1950).



Bemural (XIX)

4-amino-3' : 5'-dibromo-benzenesulphanilide.

A number of heterocyclic substituted sulphanilamides such as N^4-(2-methoxy-6-chloro-9-acridyl)-sulphanilamide (Miyatake 1953), 2-metanilamido-pyrazine ; 2-metamilamido-5-chloro-pyrimidine (metachloridine-SN11437) (American Cynamide Co., 1948) ; 2-sulphanilamido-quinoxalines ; 2 (or 4-)-sulphanilamido-quinazolines (Wolf, 1949) and N(p-sulphonamidophenyl)-4-(p-aminophenyl-sulphonimino)-3-phenylpyridine-2 : 6-dicarboxylic acid (Neelakantan *et al.*, 1952*a*) have been reported to be usually active. An interesting series of sulphabiguanide derivatives have already been discussed on pages 198-199.

Amongst sulphones, various 6-quinolyl-p-amino-(or nitro)-phenyl sulphides and their sulphones (Gilman and Gainer, 1949) as well as certain 4'-4-di-(arylbiguanido) diphenyl sulphides and sulphones (Roy *et al.*, 1950) have been reported as potential antimalarials.

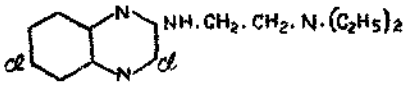
Biochemical studies.—It has been revealed that *P. gallinaceum* strain resistant to sulphadiazine was also resistant to paludrine though not to mepacrine. Such a cross-resistance could not be explained on the grounds of structural similarity or

similar mode of action of these compounds (Bishop and McConnachie, 1950 : 1951). Another interesting observation has been the potentiation of antimalarial action of sulphadiazine by paludrine (Greenberg *et al.*, 1948 ; Greenberg, 1949*b*), 2 : 4-diamino-pyrimidines (Greenberg and Richeson, 1950) and certain pterins (XXII) (Greenberg, 1949*a*) which was also accompanied by addative toxicity of the two drugs involved (Greenberg *et al.*, 1948). These findings cannot be explained on the basis of structural similarity between sulphadiazine and rest of the drugs.

MISCELLANEOUS.

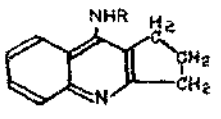
A variety of chemical structures, usually hetrocyclic rings with nitrogen, displaying all shades of antimalarial activity have been described in this chapter. Some of the promising leads could be further developed with advantage. General failure of antibiotics against malarial infections is also noteworthy.

Chemical studies.—Haworth and Robinson (1948) studied a number of substituted 3-dialkylaminoalkylamino-quinoxalines and amongst these 2 : 6-dichloro- 3-beta-diethylaminoethylamino-quinoxaline (XX) displayed good antimalarial activity against *P. gallinaceum.* Increasing the complexity of the side chain and removal or replacement of 2-chloro radical in this compound (XX) resulted in feebly active compounds. Similarly 2-chloro-3-dialkylaminoalkylamino-quinoxalines were also only slightly active (Crowther *et al.*, 1949). Further variation in 2-chloro-6-substituted-3-dialkylaminoalkylamino-quinoxalines did not offer a highly active compound except for 2 : x-dichloro-3-beta-diethylamino-ethylamino-6 (or 7)-methoxy-quinoxalines (Curd, Davey and Stacey, 1949). Similarly various substituted 6-amino-quinoxalines (Gilman and Broadbent, 1948) ; 2-aryl-4-arylamino-quinazolines (Curd, Landquist and Rose, 1948*a* ; Dass *et al.*, 1952) ; 4-(or 2)-dialkylaminoalkylamino-2-(or 4)-arythio-quinazolines (Curd, Hoggarth *et al.* 1948*d*) ; and 2-p-chloro-anilino-4-beta-diethylaminoethylamino-quinazolines (Curd, Landquist and Rose, 1948) were prepared as potential antimalarials out of which the last two groups when tested, were found to be inactive.



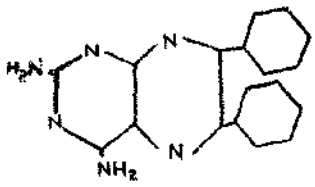
Quinoxaline (XX)

2 :6-dichloro-3-β-diethylaminoethylamino-quinoxaline.



Quinindenes (XXI)

1 2-substituted-amino-2 :3-dihydro-β-quinindene.



Pterin (Dr 15791) (XXII)

2 :4-diamino-6 :7-diphenyl-pyrimido (4, 5b) pyrazine.

Definite prophylactic activity as displayed by 9-chloro (or methoxy)-12-hydroxy-2 : 3-dihydro-beta-quinindenes (Stephenson *et al.*, 1947) prompted further studies in this group and Chadha *et al.* (1951*a* : 1951*b*) synthesised a number of 12-(arylamino or dialkylaminoalkylamino)-2 : 3-dihydro-beta-quinindenes (XXI) as well as certain 12-hydroxy-2 : 3-dihydro-beta-quinindenes having mono substituents at position 7-, 8-, 9- or 10-. These compounds were generally inactive when screened against *P. gallinaceum* (Bami, 1953*b*).

Certain substituted 1-dialkylaminoalkyl-isoquinolines (Haworth and Robinson, 1948) ; 4-amino-1-dialkylaminoalkylamino-phthalazines (Haworth and Robinson, 1948) and 4 : 5-dihydroglyoxaline derivatives (James and Turner, 1952) have been found to be inactive. Noreldrin (1951) has clinically tried a new drug "Abadol" (amino-2-thiazole) but it was found to be less active and more toxic than chloroquine.

Benziminazole derivatives substituted at 1, 2, 4, 5 and 6-positions were usually inactive except for certain 1-(2-pyridyl-aminomethyl) benziminazols which displayed some activity (Hall and Turner, 1948 ; James and Turner, 1952). A number of 2 : 6-bis-(glycolylamino)-3-substituted pyridines (Lott and Brenstein, 1949) and N-2-pyridylalkanolamines were also prepared, out of which the latter group was devoid of activity (Weiner and Kaye, 1949). Amongst pterins, 2 : 4-diamino-6 : 7-diphenylpyrimido (4, 5b) pyrazine (DR15791) (XXII) showed activity as good as quinine in *P. gallinaceum* and resembled paludrine, as regards potentiation of its antimalarial action by sulphadiazine (Greenberg, 1949*a*). Both activity and its potentiation could be attributed to the presence of a biguanide linkage in this pterin (XXII). Certain pantoyltauramides (Mill and Roblin, 1949) and isoalloxizines (McCoubrey and Websters, 1948*a*) have also been reported but results of their antimalarial screening are not indicated. Thiopegene-9, 4-one (Narang, 1953) has been found to be as active as quinine when tested against *P. gallinaceum*.

A number of substituted ketones *viz.*, 2: 5-diphenyl-3-furyl amino-ketones (Lutz and Rowlett, 1948) ; alpha-beta-dimorpholinyl ketones (Lutz *et al.*, 1949) and substituted acetophenones (Mathieson and Newbery, 1949) have been studied as possible antimalarials but the activity has been usually very feeble. Amino-carbinols obtained from acetylphenantherene (Sargent and Small, 1948*a*) and 4-(3-diethylamino-propylamino)-1-ethoxy-naphthalene (Belotsvetor, 1949) were found to be devoid of activity when tested against avian malaria. Leffler and Matson (1948) studied a number of substituted carbamates but the activity was usually moderate, the best compounds of this series being p-carbobutoxyphenyl-p'-methoxy carbamate (SN1048) and p-sulphamylphenyl-p'-methoxy-carbamate (SN4178) (Wiselogle, 1946).

Antibiotics have been conspicuous in their ineffectiveness against malaria parasites but in spite of this, Imboden *et al.* (1950) tried several of the new antibiotics against *P. vivax* infection for evaluating their curative and prophylactic action. Aureomycin and chloromycetin showed some prophylactic activity but they were not curative, while penicillin and dihydro-streptomycin were completely inactive. In short, antibiotics in general have practically very little use in the treatment of

malaria (Findley, 1951 ; Coatney *et al.*, 1953). A recent monograph by Coatney *et al.* (1953) details the screening results of a number of miscellaneous compounds.

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Indian Journal of Malariology, **7**, 2, *June* 1953.

ABSTRACT.

A FURTHER NOTE ON GONOTROPHIC DISCORDANCE IN *A. ANNULARIS.**

BY

V. VENKAT RAO

AND

A. V. ANNAJI RAO,

(June 30, 1953.)

In discussing the phenomenon of 'Gonotrophic discordance' described by the senior author (Venkat Rao, 1947), Muirhead-Thomson (1951) observed that it should have been ascertained whether the wild caught female Anopheles used in these experiments were fertilized or not as *A. annularis*, in particular, is one of those anophelines in which the development of ovaries after a blood meal takes place irrespective of fertilization.

This criticism was recognised as valid but, owing to the absence of the senior author in Burma, he requested the junior author to carry out further observations in *annularis* to clarify the issue.

Accordingly, a number of *annularis* ♀♀ showing evidence of gonotrophic discordance were collected from the Khurda Road area of Orissa for these observations. Out of them, 34 survived to oviposit and their eggs hatched as larvæ in about the normal time, indicating fertilization in every case.

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*The original manuscript (4 typescript pages) has been placed in the library of Malaria Institute of India, Delhi, and is available on loan to workers who may wish to read it.

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The following contractions are in use in the *Indian Journal of Malariology*, whether the number to be expressed is one or more :—

gramme, gm ; kilogramme, kg ; centigramme, cg. ; milligramme, mg. ; metre, m. ; kilometre, km. ; centimetre, cm. ; millimetre, mm. ; cubic centimetre, c.c. ; cubic millimetre, c.mm. ; cubic foot, c.ft.

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The following are some of the abbreviations in use :—

Amer. J. Hyg.
Ann. Trop. Med. Parasit.
Brit. Med. J.
Bull. Ent. Res.
Bull. Soc. Path. Exot.
C. R. Soc. Biol.
Geneesk. Tijd. Ned.-Ind.
Ind. J. Mal.
Ind. J. Med. Res.
J. Mal. Inst. Ind.
Jl. R. A. M. C.
J. Trop. Med. Hyg.
Meded. Volk. Ned.-Ind.
Rec. Mal. Surv. Ind.
Rev. App. Ent.
Riv. Malariol.
Trans. Roy. Soc. Trop. Med. Hyg.
Trop. Dis. Bull.
U.S.A. Pub. Hlth. Rpts.

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Indian Journal of Malariology, **7**, 3, *September* 1953.

OBITUARY.

Dr. M. A. BARBER.

It is with profound regret that we have to record the death on January 15, 1953, of Dr. Marshall A. Barber, at the age of 85.

Dr. Barber is perhaps best remembered for his discovery of Paris Green as an anopheline larvicide in 1921. He also discovered the gametocidal properties of pamaquin (Plasmochin) during the early days of the use of this drug.

He developed the use of the micropipette for isolating single microscopic organisms in 1914, for which he received the Theobald Smith Medal. He was also awarded the John Scott Medal, and the Mary Kingsley Medal from the Liverpool School of Tropical Medicine. He developed a rapid method of examining the salivary glands of mosquitoes for malaria infection. Dr. Barber is also the author of the technique to determine food preferences of mosquitoes.

After 1929, when he left the U.S. Public Health Service to become associated with the International Health Division of the Rockefeller Foundation, he travelled extensively, visiting Brazil, Africa, India, and parts of the Near East.

Dr. Barber was a man of enormous ingenuity. Much of his philosophy of life, and interesting side-lights on his chosen work, are contained in his "*A Malariologist in Many Lands*", published in 1946 by the University of Kansas Press.

We convey our deep sympathy to the bereaved family, and his friends all over the world.

J.S.

Indian Journal of Malariology, **7**, 3, *September* 1953.

STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

*XIII. Effect of glucose, biotin, para-aminobenzoic acid and methionine on the course of blood-induced infection in starving albino rats.

BY

S. P. RAMAKRISHNAN,

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AND

CHANAN SINGH.

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(July 17, 1953.)

McKee and Geiman (1948) reported that when experimentally inoculated monkeys were starved for 24 to 48 hours during different phases of *P. knowlesi* infection, the course of parasitæmia was strikingly ameliorated. They also found that administration of sucrose and PABA, sucrose, methionine and vitamin C, and sucrose in combination with methionine, PABA and vitamin C during prolonged starvation, enhanced parasite growth. Ramakrishnan (1953) reported that starvation of albino rats rendered conditions inhospitable to *P. berghei* and concluded that it was probably due to deficiency of some nutrients essential to the parasite. This paper deals with the administration of a few chemically pure nutrients and their effects on the course of parasitæmia in starved infected rats.

MATERIAL AND METHODS.

A strain of *P. berghei*† maintained by blood passage in albino rats was used in these experiments. The dose of inoculum was invariably one million parasites per rat given by the intraperitoneal route. Thin blood smears of all animals

*Financed by a grant from the Indian Council of Medical Research.

†Received in 1952 through the kind courtesy of Brigadier J. S. K. Boyd of the Burroughs Welcome Laboratories, London.

were examined daily and when positive, parasites were enumerated against 10,000 erythrocytes. Slides were declared negative when no parasites were seen in at least 100 oil immersion fields.

Thirty albino rats of both sexes approximately six months old were used. The animals were housed in special individual cages and had no access to their excreta at any time.

Although the preliminary observations reported by Ramakrishnan (*loc. cit.*) had shown that normal rats can tide over a period of about 20 days of starvation it was limited in this case to 10 days from the day of inoculation. The animals were divided into four experimental groups and four control groups of which one was on standard* diet. All of them were provided with water in bottles. Rats in each of the experimental groups, received daily, during the 10 days of starvation, one of the following chemically pure nutrients in one to two c.c. of distilled water.†

Nutrient	Quantity per day.	Route of administration.
PABA	0·75 mg.	Intraperitoneal.
Biotin	0·05 mg.‡	,,
Methionine	72·0 mg.	Subcutaneous.
Glucose	12·0 gm.	Oral.

The quantities of vitamins and amino acid were based on the daily requirements for rats (Williams *et al.*, 1950 ; Farris and Griffith, 1949) and that of glucose was calculated on the daily calorific requirements. Attempts to feed methionine orally on the first day were not completely successful ; so on subsequent days it was injected subcutaneously on the lateral side of hind leg. In the course of two to three days, injections gave rise to local punched out ulcers in majority of the animals, followed in a few cases by transient paralysis. Two of the control groups received normal saline and distilled water respectively through the intraperitoneal route.

RESULTS.

Average daily parasitæmia during the ten day starvation period in the four experimental and three control groups is shown in Tables I and II respectively. Growth of parasites in starved animals was extremely poor and confirmed previous results reported by Ramakrishnan (*loc. cit.*). The trauma of injection in Groups 2 and 3 of Table II which received distilled water and normal saline respectively, did not appear to influence the multiplication of parasites as compared with the parasitæmia in starving animals without any interference (Group 1 of Table II).

*Standard diet :—

Whole wheat flour	... 72 parts
Skimmed milk powder	... 23 ,,
Brewers yeast (dry)	... 3 ,,
Calcium carbonate	... 1 part
Common salt	... 1 ,,

†Glucose was served in a cup as paste made with about 10 c.c. of water.

‡Reported daily requirement 0·001-0·003 mg. per rat. A larger dose was given to ensure adequate supply.

TABLE I.

Effect of some essential nutrients on the daily average parasitæmia in starved albino rats.

Experimental groups.	Number of animals.	Nutrient administered daily during starvation.	Parasite count per 10,000 erythrocytes on days of starvation following infection.										Remarks.
			1	2	3	4	5	6	7	8	9	10	
1	4	Glucose	0	1	1	0	0	0	0	0	0	0	Only one animal showed patent parasitæmia.
2	3	Biotin	0	2	0	0	1	0	7	0	0	0	—do.—
3	3	PABA	0	0	2	5·7	8	4	4	1	1	1	Animals showed patent parasitæmia on 8th, 9th and 10th day respectively.
4	5	Methionine	0	2	21	22	D.1 29	D.2 103	100	D.3 108	172	D.4 and 5	

D = Death of animals.

TABLE II.

Daily average parasitæmia in starved and control albino rats.

Control group.	Number of animals.	Parasite count per 10,000 erythrocytes on days of starvation following infection.										Remarks.
		1	2	3	4	5	6	7	8	9	10	
1	2	0	2	1	0	0	0	0	0	0	0	Patency in one animal.
2	5	0	1	3	6	5	0	D.1 1	D.2 0	D.3 and 4 0	D.5	1 c.c. distilled water intraperitoneally daily.
3	3	0	3	0	0	0	0	0	0	0	0	1 c.c. normal saline intraperitoneal daily. Only one animal showed patent parasitæmia.
4	5	...	1·5	25	361	310	211	462	224	145	58	

Fed on standard diet.

D = Death of animals.

Of the four nutrients administered to infected animals during starvation, glucose and biotin did not in any way influence parasitic growth, as it was similar to that in starved controls. Administration of PABA during starvation resulted not only in slightly increased parasitæmia as compared to the controls, but it remained patent almost throughout the period of observation.

The group on methionine showed significantly increased parasitic growth as compared to the controls. Unfortunately the animals died before the tenth day probably due to ulceration caused by methionine but there was an indication that methionine was one of the essential nutrients to *P. berghei*, which was not available to them when rats were starved.

CONCLUSIONS.

Results of these investigations on albino rats and *P. berghei* confirmed some of the observations of McKee and Geiman (*loc. cit.*) on *P. knowlesi* in monkeys. Starvation of the host seemed to deprive the parasites of nutrients like PABA and methionine essential for their growth.

It appeared that glucose alone administered during starvation was not sufficient for growth of *P. berghei* in rats. Similar results were observed by McKee and Geiman (*loc. cit.*) with reference to *P. knowlesi* in monkeys fed with sucrose and vitamin C during starvation. The fact that administration of methionine alone during starvation resulted in increased growth of parasites, indicated that glucose was probably available in the plasma of starved animals.

Four chemically pure nutrients namely glucose, biotin, PABA and methionine were administered to starving albino rats infected with one million parasites. Glucose or biotin did not in any way influence parasite growth in starving animals.

In animals given PABA or methionine during starvation, enhanced growth of parasites was seen as compared to that in controls. The growth was appreciably higher in the methionine series.

It appeared that during starvation of host, essential nutrients like PABA and methionine were not available to parasites.

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Indian Journal of Malariology, **7**, 3, *September* 1953.

STUDIES ON *PLASMODIUM BERGHEI* N. SP. VINCKE AND LIPS, 1948.

*XIV. Reaction of blood-induced infection in albino mice to proguanil and dihydrotriazine metabolites.

BY

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SATYA PRAKASH,

H. L. BAMI

AND

S. P. RAMAKRISHNAN.

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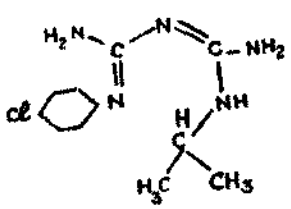
(July 13, 1953.)

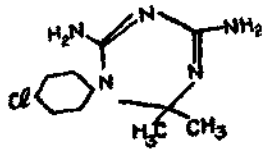
SINCE the discovery of proguanil (paludrine ; N¹-p-chlorophenyl-N⁵-isopropyl-biguanide) (Curd *et al.*, 1945), there were strong indications that perhaps this drug has a mode of action quite dissimilar from those of other potent antimalarials. In vitro studies by Hawking and Perry (1948) and Madinaveitia and Raventos (1949) suggested that high antimalarial activity of proguanil is probably due to its conversion into an active metabolite in the host. Stimulated by these results, Crounse (1951) studied the metabolic fate of proguanil in monkeys, but all the products isolated by him were completely devoid of any antimalarial activity. Carrington *et al.* (1951) and Crowther and Levi (1953) have successfully isolated from the urine of man and rabbit a degradation product of proguanil said to be ten times more active than the parent drug against *P. gallinaceum*. This active metabolite is 2 : 4-diamino-1-p-chlorophenyl-1 : 6-dihydro-6 : 6-dimethyl-1 : 3 : 5 triazine (designated '*A*' in the current trials) which is easily converted into an inactive form previously isolated by Crounse (*loc. cit.*). Modest *et al.* (1952) tested this against *P. lophurae* and found it to be six times as active as quinine, twice as active as mepacrine or proguanil and half as active as pamaquin. On the contrary, screening against *P. cynomolgi* in monkeys, Schmidt *et al.* (1952) observed that the proguanil metabolite '*A*' is only half to one quarter as active as the parent

*The investigations were partly financed by the Indian Council of Medical Research.

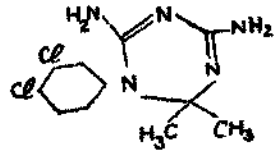
drug. These findings are not in conformity with those obtained in avian malarias. Inherent differences in susceptibilities of the plasmodia or variations in the physiological disposition of the metabolite may be responsible for this disparity (Schmidt *et al.*, 1952).



Proguanil



Proguanil active metabolite '*A*'



Metabolite '*B*'

During the course of extensive investigations on paludrine-like compounds, a 3 : 4-dichloro analogue of plaudrine (N^1-3 : 4-dichlorophenyl-N^5-isopropyl-biguanide) was found to be several times more active, but unfortunately also more toxic than proguanil both in experimental (Curd *et al.*, 1945 ; Crowther *et al.*, *loc. cit.*) and human malarias (Chaudhuri *et al.*, 1951 : 1952). Studies with the above compound on the lines similar to those of proguanil, led to the isolation of an active metabolite *viz.*, 1-(3 : 4-dichlorophenyl)-2 : 4-diamino-6 : 6-dimethyl-1 : 6-dihydro-triazine (called '*B*' in these studies). This is a 3 : 4-dichlorophenyl analogue of '*A*' (Crowther and Levi, *loc. cit.*), and was found to be 100 times as active as proguanil and ten times as active as '*A*' when tested against *P. gallinaceum* (Crowther and Levi, *loc. cit.*; Carrington *et al.*, *loc. cit.*). Against *P. cynomolgi*, however, '*B*' was even less active than proguanil, although more active than paludrine metabolite '*A*' (Schmidt *et al.*, *loc. cit.*).

Suitability of *P. berghei* as a test parasite for the screening of antimalarials has been well established (Goodwin, 1949 ; Thurston, 1950 ; Ramakrishnan *et al.*, 1951). The above conflicting results in avian and simian malarias led to the studies reported here to see how this plasmodium would respond to these metabolites when compared to proguanil.

METHODS AND MATERIALS.

The strain of parasites and the albino mice were from the same source as reported before (Ramakrishnan and Satya Prakash, 1950). Estimation of dose and route of inoculation and methods of drug administration were the same as reported previously (Ramakrishnan *et al.*, 1951 ; Jaswant Singh, *et al.*, 1952).

The dose of proguanil and dihydrotriazines '*A*' and '*B*' in every case was calculated in terms of base content. All these three compounds were white crystalline solids soluble in water. Their aqueous solutions were administered orally. Metabolites '*A*' and '*B*' were first obtained in small quantities through the courtesy of Messrs. Imperial Chemical Industries but later on these compounds were specially synthesised in the Chemistry Laboratory at the Malaria Institute,

The M.E.D. for Class I, II and III effects were determined in each case. A control group of animals was invariably maintained and the natural course and intensity of infection were similar to those already reported (Ramakrishnan *et al.*, 1951).

RESULTS.

Details of Class I, II and III effect of proguanil and metabolites '*A*' and '*B*' have been presented in Tables I, II and III respectively. M.E.D. of the three drugs as also of other antimalarials reported earlier (Jaswant Singh *et al.*, 1952) for all the three classes are given in Table IV. For Class I effect, metabolite '*A*' was found to be 25 times as active as proguanil while metabolite '*B*' was 20 times as active as '*A*' and 500 times as active as its parent drug. For Class II and III effects, metabolite '*A*' was almost just as active as proguanil while metabolite '*B*' was 500 times and 33 times respectively more effective than metabolite '*A*'.

TABLE I.

Effect of paludrine on the course of P. berghei *infection in mice.*

Dose mg. paludrine per 20 gm.	Number of mice	Average daily parasite count per 10,000 R.B.C. on days from first day of drugging										Remarks.
		1	2	3	4	5	6	7	8	9	10	
0·005	3	239	343	223	102	324	502	421	593	633	836	Class I effect in all animals.
0·05	6	312	248	124	39	210	581	777	615	688	825	
0·1	4	346	343	61	52	126	641	640	646	657	765	
0·2	2	309	190	62	49	39	374	774	336	334	534	
0·3	2	337	237	49	56	144	450	1360	1222	1770	1140	
0·4	5	245	172	79	4	0	0	0	3	43	78	Class II effect in all the animals.
0·5	2	250	143	6	0	0	0	0	0	25	216	
0·6	4	250	102	5	0	0	0	0	0	0	0	Became positive after 12th day.
1·0	2	300	80	10	1	0	0	0	0	0	0	Became positive after 13th day.
2·0	3	324	100	11	0	0	0	0	0	0	3	
2·5	5	261	82	6	0	0	0	0	0	0	0	Class III effect. Sub-inoculation negative in all animals.
3·0	4	304	153	42	0	0	0	0	0	0	0	Sub-inoculation done in one animal only, negative.

Table II.

Effect of Metabolite 'A' on the course of P. berghei *in mice.*

Dose mg. metabolite per 20 gm.	Number of mice	Average parasite count per 10,000 R.B.C. on days from first day of drugging.										Remarks.
		1	2	3	4	5	6	7	8	9	10	
0·001	2	284	149	151	228	433	1140	940	480	505	668	
0·002	5	333	147	101	146	190	382	531	739	698	792	Class I effect in all the animals.
0·005	4	368	170	51	90	119	110	80	40	115	89	
0·01	2	456	266	161	122	359	320	1150	602	543	1399	
0·05	2	275	160	84	88	260	266	373	299	544	624	
0·5	5	281	123	33	0	0	2	16	112	353	452	Class II effect in all animals.
1·5	2	480	146	12	0	0	0	0	0	0	0	Became positive on 12th and 13th days.
1·0	5	271	89	5	0	0	0	0	0	0	0	Class III Sub-inoculations negative in all.
2·5	1	540	24	0	0	0	0	0	0	0	0	

Table III.

Effect of Metabolite 'B' on the course of P. berghei *in mice.*

Dose mg. 10732 per 20 gm.	Number of mice	Average parasite count per 10,000 R.B.C. on days from first day of drugging.										Remarks.
		1	2	3	4	5	6	7	8	9	10	
0·0001	4	295	243	176	120	256	378	433	595	639	771	Class I effect.
0·004	6	287	167	27	0	0	0	11	41	184	379	Class II effect. (Two animals remained negative for four days).
0·05	4	311	52	3	0	0	0	0	0	5	95	
0·06	6	332	116	16	0	0	0	0	0	0	0	Class III sub-inoculation done in 5. All negative. One died before sub-inoculation.
0·07	3	312	96	8	0	0	0	0	0	0	0	Sub-inoculations negative in all.

TABLE IV.

M. E. D. of some antimalarials against P. berghei *in mice.*

Drug.	M.E.D. (MG./KG. BODY WEIGHT).		
	Class I	Class II.	Class III.
Quinine	1200	4800	>6000
Sulphadiazine	1·5	6·0	60·0
Daraprim	0·00005	0·0005	4·0
Paludrine	2·5	20·0	125·0
Metabolite '*A*'	0·1	25·0	100·0
Metabolite '*B*'	0·005	0·05	3·0

In Table V, quinine and sulphadiazine equivalents of the drugs under investigation as well as that of daraprim from earlier trials (Jaswant Singh *et al.*, 1952) have been listed.

TABLE V.

Quinine and sulphadiazine equivalents of antimalarials against P. berghei.

Drug.	QUININE EQUIVALENT.		SULPHADIAZINE EQUIVALENT.		
	Class I.	Class II.	Class I.	Class II.	Class III.
Quinine	...	...	0·00125	0·00125	<0.01
Sulphadiazine	800	800	...	...	...
Daraprim	2,40,00,000	96,00,000	30,000	12,000	15
Paludrine	480	240	0·6	0·3	0·48
Metabolite '*A*' ...	12,000	192	15	0·24	0·6
Metabolite '*B*' ...	2,40,000	96,000	300	120	20

Metabolite '*B*' was 20 and 500 times as active as '*A*' for Class I and II effects respectively. It was also evident that metabolite '*A*' and '*B*' have antimalarial activities (both Q.E. and sulphadiazine equivalent) lying somewhere between those of proguanil and daraprim. Comparing the doses required for radical cure, dihydrotriazine '*B*' was 20 times as active as sulphadiazine, while daraprim was 15 times as effective. Proguanil and metabolite '*A*' were less active than sulphadiazine.

DISCUSSION.

P. berghei and *P. gallinaceum* were equally sensitive to proguanil (Thurston, 1950). In its sensitivity to sulphadiazine, *P. berghei* resembled the malaria parasites of monkey and man (Ramakrishnan *et al.*, 1951). Daraprim has also been found to be highly effective against *P. berghei* (Jaswant Singh *et al.*, 1952). Daraprim has some structural resemblance with proguanil while the metabolites '*A*' and '*B*' are very much akin to daraprim chemically (Crowther and Levi *loc. cit.*; Jaswant Singh *et al.*, 1951). High antimalarial activity as displayed by these metabolites is, therefore, to be expected. However, it is significant that as regards the action of these compounds on *P. berghei* the results were more similar to those obtained with the avian plasmodia, namely *P. gallinaceum* and *P. relictum* than with *P. cynomolgi* or *P. knowlesi* in monkeys.

Metabolite '*A*' though more active than proguanil itself, is considerably less active than its 3 : 4-dichloro-analogue '*B*'. This can probably be explained on the basis that the proguanil itself is considerably less active than its 3 : 4-dichloro-analogue. It is interesting that while the dose of metabolite '*B*' required for temporary suppression is 100 times more than that of daraprim for a similar effect, the dose of the former drug for radical cure is slightly less than that of daraprim, their sulphadiazine equivalents for Class III effect being 15 and 20 respectively. This is contrary to expectations where even a comparatively lower dose of a less active drug was sufficient for a Class III effect as compared to the dose for Class II effect. Apart from the factors like drug metabolism, absorption and excretion of a drug as was discussed by Jaswant Singh *et al.* (1952), another factor may be that the range of effective dosage of compound '*B*' is much narrower as is to be expected from compounds of higher toxicity. The parent compound of this metabolite '*B*' is already reported to be more toxic than paludrine (Chaudhuri *et al.*, 1951 : 1952 ; Curd *et al.*, 1945 ; Crowther *et al.*, 1951) and it is probable that the toxicity of the metabolite is in proportion to that of the parent drug.

SUMMARY.

1. Chemotherapeutic studies with paludrine, its metabolite, and that of 3 : 4-dichloro-analogue of paludrine against *P. berghei* are reported.

2. The metabolites were more active than paludrine. The metabolite of dichloro-analogue was more active than that of paludrine.

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TOXIC MANIFESTATIONS OF REPEATED DOSES OF PYRIMETHAMINE IN *RHESUS* MONKEYS.

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DURING earlier investigation on pyrimethamine against simian malaria in *rhesus* monkeys, Jaswant Singh *et al.* (1951) had observed that the compound was four times as active as proguanil against *P. knowlesi* but in doses higher than the therapeutic level, it was four times as toxic. Tissues of monkeys which died due to single heavy doses of 50 mg./kg. or above of the drug, showed degenerative changes in the convoluted tubules of kidneys and cloudy swelling of the parenchyma cells of liver.

During the present investigation, the authors have recorded their observations on the general health and histopathological changes in kidneys and liver of *rhesus* monkeys when pyrimethamine was administered in varying doses for a period of 1 to 6 weeks.

MATERIALS AND METHODS.

Twenty-four *rhesus* monkeys were used for the chronic toxicity test. To determine the effect of the drug on the general health as indicated by loss of body weight, the animals were weighed before commencing the treatment and once again after completion of the course.

Monkeys were closely observed every day for any untoward toxic manifestations like anorexia, rapid emaciation, diarrhœa, vomiting, irritability or depression, convulsive seizures, etc.

Biopsy materials were collected from monkeys which survived the course, while immediate postmortem examination was carried out in those which died during treatment. Tissues were removed only from those animals which did not show lesions due to other diseases like tuberculosis, septic conditions, etc., and were fixed in 10 per cent formal saline. After necessary processing, sections were stained with hæmatoxylin and eosin.

FINDINGS.

Effects of pyrimethamine in doses of 5 to 40 mg./kg. for periods varying from 1 to 6 weeks were observed as per Table I.

TABLE I.

Effects of pyrimethamine.

Schedule number.	Dosage mg./kg.	Duration in weeks.	Number of animals.	Number survived the period of observation.	Changes in body weight in kg. (average)	Remarks.
I. (*a*)	5	3	3	2	0·0	One died after 9 days due to extension miliary tuberculous lesion.
(*b*)	5	6	3	3	+0·2	
II. (*a*)	10	3	6	5	−0·3	Postmortem examination of the one which died before the period showed broncho-pneumonic condition of both lungs.
(*b*)	10	6	6	2	−0·7	
III. (*a*)	20	1	3	3	−0·4	
(*b*)	20	2	3	Nil	...	All died by the 11th day.
IV.	30	1	3	1	−0·4	
V.	40	1	3	1	−0·6	

It will be observed from the table that with doses of 30 or 40 mg./kg., four out of six animals died within a week. Similarly with 20 mg./kg. dose under Schedule III(*b*), all the three animals died by the 11th day. In all these cases, anorexia was a common feature, later associated with general depression. Prior to death a certain degree of irritability was noticeable and this was followed by convulsions similar to those observed during acute toxicity studies. In cases which survived, there was loss of weight varying from 0·4 to 0·6 kg.

The three monkeys placed under Schedule III(*a*) survived the dose of 20 mg./kg. for a period of three weeks though there was some degree of anorexia and loss of body weight (0·4 kg. average).

Under Schedules I(*a*) and II(*a*), seven out of nine monkeys survived a period of three weeks. The one which died a few days after the commencement of treatment showed extensive tuberculous lesion of the miliary type. Although certain degree of anorexia was observed in animals under Schedule II(*a*), there was no appreciable loss of body weight. None of the three monkeys under Schedule I(*b*) suffered from any ill-effects and there was no loss of weight at the end of six weeks. On the contrary, a dose of 10 mg./kg. for six weeks caused considerable anorexia in both the animals which survived. They were emaciated and there was considerable loss of body weight (0·7 kg. average).

HISTOPATHOLOGICAL FINDINGS.

Tissues collected from animals under Schedules III to V soon after death or by biopsy from those which survived, showed cloudy swelling of the cells of the convoluted tubules of the kidneys and the parenchyma cells of the liver. The lesions appeared to be somewhat similar to those observed during acute toxicity studies reported earlier.

The swelling was considerable in respect of the cells of the convoluted tubules of kidneys from the animals placed under 10 mg./kg. daily dosage schedule for six weeks (II*b*). The swollen cells either narrowed or completely occluded the lumen of the tubules. Occasionally fatty globules in the cytoplasm of cells was detectable. In some cases the debris of the degenerated cells were found in the lumen. Various stages of degeneration of nuclei were observed and infrequently they had disappeared from some cells.

Changes observed in the liver were striking in that general hepatic architecture seemed to be lost and the lobular arrangement in some was no longer detectable on account of necrotic changes. The cells of most of the lobules appeared swollen, and fatty infiltration was detectable in many. Varying degrees of nuclear degeneration was also present.

In contrast, lesion in the tissues of animals treated for three weeks (II*a*) showed very slight swelling of the cells of the convoluted tubules of the kidney and of the parenchyma cells in the liver. Comparatively the changes observed were milder under this regime than those described earlier.

Sections of tissues removed from the animals placed under Schedule I(*a*) or (*b*) showed no detectable changes either in the kidneys or liver.

DISCUSSION.

Goodwin (1952) reported that a *rhesus* monkey receiving 5 mg./kg. of pyrimethamine for five days every week for six weeks did not show any toxic symptoms as reflected on its general health, appetite and blood picture. In the present series, somewhat similar observations were made. None of the six animals died due to toxic effects of the drug, nor was there any loss of body weight. Further, histopathological studies of liver and kidney did not reveal any lesion. But with

10 mg./kg. dosage, the overall mortality rate was 50 per cent. Pathological changes of liver and kidneys were severe, particularly in those cases where drug administration was continued for six weeks. The type of lesions observed was similar to that studied by Schmidt *et al.* (1953). They also reported of serious lesions in the spleen, lymph nodes, adrenal cortex and bone marrow.

With doses higher than 10 mg./kg., the monkeys did not survive beyond seven to 11 days and the lesions in liver and kidneys were similar to those observed in animals treated with a single massive dose of 50 mg./kg. or above as mentioned earlier by Jaswant Singh *et al.* (1951).

SUMMARY.

Twenty-four *rhesus* monkeys were treated with daily doses of pyrimethamine varying from 5 mg. to 40 mg. for periods of 1 to 6 weeks. In 5 mg. dosage administered for 3 to 6 weeks, there was neither any loss of weight nor any apparent histopathological changes.

There was slight loss of body weight in monkeys treated with 10 mg. dosage for three weeks as against a significant loss in those treated for six weeks. While in the former group histopathologial changes were of mild nature, in the latter damage to the convoluted tubules of the kidneys appeared to be considerable. Changes in the liver were striking as the parenchyma cells were found to be in different stages of degenerative process and the portal space was interrupted by the reticular tissue.

In the subsequent schedules, the drug administration could not be continued for prolonged period. Most of the animals died within the observation periods of 1 to 2 weeks. There was considerable loss of weight in those that survived. Histopathological changes in kidneys and liver comprised mainly of cloudy swelling but not of such a nature as was observed under 10 mg. dosage for six weeks. This is due probably to the shorter course of treatment under Schedules III to V although the dose of pyrimethamine was much higher.

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COMPARATIVE STUDY ON 4—AMINOQUINOLINES AGAINST *P. CYNOMOLGI* IN *RHESUS* MONKEYS.

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JASWANT Singh *et al.* (1949) observed that in *P. knowlesi* infection in *rhesus* monkeys, parasite clearance was attained quickest after administration of chloroquine or amodiaquin (comoquin) as compared to mepacrine, proguanil, aphacrine or metachloridine. In a subsequent study (Jaswant Singh, Ray *et al.*, 1951), it was reported that chloroquine diphosphate (Winthrops') and resochin (Bayer's) were more or less of equal value both against *P. knowlesi* in *rhesus* monkey and *P. vivax* and *P. falciparum* in man. In these investigations, the chief criterion of activity was the rate of clearance of parasites from the peripheral circulation.

During the present studies, antimalarials of the 4-aminoquinoline series like chloroquine (diphosphate), a product of Winthrops & Co., U.S.A., nivaquine (chloroquine sulphate) of May and Baker ; amodiaquin (camoquin) of Parke, Davis & Co., and avloclor (chloroquin diphosphate) of Imperial Chemical Industries, were assayed against *P. cynomolgi* in *rhesus* monkeys, and their quinine equivalent and M.E.D. were determined for evaluation of their relative merits.

METHODS AND MATERIALS.

Monkeys.—112 healthy *rhesus* (*M. mulatta mulatta*) monkeys, weighing between 2·5 and 6·0 kg., were utilized for these investigations, including 5 for comparison studies. They were all subjected to the routine procedure of blood examination and tuberculin tests (Jaswant Singh, Balbir Singh *et al.*, 1951 ; Nair and Ray, 1953) as adopted in these laboratories.

At least three monkeys were placed under each treatment.

Parasite.—A strain of *P. cynomolgi* isolated by Sinton and Mulligan (1933) and maintained through blood passages, was used.

The dose of inoculum was calculated on the basis of 5×10^6/kg. and injected intravenously in all cases.

Blood smears, thick and thin, were stained with J.S.B. stain (Jaswant Singh and Bhattacharji, 1944) and parasites were counted against 10,000 erythrocytes.

ANTIMALARIALS.

Chloroquine and avloclor, the diphosphate salts of chloroquine, are available in the form of 0·25 gm. base* per tablet and 0·15 gm. base (0·25 gm. salt) per tablet respectively. Nivaquine, the sulphate salt of chloroquine, is available in the form of 0·15 gm. base (0·2 gm. salt) per tablet. The base content of amodiaquine is 0·2 gm. per tablet. Quinine was used in the form of sulphate salt.

Treatment was commenced in all cases when parasitæmia was between 0·1 and 0·2 per cent cell infection. The dose of the antimalarial was calculated in milligramme per killogramme of the body weight and administered orally once a day for a period of seven days through a rhyles tube attached to a 10 c.c. record syringe.

Criteria of activity.—A dosage schedule was considered to be active only when there was complete clearance of parasites from the peripheral circulation by the day following the administration of the last dose of drug, a procedure adopted earlier by Jaswant Singh, Misra *et al.* (1951). At least one hundred fields of thick films were examined before recording a negative finding.

The minimum dose of an antimalarial which produced parasite clearance by the day following the last dose, *viz.*, Class II effect of Shannon (Wiselogle, 1946), was taken as the minimum effective dose (M.E.D.). The quinine equivalent of a drug was determined by dividing the M.E.D. of quinine by the M.E.D. of the test compound. The dose which caused complete sterilization of the blood-induced infection, as determined by follow-up for a period of three to four weeks and splenectomy thereafter, was taken as the M.E.D. for Class III effect of Shannon (Wiselogle, 1946).

OBSERVATIONS AND RESULTS.

Comparison group.—The course of parasitæmia in uncontrolled infection in five monkeys has been demonstrated in Chart 1 which shows that after inoculation (O-day), patency was established within a day or two. As a rule, cell infection of 0·1 to 0·2 per cent was reached by the second or third day and the peak was attained in most cases between the eighth and tenth day when cell infection reached between 13 and 17·5 per cent. While one monkey died on account of extreme anæmia on the ninth day, the infection became chronic in four others with periodical recrudescences.

*From a sample originally received in 1946.

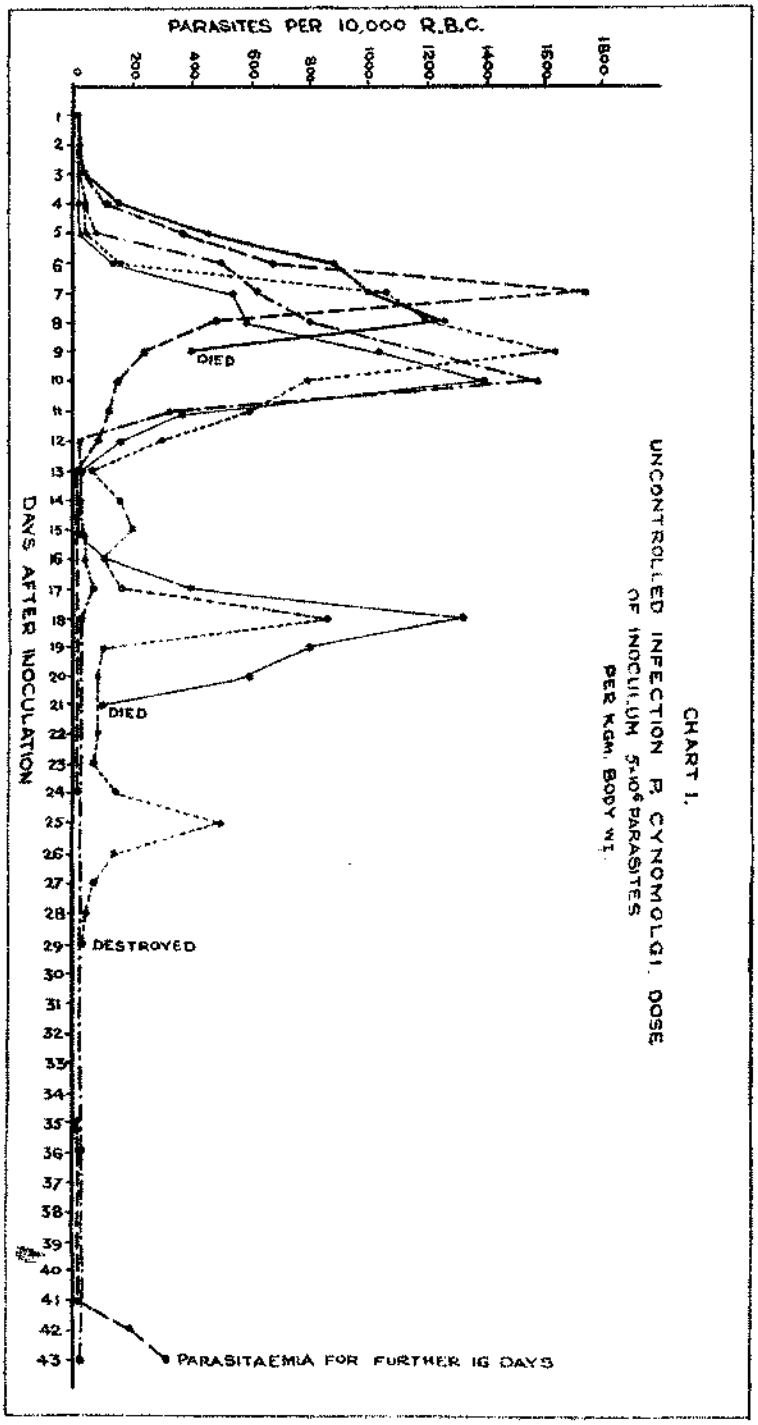

CHART 1.

UNCONTROLLED INFECTION P. CYNOMOLGI. DOSE OF INOCULUM 5×10^6 PARASITES PER KGM. BODY WT.

Experimental group.—The effect of quinine sulphate in doses varying from 8 to 32 mg./kg. is shown in Table I, from which it will be observed that there was complete clearance of parasites in all the three monkeys under Serial Numbers III, IV and V during the course of drug administration. On the other hand, a dosage schedule of 8 mg./kg. proved refractory while only one out of the three responded to 16 mg./kg dosage. Thus the M.E.D. of quinine was considered to be 20 mg./kg. Class III effect was not observed in any of them.

TABLE I.

Effect of quinine sulphate in P. cynomolgi.

Serial number.	Dosage schedule in mg./kg.	Number of monkeys.	Number showing Class II effect.	Recrudescence		Number showing Class III effect.	Remarks.
				Number of animals.	Days.		
I.	8	3	Nil	Parasitæmia persisted		Nil	Inactive.
II.	16	3	1	1	7	Nil	Deceleration (Class I effect) in 2.
III.	20	3	3	3	6–19	Nil	M.E.D.
IV.	24	3	3	3	16—24	Nil	
V.	32	3	3	3	13—27	Nil	

The effect of the 4-aminoquinolines on the plasmodium is shown in Table II from which it will be observed that 92 monkeys were placed under nine different dosage schedules ranging from 0·25 to 4·00 mg./kg. All the four antimalarials were assayed under Schedules 0·25 to 2·5 mg./kg. But with regard to amodiaquin the dosage schedules were increased up to 4·0 mg./kg. as its M.E.D. was found to be higher than the other three.

In Serial Numbers I and II (Schedules 0·25 and 0·5), in majority of the cases the drugs proved ineffective, though in a few there was deceleration in the course of parasitæmia. But complete clearance of parasites was never attained on the day following the last dose. Similar results were also observed in Serial Numbers III and IV in respect of amodiaquin.

On the other hand, in Serial Number III (Schedule 1·0 mg./kg.), Class II effect was observed in respect of all the five monkeys treated with avloclor. Thus this dose was considered to be its M.E.D. (Class II effect). The M.E.D. for chloroquine and nivaquine were found to be slightly higher (1·5 mg./kg.) as shown under Serial Number IV. But complete clearance of parasites in the peripheral blood in animals treated with amodiaquin was not observed until the dose was raised to 3·5 mg. (Serial Number VIII).

Thus, M.E.D. of quinine being 20 mg./kg., the quinine equivalent of avloclor, chloroquine, nivaquine and amodiaquin was determined to be 20, 13·3, 13·3 and 5·7 respectively.

TABLE II.

Effect of 4-aminoquinolines in P. cynomolgi.

Serial number.	Dosage schedule mg./kg.	Antimalarials.	Number of monkeys.	Number showing Class II effect.	Recrudescence after initial clearance.		Number showing Class III effect.	Remarks.
					Number of animals.	Days.		
I.	0·25	Chloroquin	3	Nil	...	...	...	Mostly inactive. In a few, slight deceleration in the course of parasitæmia (Class I effect) observed.
		Avloclor	3	Nil	...	...	...	
		Nivaquine	3	Nil	...	...	...	
		Amodiaquin	3	Nil	...	...	...	
II.	0·5	Chloroquin	3	Nil	..	...	...	—do.—
		Avloclor	3	Nil	...	...	...	
		Nivaquine	3	Nil	...	...	...	
		Amodiaquin	3	Nil	...	...	...	
III.	1·0	Chloroquin	5	2	2	4–6	Nil	
		Avloclor	5	5	5	7–15	Nil	M.E.D. (Class II effect).
		Nivaquine	5	2	2	9–14	Nil	
		Amodiaquin	3	Nil	...	...	...	
IV.	1·5	Chloroquin	3	3	3	9–14	Nil	M.E.D. (Class II effect).
		Avloclor	3	3	...	...	3	
		Nivaquine	3	3	3	11–13	Nil	—do.—
		Amodiaquin	3	Nil	...	...	...	
V.	2·0	Chloroquin	5	5	3	8–24	2	
		Avloclor	3	3	1	16	2	
		Nivaquin	3	3	2	16	1	
		Amodiaquin	3	2	2	6–13	Nil	
VI.	2·5	Chloroquin	3	3	1	6–20	2	
		Avloclor	3	3	...	...	3	
		Nivaquine	3	3	2	13–14	1	
		Amodiaquin	6	3	3	2–11	Nil	Class I effect in 3.
VII.	3·0	Amodiaquin	3	2	2	3–8	Nil	Class I effect in 1.
VIII.	3·5	Amodiaquin	3	3	3	9–16	Nil	M.E.D. (Class II effect).
IX.	4·0	Amodiaquin	3	3	3	13–14	Nil	

Class III effect could be observed in eight out of nine cases treated with avloclor in doses ranging from 1·5 to 2·5 mg. Under chloroquine and nivaquine, similar effect was observed in 33 to 66 per cent of cases when treated with doses between 2·0 and 2·5 mg. On the other hand, even with a dose of 4 mg./kg., complete sterilization was not attained in any of the monkeys treated with amodiaquin.

DISCUSSION.

Working with *P. gallinaceum* in chicks, Jaswant Singh *et al.* (1952) reported that the M.E.D. for quinine, mepacrine, proguanil, chloroquine and amodiaquine was respectively 1·6, 0·4, 0·1, 0·1 and 0·05 mg. per 50 gm. body weight of the chick. Quinine equivalents (Q.E.) of these compounds were thus calculated to be 1, 4, 16, 16 and 32. Compared to chloroquine, amodiaquine was found to be twice as effective. The reverse seems to be the case against *P. cynomolgi* in the present series as the Q.E. of amodiaquin was observed to be 5·7 against 13·3 in respect of chloroquine or nivaquine. It is well known that a drug may act differently against different species and even different strains of plasmodia (Findlay, 1951). It is then not surprising that these drugs act differently against *P. gallinaceum* and *P. cynomolgi.* On the other hand Jaswant Singh, Ray and Misra (1953) demonstrated that in *P. falciparum* infection, both chloroquine salts (resochin and nivaquine) and amodiaquin were of equal value for all practical purposes. However, the results observed during the first 24 hours showed that action of amodiaquin was somewhat faster. The present investigation confirms the view that efficacy of the diphosphate and the sulphate salts, as determined by the parasite clearance rate, is more or less the same.

But surprisingly the better results obtained with avloclor are unexplainable as structurally it is similar to chloroquine or nivaquin. This has been further reflected in its effect in causing the sterilization of blood-induced infection. With avloclor, Class III effect was produced in 88 per cent of the monkeys in doses varying from 2·0 to 2·5 mg., as against 66 per cent with chloroquine and 33 per cent with nivaquin. Thus it would appear that, against *P. cynomolgi* infection amodiaquin is the least effective out of the four compounds whereas avloclor gave the best results ; chloroquine and nivaquine being the two intermediates.

SUMMARY.

One hundred and twelve monkeys were utilized for evaluation of the relative merits of the four 4-aminoquinoline compounds. The minimum effective doses (M.E.D.) of chloroquine, nivaquine, avloclor and amodiaquin were found to be 1·5, 1·5, 1·0 and 3·5 mg. and their Q.E. was determined as 13·3, 13·3, 20·0 and 5·7 respectively.

Class III effect was produced in eight out of nine monkeys treated with 1·5 to 2·5 mg./kg. dosage of avloclor, whereas none was seen in those treated with amodiaquin in doses up to 4 mg./kg. With nivaquine and chloroquine, 33 to 66 per cent of monkeys were completely free from infection (Class III effect).

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AVIAN PLASMODIUM IN INDIAN BIRDS (*P. POLARE*).

Part II.

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MANWELL (1936) described a species of plasmodium *P. polare* obtained from a cliff sparrow (*Petrocheliden luniforns luniforns* Say). Later he detected this plasmodium in three other birds of same species but subsequent search in about thousand birds belonging to fifty different species (including 41 swallows of four other species) proved a failure (Manwell, 1935 : 1936).

Early this year, during routine survey of blood smears from commonly available birds in the vicinity of Delhi, Ray *et al.* (1953) and Ray and Bhatnagar (1953) reported their findings of two species of plasmodia in the common Indian partridges (*Francolinus pondicerianus interpositus*, Hartet), locally known as '*titar*' (Hindi). Out of 62 partridges, *P. rouxi* was detected in one, while a second species resembling *P. polare* was found in 21.

In the present paper, the authors have described the morphological characters and transmission experiments with the second species.

MORPHOLOGY.

All stages of the asexual forms of the parasites are detectable in the peripheral blood. The characteristic features of this parasite are that the gametocytes are elongated and rarely displace the host cell nuclei, and larger trophozoites and schizonts are invariably at one end (polar) of the nuclei which are not displaced.

TROPHOZOITES.

The early forms appear as rings or occasionally as solid bodies. The chromatin is proportionally large. The parasite is usually situated lateral to the nucleus of the host cell but rarely in apposition to it.

Some of the larger trophozoites are seen either lateral to the nuclei or near one end. The chromatin is fairly prominent and a few brown pigment granules are visible.

SEGMENTERS.

The segmenters nearly always assume a polar position. Quite often the parasite embraces one end of the nucleus (which is not displaced) in a V shaped manner.

Although many of the schizonts appear compact, some appear to be peculiarly scattered and the chromatin bodies attached to one another with strands (Plate III, figures 14-20).

The pigment granules are large, usually clumped in one to three masses. The average number of merozoites was found to be 10 with a range from 8 to 16.

GAMETOCYTES.

The micro and macro gametocytes are elongated and quite often broaden near one end. In some cases, the end is hooked or blunted. They are situated to the lateral side of the nucleus of the host cell which is seldom displaced. Some of the gametocytes are apparently found to be apposed to the nucleus. Quite frequently, they bend round the nucleus in a semi lunar manner and both ends of the parasite hook round the poles.

In macrogametocytes, the pigment granules are usually found to be dark and clumped at one end, while in microgametocytes their number vary from four to ten.

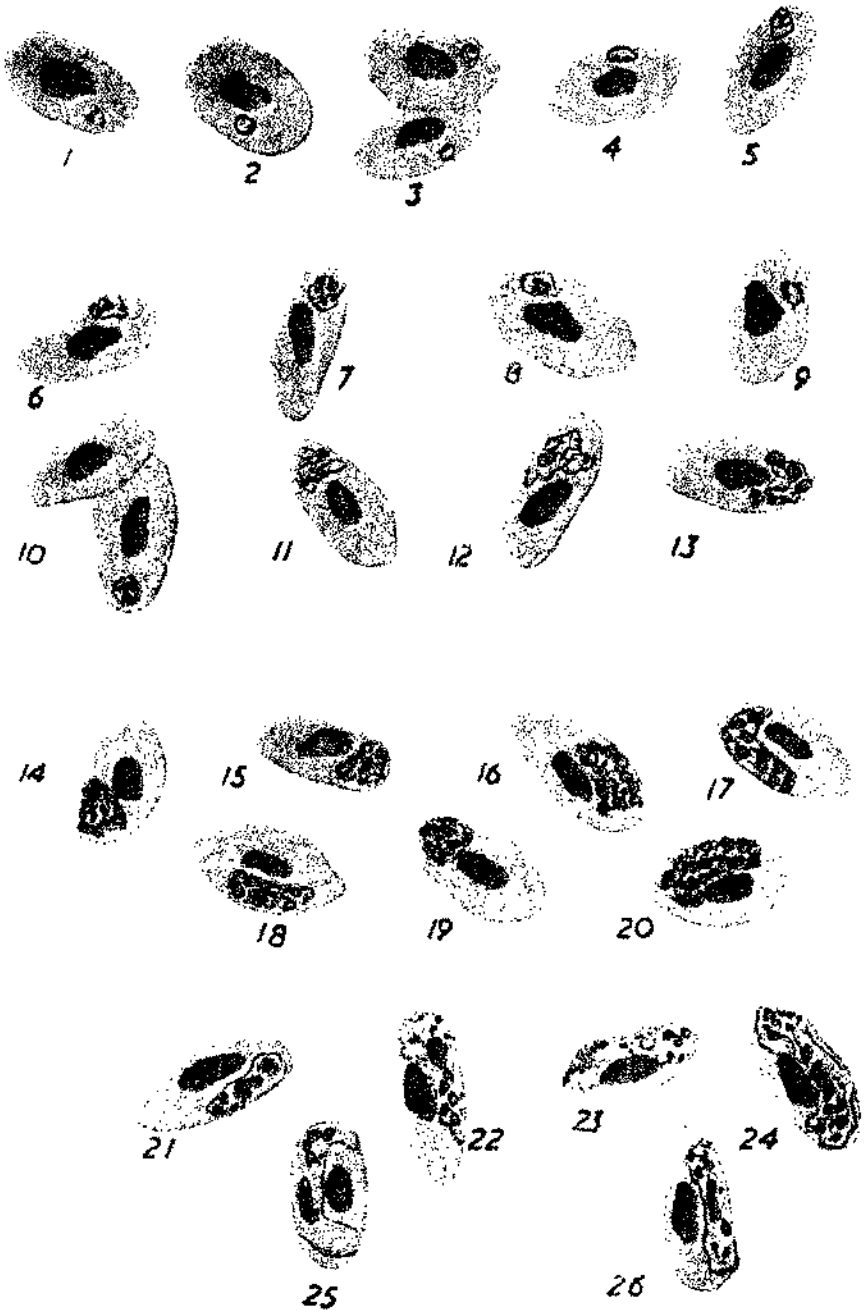
TRANSMISSION.

Blood passage.—2 c.c. of blood from a partridge showing scanty infection of the plasmodia was inoculated intravenously to two fowls, each receiving 1 c.c. of infected blood. While extremely scanty infection was detectable in one fowl after four days, infection was well established in the second one in two to three days. The parasitæmia reached a maximum of only two to three per cent cell infection. Subsequent passages in fowls did not alter the situation appreciably, though during later sub-passages the percentage of cells infected increased somewhat.

When the strain was passaged to seven to ten days old chicks, the intensity of infection was higher and in most cases 20 to 30 per cent of erythrocytes were found to be parasitized. At this stage majority of them succumbed. Sub-passages from chick to chick resulted in increased infection rate up to about 50 per cent.

PLATE III.

P. POLARE



FIGURES 1–5 Rings ;
6–13 Trophozoites and immature schizonts ;
14–20 Mature schizonts ;
21–26 Gametocytes.

Subsequently the strain was inoculated to two ducks and two guinea fowls. Although transient parasitæmia of very low degree could be detected for two to three days in one of the ducks, the other three birds remained negative throughout.

SPOROZOITE-INDUCED INFECTION.

Laboratory hatched *Aedes aegypti* from the colony were fed on the partridge which served as the donor to the two fowls mentioned earlier. Oöcysts were detectable on the sixth day and sporozoites appeared on the ninth day.

After the sudden death of the partridge on the second day after the *Aedes* were fed, another lot of freshly hatched *Aedes* were fed on a fowl which showed somewhat moderate infection. Both oöcysts and sporozoites were detectable after five and eight days respectively. When sporozoites were inoculated to fowls or chicks, patent parasitæmia was established in all of them.

DISCUSSION.

From the elongate type of gametocytes, non displacement of the nuclei of host cells and the position of the parasite in them, it was evident from the beginning that the present plasmodium belonged to group II of avian parasites as described by Giovannola (1939). As the average number of merozoites in mature schizonts always exceeded six, *P. rouxi* and *P. hexamerium* could be easily excluded. The former has always the fixed number of four merozoites while the latter has six as a rule. As more often than not the number of merozoites in mature schizonts was more than eight and the parasite was fairly large, it was evident that it differed very much from *P. vaughani* which has four to eight, but usually four merozoites per schizont, and a little larger than *P. rouxi* (Manwell, 1938). Further detailed study showed that the distinguishing features of the parasites were similar to *P. polare* described by Manwell (1935).

From the characteristically polar position of the parasite and the peculiar " stranded " appearance of many of the schizonts in which the chromatin dots are attached to one another with connecting strands, as described by Manwell (1935) and Hewitt (1940), the identity of the present plasmodium could be well established as *P. polare*.

Manwell (1935) reported that although infection with *P. polare* in one cliff swallow was moderately heavy, parasite level was always low in canaries when inoculated with a large dose of infective inoculum (Manwell, 1938). In this respect the low grade infection detected in fowls in the present series appears to be similar. But chicks appeared to be more susceptible particularly after repeated sub-passage. On the other hand the ducks and the guinea fowls proved almost refractory.

The transmission of the species from partridge to fowls through *Aedes aegypti*, as demonstrated during the current studies, brings to light the problem which one is likely to encounter during investigations on country fowls which may acquire infection from nearby wild partridges. Further it is considered that during such studies repeated blood examination of normal fowls would be essential before undertaking any investigation.

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STUDIES ON NURI STRAIN OF *P. KNOWLESI*.

I. Effect of milk diet on blood-induced infection.

BY

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MAEGRAITH *et al.* (1952) have observed that white rats infected with blood forms of *P. berghei* when fed on milk (cow's or human or reconstituted Australian dried milk or Ostermilk) developed low parasitæmia as compared to the rats fed on stock diet. Ramakrishnan *et al.* (1953) working independently on similar lines have also reported that the course of *P. berghei* infection in rats fed on an exclusive milk diet, was milder than in those on milkless diet as well as those on diet which included some milk. Bray and Garnham (1953) record a similar low grade parasitæmia in blood or sporozoite-induced *P. cynomolgi* infection in *rhesus* monkeys kept on milk diet. Hawking (1953) in a preliminary report on his experiments using milk diet has found that para-aminobenzoic acid when added to milk, could produce the usual virulence of *P. berghei* and *P. cynomolgi* in their respective hosts. The present paper records the results of an investigation dealing with the effect of milk in *S. rhesus* monkeys infected with the blood forms of a recently isolated highly virulent (Nuri) strain of *P. knowlesi* (Jaswant Singh *et al.*, 1953).

METHODS AND MATERIALS.

Fourteen adult *S. rhesus* monkeys weighing 3 to 5 kg. were selected for the experiment. Of these, 3 served as controls and 2 were utilized for subinoculations

from the experimental ones during the course of the experiment. The control monkeys received the usual normal diet* throughout the investigation. Two monkeys were starved both before and after inoculation. The preinoculation starvation in one case was for one day, and three days for the other. Effect of milk diet was observed in seven monkeys divided into two batches. Three monkeys in one batch were preconditioned by giving them milk diet exclusively, for eight days prior to inoculation. Three out of the four monkeys in the second batch were given milk from either the day of inoculation or subsequent to it, and the remaining one received normal diet plus milk from the day of inoculation.

The milk powder used in these investigations was Cowlac dried full cream milk (whole milk powder) at the rate of 1½ oz. three times a day per monkey (total of about 671 calories a day per monkey). Each ounce of the milk powder was well mixed in about 10 ounces of water and was administered by means of a stomach tube.

Each animal was inoculated intravenously with the Nuri strain of *P. knowlesi* (Jaswant Singh *et al.*, 1953) using a standard inoculum of five million parasitized R.B.Cs. per kg. body weight. Thick and thin blood smears were taken from these animals twice a day at 8·00 and 17·00 hours for parasite counts. The stain used was J.S.B. (Jaswant Singh and Bhattacharji, 1944). The density of parasites was estimated in terms of the numbers of parasitized R.B.Cs. per 10,000 cells. A smear was declared negative only if no parasites could be detected in about 100 fields of a thick film.

The criteria for the assessment of results were based on the various parasitological considerations such as (1) the incubation period, (2) duration of parasitæmia, (3) average daily parasitæmia, (4) peak parasitæmia, (5) day of peak, and (6) day of death of the animal as compared to the control series.

In some cases when the monkeys did not show any patent parasitæmia after inoculation, they were subjected to splenectomy or super inoculation.

RESULTS.

The course of infection in the experimental and the comparison groups are shown in Table I and also in Charts 1, 2 and 3. In Chart 1, the effect of milk on the three preconditioned monkeys (Numbers 3631, 3632 and 3633) is given. Chart 2 shows the results obtained in monkeys (Numbers 3629, 3630, 3700 and 3737) which received milk diet either solely or inclusive of the standard diet, from the day of, or subsequent to inoculation. Chart 3 gives the course of parasitæmia in the comparison, starved, and subinoculated monkeys (Numbers 3621, 3624 and 3628 which were fed on standard diet ; 3797 and 3840 that received no diet ; and 3682 and 3683 that received subinoculation from 3632 and 3633 respectively).

*Normal diet consisted of gram 168 grammes, wheat flour 22·4 grammes and vegetables or fruit 235·2 grammes (a total of 767 calories per monkey per day.)

TABLE I.

Effect of milk and standard diet against Nuri strain of P. knowlesi *infection in* S. rhesus *monkeys.*

A. *Course of parasitæmia in exclusively milk fed monkeys which were preconditioned prior to inoculation and in the ones that were subinoculated from these.*

Monkey number.	Experimental details.	Prepatent period. (days**).	Relapse (days**).	Duration of parasitæmia (days).	Parasite count per 10,000 R.B. Cs.			Day of death after inoculation.
					Daily average.	Peak.		
						Day after inoculation	Number.	
3631	Milk 7+1+7*	18	...	3	<1	18	<1	...
	Superinoculation on 27th day of initial inoculation.	2	...	5	2426	34	7800	34
3632	Milk 7+1+11	21	...	2	<1	21	<1	...
			45	1	<1	45	<1	...
	Splenectomy 45 days after milk.		63	4	<1	64	<1	...
3633	Milk 7+1+34	35	...	2	<1	35	<1	...
	Splenectomy 21 days after inoculation.		43	1	<1	43	<1	...
3682	Subinoculated from Number 3632 on 9th day of inoculation.	14	...	6	1690	20	6800	20
3683	Subinoculated from Number 3633 on 9th day and 20th day of initial inoculation.	30	...	4	3114	34	7800	34

B. *Course of parasitæmia in monkeys on milk diet from the day of or subsequent to inoculation.*

3630	Milk 0+1+11	2	...	6	390	5	1900	...
		...	19	5	2544	24	6400	24
3629	Normal diet+milk 0+1+11	3	...	5	27	5	101	...
		...	13	30	98	16	1250	...
3737	Milk 0+0+6†	1	...	5	42	3	80	6
3700	Milk 0+0+2‡	2	...	4	2907	6	8400	6

*Seven days before inoculation, on the day of inoculation and 7 days after inoculation.
**From the day of inoculation. †Died due to intercurrent disease.
‡Milk diet from 4th day of inoculation.

TABLE I.—(*Contd.*)

C. Course of parasitæmia in control and fasting monkeys.

Monkey number.	Experimental details.	Prepatent period. (day**)	Relapse (days).	Duration of parasitæmia (days).	Parasite count per 10,000 R.B. Cs.			Day of death after inoculation.
					Daily average.	Peak.		
						Day after inoculation	Number.	
3621	Normal diet	2	...	4	3424	6	9250	6
3624	Normal diet	1	...	4	2060	5	9000	5
3628	Normal diet	2	...	4	2547	6	9000	6
3797	Starvation 1+1+5§	1	...	5	2017	6	9600	6
3840	Starvation 3+1+6	1	...	6	1584	7	8000	7

**From the day of inoculation.

§Starvation one day previous to inoculation, on the day of and 5 days after inoculation.

CHART 1

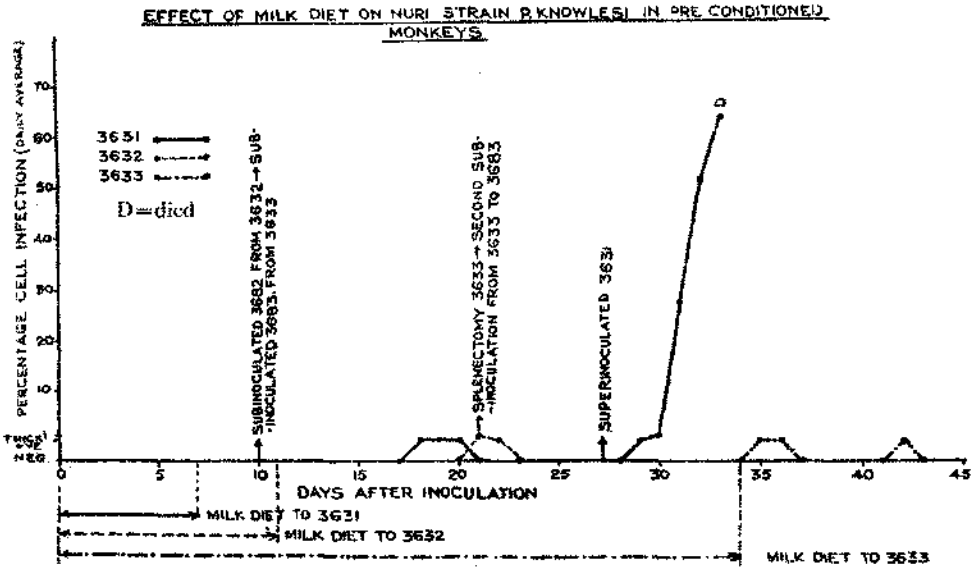


CHART 2

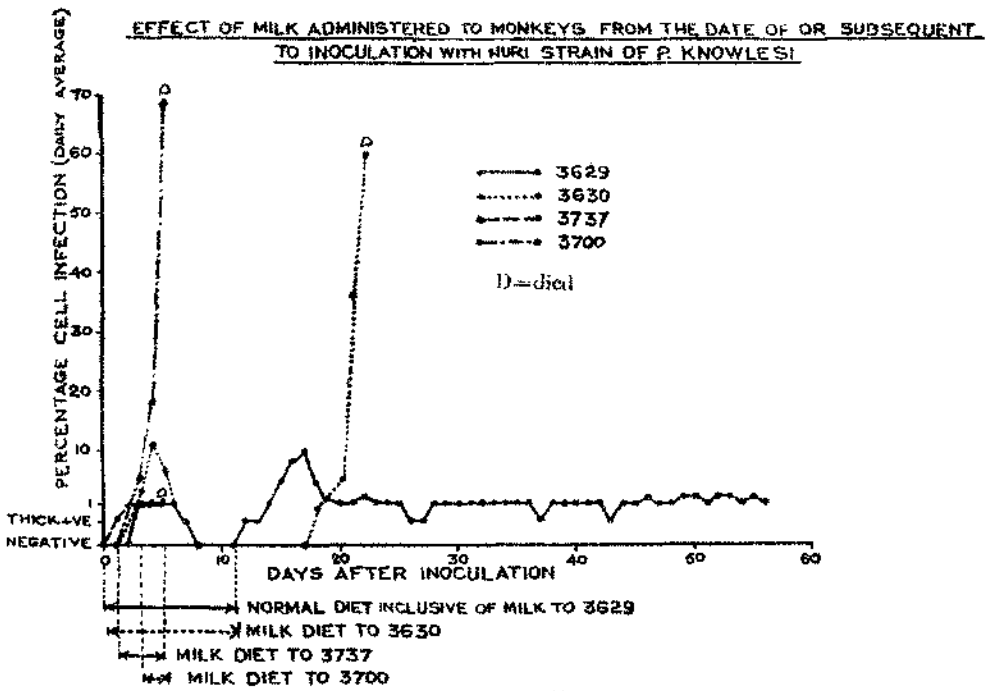
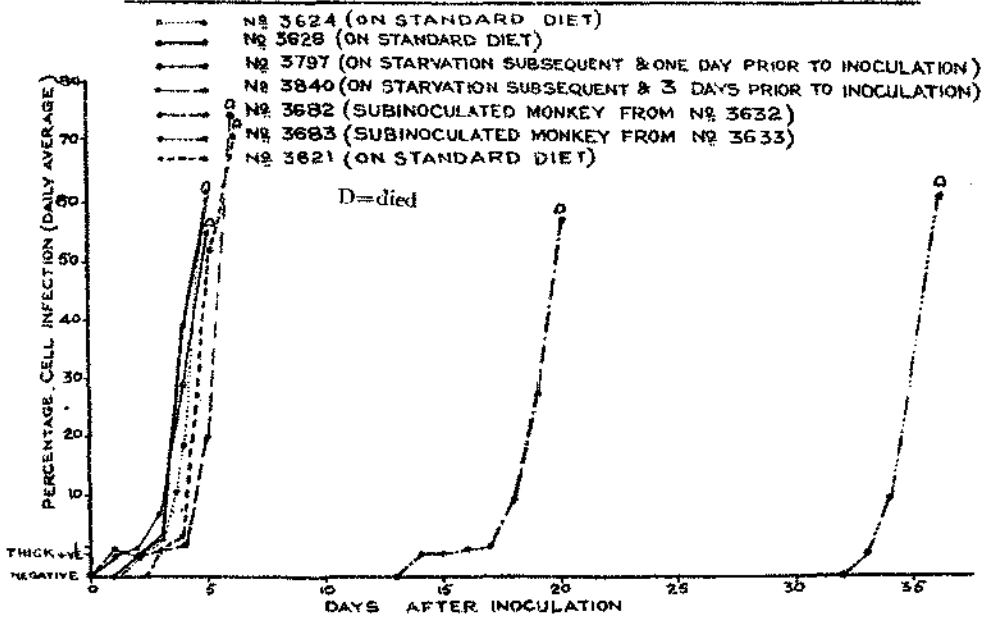


CHART 3



Number 3631 showed a very low parasitæmia for 3 days after an inoculation period of 18 days. The parasitæmia started eleven days after cessation of milk diet. Seven days later, super inoculation with a homologous strain of parasite using the same dosage of inoculation as on the first occasion, resulted in the manifestation of a very high infection resulting in death.

A similar low parasitæmia for a brief period of two days, occurred in Number 3632, once, eleven days after, and for the second time 34 days after the discontinuance of milk diet. Number 3682 which was subinoculated with 10 c.c. blood during the period of milk diet to Number 3632 (nine days after it was inoculated with blood forms of parasite), showed no parasites in its peripheral blood for a period of 14 days after which it developed a very high infection and succumbed to it.

Removal of spleen from Number 3632, 45 days after the cessation of milk diet, produced an extremely mild infection for a period of 4 days within a week after the operation (not depicted in the Chart).

Number 3633 was exclusively fed on milk diet for a period of 34 days after it was inoculated. On the 21st day when it was still on milk diet, splenectomy was performed. No patent parasitæmia was observed in its peripheral blood while it was receiving milk but transient parasitæmia occurred for a period of two days and one day, respectively, one and eight days after the cessation of milk diet. Number 3683 which was subinoculated twice from Number 3633, nine and twenty days respectively from the day of inoculation of the latter, after a long prepatent period of thirty days, developed a severe infection and died on the fourth day of parasitæmia.

Number 3700 which was placed on milk diet on the fourth day after inoculation at the time of 9 per cent cell infection, had no change in the course of parasitæmia as compared to control and it died two days later.

Parasitæmia did not exceed 0·8 per cent cell infection in Number 3737 when it was placed on an exclusively milk diet about 15 hours after its inoculation. This monkey, however, died on the sixth day of inoculation at a time when parasitæmia was on the decline due to an intercurrent disease.

Number 3629 which received milk for eleven days in addition to the standard diet had an initial infection for 5 days during which period the highest parasitæmia recorded was only about one per cent. When milk was withdrawn, it relapsed two days later and developed a chronic infection, parasitæmia persisting for the entire period of about two months' observation period. The maximum count recorded was 12·5 per cent on the third day of relapse, and the average cell infection during the whole period being less than 0·98 per cent.

An exclusive milk diet for a period of eleven days from the day of inoculation produced more or less the same initial infection in Number 3630 as in Number 3629, with the difference that in the former a higher degree of parasitæmia was recorded than in the latter. When milk was discontinued, that monkey relapsed eight days later and died of severe infection within six days.

Numbers 3621, 3624 and 3628 which were fed on standard diet became positive within 48 hours after inoculation and died four days later with high

parasitæmia. The highest parasitæmia recorded before death was 90 to 92·5 per cent cell infection and the daily average count 20·6 to 34·2 per cent.

Starvation for one day (Number 3797) and three days (Number 3840) prior to inoculation and subsequent to that, did not change the course of parasitæmia as compared to that of the controls which received standard diet.

DISCUSSION.

The infection caused by the Nuri strain of *P. knowlesi* in *S. rhesus* monkeys inoculated intravenously with 5 million parasitized cells per kg. body weight and kept on a standard diet is characterized by the rapid growth of parasites and death of the animal in 5 to 6 days with a peak parasitæmia of more than 90 per cent cell infection (Monkey Numbers 3621, 3624 and 3628). There was no clear indication of any alteration in this virulence of the parasite or the time of death of the animals when they were kept starving one to three days prior and for the entire period subsequent to inoculation (Numbers 3797 and 3840).

McKee and Geiman (1948) found that by starving monkeys for 24 to 48 hours, parasite infection caused by *P. knowlesi* was strikingly ameliorated and if the starvation was continued for more than eight or nine days before the animals were given food, there was spontaneous control of the infection. Similarly Ramakrishnan (1953) found that *P. berghei* infection was almost totally suppressed in starved rats. The authors' present findings do not confirm these but since the number of fasting animals used in the present series is not large, final conclusion should await further trials. In the meanwhile, it leads one to speculate whether the difference in behaviour of *P. knowlesi* in the same species of animal host lies in the Nuri strain being different physiologically and immunologically from the original *P. knowlesi* (Sinton and Mulligan, 1932) strain that was in common use until recently.

Milk administered from the day of inoculation or 15 hours after, was not able to stop the appearance of a patent phase of parasitæmia but the infection that was initiated was spontaneously controlled within 5 to 6 days. Return to normal diet, eleven days after milk diet, ended in a fatal breakthrough. Milk in addition to standard diet behaved more or less in the same manner as the one exclusively on milk diet but in this case, the breakthrough that occurred after stopping milk, took a striking course of long prolonged chronic infection. Milk, therefore, when continued with the standard diet appeared to help the production of premunition but milk by itself could not bring about this effect.

Preconditioning of the animals before inoculation prevented the infection from becoming patent during the entire period of milk diet. There was, however, no complete cure of the infection because the subinoculated monkeys took up the infection though after an unusually long prepatent period. Moreover all the three monkeys showed a transient parasitæmia of two days, one to eleven days after switching over to standard diet. Bray and Garnham (1953) observed a similar effect of milk on *P. cynomolgi* infection in monkeys with the difference that in their case, suppression was never complete. Ramakrishnan *et al.* (1953), Hawking (1953) and Editorial of the *British Med. Jour.* (1953) are more in favour to believe

that the mechanism responsible for this control of parasites is as a result of milk being deficient in nutrients required by the malaria parasites than due to the presence of any special antiplasmodial substance or substances in the same. It is well known that nutritional requirements of all species of plasmodia are not the same. Further, the relative importance of any of the special nutrients common to all, also may vary with individual plasmodial species. These perhaps explain why milk was able to control *P. knowlesi* better than *P. cynomolgi* infection.

The effect of milk on parasitæmia in preconditioned monkeys in the present experiments was to such an extent that, in one case (Number 3631) superinfection with the homologous strain of parasites after stopping milk, resulted in acute infection followed by death, showing thereby that no immunity had developed in the animal as a result of the previous infection. In another (Number 3633), splenectomy towards the end of a five weeks milk diet, did not produce any remarkable parasitæmia even after return to normal diet. In the third monkey (Number 3632) though one or two parasites appeared in the thick film after milk was withdrawn, the subsequent removal of spleen did not bring about any appreciable infection. Further trials may indicate whether milk diet given over a longer period prior to inoculation could be effective in bringing about complete prevention of the infection with this strain of the parasite.

SUMMARY.

The effect of milk on the course of blood induced Nuri strain of *P. knowlesi* was studied using 14 monkeys for the investigation.

The course of parasitæmia in the monkeys on standard diet is given.

Monkeys, exclusively fed on milk diet both before and after blood inoculation, were free from patent prasitæmia during the period.

Milk diet given from the day of inoculation or soon after it, though could not prevent the appearance of patent parasitæmia, was able to control the infection within the course of four to five days. Fasting had no effect on the amelioration of the infection.

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EFFECT OF MILK DIET ON *PLASMODIUM GALLINACEUM* INFECTION IN ITS VERTEBRATE AND INVERTEBRATE HOSTS.

BY

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(September 30, 1953.)

"*One man's meat is another's poison*".

English Proverb.

INVESTIGATIONS on *Plasmodium berghei* in albino rats by Maegraith, Deegan and Jones (1952) and Ramakrishnan *et al.* (1953) have revealed that milk is deficient in some essential nutrilite or nutrilites required by the parasite, with the consequent result that parasite multiplication in rats on milk diet is poor as compared to that in controls. Similar results were observed by Jaswant Singh *et al.* (1953) in monkeys on milk diet, infected with *Plasmodium knowlesi.* Hawking (1953) in experiments on *P. Berghei* in rats has conclusively shown that PABA is one of the factors deficient in milk and that this deficiency is responsible for restricted parasite multiplication.

Bray and Garnham (1953) have observed that *Plasmodium cynomolgi* shows restricted multiplication in monkeys on milk diet as compared to their controls though multiplication was not totally absent as in case of Maegraith's experiments. Further, they concluded that deficiencies in a milk diet did not affect the pre-erythrocytic development of parasites but their effects were most pronounced on immature schizonts in erythrocytes.

As the physiological characteristics of the different species of malaria parasite are known to vary, it was considered that investigations similar to the above on an avian malaria parasite may be of some interest. The present report

deals with the effects of milk diet on sporozoite as well as blood-induced infections of *Plasmodium gallinaceum* in fowls and on the sporogony cycle in the insect host.

EXPERIMENTAL DETAILS.

Eleven adult (Rhode Island Red) fowls weighing two to three pounds each were used in this investigation. Six were put on milk, and the rest on standard diet. Of these, two served as controls for blood and three for sporozoite-induced infections.

A balanced standard diet for the control birds, E. 596, 606, 615, 643 and 652, was as prescribed by the Government Poultry Expert, and did not contain any milk. Its calorific value was approximately 53 calories per ounce. Preliminary observations revealed that on an average a bird consumed about seven ounces of the food or about 375 calories during the day. The average consumption of milk per bird per day was determined to be equivalent to 36 gm. of desiccated whole milk (Nespray). In order to ensure that the diet of both experimental and control birds were isocalorific, each control bird was given 3·5 ounces of the standard diet, calorifically equivalent to approximately 36 gm. of milk powder (188 calories).

The pre-conditioning period on milk for the experimental birds, prior to inoculation, was two days in each case except E.598, for which it was prolonged to four days. Each bird was given 18 gm. of milk powder dissolved in two ounces of water twice daily by means of a pipette.

The dose of inoculum for blood-induced infection was one million prasitized erythrocytes per kg. weight of recipient fowl. For sporozoite infection, each fowl was inoculated intravenously with four mosquito equivalent of positive gland dissections carried out in normal saline. Thin blood smear of every fowl was examined daily and when positive, parasites were enumerated per 10,000 erythrocytes.

Three lots of laboratory bred *Ædes ægypti* mosquitoes were fed on suitable gametocyte carriers. Each lot of fed mosquitoes were divided into two batches and stored in the insectary where the average maximum and minimum temperatures were 86°and 83° F., respectively, and the relative humidity was between 75 and 80 per cent.

Three of the batches of mosquitoes were on a maintenance feed of 5 per cent glucose dissolved in fresh buffalo's milk daily and soaked in cotton wool. The remaining three batches served as controls and were on maintenance feed of 5 per cent glucose solution in water. Dissection of the mosquitoes was carried out from the 5th to 10th day of infective feed.

RESULTS.

No loss in weight of birds either in the experimental or control group was observed during the observation period of 20 days, and the birds showed no signs of undernourishment.

Milk diet to fowls seemed to provide all the nutritive requirements of parasite. Blood-induced infection in three fowls on milk diet (Table I) showed a high degree of multiplication of parasites in blood. Indeed, the parasitæmia in the experimental birds was much greater than in their controls and resulted in the death of the hosts on 6th, 7th and 8th day of infection respectively, while the two control birds were able to overcome their parasitæmia and survived with latent infections.

TABLE I.

Daily parasitæmia in fowls with blood-induced infection on milk and standard diets.

Group.	Fowl number.	Parasites per 10,000 erythrocytes on days following blood inoculation.												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Milk diet ...	E. 608	...	...	144	248	720	11,200	D	...	...	...	...	...	...
	E. 648	...	...	128	280	880	D	...	...	...	...	...	...	...
	E. 649	...	3	1	3	6	880	24,400	D	...	...	...	...	...
Standard diet	E. 606	...	1	6	8	22	904	808	980	800	880	30	18	3
	E. 643	...	...	2	3	28	712	380	280	200	190	1	1	...

D=died.

The course of sporozoite-induced infection (Table II) in the three control birds was similar and in all of them it became latent after 8 to 12 days of patency. None of the birds died from acute infection. Each of the three birds on milk diet showed a much higher degree of prasitæmia than any of the controls and succumbed.

TABLE II.

Daily parasitæmia in fowls with sporozoite-induced infection on milk and standard diets.

Group.	Fowl number.	Parasites per 10,000 erythrocytes on days following sporozoite inoculation.																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Milk diet	E. 598	...	...	...	...	...	...	...	1	4	13	30	328	480	1280	960	620	540	492	108	18	2
	E. 647	...	...	...	...	...	...	1	6	22	120	820	2800	2200	D	...	...	...	...	...	...	...
	E. 651	...	...	...	...	...	...	...	...	...	4	48	280	720	960	1080	840	220	18	6	1	...
Standard diet ...	E. 596	...	...	...	...	...	1	1	1	3	92	160	260	180	38	8	6	1	...	...	...	...
	E. 645	...	...	...	...	...	...	...	24	52	140	120	28	6	8	1	...	...	...	...	...	...
	E. 652	...	...	...	...	...	...	...	30	48	380	480	68	8	1	1	...	...	...	...	...	...

D=died.

Milk diet to the infected fowls did not seem to affect gametocytes, as their density was about equal in the blood of experimental as well as control fowls. Laboratory bred female *Ædes ægypti* mosquitoes were fed on E. 598 which was fed throughout on milk diet. 55 per cent of the mosquitoes showed gland infection on the 10th day, and 16 mosquito-equivalent of the sporozoites injected intravenously into a clean fowl fed on normal diet established a patent infection on the 9th day.

The quantity of milk consumed by the experimental mosquitoes was an unknown variable. Considering this limitation of the experiment, milk diet to mosquitoes did not seem to influence in anyway the sporogony cycle of the parasites in them (Table III). Differences between experimental and control mosquitoes in respect of oöcyst and sporozoicte rates are too minor to require any comment.

TABLE III.

Effect of milk diet to Ædes ægypti *on sporogony cycle of* Plasmodium gallinaceum.

Experiment number.	Maintenance feed.					
	Five per cent glucose in water.			Five per cent glucose in fresh buffalow's milk.		
	Number dissected.	Number with oöcysts.	Number with sporozoites.	Number dissected.	Number with oöcysts.	Number with sporozoites.
1	42	6	17	32	3	13*
2	21	3	7	10	1	1
3	46	4	14	12	1	2
TOTAL	109	13	38	54	5	16

*4 mosquito-equivalents inoculated intravenously into a clean fowl resulted in a patent erythrocytic infection eight days later.

DISCUSSION.

The results of this experiment showed that *P. gallinaceum* in fowls on milk diet was not affected in any way similar to *P. berghei* in rats or *P. cynomolgi* and *P. knowlesi* in monkeys in the experiments quoted earlier. On the contrary, *P. gallina eum* infection was considerably more intense in fowls on milk diet than in their controls. Milk as a sole diet to rats and monkeys was found deficient in so far as their respective malaria parasites were concerned, but it was not only not deficient in any requirements of *P. gallinaceum* in fowls, but apparently provided better nourishment to it than a balanced diet without milk. The results more than confirmed the specificity of physiology of individual plasmodial species.

Physiological specificity as a characteristic of individual plasmodial species in all probability is applicable to their individual biological strains also. In the experiments on *P. berghei* quoted earlier, Ramakrishnan *et al.* (*loc. cit.*) found the

multiplication of *P. berghei* less in milk-fed rats than in their controls. But the parasitæmia in their experimental animals was considerably more intense than in those of Maegraith, Deegan and Jones (*loc. cit.*) It is possible that the difference was due to the physiological variation of the parasite strains employed in the two investigations.

The physiology and metabolism of different hosts may also be factors in determining the effect of any diet on their respective parasites. In this connection it has to be borne in mind that milk is an unnatural diet to birds and mosquitoes unlike to monkeys and rats which are mammals.

It is noteworthy that although the effect of milk on the asexual erythrocytic phase of *P. gallinaceum* in fowls was in contrast to similar phases of *P. berghei*, *P. cynomolgi* and *P. knowlesi* in rats and monkeys, respectively, the effect of milk was identical on the pre-erythrocytic phase as well as gametocytes of both avian and mammalian parasites. Gametocytes of *P. gallinaceum* in the blood of milk-fed fowl E.598 were infective to mosquitoes as were gametocytes of *P. cynomolgi* in the experiments of Bray and Garnham (*loc. cit.*). Further the sporogony cycle was complete in mosquitoes maintained on milk after the infective feed and the sporozoites in them were found to be infective.

SUMMARY.

The course of blood as well as sporozoite-induced infections of *Plasmodium gallinaceum* was found to be considerably more severe in fowls on milk diet than in their controls.

Gametocytes were not affected by milk diet in fowls, and were infective to mosquitoes. A similar diet to *Ædes ægypti* did not influence the sporogony cycle of the parasite in them.

The difference in the effect of milk diet when fed to the respective hosts of *P. berghei*, *P. cynomolgi*, *P. knowlesi* and *P. gallinaceum* emphasised the well-known individuality of physiology of the different plasmodial species.

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J. S. B. STAIN—ITS PREPARATION IN THE POWDER FORM AND THE STAINING TECHNIQUE.

BY

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JASWANT Singh and Bhattacharji (1944) introduced a water soluble stain (J.S.B.) for the rapid staining of malaria parasites. This stain has been found to be equally comparable to the standard ones like Leishman or Giemsa which have been completely replaced by J.S.B. in these laboratories for the last ten years. In summarizing the utility of this stain, Manwell (1945) concluded that this stain was found to be superior in most respects to any of the other commonly used processes for the staining of blood and blood protozoa. To quote in his own words " The technique is simple and the staining process can be completed in less than two minutes for thin smears and in less than one minute for thick films. The staining solutions are not difficult to make up, are relatively inexpensive and keep well for weeks or months, even in hot weather. Preparations stained by this process appear very much like those made by Geimsa's method, cytoplasm and chromatin of blood cells and parasites being differentiated with equal clearance and having similar colour values. J.S.B. preparations are somewhat less resistant to fading but will stand much more exposure to light than they would ordinarily receive ".

Russell *et al.* (1944) described various stains and staining techniques including J.S.B. Subsequently, Russell (1952) in discussing the diagnosis of malaria parasites mentioned of only two stains important for microscopic diagnosis of malaria parasites. These are Giemsa and J.S.B.

In this note, a short account is given of certain modifications that have been introduced in our laboratories in recent years in the preparation of J.S.B. stain

and its staining technique with the hope that the utility and advantages of this stain are furher enhanced.

PREPARATION OF THE STAIN SOLUTIONS I AND II.

J.S.B. stain solution I can now be prepared conveniently in powder form. For this 0·5 gm. medicinal methylene blue is thoroughly dissolved in 500 c.c. tap water in a flask and 3 c.c. of one per cent sulphuric acid added to it. 0·5 gm. of potassium dichromate is then added. A heavy amorphous purple coloured precipitate of methylene blue chromate forms. The resulting mixture is heated either (1) over gentle flame of a spirit lamp or (2) over a very low flame of stove or (3) in water bath or (4) in an autoclave. To prevent excessive evaporation, a reflex condenser or a long glass tubing fitted to the cork of the flask is recommended.

The duration of heating is always guided by the appearance of deep blue colour and not by any fixed time factor. Sometimes though the solution may appear blue while boiling, it may turn greenish on shaking or cooling. In that case the solution should be boiled again for a further period. The indication that the solution has been heated properly can be had by finding a blue filtrate on filtering a few c.c. of the boiling solution. The same can also be ascertained by drawing the boiling solution in capillary tubes and finding whether blue colour of the solution is retained when the tubes are cooled. The end point is mostly reached after 3 to 4 hours of heating in an autoclave, 4 to 6 hours on a stove or a spirit lamp and 5 to 6 hours in a water bath. For small scale preparations, spirit lamp will be found convenient. While using autoclave for preparation of large quantities of this stain, care should be taken to maintain the temperature from 100 to 109°C., and pressure 0 to 5 lbs. If temperature is allowed to rise above 110°C., oxidation of methylene blue may be carried too far and in that case solution will turn violet purple.

When the solution turns deep blue, the flask is cooled at room temperature and filtered. The precipitate on the filter paper is dried in a petri dish at room temperature or in a vacuum dessicator. The precipitate is carefully collected from the filter paper, mixed and powdered thoroughly with 1·75 gm. of disodium hydrogen phosphate (Na_2HPO_4, $2H_2O$) in a glass mortar and stocked in small specimen tubes. Whenever required, the powder in the specimen tube is dissolved in 450 to 500 c.c. distilled water and used after allowing to mature for 4 to 7 days. The yield of the powder (without disodium hydrogen phosphate) from different batches varies from 0·35 gm. to 0·5 gm. When only small quantities of the stain are required, it is preferable to mix 1/5 of the stain powder with 0·35 gm. of disodium hydrogen phosphate and dissolve in 100 c.c. distilled water. The dried powder keeps well.

In experienced hands, the filtrate after addition of 2·5 gm. of disodium hydrogen phosphate, and allowing to mature for about two weeks, can be used as Solution I.

For preparing J.S.B. stain Solution II, 1 gm. of water soluble yellow eosin is dissolved in 500 c.c. of ordinary water.

STAINING TECHNIQUE.

According to the new method of staining, the smear is first dipped in the coplin jar containing solution II (Eosin solution) for 1 to 2 seconds. Excess of eosin stain is removed from the smears by dipping slides in a jar containing wash water (*p*H 6·2 to 6·6). It is then immediately transferred to solution I and kept for 40 to 45 seconds. After this, it is finally washed by dipping in the same wash water 3 to 4 times. For obtaining uniformity in the staining, a buffered wash water is preferable to acidulated water. For this 0·22 gm. of disodium hydrogen phosphate (Na_2HPO_4, $2H_2O$) and 0·74 gm. of pot. acid phosphate are dissolved in 1,000 c.c. distilled water. This is kept in a green bottle (preferably *Pyrex* bottle) and used whenever required. One coplin jar full of wash water is enough for one day's use without changing and will suffice to stain about 50 blood smears.

DISCUSSION.

Since describing this stain in 1944, Jaswant Singh and David (1949) used it for staining oöcysts in the midgut of mosquitoes and sporozoits in the salivary glands. Use of this stain has been advocated for staining organisms such as *Toxoplasma gondii*, *Hæmoproteus columbæ* (Ray, 1949), microfilaria (Raghavan and Krishnan, 1949), trypanosomes, *Triponema anserinum* Hepatazoon (Nair and Basu, 1950), *Leishmania donovani* (Manwell *loc. cit.*), *Pasteurella septica*, *P. pestis*, spriochactes, and anthrax (Das, 1953). Ramakrishnan and Satya Prakash (1950) obtained stippling in cells infected with *P. berghei* to a great extent in smears stained by the J.S.B. method than with Giemsa.

The fact that the stain solution can now be prepared in the powder form is an additional advantage to the workers in the field. Preparation of the stain powder more or less on the same lines as practised here, has also been described by Manwell and Feigelson (1948). The powder is not difficult to make and the use of the stain with all the modifications suggested, remains still an inexpensive one. To quote a concrete example, the approximate cost of Giemsa stain sufficient enough to stain 100 slides comes to 11 annas, whereas to stain an equal number of smears with J.S.B., the same comes only to 0·22 annas. J.S.B. stain, therefore, is about 50 times cheaper than Giemsa. Further, by following the modified staining technique it is now possible to stain a smear in less than one minute and at the same time obtain highly satisfactory results.

*p*H of the water used for staining and washing blood smears is an important factor with all Romanowsky stains. While with Giemsa or Leishman a neutral or slightly alkaline *p*H is required for staining as well as washing, wash water with a *p*H of 6·2 to 6·8 is considered the optimum for J.S.B. stain. This variation in the *p*H range required for staining with these two different types of stains is due to the differences in the *p*H of the respective stock solutions and, therefore, has nothing to do with the particular dyes that are used for the preparation of the stains.

Recently, Nair (1953) has found that in the species identification of intestinal amœbæ, plain *p*H solutions or the easily available basic dyes such as methylene blue and pyronine, gave results as good as or better than those obtained with complex dyes like Velat's or Quensel's provided the *p*H of the solution was maintained

at an optimum. This is an indication that in the morphological study of all protozoan parasites, a basic understanding of the sensitivity to particular *p*H ranges by the different species of parasites is an essential pre-requisite.

While simple acetic acid can be used for acidulating the wash water for J.S.B. stain, it cannot be expected to give a constant result under all circumstances unless there is facility to estimate the exact quantity of acetic acid that is required to bring down the *p*H of the local water supply to the required level. But this difficulty is easily overcome in the present modification of the staining technique by using buffered salts which have the property to maintain the *p*H level when added in suitable proportions.

SUMMARY.

An account is given of a simple method of preparing J.S.B. stain solution I in powder form and also of a modified staining technique by which smears can be stained with better results and greater speed.

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PRELIMINARY REPORT ON LIVER INJURY ASSOCIATED WITH MALARIA.*

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(September 30, 1953.)

REPORTS on the functional and pathological lesions of the liver in malaria have appeared earlier. Although at one time it was believed that repeated attacks of malaria caused diffuse hepatic fibrosis in the tropics (Findlay, 1951), so far no definite co-relation between the incidence of fibrosis and prevalence of malaria could be established (Himsworth, 1950). Wahi and Arora (1951) reported that in human malaria the structural changes are confined to the reticulo-endothelial proliferation and patchy necrosis and that " extensive destruction of liver parenchyma and fibrosis of the extensive diffuse hepatic fibrosis type are never seen ". Maegraith (1951) observed that hepatic lesion like centrilobular necrosis occurs in malaria and the pathogenic factors responsible for such changes are due to intralobular blood flow.

However, conditions like diffuse hepatic fibrosis are often encountered where there is poverty and malnutrition (Himsworth, *loc. cit.*). Hill (1951) observed that fibrotic liver disease amongst Jamaican children is commonly associated with low protein diet. Histopathological studies of such cases revealed fatty metamorphosis in some, while fibrosis in others. Similar evidence has been put forward by Cayer (1951) who reported that in all his series of cases with fatty infiltration or fibrotic changes in the liver, there was evidence of low protein dietary conditions. Himsworth (*loc. cit.*) reported that in experimental animals, withdrawal of lipotropic factors from diet caused fatty infiltration of the liver and that prolonged fatty infiltration, irrespective of the cause, produced nutritional hepatic fibrosis. The lipotropic factors commonly present in human diets are inositol, choline, choline precursors, betaine, methionine and proteins (Atrom, 1951). Further, Hartroft and Ridout (1951) recently demonstrated fatty infiltration resulting in fatty cysts in the livers of rats fed on choline deficient diets.

*These studies form a part of a thesis.

In the current series of investigations, the effects of malarial infections on liver of *rhesus* monkeys under different dietary conditions are being studied, and this communication refers to only some aspects of such experimental observations.

At the initial stage the effect of acute infection with graded doses of inoculum (blood-induced *P. cynomolgi*) was compared to the normal liver structure. Subsequently biopsy materials were collected from uninfected monkeys kept on low protein diet for varying intervals. Some of these animals were then inoculated with *P. cynomolgi* with a single dose, while in some others it was repeated at intervals with higher doses of inoculum. The others served as the comparison group.

Unlike in *P. knowlesi* infection lesion due to *P. cynomolgi* is negligible. Apart from some degree of cloudy swelling, no other changes could be found in the liver of the uninfected monkeys kept on fat-free low protein diet for periods varying from 6 to 12 weeks. But when such animals were inoculated with *P. cynomolgi*, liver materials collected after the peak of infection had attained, showed marked fatty infiltration with formation of characteristic fatty cysts. After reinoculation with a heavier dose, infiltration and cysts were found to be extensive (Plate IV, Figs. 1-3). Silver impregnation showed that besides fatty infiltration in certain areas there was condensation of the reticulin framework of the hepatic lobules. But there was no apparent collapse of any part of the hepatic lobules.

Further details will be reported later.

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CHOICE OF METHOD OF MALARIA CONTROL FOR RURAL AND URBAN AREAS.

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RESIDUAL insecticides are powerful weapons for the control of malaria. The great advantage accrued from them lies in the fact that spraying operations need be carried out only at extended intervals against the adult mosquitoes. As a result, the control of malaria in vast rural areas has now become economically and technically feasible.

Among the new insecticides, D.D.T. has so far given the most outstanding performance. There is no doubt now that the use of this insecticide can be relied on as a basis of a wide-spread attack on malaria. Its effectiveness has been demonstrated against anopheline vectors of malaria with widely different habits. Time is ripe now to give serious thought to problems of cost of control. A country-wide programme of D.D.T. house-spraying depends on the budgetary facilities of any country. It is more so with India which is a poor country whose malaria problem covering vast areas is interlinked with many other developmental projects of vital importance.

Methods available for malaria control are mainly anti-adult or anti-larval which may further be classified as permanent or recurrent measures. In practice generally, indoor residual insecticide spraying is considered the method of choice for adult control in rural areas while anti-larval measures are recommended for urban areas. Also in the latter case, permanent anti-larval measures have definitely minimized the cost on recurrent measures.

PLATE IV.

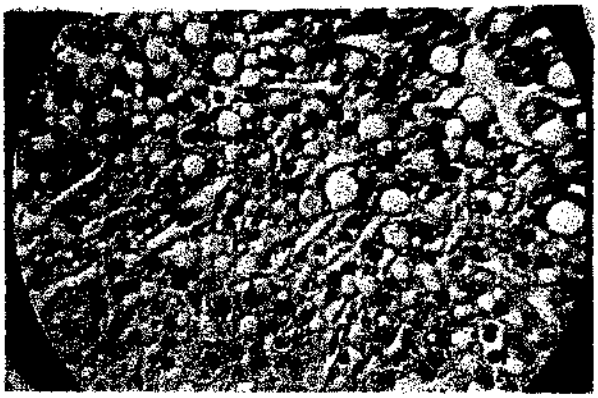


FIG. 1. Fatty changes.

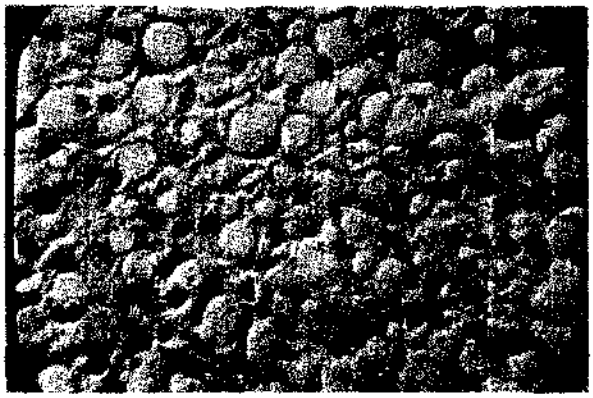


FIG. 2. Large fatty cysts.

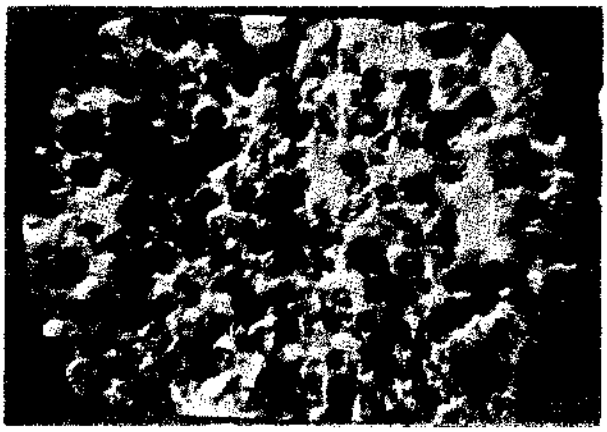


FIG. 3. Fatty changes-Stained with Sudan Black.

As already mentioned, the advantage of D.D.T. for anti-adult measures mainly lies in the fact that it requires repetition only at extended intervals. Its use as a larvicide, however, affords an advantage of a different nature. It does not exert any appreciable residual action but brings about a reduction in cost by minimizing the large quantities of oil otherwise required for the control of larvæ.

It is the purpose of this note to examine the criteria for the choice of specific measures in a given area ; it is particularly important in an under-developed country where the process of urbanization is gradually evolving in a large number of municipalities occupying a border line position between rural and urban areas. The most important consideration in determining the method of choice will naturally be the cost of effective control.

In making a decision, the underlying principle to be borne in mind in a general way is that the cost of anti-larval measures is inversely proportional to the density of population in an area, whereas, that of anti-adult measures is directly proportional to it. The fundamental requirement for a proper answer to the problem is therefore to determine a reasonable population figure to denote a line of demarkation between urban and rural areas.

Cost of larval control in relation to population.—1. Trends of cost of anti-larval measures in relation to population, as demonstrated by Sweet and Rao (1934) by employing recurrent application of Paris green, were as follows :—

Range of population		*Range of cost per capita*
Village of	500-2000	Rs. 6/- to Rs. 2/-
,,	2000-5000	Rs. 1/8/- to Re. 0/12/-
,,	5000-10000	Re. 1/- to Re. 0/6/-
Cities above	10,000	As. 6 to pies 6

The cheapest control was obtained in Bangalore City which had a population of about 179,600. The cost was under 6 pies per head and well under 1 per cent of the normal receipts of the municipality.

2. Covell and Afridi (1939) adopted recurrent anti-larval measures viz. larvicides, removal of vegetation from breeding places, minor levelling and drainage operations, and arrived at the following figures for the Delhi Urban Area :—

Range of population.	*Cost per capita.*
486,074 (in 1938)	2 annas

3. Covell (1941) estimated the capital cost of permanent engineering works for malaria control in Delhi, in relation to a population of 500,000 (1941), as Rs. 3-8-0 per head. This seems high but it does not represent recurrent expenditure on anti-larval plus anti-adult measures. This is computed at about annas 4·5 per head per annum, the residual spraying, however, being limited to the riverain tract. Collateral benefits derived from it in the form of general improvement in sanitation of the place and consequent reduction in flyborne diseases in itself yields colossal dividends. In many instances valuable land has been reclaimed for building sites and value of property has been greatly enhanced. It is also of value from an æsthetic point of view, as it beautifies the place.

4. Viswanathan (1946) in his consideration of control of malaria in Haliyal by recurrent larvicidal measures by clean-weeding of streams and copper cyaniding paddy fields, gives the cost per capita in relation to population as follows :—

Range of population.	*Cost per capita.*
7,000	8 annas

Cost of anti-adult control in relation to population.—Figures for cost of successful anti-adult D.D.T. measures in relation to population give the following picture :—

TABLE I.

Cost per capita.	Number of rounds of spray.	Range of population of communities.	Author.
Rs. as. p.			
0 1 8	2 rounds.	288 (Actual).	Senior White (Jeypore Hills), 1945.
0 2 4	2 rounds.	1000 (Average)	Afridi and Bhatia (Baluchistan), 1947.
0 5 6	Partly 2 Partly 1	1098 (Average)	Afridi and Dalip Singh (Delhi Villages), 1947.
0 5 10	—do.—	1050 (Average)	Jaswant Singh and Dalip Singh (Delhi villages), 1949.
0 6 11	3 rounds.	839 (Average)	Viswanathan and Ramachandra Rao (Kanara and Dharwar, Bombay), 1947.
0 8 0	Greater part 3 ; lesser 2 and 1 rounds.	37-700 (Actual) 488 (Average)	Viswanathan and Parikh, (Bombay), 1946.
0 10 7	3 rounds.	908 (Average)	Ramakrishnan *et al.* (South Kanara, Madras), 1948.
0 10 8	3 rounds.	7102 (Actual)	Jaswant Singh and Kariapa (Mercara, Coorg), 1949.

It is true that there are a number of variables in the calculations of costs in different parts of the country by different authors. But in the examples cited above, there is a fair amount of homogeneity in the sense that in all of them only recurring charges for materials, labour and transport are taken into account excluding salaries and expenses of public health personnel in permanent service of the governments concerned. These figures on the whole do show that higher the population of a community, higher will be the cost per capita for anti-adult measures. It will be seen from the above figures that the community with highest cost of about annas 10 per capita has a population of about 7102 (Coorg). The question arises how much above this limit of population one could still employ anti-adult measures economically. Viswanathan (1950) arbitrarily fixed the limit at 40,000 population as the dividing line. The senior author of this paper on the other hand after the advent of residual insecticides at an early stage considered a limiting figure of 20,000

as more appropriate. Communities above this limit were to be regarded as suitable for anti-larval and below for anti-adult measures. Available data from Mysore State furnishes some information for a direct comparison of costs of the two methods applied to given communities. It is briefly as follows :—

(*a*) Mandya Town occupying approximately half a square mile area has a population of about 40,000. Equally effective malaria control could be brought about by either anti-larval or anti-adult measures with modern insecticides. The total annual cost on anti-larval measures is about Rs. 6,800, whereas for anti-adult measures the estimated cost is Rs. 30,000 which is a remarkable difference in favour of anti-larval measures. The anti-larval measure employed was B.H.C. (P. 520) one ounce in a gallon of water disbursed at 15 gallons per acre. The estimated cost of anti-adult measure is based on two sprays of D.D.T. wettable powder, applied at 100 mg. per sq. ft.

(*b*) Same measures in Dodbalapur with a population of 18,000 cost about Rs. 4,800 on anti-larval and Rs. 9,000 on anti-adult measures.

The expenditure expressed in terms of cost per capita in the two communities can be compared as follows :—

Community	*Cost per capita*	
	Anti-adult	Anti-larval
40,000	Re. 0-12-0	Re. 0-2-9
18,000	,, 0- 8-0	,, 0-4-3

Inferences to be drawn from these broadly are that when the population of the community is about 40,000 the cost on anti-adult measure is about 4 times that of anti-larval. On the other hand, when the community is about half that strength *viz.*, about 20,000-18,000 the cost on anti-adult measures comes down to about twice the cost of anti-larval measure. It is, therefore, fair to assume that in a still smaller community representing a population of about 10,000 the cost on anti-adult or anti-larval measure will become almost equal. In other words a community with a population of about 10,000 would appear to represent the border line where anti-adult or anti-larval costs will be almost equal.

Further definition.—The sequence of gradual change from rural to urban conditions which take place have been illustrated in Figs. 1-6 to show the evolution of the latter from the former.

A rural community comprises a small population group living in a cluster of discrete houses. More often the houses are widely scattered. Such a community, depends primarily on the produce of their lands. Its occupations are essentially agriculture, poultry farming, dairy and cattle rearing. The land, if in malarious belts, is traversed by streams or a net work of canals or irrigation channels and fallow fields which collectively present clear water surfaces, moving as well as stationary, ideal for abundant breeding of anopheline mosquitoes. Human habitations are sparse but larval habitats are many.

Urban communities on the other hand are concentrated populations in limited areas. The houses are of various types, situated in close proximity to one another. Occupations of the people are predominantly trade, commerce and industry and their interest in land is mainly in its exploitation for constructing buildings, workshops, shops, factories and warehouses, with only patches of ornamental gardens of limited dimensions. Water supply and drainage are generally controlled in a fully developed urban community.

An urban community seldom springs up suddenly *de novo*. It is generally evolved from rural surroundings on account of development of trade and industrial enterprises. The conditions that present themselves in the transitional phases in the course of development of an urban community, are diagramatically represented by Figures 1-6 on page 279.

Fig. 1 shows a rural community composed of a few hundred people and cattle distributed in scattered houses indicated by symbol 'X' in a given area. 'S' shaped lines represent the primary waters which may be taken as minor rivers, irrigation channels, streams, canals or any other type of clear water surfaces such as fallow fields which breed vector anophelines. The circle is the outer boundary of the half a mile zone beyond the periphery of houses up to which anti-larval measures must be extended. At a glance one can see that water surface which implies the extent of anti-larval effort is many times greater than the number of houses which require treatment by anti-adult measures. The relative difference in effort is better appreciated if one remembers that anti-larval measure needs to be repeated at short intervals while residual anti-adult sprays are to be applied at extended intervals.

Fig. 2. shows the community to have grown a little with increased population (animal or human) and in consequence adding a few houses. No change has taken place in vector breeding water surface. Cost of control on anti-larval measure remains the same as before, but the work required on houses (anti-adult) is increased a little although still remaining many times cheaper than anti-larval.

Fig. 3 represents a still further stage when more population and houses are added to the old community. Concentration of population starts usually in one or more focal points which are more or less in the centre of the area. Hardly any change has taken place at the periphery and the half mile zone surrounding the peripheral houses, therefore, continues to remain in the old places for application of anti-larval measures if adopted. Towards the centre of the area a great change is taking place in vector breeding waters which come in close proximity to habitations. Quite a good part of them begin to get polluted or interfered with by man and/or animal in many ways and become unfit for vector breeding. The ends of 'S' shaped lines converging towards the centre of the area are for this reason shown to be fading. While considerable reduction in vector breeding water surfaces is taking place in this way, conditions are no doubt being created for abundant production of culicines and non-vector anophelines in the polluted waters. Very often, water courses which were originally streams carrying clear water, have become nothing more than haphazard drains of foul smelling highly polluted waste water. Here and there new types of clear water collections also crop up to some extent in the form of depressions and borrowpits created during the process of

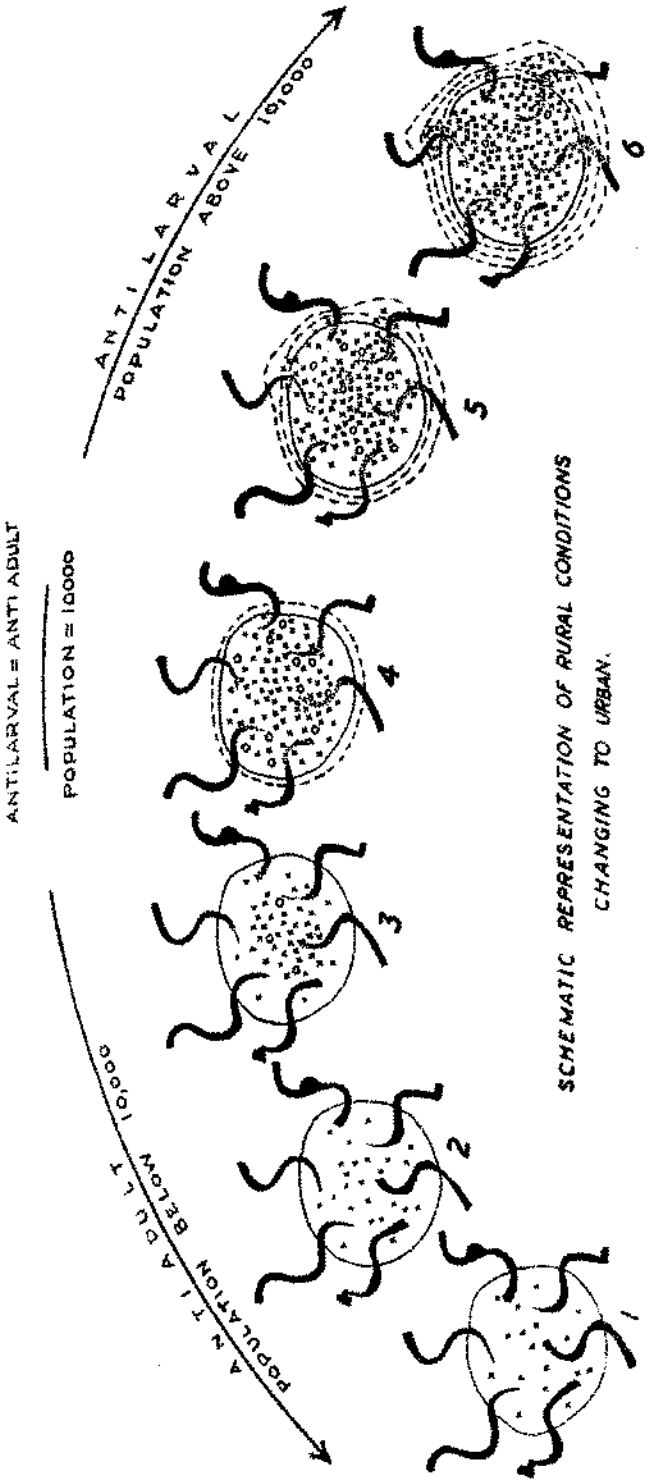
building roads and houses. These are the secondary water collections which yield in some cases vector species, but in most others, non-vector mosquitoes, and are shown as small 'o' (circles) in the Figure. Anti-larval measure (for malaria control) on the whole still entails much higher expenditure although of a lower order than it was in the first two stages (Figs. 1 and 2) and anti-adult measure continues to be cheaper.

Fig. 4 shows the community to have grown to such a stage that its population has risen to about 10,000 people. The primary vector breeding waters have become significantly altered so as to present only very small parts of them suitable for vector breeding. Secondary water collections have increased a little more but from the point of view of control of malaria carrying species, the work required on anti-larval measures has decreased significantly and there is a corresponding increase with scope for anti-adult measure due to further increase in the number of houses. Peripheral houses have moved further out and in consequence the half a mile zone embracing the habitations for anti-larval measure is correspondingly moved outwards. Further, the cost of operation is being distributed over a larger number of people bringing down the per capita cost. Now a condition has reached, when the cost of anti-larval measures is about the same as that on anti-adult measures.

Figs. 5 and 6 show further concentration and expansion of the community consisting of more than 10,000 people. The half a mile zone around the peripheral houses has been shifted out more and more. The primary vector breeding waters in the interior of the community have been reduced to a very low state and the institution of proper drainage and sanitation have come into existence, thereby the hitherto haphazard watercourses are properly trained and maintained. Thus, many of the secondary water collections have been eliminated, with the result, the water surfaces to be treated for anti-larval measures are far less than the house surfaces to be treated by anti-adult measures. In other words conditions have been reversed making anti-larval measures cheaper.

An appropriate example of what has been described above is to be found in the latest planning of malaria control on a country-wide basis in the Philippine Islands. In that country the concentration of population even above 1,000 in a community is reported to bring about such a change in malariogenic conditions that it is no longer regarded malarious although no deliberate effort was made by man to reduce malaria from such communities. On account of this reason majority of communities (Barrios) with a population above 1,000 are excluded from the operational areas for residual spraying against adult mosquitoes. This perhaps is an extreme example and may be regarded specially applicable to Philippine Islands where there is only one predominent vector *A. minimus* var. *flavirostris* which breeds mainly in slow running clear water streams with grassy edges. In India, however, communities with much higher population do exhibit malaria hyperendemicity and a line has to be drawn to choose between anti-larval and anti-adult measures not only from the point of view of economy but also from collateral benefits that may accrue.

The question that now arises is to determine the upper limit of population for anti-adult measures taking all aspects into consideration. Obviously, fixing an upper limit of 10,000 merely on grounds of comparative cost of malaria control



SCHEMATIC REPRESENTATION OF RURAL CONDITIONS CHANGING TO URBAN.

only would not do, as there are large areas in this country where tremendous collateral advantages are to be derived from residual spraying in the control of other insect-borne diseases such as filariasis, sandfly fever, dengue, plague, and fly-borne diseases as enteric, etc. Poona City, for instance, has a population of approximately 600,000 which far exceeds the limit for the adoption of anti-adult measures. It has nevertheless been found to be economical to undertake anti-adult measures on account of co-existence of plague and other insect-borne diseases (Viswanathan, 1950). Although anti-larval control of malaria here costs only about Rs. 64,000 a year, higher expenditure on anti-adult residual spray costing about Rs. 300,000 per year (calculated @ 8 annas per head—Viswanathan, 1953) is considered worthwhile. Justification of this may to some extent be gauged from the fact that Poona City Municipality in the past has been bearing an annual expenditure of Rs. 100,000 on plague control alone without achieving fully satisfactory results. In fact, Viswanathan (1953) goes to the extent of supplementing anti-adult residual spray with anti-larval measures in special areas of this city. Combined anti-larval and anti-adult measures have been practised with a view to obtain maximum collateral benefits in Delhi urban area from 1947 and more recently in the larger municipal areas in Mysore State. An integrated programme for the control of more than one insect-borne disease by a common measure when practicable is, therefore, bound to be cheaper and more effective in the long run.

When the aim of control is to eradicate malaria, anti-adult residual spraying needs to be combined with anti-larval measures irrespective of whether a community is rural or urban. It would, therefore, appear that while it would be more economical to control malaria only by anti-adult measures for population perhaps upto 10,000, taking other collateral effects into consideration, such measures would still be reasonable in population groups between 20-40 thousand, anti-adult measures being recommended upto 20,000 as a routine and for population between 20-40 thousands each case being examined individually in relation to the incidence of other insect-borne diseases and a combination of both measures to suit individual needs, is suggested.

Another circumstance under which an urban community (exceeding the limit of 10,000 population) may be protected from malaria essentially by anti-adult measures, is during a year of epidemic. The control of malaria during an epidemic in any community is a point on which the authors of this note wish to focus the attention of the public health worker. A view is often put forward that pyrethrum space spraying is the method of choice during an epidemic but critical analysis of the information on the subject makes one hesitate to accept it as such.

Senior White (1945) has shown that malaria control by D.D.T. residual application is 20-25 times cheaper than pyrethrum space spraying. He also states that in a given village where D.D.T. residual application brings about 81 per cent reduction of *A. culicifacies*, pyrethrum space spraying twice weekly reduces the density of the same species only by 49 per cent. He further explains that pyrethrum as a method of protecting population from malaria is useless when *A. fluviatilis*, *A. varuna* and *A. minimus* are the vectors. Twice weekly space spray with pyrethrum of Chatikona Village did not arrest the high infectivity rate among these species. Covell (1943) also is of opinion that during epidemic years in areas where

A. culicifacies is the vector, space spraying by pyrethrum to be effective, must be repeated daily. Viswanathan *et al.* (1944) have concluded from their observations in North Kanara on *A. fluviatilis* that, when this species has a gonotrophic cycle of 48 hours, 60 per cent of the females leave the houses every night and when the cycle takes 72 hours, 40 per cent of them leave every night. The result of this is that during various pyrethrum space spraying intervals, certain percentages of females keep escaping the effects of the spray. They assert that with an efficient vector like *A. fluviatilis*, even a very low escape rate is sufficient to maintain malarial hyperendemicity. Senior White *et al.* (1945) have further confirmed this finding. In Kanara District, space spraying with pyrethrum even 4 times per week failed to stop transmission of malaria by *A. fluviatilis* (Viswanathan, 1946).

It is true that pyrethrum has quick knock-down effect on mosquitoes but this aspect would appear to be over-stressed if one bears in mind that all anti-adult spray operations whether residual or space spray are usually carried out during day time when malaria vectors seldom bite. As such it makes no difference whether the mosquito takes ten minutes or three hours to die.

The vital need under such a situation is to complete the residual spraying of houses and cattle-sheds in the shortest possible time. A suitable organization should be mobilized to cover the entire affected area in as short a time as possible by putting more spraying squads into the area. The space spraying of houses by the residents themselves as an additional measure of protection might be encouraged while the concerted effort of reinforced spray squads finish the D.D.T. residual application in record time.

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METABOLIC STUDIES WITH BROMOGUANIDE*.

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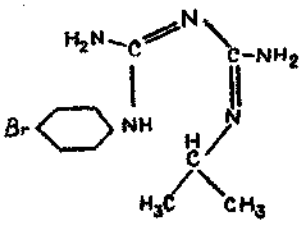
BROMOGUANIDE (I) a *p*-bromophenyl analogue of proguanil was synthesised to ascertain the effect of replacement of chlorine with bromine in proguanil molecule (Bami and Guha, 1948 ; Curd *et al.*, 1948 ; Ainely *et al.*, 1949). Preliminary studies against *P. gallinaceum* and *P. knowlesi* have revealed that bromoguanide possesses high antimalarial activity (Bami *et al.*, 1949 ; Ainely *et al.*, 1949 ; Jaswant Singh *et al.*, 1949) and this led to a further examination of this potential antimalarial by Jaswant Singh *et al.* (1950) against avian and simian malarias. According to these investigations, the comparative efficacy of bromoguanide in terms of time taken for clearance of all stages of parasites, was in the following order of increasing magnitude, *P. gallinaceum*, *P. cynomolgi*, *P. imui* and *P. knowlesi*. The antimalarial activity itself was generally equal to that of proguanil but bromoguanide was definitely inferior to proguanil as regards acute toxicity and prevention of relapses in these strains of plasmodia. It was, however, observed that in certain cases there was no correlation between its dosage and the biological response, which could be explained on the basis of toxicity of bromoguanide and/or due to particular mechanism of its absorption and degradation *in vivo*.

Proguanil had very little *in vitro* antimalarial activity but the serum obtained from animals treated with proguanil was found to be active against *P. gallinaceum* *in vitro* (Tokin, 1946 ; Hawking, 1947 ; Hawking and Perry, 1948). These observations and studies on adenosine antagonism *in vitro* (Madinaveitia and Raventos, 1949) led to the belief that proguanil is converted into an active metabolite prior to exerting its antimalarial activity. Certain hypothetical metabolites of paludrine have been suggested (Fraser and Kermach, 1951 ; Chase *et al.*, 1951 ; Britwell, 1952) but Crounse (1951) first isolated two inactive metabolites from the urine of monkeys receiving proguanil. One of these compounds was simple p-chloro-phenyl-biguanide while the other was an inactive triazine (II, R=Cl), which had

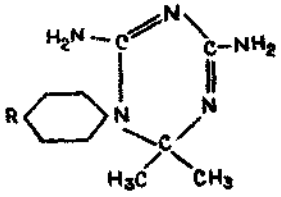
*Bromoguanide is a *p*-bromo-phenyl analogue of proguanil *viz.*, N^1-*p*-bromophenyl-N^5- isopropyl-biguanide, hydrochloride.

previously been synthetically prepared by Britwell *et al.*, (1948). Further investigations in rabbits and human-beings by Carrington *et al.*, (1951) and Crowther and Levi (1953), led to the isolation of a highly active proguanil metabolite viz., a dihydrotriazine (III, R=Cl), very much similar and easily convertible to the inactive triazine (II, R=Cl). This active metabolite (III, R=Cl) proved ten times as active as proguanil in *P. gallinaceum* (Carrington *et al.*, 1951 ; Crowther and Levi, 1953), while six times as active as quinine, twice as active as mepacrine or proguanil and half as active as pamaquin when tested against *P. lophurae* (Modest *et al.*, 1952). Against *P. berghei*, it was almost as active as proguanil (Krishnaswami *et al.*, 1953) while against *P. cynomolgi* in monkeys it was only half to quarter as active as proguanil (Schmidt *et al.*, 1952).

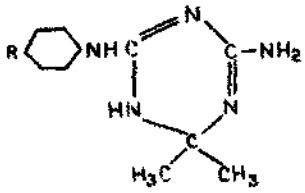
The disparity between the action against avian and simian malarias indicated above, suggests that this may be due to inherent differences in susceptibilities of the two types of strains and/or differences in the physiological disposition of the metabolites itself. However, as the metbolites isolated by Crounse (1951) were only screened against *P. cynomolgi*, it is argued that hopeful results were missed Schmidt *et al.*, *loc. cit.*). Easy convertibility of the active dihydrotriazine (III) into inactive triazine (II) with exposure to heat and alkali, may have been responsible for failure to isolate the active metabolite, as Crounse's technique involved more intensive exposure of the metabolities to alkali.



Bromoguanide. (I)



Dihydrotriazine (III) (Active proguanil metabolite; R=Cl) ("Bromoguanide" metabolite; R=Br).



Triazine (II) (Inactive proguanil metabolite R=Cl).

In other words, the active metabolite of progaunil could not be isolated from monkeys, an animal more readily available and nearer to man. Considering the high antimalarial activity of Bromoguanide (I) and other interesting observations discussed before, it was though worthwhile to study its metabolic degradation *in vivo* using monkeys, in order to determine if similar active metabolites are produced in monkeys and what is their relative biological efficacy. The choice of general technique was one, adopted by Crowther and Levi (1953), as it involved least exposure of the metabolites to heat and alkali.

A number of experiments were carried out using at least two healthy *rhesus* monkeys each weighing about five to ten lbs. In preliminary experiments a dose of 40 mgs. base per kg. body weight was administered orally in the form of aqueous solution of bromoguanide hydrochloride but in some animals severe symptoms of chronic toxicity were noticed. The dosage was then reduced to half and was well

tolerated by all animals. The drug was administered once daily for 6 to 10 days, and the urine collected over chloroform. The urine was neutral in reaction and was preserved at 0-5° C. It was treated as early as possible to avoid further decomposition.

The active metabolite viz., 1-p-bromophenyl-6 : 6-dimethyl-2 : 4-di-amino-1 : 6- dihydro-1 : 3 : 5-triazine (III, R =Cl) was ultimately isolated as a picrate and this product was found to be identical with the picrate of the same compound prepared synthetically by the auther. Yield of pure metabolite from the monkey urine on the basis of proguanil was 5 per cent. About 2·5 per cent of the bromo-guanide (1) was recovered as such during the separation while the inactive triazine (II, R =Br.) could not be detected. In one case during the course of isolation, the active material got excessively exposed to heat and alkali and consequently the final picrate was found to be a mixture of the picrates of metabolite (III, R =Br) and its modified triazine form (II, R =Br).

The active metabolite of bromoguanide (III, R =Br) was found to be four times as active as the paludrine metabolite (III, R =Cl) and 32 times as active as proguanil when screened against *P. gallinaceum* in young chicks (Bami, 1953 ; Jaswant Singh *et al.*, 1953). However, preliminary trials against *P. knowlesi* (Nuri strain) in *rhesus* monkeys, indicated that bromoguanide metabolite (III, R =Br) was probably a little less active than even proguanil (Ray and Nair, personal communication).

EXPERIMENTS.

Two monkeys weighing 5·5 kg. each were put in a metabolic cage and given orally once a day 20 mgs. base per kg. body weight of bromoguanide (1) in the form of a 0·4 per cent aqueous solution, by means of stomach tube. Drug ad-ministration was continuid for six days, the urine collected over chloroform daily and preserved. Total urine (1,000 ml.) was finally pooled and worked up as below :—

Isolation of 1-p-chlorophenyl-2 : 4-diamino-1 : 6-dihydro-6 : 6-dimethyl-1:3: 5-triazine (III, R =Br).—Lead acetate was added to the urine (1,000 ml.) till further addition caused no precipitation. The mixture was filtered through kieselguhr and clear solution extracted with butanol three times (400 ml., 200 ml. and 200 ml.) using a mechanical shaker. The total butanol extract was concentrated under reduced pressure at room temperature and near the end, the butanol phase was displaced with water (by repeating the dilution of the concentrated butanol extract with water and evaporation *in vacuo* twice). The aqueous phase (50 ml.) was made faintly acidic with N-hydrochloric acid, the precipitated lead chloride filtered, and the clear filtrate treated with hydrogen sulphide when all the remaining lead was precipitated. The suspension was filtered and the clear filtrate aerated for 10-15 minutes to remove excess of hydrogen sulphide. The aqueous solution at this stage was cooled to 0°C. (small pieces of ice were also added to solution itself) and basified with dilute alkali. The alkaline solution was then extracted with ether three times (100 ml., 50 ml. and 50 ml.) and total ether extract kept aside for later examination. The aqueous alkaline solution, was neutralized in cold

and again extracted with butanol three times (100 ml., 50 ml. and 50 ml.). This butanol extract was shaken with one per cent sodium hydroxide (15 ml.), the coloured aqueous layer removed, and organic as well as aqueous phase again made neutral. The neutral butanol extract was separated and evaporated in vacuum. The organic phase was replaced by water as described before and the final aqueous solution (15 c.c.) treated with alcoholic picric acid (2-3 ml.). A crystalline yellow precipitate was obtained which was filtered after 24 hours and crystallized from alcohol twice, as long yellow needles of 1-p-bromophenyl-2 : 4-diamino-1 : 6-dihdro-6 : 6-dimethyl-1 :3 : 5-triazine picrate with m.p. 201° C (uncorrected). Mixed m.p. with an authentic synthetic sample was 201° C. (Found C, 38·6, H.3·5, N 21. 2, 2·18, $C_{11}H_{14}N_5$ Br., $C_6H_3O_7N_3$ required C 38·8, H. 3·2. N, 21·3 per cent.).

Recovery of bromoguanide and other materials.—The ether extract obtained above was evaporated in a current of air and the residue titurated with dilute hydrochloric acid (10 ml.). The acid solution was filtered and made slightly alkaline with ammonia. On cooling and scratching a white crystalline precipitate was obtained which was filtered, washed and dried. This was identified to be bromoguanide hydrochloride m.p. 249° C. (mixed m.p. with synthetic sample 249° C.). The filtrate at this stage was basified and treated with ammoniacal copper-sulphate. A small amount of copper complex of bromoguanide was obtained, which was filtered and the product regenerated. The clear basic filtrate was acidified and treated with hydrogen sulphide to remove the excess of copper ions. After the removal of the precipitated copper sulphide, the clear solution was treated with alcoholic picric acid but no other product was obtained.

DISCUSSION.

Isolation of an active metabolite (III, R=Br) from monkeys receiving bromoguanide indicates that mode of degradation of this drug is parallel to that of proguanil in rabbits and human beings. Thus monkeys can be profitably used for such metabolic studies as these results may well be applicable to human beings satisfactorily. It also confirms that failure to isolate an active metabolite (III, R=Cl) of proguanil by Crounse (1951), was mainly due to experimental techniques involved. Production of this active metabolite may be a reason for high antimalarial activity of bromoguanide, but this being less active than proguanil when tested against *P. knowlesi* in monkeys is somewhat surprising. Against *P. gallinaceum*, however, bromoguanide metabolite is 32 times as active as proguanil and above disparity in screening results may be explained due to different physiological disposition of these products or inherent differences in the susceptabilities of different strains (*Schmidt et al.*, 1952). Considering, that metabolic degradation processes in case of bromoguanide, proguanil and 3 : 4-dichlorophenyl analogue of proguanil (Crowther and Levi, 1953) are the same in different animals, differences in susceptability of various strains of plasmodia to these metabolites may be more likely possibility. Structural similarities between pyrimethamine (daraprim) (Falco *et al.*, 1951 ; Russel and Hitchings, 1951 ; Jaswant Singh *et al.*, 1951) and these active metabolites (type III) also do not justify great disparity between the results of simian and avian malaria screening.

A 3 : 4-dichlorophenyl analogue of proguanil metabolite has also been obtained from the urine of rabbits receiving N^1-3 : 4-dichlro-phenyl-N^5-isopropyl-biguanide (Crowther and Levi, 1953). The above metabolite was ten times as active as proguanil metabolite (III, R=Cl) against *P. gallinaceum* while bromoguanide metabolite (III, R=Br) was only four times as active as proguanil metabolite (III, R=Cl). These results are explainable on the basis that the parent compounds viz., 3 : 4-dichloro-analogue of proguanil (Curd *et al.*, 1950, Crowther *et al.*, 1951) and bromoguanide are also usually more active than proguanil. Thus the activities of these metabolites are generally parallel to those of corresponding biguanides with the exception, that simian malarias do not respond to these metabolite satisfactorily.

SUMMARY.

Metabolic studies with bromoguanide (N^1-p-bromophenyl-N^5-isopropyl-biguanide) in monkeys have led to the isolation of 1-p-bromophenyl-2 : 4-diamino-6 : 6- dimethyl-1 : 6-dihydro-1 : 3 : 5-triazine from the urine. This metabolite has been found to be 32 times as active as proguanil and 4 times as active as proguanil metabolite against *P. gallinaceum*.

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PRELIMINARY STUDIES ON 8-AMINOQUINOLINES.

BY

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(July 14, 1953.)

SINTON *et al.* (1930) established antirelapse properties of a combined dosage schedule of quinine and pamaquin but the regime has not been followed extensively in India due to the toxic nature of the compound. Further, as extensive mosquito control measures had not been undertaken before, re-infection after cure was quite frequent, thus defeating the object for which antirelapse treatment was instituted. But in view of the nation-wide control measures about to be launched throughout India (Jaswant Singh, 1953) it has now become imperative to take up the problem of radical cure in *P. vivax* infection. Prevention of relapses would automatically cut down the carrier rates and would thus minimize the possibility of further transmission.

In view of the highly toxic nature of the compounds of the 8-aminoquinolines like pamaquin, during recent years several compounds of the same series, but less toxic, have been synthesized. These are pentaquine (Drake *et al.*, 1946), isopentaquine and primaquine (Elderfield *et al.*, 1947).

This preliminary report is based on observations made on 45 cases of *P. vivax* infection treated in two hospitals in Delhi. Twenty cases were treated with quiniplex* (Schedule II) and a similar number with primaquine (Schedule I).

*Each tablet of quiniplex contains 150 mg. (2·25 grains) of quinine and 6·5 mg. (5·0 mg. base) pentaquine.

For comparison 5 patients received a course of quinine and pamaquine (Schedule III). During the first two days the daily dose of quinine under Schedules II and III (Table I) was brought upto 20 grains. For the rest 5 days it was 10 grains only.

TABLE I.

Dosage schedules.

Schedule Number	Number of cases.	Antimalarials.	Dose/day.	Duration (days).
I.	20	Primaquine	10 mg. (base)	7
II.	20	(i) Quiniplex 2 tablets b.d. } Pentaquine + Quinine	10 mg. (base) + 9 grains	7
		(ii) Quinine	10 grains	3*
III.	5	Pamaquine	20 mg. (base)	
		Quinine	10 grains	7
		,,	10 grains	2*

*10 grains for the first two days concurrently with quiniplex or pamaquine-quinine, thus making the total dose during this period upto 20 grains a day.

The study on the relapse pattern was made during an observation period from 6 months to 2 years. In addition, speed of clearance of parasites and relief of clinical symptoms during the course of treatment were taken into consideration.

Tables II and III below show the relative merits of the different schedules *in relieving* clinical symptoms and in clearing parasites, asexual and gametocytes, from the peripheral circulation.

TABLE II.

Clinical response to treatment.

Schedule Number.	Number of cases.	Relief of clinical symptoms (hours).				Remarks.
		24	48	72	96	
I.	20	10 / (50)	9 / (95)	...	1 / (100)	
II.	20	9 / (45)	6 / (75)	3 / (90)	2 / (100)	
III.	5	2 / (40)	2 / (80)	1 / (100)	...	

(Figures in bracket represent percentage.)

Response to treatment appears to be satisfactory under all schedules as 90 to 100 per cent of the cases were afebrile within 72 hours. Further, at least 40 to 50 per cent of cases were relieved of clinical symptoms even within 24 hours (Table II). In this respect the results achieved with primaquine as per Schedule I are more striking than the others as clinical cure was attained in 95 per cent of the cases within 48 hours, against 75 and 80 per cent under Schedules II and III respectively.

TABLE III.

Effect on parasitæmia.

Schedule number.	Number of cases.	Parasite clearance (within hours).								
		Asexual.					Gametocytes.			
		24	48	72	96	Number of cases with gametocytes.	24	48	72	96
I.	20	5 (25)	11 (80)	4 100	...	18	3 (16·6)	9 (66·6)	6 (100)	...
II.	20	7 (35)	5 (60)	5 (80)	4 (100)	10	1 (10)	3 (40)	2 (60)	4 (100)
III.	5	2 (40)	2 (80)	...	...	3	2 (66·6)	1 (100)	...	...

(Figures in bracket represent percentage.)

Although clearance of asexual parasites occurred in all cases under the three schedules within 96 hours, parasites were not detectable beyond 72 hours in those treated under Schedules I and III. In 80 per cent of cases under the last two schedules, parasite clearance was attained within 48 hours, against 60 per cent under Schedule II (Table III).

The rate of gametocyte clearance appeared to be slow during the first 24 hours in cases treated under Schedules I and II, but within 48 hours speed of clearance was increased 4 fold (Table III). Surprisingly the clearance rate was remarkably slower in those treated with quiniplex as only in 60 per cent of cases there was complete clearance within 72 hours as against 100 per cent in the other schedules.

RELAPSES.

Out of 45 cases treated, 40 (88·8 per cent) were under observation for periods varying from 1 to 2 years. The rest five were observed for over 6 months. Out of the former group there have been only three cases of relapses so far. One case under Schedule I relapsed after 2 weeks while two under Schedule II after 7 and 8 weeks respectively.

Untoward side effects.—In none of the cases was any of the untoward side effects observed. There was no evidence of cyanosis or gastrointestinal disorder in any of the cases.

DISCUSSION.

Pamaquin was used extensively in the past for its powerful gametocytocidal action (Green, 1929 ; Aimes, 1930 ; Clemesha and Moore, 1930) but gametocyte prophylaxis did not meet with much success (Senior White and Adhikari, 1937 ; Field, 1939) mainly because prolonged use of this drug produced toxic manifestations. Subsequent to the detailed investigation by Sinton and Bird (1928) and Sinton *et al.* (1930), the main use of the drug was restricted to anti-relapse treatment under strict medical supervision preferably under hospital conditions.

In view of the potentialities of this compound, a few more of the 8-aminoquinolines like pentaquine, isopentaquin and primaquine have been synthesized and claimed to be less toxic than pamaquin. Primaquine is considered to be the least toxic of the series.

The relapse rates observed in the present series were 5 per cent under primaquine regime and 10 per cent with quinine and pentaquine (Quiniplex).

These observations are in conformity with those reported by Edgcomb *et al.* (1951) in respect of primaquine, and by Alving and Coggeshall (1947), Loeb *et al.* (1946), Spicknall and Terry (1948) for quinine and pentaquine regime.

None of the five cases treated with quinine and pamaquin had relapsed.

The redeeming feature was that no toxic manifestation of any kind was detectable in a single case even in those treated with quinine and pamaquin. This may be due mainly to the much smaller doses administered in the present series than are usually recommended. With regards to pamaquin, Sinton *et al.* (1930) observed that a daily dose should not exceed 40 mg. and even 30 mg. or less should be effective without causing much side effects. As such it is not surprising that no ill effects were observed after 18 mg. of pamaquine a day for 7 days. Toxic manifestations like cyanosis and mild abdominal discomforts have been reported in cases treated with 60 mg. pentaquin daily (Findlay, 1951) but in the present series the dose was only one sixth. Even for primaquine the dose was lower (10 mg.) than recommended (15 mg.) by Alving (1952).

It had been the common practice to administer quinine concurrently with pamaquine (Sinton *et al.*, 1930) and pentaquine (Alving and Coggeshall, 1947 ; Alving, 1952). This is because firstly these latter compounds are poor schizonticidal drugs and thus act slowly during an acute attack. Moreover quinine is believed to act synergistically with 8-aminoquinolines and also reduces toxicity of these compounds. But Alving (1952) considers that primaquine in 15 mg. dosage is adequate for the purpose without any addition of quinine.

In view of this, while quinine was administered along with pamaquine or pentaquin, in the present series it was not so done in respect of primaquine. But yet it may be observed that within a period of 48 hours relief of clinical symptoms

was attained in 95 per cent of the cases as against 80 and 75 per cent of cases under quinine and pamaquin, and quiniplex respectively.

From these observations it would therefore seem that primaquine in addition to its anti-relapse properties has also rapid schizonticidal action.

If these results are substantiated in large scale field investigations, the future rôle of 8-aminoquinolines, particularly primaquine, becomes quite obvious more so as concurrent administration of a schizonticidal drug may be wholly unnecessary.

SUMMARY.

Forty five cases were treated with anti-relapse drugs, viz., 20 cases with primaquine alone, 20 with quinine and pentaquin (Quiniplex) and 5 with quinine and pamaquin.

There were 3 cases of relapses, one under primaquine and 2 under quiniplex, during an observation period of 6 months to 2 years.

The rate of asexual parasite clearance with primaquine alone was more rapid than the other two regimes even though 20 grains of quinine was administered daily for the first two days along with pentaquine or pamaquin. Primaquine seems to have a future.

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The following contractions are in use in the *Indian Journal of Malariology*, whether the number to be expressed is one or more :—

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Amer. J. Hyg.
Ann. Trop. Med. Parasit.
Brit. Med. J.
Bull. Ent. Res.
Bull. Soc. Path. Exot.
C. R. Soc. Biol.
Geneesk. Tijd. Ned.-Ind.
Ind. J. Mal.
Ind. J. Med. Res.
J. Mal. Inst. Ind.
Jl. R. A. M. C.
J. Trop. Med. Hyg.
Meded. Volk. Ned.-Ind.
Rec. Mal. Surv. Ind.
Rev. App. Ent.
Riv. Malariol.
Trans. Roy. Soc. Trop. Med. Hyg.
Trop. Dis. Bull.
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SYMPOSIUM ON PYRIMETHAMINE (DARAPRIM, 50-63) HELD ON NOVEMBER 21, 1953, AT G.R. MEDICAL COLLEGE, GWALIOR, UNDER THE AUSPICES OF INDIAN COUNCIL OF MEDICAL RESEARCH.

THE Chairman, Colonel Jaswant Singh, in opening the symposium observed that pyrimethamine, a new addition to the series of synthetic antimalarials, has received considerable attention both in India and abroad.

In view of intensive studies undertaken during the past two years in this country, the Chairman hoped that this symposium will not only reveal the nature and the various aspects on which investigations have been carried out but it will also throw light in assessing the rôle of pyrimethamine in the treatment of malaria in India.

The papers which were read and those from which summaries or extracts were reported in the meeting, are published in this issue of the Journal.

Indian Journal of Malariology, 7, 4, *December* 1953.

PYRIMETHAMINE (DARAPRIM) AND ITS ESTIMATION.*

BY

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(*Malaria Institute of India, Delhi.*)

(November 21, 1953.)

DARAPRIM, now called pyrimethamine (II) is the outcome of collaborative efforts of two teams of investigators, working on either side of Atlantic. Hitchings, Falco, Russell and colleagues working in the Wellcome Research Laboratories, Tuckahoe, New York, developed the idea of 2 : 4-diamino-pyrimidines as potential antimalarials and synthesised nearly three hundred of these new derivatives. These compounds were sent for antimalarial testing against *P. gallinaceum* and *P. berghei* at Wellcome Laboratories of Tropical Medicine, London, by Rollo, Goodwin and associates. Their combined efforts established pyrimethamine as a highly active antimalarial and stimulated further detailed laboratory and field trials by several research workers all over the world.

High antimalarial activity of sulpha-pyrimidines (sulphadiazine, sulphamerzine, sulphamezathine, etc.) against avian and simian malarias and the importance of pyrimidine ring systems as components of certain nucleoproteins (chief constituents of cell nucleus where changes associated with growth occur), led to intensive investigations of suitably substituted pyrimidines as potential antimalarials by a group of Imperial Chemical Industries workers headed by Curd, Rose, Davey and associates. These workers encountered slight activity in the case of compound M-2666 (Curd and Rose, 1946*a*) and further variation in this pyrimidine molecule led to the discovery of M-3349 from which proguanil was ultimately derived by replacing the guanidino-pyrimidine system of M-3349 with a substituted biguanide chain (Curd and Rose, 1946*b*). Thus the discovery of proguanil (I) was the direct outcome of researches on pyrimidines. In fact the most active precursor of proguanil *viz.*, M- 3349 had 2 : 4-diamino-pyrimidine as its basic structural unit, as in the case of pyrimethamine (II), although in the latter case both of its amino groups are non-substituted. High antimalarial activity associated with proguanil led to extensive researches on biguanides and pyrimidines but unfortunately further variations in the pyrimidine molecule offered little promise in the hands of these workers (Rose, 1951 ; Bami, 1953).

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G.R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

Hitchings and his co-workers had been independently interested in a group of 2 : 4-dimino pyrimidines since 1948 which displayed competitive antagonism towards folic and folinic acids in the growth of *Lacto bacillus casei* (Hitchings, Elion, Vanderwerff and Falco, 1948 ; Hitchings, 1952). Such of the 2: 4-amino-pyrimidines which carried substituents in position 5- were found to be even better inhibitors under the above conditions and they also displayed only moderate toxicity. It was argued that this class of compounds may offer chemotherapeutic agents. In fact one such compound *viz.*, 2 : 4-dimino-5-*p*-chlorophenoxy-pyrimidine was found to be somewhat structurally related to proguanil and both these compounds in turn, were powerful antagonists of pteroylglutamic acid (folic acid) in cultures of *Lacto bacillus casei* (Falco *et al.*, 1949). Considering the high antimalarial activity of proguanil, it was thought that 2 : 4-diamino-5-p-chlorophenoxy-pyrimidine may also have some antimalarial activity. On screening, this was found to be true in the above case (Falco *et al.*, 1949) as well as in the case of its 6-methyl homologue (Goodwin, 1949). It may be pointed out here that the degree of antagonism between the above compounds and pteroylglutamic acid was in no way proportional to their antimalarial activity (Falco *et al.*, 1949). However, this offered the necessary lead for further extensive chemical and biological studies for improving antimalarial activity of the above type of compounds. Three series of compounds *viz.*, 2 : 4-diamino-6-alkyl-5-phenoxy-pyrimidines (Falco, Russell and Hitchings, 1951), 2 : 4-diamino-6-alkyl-5-benzyl-pyrimidines (Falco, Du Breil and Hitchings, 1951) and 2 : 4-diamino-6-alkyl-5-aryl-pyrimidines (Russell and Hitchings, 1951) were investigated and high antimalarial activity was encountered in the case of last mentioned group to which pyrimethamine belongs. Within each of the above three types of compounds, the effect of different 6-alkyl groups as well as that of the different substituents in the 5-benzene ring, on their antimalarial activity, was studied in detail. Some general conclusions ragarding the relationship of chemical structure to antimalarial activity, are as follows (Falco, Goodwin *et al.*, 1951 ; Rollo, 1952 and Bami, 1953).

(*i*) 2 : 4-diamino-group in the above class of compounds is most essential for activity, and substitution of either or both amino groups resulted in loss of activity.

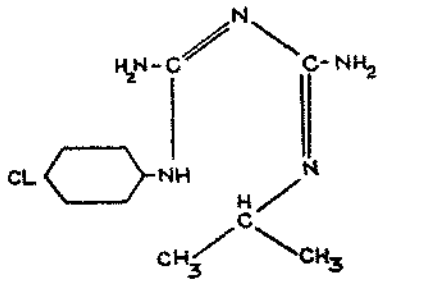
(*ii*) 2 : 4-diamino-6-alkyl-pyrimidines with 5-phenyl substituents were most active, followed by 5-benzyl and 5-phenoxy isomers, respectively.

(*iii*) Alkyl groups at 6-position enhanced antimalarial activity. In the case of 2 : 4-diamino-5-aryl-6-alkyl-pyrimidines, peak activity was encountered in the case of 6-ethyl isomers while lengthening of the 6-alkyl chain adversely affected the activity.

(*iv*) Halogen and nitro groups at para position of the 5-phenyl ring yielded most active compounds while similar substituents in meta or para position as well as other substituents in different positions, resulted in considerable loss of activity.

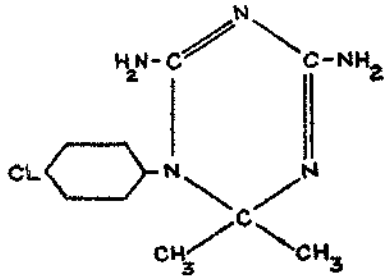
It was evident that pyrimethamine was the most outstanding compound of the series and hence, chosen for further extensive laboratory and field trials. Several 2 : 4-diamino pyrimidines comparable to those described above had been investigated previously by Curd, Richardson and Rose (1946) and Hull *et al.* (1946 : 1947), and in most of these cases, the two amino groups were substituted

and a basic alkyl-amino side chain was also considered pre-requisite for activity. This is evidently quite contrary to the essentials for activity in the present series of 2 : 4-diamino pyrimidines (II). Most of the former types of compounds were poorly active and it is quite likely that their mode of action is different from those of the 2 : 4-diaminopyrimidines investigated by Hitchings and co-workers (Greenberg and Richeson, 1950).



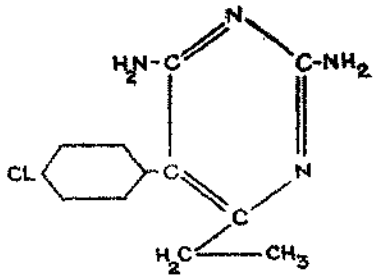
PROGUANIL (I)

N^1-*p*-CHLOROPHENYL-N^5-ISOPROPYL BIGUANIDE



ACTIVE PROGUANIL METABOLITE (III)

1-*p*-CHLOROPHENYL-2:4-DIAMINO-6:6-DIMETHYL-1:6-DIHYDRO-1:3:5-TRIAZINE



PYRIMETHAMINE (II)

5-*p*-CHLOROPHENYL-2:4-DIAMINO-6-ETHYL-PYRIMIDINE

Structural similarity between proguanil and 2 : 4-diamino-pyrimidines like pyrimethamine can be demonstrated by writing the linear compound (proguanil) as a ring. Moreover, Carrington *et al.* (1951) and Crowther and Levi (1953) have been able to isolate an active metabolite of proguanil *viz.*, 1-p-chlorophenyl-2 : 4-diamino-6 : 6-dimethyl-1 : 6-dihydro-1 : 3-5-triazine (III) which bears a strong resemblance to pyrimethamine and in fact can be considered as a structural hybrid between proguanil (I) and pyrimethamine (II). It indicates further that although proguanil and pyrimethamine have developed through seemingly different routes, their similar mode of action can be attributed to their structural similarities. These ideas have received further support from the studies on asymmetrical triazines, closely allied to dihydrotriazine and pyrimethamine, which are antagonists of folic

and folinic acids as well as displaying rather high antimalarial activity (Hitchings, 1952).

A brief account of the similar biological responses which may have resulted by virtue of structural similarities between pyrimethamine and proguanil, is given limited below :—

(1) Pyrimethamine and proguanil are slow acting schizonticides (suppressives) producing clinical cures. They may also serve as causal prophylactics having limited curative properties against human malarias.

(2) Flat dosage response curve in the case of pyrimethamine and proguanil limits their usefulness (Schmidt and Genther, 1953; Goodwin, 1952), because even if a very small dose is capable of reducing the parasitæmia, the dose required to completely remove the parasites from the blood may proportionately be very large. The effective dose regime in the case of pyrimethamine also showed wide variations, i.e. if a smaller dose is capable of reducing the parasitæmia in some, in others a comparatively higher dosage may be necessary to obtain the same results.

(3) Like proguanil, it has also been observed that repeated dosage of pyrimethamine will be required in order to achieve the desired therapeutic effects. This is a distinct disadvantage when compared to clinical suppression with 4-aminoquinoline group of drugs (Schmidt and Genther, 1953).

4. Although the gametocytes of *P. vivax* are equally affected by both these drugs, the sexual forms of *P. falciparum* are not destroyed by any of the two drugs.

(5) Pyrimethamine and proguanil have been known to interfere with the nuclear division of malaria parasites which can be explained on the basis that both these drugs are inhibitors of folic and folinic acid group of vitamins which are considered essential for neuclic acid synthesis. It is quite likely, therefore, that these two drugs may be acting through interference with the utilisation of these essential metabolites during growth and multiplication.

(6) Different strains of plasmodia acquired resistance to pyrimethamine with the same ease as in the case of proguanil. It has also been observed that a strain of plasmodia resistant to one of these drugs, may also be cross resistant to the other (Jaswant Singh *et al.*, 1952 : 1953). Like proguanil, pyrimethamine is also not equally affective against various geographical strains of human plasmodia.

(7) Taylor *et al.* (1952) have shown that pyrimethamine is inactive against dividing forms of *P. gallinaceum in vitro* which means that like proguanil, perhaps pyrimethamine is also converted into an active metabolite prior to its action. Recent studies by Goodwin (1952) and Schmidt, Hughes and Schmidt (1953) have also indicated a similar possibility, although no definite active degradative product has been isolated.

(8) Comparative studies on the tissue distribution of pyrimethamine and proguanil have shown that at a given dose, tissue concentrations of the former drug are only slightly greater than those with the latter. Compared to chloroquine, the above two drugs are only moderately localized in tissues like lung, liver, kidney and spleen (Schmidt, Hughes and Schmidt, 1953).

In spite of the similarities of biological actions discussed above, toxic characteristics of pyrimethamine are wholly different from those of proguanil. Pyrimethamine is comparatively more toxic than proguanil in man and monkeys but in rats it is only half as toxic. Lower dosage of pyrimethamine when compared to that of proguanil, however, makes it safe enough, although according to some its toxicity deserves a serious consideration (Schmidt, Hughes and Schmidt, 1953). To sum up it can be said that the mode of action of pyrimethamine is qualitatively very similar, if not exactly identical, to that of proguanil and these two drugs stand as a class by themselves in contrast to drugs like chloroquine, camoquin, etc.

Considering that laboratory and field evaluation of pyrimethamine was receiving active attention of the workers at the Malaria Institute of India, Delhi, it was thought worthwhile to commence studies on the concentrations achieved in the blood subsequent to oral administration of pyrimethamine. For the present, the work has been started in monkeys.

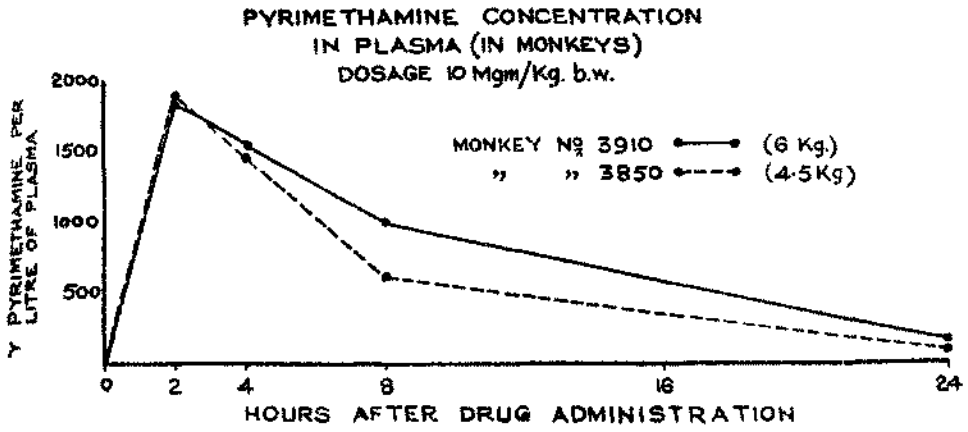
"Methyl orange" method of Brodie *et al.* (1947) as applied to estimation of amino-heterocyclic compounds was considered to be a suitable technique and the experimental details followed were more or less similar to those described by Schmidt, Hughes and Schmidt (*loc. cit.*). According to this method, pyrimethamine as well as its closely related degradation products which have power to combine with methyl orange, got estimated jointly and hence "pyrimethamine concentration" in the present case did not necessarily mean the presence of pyrimethamine alone. This method appeared to be sensitive enough but considerable care has to be excercised at every extraction in order to avoid loss, contamination and subsequent errors in results. After standardisation of the method, the following typical procedure was adopted for analysis :—

Two normal *rhesus* monkeys (Numbers 3910 and 3850) weighing 4·5 kg. and 6·0 kg. respectively were given 10 mg./kg. b.w. dose of pyrimethamine in aqueous suspension orally by means of a stomach tube. Blood samples were drawn from the cephalic vein 2, 4, 8 and 24 hours after drug administration, immediately centrifuged and plasma preserved in cold storage. Blood samples were also drawn from the monkeys, prior to drug administration, in order to obtain black plasma samples.

A two ml. aliquot of each plasma sample including blank, was taken into 50 ml. glass-stoppered bottles. To each of these bottles was added one ml. of 0·1N sodium hydroxide and eight ml. of ethylenedichloride which had been previously treated with charcoal and alkali and then thoroughly washed with water. The mixtures were shaken mechanically for half an hour and centrifuged at 3,000 to 4,000 r.p.m. for 10 to 15 minutes. The aqueous layers were removed by aspiration and four drops of iso-butanol added to each of the tubes in order to reduce the adsorption of drug on the glass surface. Ethylenedichloride extracts were returned to fresh 50 ml. bottles and 8 ml. of alcholic alkali (200 ml. of alcohol added to 800 ml. of 0·1 N sodium hydroxide) was added to each of them. The resulting mixtures were shaken, centrifuged and the aqueous layer removed by aspiration. The organic phase obtained in each case was again transferred to 50 ml. bottles and 1·5 ml. of methyl orange solution (Brodie and Udenfriend, 1945) added to each of them. The mixtures at this stage were shaken and centrifuged as described above.

The aqueous methyl orange layer in each case was completely removed by aspiration with capillary pipette and care was taken not to disperse the aqueous phase in ethylene-dichloride. From each tube, 2 ml. aliquot of organic phase was taken into a micro-colorimeter tube containing 0·2 ml. of acidic ethanol (2 ml. concentrated hydrochloric acid in 100 ml. of ethanol). The contents of colorimeter tube were well mixed and the color reading taken in a klett-summerson photoelectric colorimeter fitted with filter Number 54 and adjusted to zero with blank plasma sample, passed through the procedure outlined above. The actual "pyrimethamine" concentrations were read from the reference graph which was obtained by readings of known quantities of pyrimethamine subject to the above analytical procedure.

GRAPH 1.



From Graph 1, it will be evident that a peak concentration of pyrimethamine is reached at least two hours after the drug administration and that concentration rapidly falls by the end of 24 hours. These results are in conformity with those reported by Schmidt, Hughes and Schmidt (*loc. cit.*) and it appears that the drug does not accumulate in the plasma to any appreciable extent.

Author's thanks are due to Mr. Chanan Singh for his valuable assistance in the present studies.

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THE EFFECT OF VITAMIN B_{12} ON THE TOXICITY OF PYRIMETHAMINE (DARAPRIM) IN THE MONKEY.*

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(November 21, 1953.)

THE metabolic inter-relationships of the anti-megaloblastic hæmopoietic substances—folic acid, vitamin B_{12} and citrovorum factor (C.F.)—are complex and little understood (Girdwood, 1952). Hitchings (1952) discovered that pyrimethamine behaves as an antagonist to folic acid and C.F. in the growth of *Lactobacillus casei*. He also found that liver extract was superior to folic acid and C.F. in alleviating the toxic effects of pyrimethamine in the rat. In the present study, the effect of vitamin B_{12} on the toxicity of pyrimethamine was investigated in young *rhesus* monkeys. The peripheral blood and bone marrow pictures and the period of survival of the animals were used as criteria.

Pyrimethamine was given daily by stomach tube to every monkey in the dose of 10 mg. per kg. of initial body weight. Six animals were given the drug alone and served as controls while another five received in addition to the drug, vitamin B_{12} by the intramuscular route in doses ranging from 50 to 200 μg. All animals were fed on an adequate stock diet. The untreated control animals showed a progressive loss of weight and granulocytopenia and died between the 12th and 20th days. Their bone marrow showed a maturation arrest of the myelocytes with the appearance of large numbers of abnormal giant myelocytes. There was, however, no anæmia and no megaloblastic transformation of the marrow. In the group receiving vitamin B_{12}, neither the survival period nor the blood and bone marrow pictures were influenced favourably by treatment with vitamin B_{12}. The results suggest that the protective influence of liver extract is not due to its vitamin B_{12} content. They are in general agreement with our earlier experience in rats in which it was found that vitamin B_{12} exerts no beneficial influence

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

on the toxic effects of another anti-metabolic of the folic acid—C.F. system—4-aminopteroylglutamic acid ('Aminopterin') (Ramalingaswami and Sinclair, 1951).

These observations which are of a preliminary nature are being extended using larger doses of vitamin B_{12} and will be described in detail in a future communication.

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A BRIEF SUMMARY OF TRIALS WITH PYRIMETHAMINE (DARAPRIM) PREVIOUSLY UNDERTAKEN AT THE MALARIA INSTITUTE OF INDIA.*

BY

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[November 21, 1953.]

During laboratory investigations on pyrimethamine which had been commenced at the Malaria Institute of India as early as August, 1951, it was observed that the drug was four times more effective than proguanil against *P. knowlesi* (Jaswant Singh *et al.*, 1951) and sixty-six times more active than proguanil against *P. gallinaceum* in chicks (Jaswant Singh, Chandrasekhar *et al.*, 1953). Against P. *berghei*, Class II and III effects were obtained in very minute doses (Jaswant Singh, Krishnaswami, *et al.*, 1952).

The drug also proved effective against the sporogony cycle of *P. gallinaceum* in *Ædes ægypti* (Jawant Singh, Narayandas and Ray, 1953 ; Jaswant Singh, Misra, Sen Gupta *et al.*, 1953). In human malaria (*P. vivax* and *P. falciparum*), clinical cure was attained in majority of the cases within 72 hours in as small a dose as of 25 to 50 mg. (total). However, a few cases (*P. vivax* 1 out of 60 ; and *P. falciparum* 2 out of 80) failed to respond to treatment. Further, in 25 to 50 mg. dosage, pyrimethamine was found to be an effective suppressant. These studies were undertaken in a malarious area in Uttar Pradesh Terai (Jaswant Singh, Ray, Basu and Misra, 1952 : 1953 ; Jaswant Singh, Ray, Misra and Basu, 1952 ; Jaswant Singh, Misra and Ray, 1953).

Although no kind of toxic manifestations were encountered during trials against human malaria, acute and chronic toxicity tests in *rhesus* monkeys showed that in doses higher than therapeutic level, the drug is capable of producing various types of lesions (Jaswant Singh, Ray, Misra and Basu, 1953).

The author, thereafter, gave a summary of present studies on (*a*) Field investigation to assess antirelapse properties of pyrimethamine *vis-a-vis* primaquine, and (*b*) Laboratory and field observations on the synergistic action of quinine and pyrimethamine.

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

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Indian Journal of Malariology, 7, 4, *December* 1953.

PRELIMINARY REPORT ON THE ANTIRELAPSE PROPERTIES OF PYRIMETHAMINE (DARAPRIM) AND PRIMAQUINE.*

BY

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(November 21, 1953.)

NINETY-FIVE cases of *P. vivax* were treated with these antirelapse drugs in a few villages near Shivpuri (Madhya Bharat) where a field station was established by the Malaria Institute of India in the autumn of 1953, at a time when malaria morbidity rate was high. At the time when treatment was begun, the villages were heavily sprayed with different insecticides, some villages with D.D.T., others with D.D.T. and B.H.C., while some others with dieldrin to intercept any further transmission.

Forty-nine of these cases were treated with a combined dose of quinine-daraprim tablets (a preparation of Wellcome Laboratories) as initial treatment during acute attack. For adults, two tablets were given at 12 hourly interval up to 3 such doses. Each tablet contained 0·3 gm. of quinine hydrochloride and 5 mg. of pyrimethamine. Proportionate doses were given to children.

Subsequently the treatment was followed by a weekly dose of 25 mg. (for an adult) of pyrimethamine administered for eight weeks.

*This paper was read at the " SYMPOSIUM ON PYRIMETHAMINE " held on November 21, 1953, at G R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

Forty-six cases of *P. vivax* were treated with primaquine. For adults, the treatment regime consisted of 7·5 mg. b.d. for a period of five days only.

Besides these, another series of 61 cases were treated with quinine-daraprim regime in the Police and the local Jail Hospitals in Delhi.

Clinical response and clearance of asexual parasites from peripheral circulation were attained in all cases, though such response was somewhat slower in respect of series treated with primaquine alone. No toxic manifestation of any kind was encountered in any of the series. Up to the end of December, 1953, not one out of the total of 156 cases, relapsed. Further observations are being continued and detailed report would be available on completion of follow-up for at least six months.

Indian Journal of Malariology, **7**, 4, *December* 1953.

SCREENING OF ANTIMALARIALS AGAINST *P. GALLINACEUM** IN CHICKS.

Part III.† Synergistic action of pyrimethamine and quinine.

BY

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AND

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(November 21, 1953.)

GREENBERG *et al.* (1948) observed the efficacy of a combined treatment regime with proguanil and sulphadiazine against *P. gallinaceum* in chicks. The dose of proguanil was reduced to one-quarter of the effective dose, while that of sulphadiazine was brought down to a dose ranging from $\frac{1}{32}$ to $\frac{1}{64}$th of the effective dose. This showed that proguanil and sulphadiazine potentiate each other's action against this plasmodium.

Synergistic action between pyrimethamine and quinine, against *P. gallinaceum* in chicks, as seen in the present studies, is recorded in this paper.

MATERIALS AND METHODS.

One hundred and forty-two 7-day old laboratory hatched white leghorn and Rhode island red chicks were utilized for these studies.

*Summary of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

†This work was carried out under the scheme "Screening of antimalarial drugs" which is financed by the Council of Scientific and Industrial Research. Parts I and II of this series have appeared in *Indian Journal of Malariology*.

‡*Appointed on the staff of the Screening of Antimalarial Drugs Scheme, Council of Scientific and Industrial Research at the Malaria Institute of India.*

The dose of infective inoculum, route of inoculation, method of drug administration etc., were similar to those reported earlier by Jaswant Singh, Basu and Ray (1952). The dosage regime was considered active when the fourth day parasite count was 25 per cent or less as compared to that of the untreated group.

The dosages adopted were in fractions of the minimum effective dose (M.E.D.) of pyrimethamine and quinine which had been previously determined to be 0·0015 mg./50 mg. (Jaswant Singh, Ray and Chandrasekhar, 1953) and 1·6 mg./50 mg. (Jaswant Singh, Basu and Ray, 1952), respectively. The doses ranged from $\frac{1}{2}$ to 1/128th M.E.D. of pyrimethamine and quinine in various combinations similar to those shown in Tables I, II and III. For convenience, the M.E.D. of pyrimethamine has been represented as '*a*' and that of quinine as '*b*'.

RESULTS.

The investigations were carried out in the three stages. During the first stage the combined dosage schedule ranged from $\frac{a}{2} + \frac{b}{2}$ to $\frac{a}{16} + \frac{b}{16}$.

Table I.

Pyrimethamine and quinine combined dosage schedules.

Schedule number.	Dose of.		Number of chicks.	Average fourth day parasite count per 10,000.	Remarks.
	Pyrimethamine.	Quinine.			
	a=M.E.D.	*b*=M.E.D.			
I	$\frac{a}{2}$	$\frac{b}{2}$	3	0·3	Active
II	$\frac{a}{2}$	$\frac{b}{4}$	3	1·3	,,
III	$\frac{a}{2}$	$\frac{b}{8}$	3	0	,,
IV	$\frac{a}{2}$	$\frac{b}{16}$	4	1	,,
V	$\frac{a}{4}$	$\frac{b}{2}$	3	0	,,
VI	$\frac{a}{4}$	$\frac{b}{4}$	3	0	,,
VII	$\frac{a}{4}$	$\frac{b}{8}$	4	0	,,

TABLE I—(*Concld.*)

Schedule number.	Dose of		Number of chicks.	Average fourth day parasite count per 10,000.	Remarks.
	Pyrimethamine.	Quinine.			
	a=M.E.D.	*b*=M.E.D.			
VIII	$\frac{a}{4}$	$\frac{b}{16}$	4	2	,,
IX	$\frac{a}{8}$	$\frac{b}{2}$	3	0	,,
X	$\frac{a}{8}$	$\frac{b}{4}$	2	0	,,
XI	$\frac{a}{8}$	$\frac{b}{8}$	3	0	,,
XII	$\frac{a}{8}$	$\frac{b}{16}$	4	0.5	,,
XIII	$\frac{a}{16}$	$\frac{b}{2}$	3	0	,,
XIV	$\frac{a}{16}$	$\frac{b}{4}$	2	0	,,
XV	$\frac{a}{16}$	$\frac{b}{8}$	3	1	,,
XVI	$\frac{a}{16}$	$\frac{b}{16}$	5	2.6	,,
Comparison group			7	3,857	

From Table I above, it may be noted that while average fourth day parasitæmia in the comparison group was 3,857 per 10,000 erythrocytes, the highest average count (under Schedule XVI) was 2·6 per 10,000.

In the second stage, combination of both drugs in doses ranging from $\frac{a}{32}+\frac{b}{16}$ to $\frac{a}{128}+\frac{b}{128}$ were made and chicks were treated similarly (Table II).

TABLE II.

Pyrimethamine and quinine combined dosage schedules.

Schedule number.	Dose of		Number of chicks.	Average fourth day parasite count per 10,000.	Remarks.
	Pyrimethamine.	Quinine.			
	a = M.E.D.	b = M.E.D.			
I	$\frac{a}{32}$	$\frac{b}{16}$	3	0·5	Active
II	$\frac{a}{32}$	$\frac{b}{32}$	3	2·5	,,
III	$\frac{a}{32}$	$\frac{b}{64}$	3	5·3	,,
IV	$\frac{a}{32}$	$\frac{b}{128}$	3	3,500	Inactive.
V	$\frac{a}{64}$	$\frac{b}{16}$	3	8·0	Active.
VI	$\frac{a}{64}$	$\frac{b}{32}$	3	6·6	,,
VII	$\frac{a}{64}$	$\frac{b}{64}$	3	26	,,
VIII	$\frac{a}{64}$	$\frac{b}{128}$	3	4,500	Inactive.
IX	$\frac{a}{128}$	$\frac{b}{16}$	3	3,830	,,
X	$\frac{a}{128}$	$\frac{b}{32}$	3	4,000	,,
XI	$\frac{a}{128}$	$\frac{b}{64}$	3	3,900	,,
XII	$\frac{a}{128}$	$\frac{b}{128}$	3	5,300	,,
Comparison Group			7	4,550	

These results clearly show that activity of the combinations was retained even when the doses were reduced to $\frac{1}{64}$ of the M.E.D. of both. But when

further reduction in the doses of either pyrimethamine or quinine was made, the regime failed to be active. As activity was retained in combinations of $\frac{a}{32}$ or $\frac{a}{64}$ with quinine in 1/16 M.E.D., any increase in the dose of quinine was not considered necessary.

Therefore, in the third stage the dosage schedules of pyrimethamine were fixed at 1/128th of M.E.D. and the dose of quinine was varied at higher range. Similarly, the dose of quinine was fixed in other schedules as 1/128th of the M.E.D. (the lowest of the series) and the dose of pyrimethamine was varied accordingly as shown in Table III.

TABLE III.

Pyrimethamine and quinine combined dosage schedules.

Schedule number.	Dose of.		Number of chicks.	Average fourth day parasite count per 10,000.	Remarks.
	Pyrimethamine.	Quinine.			
	a=M.E.D.	b=M.E.D.			
I	$\frac{a}{128}$	$\frac{b}{2}$	5	42	Active.
II	$\frac{a}{128}$	$\frac{b}{4}$	5	269·2	,,
III	$\frac{a}{128}$	*$\frac{b}{8}$	5	3,025	Inactive.
IV	$\frac{a}{2}$	$\frac{b}{128}$	5	2,636·2	,,
V	$\frac{a}{4}$	$\frac{b}{128}$	5	2,750	,,
VI	$\frac{a}{8}$	$\frac{b}{128}$	5	2,400	,,
VII	$\frac{a}{16}$	$\frac{b}{128}$	5	2,520	,,
Comparison Group			5	3,636	

*For combination regime $\frac{a}{128} + \frac{b}{16}$, *See* Table II.

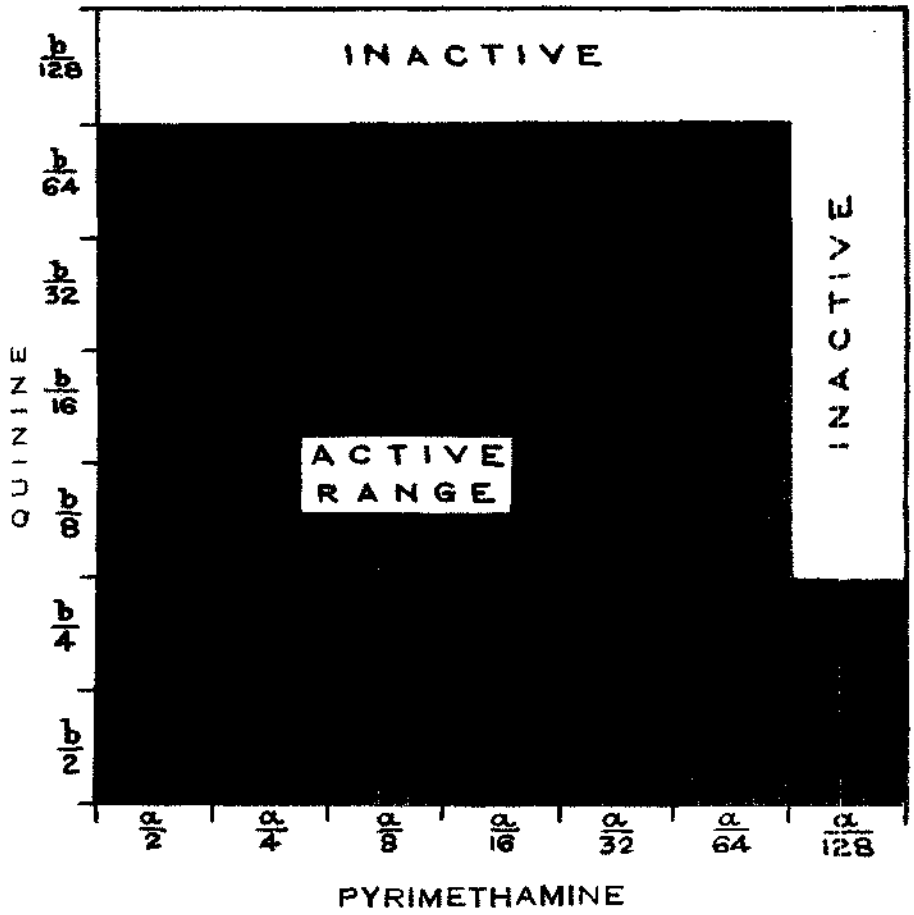
From the above Table, it may be observed that activity was retained even when the M.E.D. of pyrimethamine was reduced to 1/128th and that of quinine to 1/4th the M.E.D. But on the other hand when the dose of quinine was brought

down to 1/128th of the M.E.D., and administered concurrently with pyrimethamine in as high a dose as 1/2 M.E.D., the regime proved ineffective.

The active ranges under the combined schedules have been shown as per Chart I.

CHART 1

SHOWING THE ACTIVE RANGES UNDER THE COMBINED REGIMES IN VARYING DOSES OF PYRIMETHAMINE AND QUININE



a = M.E.D. (0·0015 mg/50gms) OF PYRIMETHAMINE

b = M.E.D. (1·6 mg/50gms) OF QUININE

DISCUSSION.

Walker and Richardson (1948) showed that when pamaquine was administered along with one of the naphthoquinones (hydrolapachol), the dose of each could be reduced to 1/10th of the effective level without interference with activity. Similar observations were made by Greenberg *et al.* (1948) in respect of proguanil and sulphadiazine as mentioned earlier. During the current studies it may be noted from the chart that although the minimum effective doses of pyrimethamine and quinine are 0·0015 mg./50 gm. and 1·6 mg./50 gm. respectively, when both the drugs were administered concurrently, a reduction up to 1/64th the M.E.D. could be effected without reducing the effectiveness of the regimes. But in the regimes where further reduction was made in the dose of quinine, they proved ineffective irrespective of the dose of pyrimethamine. On the other hand when the dose of pyrimethamine was reduced to as low as 1/128th of the M.E.D. and that of quinine reduced up to 1/4 of M.E.D., the effectiveness of the regimes was retained. Thus it is obvious that both pyrimethamine and quinine potentiate the action of each other in that a combined regime of 1/64th of M.E.D. of each is effective. But the synergistic action of quinine failed when it was reduced to 1/128th the M.E.D., whereas in respect of pyrimethamine it was still retained in dosage of 1/128 the M.E.D. and 1/4 the M.E.D. of quinine. Thus it would appear that even when a very minute dose of pyrimethamine is combined with a sub-effective dose of quinine up to 1/4 M.E.D., the regime is still active. Similar synergistic action in respect of both these drugs against *P. falciparum* has recently been reported by Jaswant Singh, Ray *et al.* (1953). These findings are of great interest and open up newer lines of study in the field of chemotherapy.

SUMMARY.

One hundred and forty-two chicks were employed to determine synergistic action of pyrimethamine and quinine.

It was observed that when these two drugs were administered concurrently, the dose of each could be reduced to 1/64th of the M.E.D., without interference of activity; but further reduction in both proved inactive.

On the other hand, the regime was found to be still active when the dose of pyrimethamine was reduced to 1/128th of the M.E.D. and that of quinine to 1/4 the M.E.D.

That pyrimethamine and quinine potentiate the activity of each other, is amply proved.

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SYNERGISTIC ACTION OF QUININE AND PYRIMETHAMINE IN *P. FALCIPARUM* INFECTION.*

BY

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SINTON *et al.* (1930) observed that "The combination of quinine with plasmoquine seemed to be more effective in the production of both clinical and radical cures of the disease than plasmoquine alone". Further, the toxicity of pamaquin was lowered under this combined regime. These results were corroborated by a number of workers (Manifold, 1931 ; Jarvis, 1932 ; Spicknall and Terry, 1948 ; Ruhe *et al.*, 1949). Similar results were shown when isopentaquin or pentaquin was administered concurrently with quinine (Alving, 1948). Further, Baranger and Filter (1948) recorded that certain combinations of the four main alkaloids of cinchona in pairs gave more favourable results than quinine, and that there appeared to be a synergistic action between cinchonidine and quinine.

Similar synergistic action was shown by Greenberg *et al.* (1948) who reported that proguanil and sulphadiazine potentiate each other's action against sporozoite-induced *P. gallinaceum* infection in chicks, in that when the effective dose of proguanil was reduced to one-quarter and combined with 1/32 to 1/64th of the effective dose of sulphadiazine, protection was afforded to chicks equally well.

Recently Ray, Misra *et al.* (1953) have observed that in *P. gallinaceum* infection in chicks, a combined regime of quinine and daraprim in doses equal to fractions of their M.E.D. was as effective as the M.E.D. of either of the compounds.

*Summary of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

The present report records the findings of a combined regime of quinine and pyrimethamine administered in varying doses against *P. falciparum* infection in man.

Thirty cases of *P. falciparum* were treated at a field station at Shivpuri, Madhya Bharat, with a product of Wellcome Laboratories known as quinine-daraprim, each tablet containing 5 mg. of pyrimethamine (daraprim) and 0·3 mg. of quinine hydrochloride. The regime adopted consisted of three doses of two tablets each every 12 hours which was similar to that reported by Ray, Pal *et al.* (1953) for the treatment of 110 *P. vivax* cases i.e., equivalent to a total dose of 30 mg. of daraprim and 27 grains of quinine hydrochloride (equivalent to 37 grains of quinine sulphate).

These results were compared to those obtained in other series treated separately with 37 grains of quinine sulphate or 30 mg. of daraprim alone administered in three divided doses. Some other series received treatment with both the drugs administered simultaneously but the doses of each varied in the different series. The various dosage regimes adopted for treatment of 91 cases are shown in Table I.

TABLE I.

Dosage regimes for treatment of 91 cases.

Regime	number	Dosage regimes of quinine salts.	Daraprim.
I	Combined	27 grain quinine hydrochloride (37 grain quinine sulphate)	30 mg.
II		37 grain quinine sulphate	...
III		...	30 mg.
IV	Combined	37 ,, ,, ,,	25 ,,
V	,,	37 ,, ,, ,,	20 ,,
VI	,,	37 ,, ,, ,,	15 ,,
VII	,,	37 ,, ,, ,,	10 ,,
VIII	,,	37 ,, ,, ,,	5 ,,
IX	,,	35 ,, ,, ,,	30 ,,
X	,,	30 ,, ,, ,,	30 ,,
XI	,,	25 ,, ,, ,,	30 ,,
XII	,,	20 ,, ,, ,,	30 ,,
XIII	,,	15 ,, ,, ,,	30 ,,
XIV	,,	10 ,, ,, ,,	30 ,,
XV	,,	5 ,, ,, ,,	30 ,,

CRITERION OF ACTIVITY.

The rate of clearance of asexual parasites was taken as the guidance. For this, at least 50 fields of the thick film were searched before recording a negative finding. The series in which clearance occurred in all cases within 72 hours, was considered as 100 per cent effective.

RESULTS.

The amounts of quinine and/or daraprim administered in each series have already been mentioned earlier (Table I). The rate of parasite clearance and the effectiveness or otherwise of the regimes under the different series are shown in Table II.

TABLE II.

Rate of parasite clearance.

Serial number.	Number of cases.	PARASITE CLEARANCE IN HOURS.					Remarks.
		24	48	72	96	120 or over.	
I	30	...	...	...	...	...	100 per cent clearance within 72 hours.
II	5	...	1	2	1	1	60 " " "
III	4	...	...	2	1	1	50 " " "
IV	4	1	2	1	...	...	100 " " "
V	4	...	2	2	...	...	100 " " "
VI	4	...	1	3	...	...	100 " " "
VII	6	...	2	3	1	...	83 " " "
VIII	4	...	1	2	...	1	75 " " "
IX	4	...	1	3	...	...	100 " " "
X	6	...	2	4	...	...	100 " " "
XI	4	...	1	2	1	...	75 " " "
XII	4	...	...	2	2	...	50 " " "
XIII	4	...	1	2	...	1	75 " " "
XIV	4	...	...	2	...	2	50 " " "
XV	4	...	...	2	1	1*	50 " " "

*Did not respond to treatment.

From these it would be clear that the best results were obtained under Regimes I, IV, V, VI, IX and X, under which parasite clearance occurred in

100 per cent of cases within 72 hours. The rate was somewhat slower under Regimes VII, VIII, XI and XIII and significantly so under Regimes II, III, XIV and XV.

DISCUSSION.

During their earlier studies, Jaswant Singh *et al.* (1952) observed that in a series of 80 *P. falciparum* cases treated with 25 mg. pyrimethamine administered on two consecutive days (50 mg. in 24 hours), the asexual parasite clearance rate was 74·2 to 100 per cent within 48 hours and 93·6 to 100 per cent within 72 hours. Further, two cases did not respond to the drug.

The rates of clearance within 72 hours during the current studies are 50 per cent under Regime III (30 mg. pyrimethamine), 60 per cent under Regime II (37 grain of quinine sulphate) and 100 per cent under Regime I (37 grain of quinine sulphate plus 30 mg. of pyrimethamine). Further in this series (Regime I), the clearance rate was 96·6 per cent within 48 hours. As such, it is evident that the results attained under Regime I are not only superior to those observed under Regimes II and III but they are even somewhat better than those treated with 30 mg. of pyrimethamine alone as mentioned earlier (Jaswant Singh *et al.*, 1952). Further it is noted that the rates of parasite clearance due to quinine and pyrimethamine administered alone and in certain combinations like Regimes XII to XV are more or less similar. In other words, dose of 30 mg. of daraprim alone gave about the same results as a similar dose of the same drug in combination with 5 to 20 grain of quinine.

Taking 100 per cent parasite clearance within 72 hours as the index of efficiency, it would be observed that out of the other series the best results were attained under Regime IV (37 grain quinine plus 25 mg. pyrimethamine), Regime V (37 grain quinine plus 20 mg. pyrimethamine), Regime VI (37 grain quinine plus 15 mg. pyrimethamine), Regime IX (35 grain quinine plus 30 mg. pyrimethamine) and Regime X (30 grain quinine plus 30 mg. pyrimethamine), and these are similar to that observed under Regime I (37 grain quinine plus 30 mg. pyrimethamine).

These findings may be presented as follows :—

37 Q plus 25 P
or
37 Q plus 20 P
or
37 Q plus 15 P
} ≐ 37 Q plus 30 P.

Taking the lowest dosage regime, it may be observed that results attained with 39 Q plus 15 P ≐ 37 Q plus 30 P.

Similarly

35 Q plus 30 P
or
30 Q plus 30 P
} ≐ 37 Q plus 30 P.

Taking the lower dosage regime it would seem that results with 30 Q plus 30 P ≐ 37 Q plus 30 P.

Q = Quinine P = Pyrimethamine.

Thus even when the dose of pyrimethamine was reduced by 50 per cent of the original dose as in Regime I, the efficacy of the combined regime (as in Regime VI) was retained. Similarly, a reduction by 25 per cent of the dose of quinine did not alter the efficiency.

These findings would lead one to believe that quinine and pyrimethamine potentiate each other's action in the doses described above, similar to that reported by Greenberg *et al.* (1948) in respect of proguanil and sulphadiazine, and by Walker and Richardson (1948) in respect of one of the drugs of the naphthoquinone series M. 2279 and pamaquine.

SUMMARY.

Out of ninety-one cases of *P. falciparum* infection, five were treated with 37 grain of quinine, four with 30 mg. of pyrimethamine, 30 cases with 37 grain of quinine along with 30 mg. pyrimethamine and the rest with varying doses of quinine and pyrimethamine adminstered concurrently.

In the series treated with either of the drugs alone, the parasite clearance rate was significantly slower than that observed with the series treated with both the drugs (and in the dosage) administered together.

But under the combined regimes it was observed that even after reduction in the dosage of either pyrimethamine (by 50 per cent) or quinine (by 25 per cent), the results were as good as the series receiving 37 grain of quinine and 30 mg. pyrimethamine. Thus the authors contend that quinine and pyrimethamine potentiate each other.

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EFFECT OF PYRIMETHAMINE IN THE SPOROGONY CYCLE OF *P. GALLINACEUM* BRUMPT, 1935.*

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DURING their earlier observations, Jaswant Singh *et al.* (1951) had observed that action of pyrimethamine against gametocytes of *P. knowlesi* was tardy. They subsequently reported that though the drug was somewhat effective against the sexual forms of *P. vivax*, it had little effect against the crescents (Jaswant Singh, Ray, Basu and Misra, 1952 ; Jaswant Singh, Ray, Misra and Basu, 1952 ; Jaswant Singh *et al.*, 1953). Similar observations against gametocytes in *P. vivax* and *P. falciparum* were made by Schneider *et al.* (1952) and McGregor and Smith (1952).

During the present study, the authors have made observations on the effects of pyrimethamine on the developmental phases of *P. gallinaceum* in *Ædes ægypti* after the fowls showing gametocytes were treated with various doses of this antimalarial.

MATERIALS AND METHODS.

White leghorn fowls weighing 2 to 3 lbs. obtained from the Government Poultry Farm, Delhi, were used in these experiments. Two fowls were used in each of the different dosage regimes.

*Abstract of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

The experimental fowls were inoculated intravenously with 50 million parasitized (*P. gallinaceum*) erythrocytes per kg. body weight. For gametocyte count, blood smears were stained with J.S.B. (Jaswant Singh and Bhattacharji, 1944).

Freshly hatched *Ædes ægypti* obtained from a colony maintained in these laboratories were starved overnight and then fed on fowls showing gametocytes. Fully fed mosquitoes were then kept in Barraud cages at a temperature of 80° to 85° F., with humidity ranging from 75 to 80 per cent. The technique adopted for feeding mosquitoes has been described earlier (Jaswant Singh, Basu and Ray, 1952).

Batches of mosquitoes were fed on fowls prior to drug administration for two consecutive days (referred to as -2 and -1 day in Table I). These served as comparison groups. Subsequently three batches of mosquitoes were fed at 4-hourly intervals on each fowl following drug administration (zero day) and thereafter one batch of mosquitoes was fed on each fowl everyday up to the sixth day. The regimes adopted were calculated in terms of 5, 7·5, 10, 25, and 50 mg. human equivalent doses, taking average weight of an adult as 70 kg.

Sample dissections of at least five mosquitoes from each batch were made from the fourth day following the infective feed and infections of gut and gland were noted. Normally dissections were carried out for a period of 14 days but in a few cases up to 20 days. When sporozoites were detected in any batch, two mosquito equivalent dose of sporozoites was inoculated in a clean fowl, to determine viability of sporozoites.

RESULTS.

Details of dissection of *Ædes ægypti* fed on fowls receiving different human equivalent doses of the compound are shown in Table I.

It will be observed that, in all series *Ædes ægypti* fed on infected fowls prior to drug administration (-2 and -1 day) showed infection of both gut and glands.

Infection of glands from four hours onwards after drug administration was observed in Series I to III, whereas in Series IV no sporozoites were detectable in mosquitoes fed eight hours after 25 mg. of pyrimethamine. Mosquitoes fed subsequently did show infection. But the rate was significantly lower than in those fed prior to drug administration. In Series V, no sporozoites were detectable in mosquitoes fed up to 24 hours after treatment, though lower rate of infection was observed thereafter.

In each series, whenever sporozoites were detected, two mosquito equivalent doses were inoculated in normal fowls. In all such cases, patent infection was established 7 to 12 days after.

DISCUSSION.

Foy and Kondi (1952) reported that pyrimethamine was effective in rendering gametocytes of *P. falciparum* non-infective to *A. gambiæ*. The dosage regime adopted was 20 mg. initially followed by 50 mg. on two consecutive days. Shute (1952) observed that in *P. vivax* infection, oöcysts developed in batches of *A. atroparvus* fed four hours after administration of 2·5 mg. pyrimethamine to a gametocyte carrier but numerically they were fewer in number as compared to the comparison

group. But those mosquitoes fed after 27, 48 and 72 hours after treatment, showed oöcysts and sporozoites comparable to the control series. The authors concluded that the effect was merely of a transient nature.

TABLE I.

Results of dissections of Ædes ægypti *fed on fowls with different dose regimes (equivalent to adult human doses).*

Dosage - human equivalent to fowls.

Time of Feeding.	Series 1. 5 mg.			Series 2. 7·5 mg.			Series 3. 10 mg.			Series 4. 25 mg.			Series 5. 50 mg.		
	Total mosquitoes dissected.	Oöcysts rate (per cent).	Sporozoites rate (per cent).	Total mosquitoes dissected.	Oöcysts rate (per cent.)	Sporozoites rate (per cent.)	Total mosquitoes dissected.	Oöcysts rate (per cent.)	Sporozoite rate (per cent.,	Total mosquitoes dissected.	Oöcysts rate (per cent.)	Sporozoite rate (per cent.)	Total mosquitoes dissected.	Oöcysts rate (per cent.)	Sporozoite rate (per cent.)
−2 days	75	12·0	50·3	21	19·0	—	87	10·3	49·4	14	21·4	71·4	42	11·9	66·6
1 day	85	14·0	60·0	77	10·4	61·0	53	9·4	53·0	35	2·8	51·4	34	14·6	44·1
0 day — 4 hours	74	8·1	54·0	44	—	27·4	36	22·2	58·3	37	—	5·4	25	—	—
0 day — 8 hours	66	10·6	39·4	46	6·5	21·7	36	16·6	61·1	42	—	—	28	—	—
0 day — 12 hours	48	10·6	39·8	46	—	30·4	36	8·3	47·2	42	2·4	4·7	27	—	—
1 day	28	10·7	39·3	8	—	25·0	56	7·1	51·8	37	—	13·5	25	—	—
2 day	48	29·1	41·6	6		16·6	53	9·4	62·2	42	—	38·0	23	13·0	30·4
3 day	71	12·6	53·5	8*	—	—	33	6·0	57·5	48	—	23·0	16	—	18·7
4 day	30	10·0	26·6	Not known			28‡	—	—	52	—	23·0	28‡	—	—
5 day	Fed mosquitoes died before dissection.						37‡	—	—	38		5·3	22‡	—	—
6 day	15	—	77·0				Not done			23		4·3	9‡		

*Gametocyte count nil.

‡Fowls were negative for both asexual and sexual parasites.

The results observed during the present series would indicate that in human equivalent doses of 5, 7·5 and 10 mg. dosage, the drug had no inhibiting effect on the growth of *P. gallinaceum* in Ædes. With 25 mg., inhibiting effect was observed for a brief period as no development was observed in *Ædes ægypti* fed eight hours after drug administration. Normal development occurred in batches fed four hours after drug administration. When the dose was increased to 50 mg. human equivalent dose, the inhibiting influence was more pronounced as none of the batches of mosquitoes fed 4, 8, 12 and 24 hours after treatment, showed infection

of gut or glands. But even in this dosage, the effect did not last beyond that as both gut and gland infections were observed in the batches fed 48 hours after drug administration. It would thus appear that inhibiting influence is transitory similar to that reported by Shute (1952).

These results are somewhat different to those observed in respect of proguanil where the effect was more prolonged as reported by Ramakrishnan *et al.* (1952).

But on the other hand when *Ædes ægypti* were allowed to imbide pyrimethamine or proguanil from drug soaked lint with 0·01 per cent concentration or above, before and after infective feed on fowls, there was complete inhibition of development of sporozoites though not of oöcysts (Jaswant Singh *et al.* (1953).

Thus it is apparent that both proguanil and pyrimethamine have some degree of inhibiting influence on the sporogony cycle of *P. gallinaceum* in *Ædes ægypti* whether the drug is administered to the vertebrate or invertebrate hosts. This similarity of their action may very well be due to their structural resemblance ; the only difference between the two being that the inhibitory effect of pyrimethamine, when administered in vertebrate host, is of more transient nature than in the case with proguanil. This would suggest that concentration of the drug in the blood is perhaps more rapidly lowered in case of pyrimethamine than proguanil.

SUMMARY.

1. Different dosage regimes (human equivalent) were tried to study the infectivity of gametocytes of *P. gallinaceum* after drug administration in fowls. The dosage regimes adopted ranged from 5 mg. to 50 mg. (human eqivalent) administered in single doses in fowls.

2. Mosquitoes fed on fowls with gametocytes, two days prior to and six days following drug administration, became positive for sporozoites, which were viable.

3. No infection in mosquitoes fed on fowls getting 50 mg. (human equivalent) was observed either on the day of drug administration or on the day following.

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PYRIMETHAMINE (DARAPRIM) IN MALARIA.*

BY

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(November 21, 1953.)

In the School of Tropical Medicine, pyrimethamine (daraprim) was tried last year on sixty-two patients with active malaria representing *P. falciparum*, *P. vivax* and *P. malariæ* infections (Chakravarty, and Chaudhuri, 1953). The drug was at first given in a single dose of 50 mg. but as this was ineffective in some cases, the dose was doubled and given on two consecutive days. This, however, did not appreciably improve the results. The author had also presented preliminary observations thereon at the Informal Meetings of the Malaria Advisory Committee last year, and mentioned that further increase of dosage was apt to produce untoward reactions.

The immediate effect of the drug on the temperature and parasites was fairly good in a large proportion of cases but slower than that of chloroquine or camoquin. Approximately 50 per cent of the cases were free from asexual parasites in two days and 86·6 per cent in three days' time. The remaining cases took a little longer time, *i.e.* four to five days, to get rid of the fever and parasites. One *P. falciparum* case did not respond at all to a single dose of pyrimethamine for six days, but when he was given camoquin in a single dose of 0·6 gm., it acted promptly.

The late effects of pyrimethaimne were, however, not satisfactory. In *P. falciparum* infection, early recrudescences (within one to two weeks) were too common ; out of 26 cases there were 10 failures, including the case just referred to in which there was no response.

In *P. vivax* cases, fever and parasitæmia were usually controlled within three days, and in contrast to the *P. falciparum* cases, there was only one case of early recrudescence out of 34 cases. It has not been possible to follow up all the cases, but four cases reported with relapse within four to twelve weeks of the treatment.

Regarding sexual parasites, the *vivax* and *malariæ* gametocytes were not seen after five days but crescents persisted.

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

Another 10 cases (seven *vivax* and three *falciparum*) have been treated this year with two doses of 50 mg. pyrimethamine with more or less similar immediate effect. No relapse occurred during the period of observation (average three weeks). It is interesting to note that two of the *P. falciparum* cases had prior to treatment with daraprim, failed to respond to paludrine, thus suggesting absence of cross-resistance.

Pyrimethamine in the prophylaxis of malaria was also tried. This was carried out in a malarious village, where both *falciparum* and *vivax* infections have been prevalent, the majority being *falciparum*. The drug was given in weekly doses of 25 mg. to the local school boys during the transmission period which lasted from July to November. More than half the boys, i.e., 172 received pyrimethamine and the remaining 139 received dummy tablets, serving as control. A preliminary estimate of spleen and parasite rates was made. Thereafter the blood of all the boys was examined once a month, and finally after the conclusion of the trial, both blood and spleen were again examined.

The result was marked reduction of the parasite rate in the daraprim group, i.e., from six in July to less than one in November, while in the control group the rate became almost double during the same period. It was noted that in all the parasite positive cases in the treated group, only *P. falciparum* was seen throughout the period. The suppressive effect of daraprim was also reflected on the spleen rate which showed a decrease of 30 per cent in daraprim group and an increase of 50 per cent in the control group. It was not possible to get accurate information about the incidence of malaria attacks but enquiries went to show that these were much less in those who received pyrimethamine.

In conclusion it may be said that :—

(*i*) Pyrimethamine like proguanil is slower in action than chloroquine and camoquin.

(*ii*) It is effective in *P. vivax* and *P. malariæ* infections for the termination of acute attack, but relapses may occur as with other antimalarials.

(*iii*) In *P. falciparum* infection, it is not a suitable form of treatment as early recrudescences are frequent and it may fail in a certain proportion of cases. This failure may not be due to proguanil resistance.

(*iv*) Pyrimethamine may be useful for suppressive therapy among semi-immune people in weekly doses of 25 mg.

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A NOTE ON FIELD TRIALS WITH PYRIMETHAMINE (DARAPRIM) AT ARONE (MADHYA BHARAT).*

BY

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(November 21, 1953.)

UNTIL some years ago, Arone, a village near Ghatiagaon Tehsil (25 miles from Gwalior) was quite a flourishing village with a population of 5,000. But during recent years, this area has had very high incidence of malaria and gradually the village has been depopulated and turned into ruins. At present there are only 700 people. The place is undulating and there are low-lying areas and a number of insanitary tanks in the vicinity.

The spleen rate at the time of commencement of the investigation during the autumn of 1953, was 54 per cent and parasite rate 32·6 per cent.

Thirty *P. vivax* cases were treated with a combined course of quinine-daraprim tablets. Two tablets were given every 12 hours up to three such doses. The total dose of quinine hydrochloride was 27 grain and that of pyrimethamine 30 mg. This was followed by a weekly dose of 25 mg. pyrimethamine for eight weeks.

At the initial phase of the operation, the village was sprayed heavily with D.D.T. to intercept any further transmission, and thus eliminating chances of superinfection.

In most cases, asexual parasite clearance occurred within 72 hours of commencement of treatment. Subsequently blood smears were being taken every 10 to 15 days for any evidence of a possible parasitic relapse. This would be continued up to the end of March, 1954. So far none of the cases developed either parasitic or clinical relapse during an observation period of about two and a half months.

ACKNOWLEDGMENT.

The author wishes to express his thanks to the Director, Malaria Institute of India, for giving an inspiration to conduct this investigation, and for the supply of antimalarials.

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

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A NOTE ON CLINICAL TRIAL OF PYRIMETHAMINE (DARAPRIM) AND AMODIAQUIN (CAMAQUIN) AT THE MALARIA FIELD STATION, THIRMALAPUR, NIZAMABAD DISTRICT.*

BY

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AND

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UNTIL a few years ago, it was possible to get a fair number of cases of malaria. *P. malariæ* infection was quite common. But lately, due to intensive malaria control programme, malaria morbidity rates are quite low.

However, a total of 24 cases of *P. malariæ* were treated ; 7 with pyrimethamine and 17 with amodiaquin. Besides, 2 cases of *P. vivax* and equal number of *P. falciparum* cases were also treated with pyrimethamine.

Cases treated with pyrimethamine received a single dose of 50 mg. while those under amodiaquin received 0·6 gm.

In *P. malariæ* infection treated with pyrimethamine, the asexual forms disappeared between two and four days. Action of the drug against the gametocytes was found to be tardy as clearance occurred between 11 and 22 days.

In *P. falciparum* and *P. vivax* infections (both forms), parasite clearance was attained between two and five days and four and five days respectively.

Out of the 17 cases of *P. malariæ* treated with amodiaquin, seven had asexual forms while 10 others showed both asexual forms and gametocytes. Complete parasite clearance occurred between two and four days.

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

From these it would appear that as in both series of *P. malariæ* cases treated with pyrimethamine or amodiaquin, asexual parasite clearance occurred in two to four days, the action of these two compounds against this species is somewhat similar, although against gametocytes, pyrimethamine showed comparatively slower action.

As the number of cases of *P. vivax* and *P. falciparum* are very small, no definite conclusion can be drawn regarding the action of pyrimethamine against these two species.

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DRUG FEVER, LEUCOCYTE COUNTS, ERYTHROCYTE SEDIMENTATION RATE AND URINE AFTER PYRIMETHAMINE (DARAPRIM).*

BY

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November 21, 1953.

THE present work was conducted with a view to finding the following after the administration of pyrimethamine : drug fever ; total leucocyte count ; differential count of leucocytes ; erythrocyte sedimentation rate ; and urine changes.

Ten persons were selected, out of which six were patients convalescing from different diseases in the wards, and four were healthy young volunteer doctors.

They were divided into two groups, first group was given one tablet of pyrimethamine of 25 mg. daily for three consecutive days ; and the second group was given three tablets of pyrimethamine of 25 mg. each in a single dose. The above-detailed investigations were carried out before the drug was given, and 24 hours after the last tablet in the first group and 48 hours after the three tablets in the second group. Their temperature was recorded every four hours during this period.

Results are tabulated in Table I

* This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

TABLE I.

Effect of pyrimethamine

First group—25 mg. each day, for 3 consecutive days (total 75 mg.)

Number.	Name.	Age in years Nationality, and sex.	Temperature		Leucocyte count (total per c. mm. and differential in per cent.)		E. S. R. in mm., 1st hour Wintrobe.		Urine Report		Reactions. after drug.
			Before drug.	After drug.	Before drug.	After drug.	Before drug.	After drug.	Before drug.	After drug.	
1.	T. K.	36, H. M.	N.	N.	T—5,000. P. 72. L. 28.	T—6,250. P. 75. L. 20. E. 5.	39.	36.	N. A. D.	N. A. D.	Nil
2.	R. B. J.	27, H. M.	N.	N.	T—6,000. P. 84. L. 16.	T—7,000. P. 68. L. 23. E. 9.	20.	18.	N. A. D.	N. A. D.	Nil
3.	S. S.	30, H. M.	N.	N.	T—5,200. P. 62. L. 32. M. 2. E. 2.	T—6,000. P. 60. L. 30. E. 4.	28.	25.	N. A. D.	N. A. D.	Nil
4.	H. N.	23, H. F.	N.	N.	T—8,000. P. 56. L. 38. M. 2. E. 6.	T—7,600. P. 60. L. 36. E. 4.	21.	18.	N. A. D.	N. A. D.	Nil
5.	R. N. S.	26, H. M.	N.	N.	T—6,800. P. 61. L. 21. M. 2. E. 2.	T—7,250. P. 67. L. 32. E. 1.	18.	16.	N. A. D.	N. A. D.	Nil
Second group—75. mg. in single dose.											
6.	K. S.	14, H. M.	N.	N.	T—12,450. P. 75. L. 13. M. 1. E. 11.	T—12,400. P. 68. L. 16. E. 16.	10.	12.	N. A. D.	N. A. D.	Intestinal colic, vomiting.
7.	D.	38, H. M.	N.	N.	T—10,100. P. 64. L. 29. M. 1. E. 6.	T—9,800. P. 67. L. 33.	18.	22.	N. A. D.	N. A. D.	Sinus bradycardia, increased frequency of micturition.
8.	S. D. T.	25, H. M.	N.	N.	T—9,300. P. 64. L. 36.	T—8,250 P.52.L.43.M.2.E.2.	7.	3.	N. A. D.	N. A. D.	Headache.
9.	B. S. M.	23, H. M.	N.	N.	T—8,300. P. 71. L. 22. M. 1.	T—8,950. P.64.L.30.M.2.E.2.	20.	17.	N. A. D.	N. A. D.	Diarrhoea and headache.
10.	B. L.	30, H. M.	N.	N.	T—8,300. P. 64. L. 36.	T—8,900. P. 64. L. 34. E. 2.	24.	29.	N. A. D.	N. A. D.	Nil

H. M.=Hindu male. H. F.=Hindu female. N.=normal. T.=total leucocytes. P.—polymorphonuclears. L.—lymphocytes. M.=monocytes. E.=eosinophils. N. A. D.=nothing abnormal detected. E. S. R.=Erythrocyte sedimentation rate.

RESULTS WITH DISCUSSION.

In none among them, drug fever occurred.

No significant change was observed in the total leucocyte count, or in the differential counts of polymorphonuclears, lymphocytes and monocytes.

In the differential count of eosinophils, some changes were noted. In seven persons (70 per cent), there was a rise varying from two to nine per cent, while in the remaining three persons (30 per cent) there was a fall varying from one to six per cent. No definite conclusion could be drawn from this observation.

Erythrocyte sedimentation rate showed practically no change.

No change, chemical or microscopical, was found in the urine.

McGregor and Smith (1952) reported that evidence of hæmatotoxicity and renal toxicity was sought but not found. Goodwin (1952) reported that hæmatological tests were negative in his trials of pyrimethamine in human volunteers.

Reactions.—In the first group of persons, who were given one tablet of pyrimethamine of 25 mg. on three consecutive days, there was no reaction.

In the second group of persons, who were administered three tablets of 25 mg. each in single dose, reactions occurred. Case Number 6, four hours after the drug, had severe intestinal colic followed by vomiting. Vomiting and intestinal colic lasted for six and twelve hours respectively, without any relief to the usual therapies for them. The authors are of the opinion that pyrimethamine possesses parasympathomimetic properties. The colic and vomiting were results of increased tone of the stomach, arising out of vagus stimulation. Case Number 7, 24 hours after the drug, developed marked bradycardia, pulse and cardiac rates being 38 per minute, and also increased frequency of micturition without polyuria. An E.C.G. was taken, which revealed sinus bradycardia. There was no evidence of past or present cardio-vascular disease. After about 10 days, his pulse and cardiac rates mounted up to 64 per minute, and the increased frequency of micturition was also gone. Repeated examination of his urine during this period revealed no abnormality. Sinus bradycardia and the increased frequency of micturition were most probably the results of the parasympathomimetic action of the drug, which increased the tonic inhibitory action of the vagus supplying the heart and stimulated the cholinergic fibres of the parasympathetic nerves which are motor to the wall of the bladder and relaxant to the sphincter. Case Number 8, two hours after the drug, developed marked headache, not responding to analgesics and lasting for nearly 72 hours. The headache could be explained by the parasympathomimetic action of the drug, producing congestion of one or more extra- or intracranial blood vessels. Case Number 9, two hours after the drug, had diarrhœa followed by headache. They cleared away after about 24 hours. These two symptoms could be explained similarly by the action of the drug consisting of its stimulation of the parasympathetic nerves. Diarrhœa was due to increased perstalsis and the headache as explained before.

SUMMARY.

Drug fever did not occur in any person among the 2 groups. No significant change was observed in the total and differential counts of leucocytes, nor in the E.S.R. and the urine.

75 mg. of pyrimethamine in single dose produced reactions, while there was no reaction when it was given in three divided doses of 25 mg. Whether the reactions were due to hypersensitivity to or to toxicity of the drug, the authors are unable to throw much light. But there were enough clinical data to lead the authors to conclude that pyrimethamine possesses parasympathomimetic properties. And the reactions could easily be explained by this property.

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Their thanks are due to Dr. K. S. Mathur, M.D.(Luck.), M.R.C.P.E., P.M.S., Reader in Medicine, for the E.C.G. findings of case Number 7.

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ACUTE MALARIA TREATED WITH PYRIMETHAMINE (DARAPRIM).*

BY

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(November 21, 1953.)

THE present work consists of the treatment of acute malaria with pyrimethamine which is 2 : 4-diaminopyrimidine, one of the latest in the family of the new synthetic antimalarial drugs.

Archibald (1951); Schneider *et al.* (1952); McGregor and Smith (1952); Goodwin (1952); Jaswant Singh, Ray, Basu and Misra (1952 : 1953); Jaswant Singh, Ray, Misra and Basu (1952); Wilson and Edeson (1953); Chakravarty and Chaudhuri (1953); Covell, Shute and Maryon (1953); and Cameron (1953) published their observations with regard to the effects of this drug in human malaria.

PRESENT WORK.

Thirty-one patients were treated. They were suffering from acute malaria. The cases were divided into three groups, first being treated with 2×25 mg., second with 3×25 mg., and the third with 4×25 mg. of the drug. The diagnosis in each case was established by the presence of malarial parasite in the peripheral blood. Each patient was given the drug as a single dose by one of the authors. Subsequently, the blood was examined every four hours to observe the clearance of the parasite. In no case, the parasite was declared to have disappeared unless three subsequent examinations proved to be so. A careful record was kept of their temperature and other symptoms.

Results are tabulated below.

*This paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

TABLE I.

First group, treated with 2 ×25 mg.

Serial number.	Name.	Age in years, nationality and sex.	Duration of fever in days before drug.	Type of parasite.	Disappearance of fever in hours after drug.	Clearance of parasite in hours after drug.	Reactions
1	R.S.	25, H.M.	1	*vivax*	24	18	*Nil*
2	D.L.	32, H.M.	1	*vivax*	24	12	*Nil*
3	R.S.	22, H.M.	3	*vivax*	48	22	*Nil*
4	R.C.	18, H.M.	1	*vivax*	72	48	*Nil*
5	M.	15, H.M.	3	*vivax*	16	12	*Nil*
6	R.	26, H.M.	1	*vivax*	24	24	*Nil*
7	M.	32, H.M.	8	*vivax*	72	48	*Nil*
8	G.L.	46, H.M.	14	*vivax*	24	24	*Nil*
9	Y.S.	26, H.M.	2	*falciparum*	20	12	*Nil*
10	R.S.	30, H.M.	1	*falciparum*	42	22	*Nil*
11	M.	42, H.M.	3	*falciparum*	72	48	*Nil*
12	F.L.	25, C.F.	2	*vivax*	130	72	*Nil*

H.M. = Hindu male. C.F. = Christian female.

RESULTS WITH DISCUSSION.

In this group of 12 cases, nine were suffering from acute *vivax* malaria and three from acute *falciparum* malaria.

Fifty per cent became afebrile within 24 hours, 66 per cent within 48 hours, and 91·6 per cent within 72 hours. These results were very similar to those obtained by Jaswant Singh *et al.* (1953).

In this group, case number 12 did not become afebrile up to 84 hours, although the parasites had disappeared from the peripheral blood within 72 hours. From the clinical point of view, the drug, in 50 mg. dosage, proved ineffective. So, two more tablets (50 mg.) were given, and she became afebrile within a total period of 130 hours.

In 66·6 per cent of the cases, the clearance of the parasite was obtained within 24 hours, in 91·6 per cent within 48 hours, and in 100 per cent within 72 hours. These results were in some aspects different from those of Jaswant Singh *et al.* (1953). Among their patients, who became afebrile within 24 hours, 64·3 per cent had

previous attacks of malaria during the course of a year or so, as compared to 45·7 per cent without any such previous history. Their 64·3 per cent compares almost equally with the authors' 66·6 per cent. This could be explained by speculating that the authors' 66·6 per cent cases had subclinical attacks of malaria within recent years, and thereby had acquired the legacy of partial immunity.

The clinical action of the drug was slow, as evidenced by the fact that in 83·3 per cent of cases fever and other symptoms persisted from 4 to 56 hours after the clearance of the parasites from the peripheral blood.

Jaswant Singh, Ray, Misra and Basu (1952) showed that two malignant tertian cases out of eighty, proved refractory to pyrimethamine while Chakravarty and Chaudhuri (1953) reported that one patient with malignant tertian malaria did not respond to a single dose of 50 mg. Three cases of acute *falciparum* malaria in the authors' present group responded as well as the cases of acute *vivax* malaria to a single dose of 50 mg.

TABLE II.

Second group treated with 3 × 25 mg. pyrimethamine.

Number.	Name.	Age in years, nationality and sex.	Duration of fever before drug. (days)	Type of parasite.	Disappearance of fever after drug. (hours).	Clearances of parasite after drug. (hours).	Reactions.
1	S.L.	23, H.M.	3	*vivax*	24	20	*Nil.*
2	S.S.	24, H.M.	1	*vivax*	10	29	*Nil.*
3	I.	30, H.M.	2	*falciparum*	12	12	*Nil.*
4	D.S.	17, H.M.	8	*falciparum*	58	56	*Nil.*
5	S.L.S.	35, H.M.	4	*vivax*	**	**	Paroxysmal tachycardia followed by auricular fibrillation.
6	S.	35, H.M.	11	*falciparum*	36	13	*Nil.*
7	G.	30, H.M.	3	*vivax*	16	20	Hiccough.
8	R.S.	30, H.M.	1	*falciparum*	48	22	*Nil.*
9	S.K.S.	19, H.M.	7	*vivax*	**	**	Severe præcordial pain.
10	S.R.	30, H.M.	1	*vivax*	60	12	*Nil.*
11	H.G.	50, H.M.	5	*vivax*	20	24	Hæmaturia.
12	B.S.	30, H.M.	2	*vivax*	**	20	*Nil.*
13	B.S.	16, H.M.	16	*vivax* and *falciparum*	20	12	Headache.

H.M.=Hindu male.

RESULTS WITH DISCUSSION.

In this group of 13 cases, 8 were suffering from acute *vivax* malaria, 4 from acute *falciparum* malaria, and 1 from mixed acute *vivax* and *falciparum* malaria.

A detailed study regarding the therapeutic efficacy of pyrimethamine could be made only in ten cases, owing to the occurrence of reactions in two cases (Numbers 5 and 9) for whom quinine had to be resorted to, and the superimposition of acute bronchitis in case Number 12, in whom the disappearance of fever could not be observed.

Sixty per cent cases became afebrile within 24 hours, 80 per cent within 48 hours, and 100 per cent within 60 hours. These results were superior to those obtained with 50 mg.

The clearance of the parasite was studied in 11 cases. In 90·9 per cent, the clearance was within 24 hours, and in 100 per cent within 56 hours. Therefore, the clearance with 75 mg. was earlier than with 50 mg.

The clinical action of the drug with 75 mg. was faster than with 50 mg., as evidenced by the fact that in 60 per cent, the fever and other symptoms disappeared either before or simultaneous with the clearance of the parasite. Thus, the lag in the interval of the disappearance of symptoms *vis-a-vis* the clearance of the parasite, was observed in 40 per cent as compared to 66·6 per cent with 50 mg. This observation is contradictory to the observation of Schneider *et al.* (1952) who made special mention of the fact that results were not improved by using more than 50 mg. in single dose.

With 75 mg., the drug was effective against 4 cases of acute *falciparum* malaria. The conclusion of Chakravarty and Chaudhuri (1953) that the drug cannot be regarded as a suitable treatment for malignant tertian malaria, could not be substantiated. In this connection, it would be valuable to take into consideration the fact that immunological factors play potent rôles in determining the therapeutic efficacy of the drug, as it is with other synthetic antimalarial drugs.

Reactions to the drug, were observed in this group. They are being discussed in a subsequent chapter.

TABLE III.

Third group, treated with 4 × 25 mg. pyrimethamine.

Number.	Name.	Age in years, nationality and sex.	Duration of fever before drug (days).	Type of parasite.	Disappearance of fever after drug (hours).	Clearance of parasite after drug (hours).	Reactions.
1	J.S.	40, H.M.	5	*vivax*	70	20	*Nil.*
2	A.H.	34, H.M.	3	*vivax* and *falciparum*	60	60	*Nil.*
3	M.A.	18, H.M.	4	*vivax*	36	18	*Nil.*
4	J.S.	22, H.M.	4	*vivax*	60	37	*Nil.*
5	P.	25, H.F.	2	*vivax*	64	26	Intestinal colic and palpitation.
6	K.K.	45, H.M.	5	*vivax*	36	12	*Nil.*

H.M.—Hindu male. H.F.—Hindu female.

RESULTS WITH DISCUSSION.

The number of cases studied in this group was small. Out of six cases, five were suffering from acute *vivax* malaria, and one from mixed acute *vivax* and *falciparum* malaria.

In none of them, the disappearance of fever occurred within 24 hours, 33·3 per cent became afebrile within 36 hours, and 100 per cent within 70 hours. The results were not satisfactory, when compared to those obtained with 75 and 50 mg.

The clearance of the parasite was obtained within 24 hours in 66·6 per cent cases, and within 60 hours in 100 per cent. The 24-hour clearance in this group had the same percentage as that in the first group (*i.e.*, with 50 mg.), and the 100 per cent clearance in this group had almost the same figure as that obtained with 75 mg.

The clinical action of the drug was definitely slow, as the lag of interval in the disappearance of symptoms *vis-a-vis* the clearance of the parasite was markedly prolonged.

It was concluded that the overall results with 100 mg. in single dose were in no way better, rather the clinical response was slower, than those obtained with 75 or 50 mg. Here the authors were in agreement with Schneider *et al.* (1952) who, as already pointed out, made special mention of the fact that results were not improved by using more than 50 mg. in single dose. But this agreement looks paradoxical when one reviews the authors' results with 75 mg., where there was improvement. In four cases (Numbers 1, 2, 4 and 5), after an initial fall of the temperature to normal, lasting from 12 to 20 hours, there was a second rise of temperature, when a very careful examination of the blood of patients did not show any parasite. This second rise increased the total length of the period of the disappearance of fever after 100 mg. of pyrimethamine. But for this second rise, the period of the disappearance of fever would have been short, and thus the results would have been superior. Could this second rise be due to ' drug fever ' ? The authors were unable to answer this. One could also argue that the cases (at least the four referred to above) did not possess partial immunity, and hence the clinical response was poor. In the absence of any definite data, that argument would only be within the canopy of speculation.

RELAPSE.

No comment can be made about relapses, since under existing conditions it is difficult, rather impossible here, to distinguish between relapse and reinfection.

REACTIONS.

References in the literature, with regard to reactions with this drug, even where the number of cases studied was large, are conspicuous by their lean figures. But in the authors' present series, reactions constitute a very important chapter.

In the first group, where the drug was given in a single dose of 50 mg., there was no reaction.

In the second group, where the drug was given as a single dose of 75 mg., reactions occurred in 38·4 per cent cases. And some of the reactions were of acute nature. Case number 5, two hours after the drug, developed paroxysmal tachycardia, which after 12 hours, was converted into auricular fibrillation. As he became seriously ill and the symptoms were persisting, 7·5 grain of quinine bihydrochloride was injected intramuscularly, and tablets of digoxin (each 0·25 mg.) were administered orally. Twelve hours later, by which time he had taken three tablets of digoxin, the auricular fibrillation disappeared. He made an uneventful recovery, though the convalescence was somewhat protracted. There was no evidence of past or present cardio-vascular disease, nor was there any history of a similar attack in the past. Case number 7, four hours after the drug, got hiccough, which proved completely refractory to the usual therapies for the same. But it gradually subsided after about 36 hours.

Case number 9, developed sudden, severe, praecordial pain, two hours after the drug, and this gradually subsided after 15 hours. Owing to the persistence of fever, quinine was given orally. He was discharged cured. His cardio-vascular system was normal, and he never had a similar attack in the past. The skiagram of his chest was normal.

Case number 11.—Four days after the drug, had frank hæmaturia, every sample of urine containing blood. The following investigations were done.—Urine—microscopic : red blood cells, leucocytes, no casts ; *coagulation time*—four minutes 45 seconds ; *bleeding time*—one minute 20 seconds ; *platelets*—1,56,000 per c.mm. ; *prothrombim concentration*—100 per cent ; and no radio-opaque calculus was visible in plain skiagrams of the urinary tract. He was treated with coagulants and vitamins with poor response. The hæmaturia continued for seven days, after which it stopped macroscopically, and five days later microscopically. There was no history of hæmaturia or any renal complaint in the past. Except its being due to the drug, no other cause could be held responsible. In this manifestation, it simulated proguanil, which is known to produce hæmaturia in some odd cases.

Case number 13.—Four hours after the drug, complained of severe headache, not responding to any analgesic. It lasted for about 72 hours.

In the third group, treated with a single dose of 100 mg., there was reaction in one case out of six (*i.e.*, in 16·6 per cent). The patient (number 5) had intestinal colic, vomiting, and palpitation. Among them, the first to occur was intestinal colic two hours after the drug, followed by vomiting. The colic lasted for nearly 24 hours, and the vomiting for about eight hours. The palpitation had started four hours after the drug, and lasted for about 12 hours.

PATHOGENESIS OF THE REACTIONS.

The authors are of the opinion that pyrimethamine possesses parasympathomimetic properties, and the immediate reactions were due to them. Whether these properties are due to hypersensitivity or toxicity, the authors are not in a position to answer.

The cardiac arrhythmias, in case number 5 of Group II, could be explained on the basis of the stimulation of the vagus nerve supplying the heart. During the first phase of stimulation, possibly there was what is known as ' vagus escape ' which was associated with high pressure in the great veins entering the right auricle. This stimulated the sensory fibres passing up the vagus to the cardiac centre reflexly to accelerate the heart (Bainbridge or auricular reflex) which was expressed clinically as paroxysmal tachycardia. After 12 hours, during the second phase, the stimulation of the vagus was more direct, and thereby auricular fibrillation was produced. Cooke and White (1940) reported the occurrence of auricular fibrillation by direct stimulation of the vagus by parasympathomimetic drugs. The explanation of the præcordial pain in case number 9 in Group II, is as follows. The coronary vessels receive constrictor fibres from the vagus. Pyrimethamine by virtue of its parasympathomimetic action, stimulated the vagus resulting in diminution of the coronary flow, which produced coronary insufficiency whose clinical expression was the præcordial pain. Hiccough, in case number 7 of Group II, could be due to vagal stimulation, the headache in case number 13 of Group II, was probably the result of congestion of one or more extra- or intracranial blood vessels due to vagal stimulation, and colic, vomiting and palpitation in case number 5 of Group III, were also expressions of vagal stimulation.

The late reaction of hæmaturia in case number 11 of Group II, could not possibly be due to vagal stimulation. The exact pathogenesis remains obscure, except to speculate that pyrimethamine had produced selective damage to some of the nephrons.

SUMMARY.

Pyrimethamine which is 2 : 4-diaminopyrimidine, one of the latest synthetic antimalarial drugs, had been given a clinical trial in 31 cases of acute malaria, 23 among whom were suffering from acute *vivax* malaria, seven from acute *falciparum* malaria, and one from mixed acute *vivax* and *falciparum* malaria.

The cases were divided into three groups, first being treated with 2×25 mg., second with 3×25 mg., and third with 4×25 mg. of the drug, given in a single dose.

The drug was effective in all the groups, but the clinical action of the drug was slow. There were no failures in any group in cases of acute *falciparum* and mixed acute *vivax* and *falciparum* malaria.

There were no reactions to the drug with 50 mg. But with 75 and 100 mg., reactions occurred. And some of them were dangerous.

The authors concluded that pyrimethamine possesses parasympathomimetic properties, and the immediate reactions could be explained by them.

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THERAPEUTIC TRIALS WITH PYRIMETHAMINE (DARAPRIM), RESOCHIN, AMODIAQUIN (CAMOQUIN) AND QUININE.*

BY

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(November 21, 1953.)

FORTY-EIGHT cases have been treated so far of which 26 were due to *P. falciparum* and 22 due to *P. vivax* infection. Quinine was used by parenteral route in six cases of subtertian infection which presented symptoms of unconsciousness and marked toxæmia, necessitating emergent treatment. The remaining 42 cases were treated with resochin, amodiaquin and pyrimethamine. The only criteria of selection was that almost equal number of cases, both of *vivax* and *falciparum* infection, be treated by each of the three drugs as under :—

Cases of P. vivax *and* P. falciparum *treated with four antimalarials.*

Antimalarial.	Total number of cases.	*P. vivax.*	*P. falciparum.*
Quinine	6	...	6
Resochin	14	7	7
Amodiaquin	13	7	6
Pyrimethamine...	15	8	7

DOSAGE.

Resochin, four tablets, was given in a single dose to half the cases, and in the other half, it was modified as follows :—

First day : Four tablets in the morning and two in the evening.

Second day : Two tablets in the morning.

Third day : Two tablets in the morning.

*This paper was read at the " SYMPOSIUM ON PYRIMETHAMINE " held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

This modified dosage was given only to the group showing heavy infection. Amodiaquin was given in a single dose of three tablets each of 0·2 gm. Pyrimethamine was given as one tablet in the morning and second in the evening, each of 25 mg. None of the patients turned up either for relapse or recurrence of symptoms.

OBSERVATIONS.

The results of this small group of observations are presented below :—

1. *Resochin.*—The drug was found to be very effective in both the *vivax* and *falciparum* infections. The temperature touched normal within 24-36 hours. Almost complete clearance of parasites was noticed by 72 hours. Toxic symptoms, except for nausea and vomiting in two cases, were hardly any.

2. *Amodiaquin.*—This drug was found to be equally effective in both the infections. The time of clearance of parasitæmia was very much close to that obtained with resochin.

3. *Pyrimethamine.*—It was more effective in the *vivax* infection. In two of *vivax* cases, temperature touched normal within 48 hours. But in two cases of heavy subtertian infection, which fell in the lot of pyrimethamine, the temperature and parasitæmia persisted for over 96 hours, after which other antimalarials had to be used. One thing was, however, observed that its action was rather delayed compared to resochin and amodiaquin. Vomiting was present in two cases. No other toxic symptoms were observed.

Comparative response of three antimalarials.

Antimalarial.	*Vivax* infection.		*Falciparum* infection.		Remarks.
	Temporary response (hours).	Disappearance of parasitæmia (hours).	Temporary response (hours).	Disappearance of parasitæmia (hours).	
Resochin ...	24–36	72–96	24–36	72–96	Response satisfactory.
Amodiaquin ...	28–36	72–96	28–36	72–96	Response satisfactory.
Pyrimethamine ...	36–72	72–108	48–96	96–120	Two failures with subtertian infection. Delayed response.

DISCUSSION.

This trial gives an opportunity of comparing the author's results with others, who have used pyrimethamine in the treatment of both *vivax* and *falciparum* infections.

(*a*) Vivax *infection.*—Covell *et al.* (1953) gave pyrimethamine to acute cases using the Madagascar strain of *P. vivax.* They noted a slow clinical response even though it was given for five days. Wilson and Edeson (1953) in their series also observed slow progress in 11 cases of *vivax* malaria, though no failures were encountered. Jaswant Singh, Ray, Basu and Misra (1952 : 1953), however, observed effectiveness of pyrimethamine in *vivax* malaria, though its action on gametocytes was slower. The author's finding of this delayed response is confirmed by many workers.

(*b*) Falciparum *infection.*—Wilson and Edeson (*loc. cit.*) treated 26 cases of *falciparum* infection giving 300 mg. of pyrimethamine in four days. They noted seven failures and observed that the clinical action of the drug was slow, and that a third of this group had persistence of fever and symptoms even after disappearance of trophozoites. Bruce-Chwatt and Archibald (1953) in their comparative study of chloroquine, amodiaquin, pyrimethamine and azacrin in the treatment of acute malaria, found all the four drugs equally good schizonticides. In the present trials, two failures were observed in heavy subtertian infection treated with pyrimethamine similar to those reported by Jaswant Singh, Ray, Misra and Basu (1952) and Chakravarty and Chaudhuri (1953). Besides, its delayed action was again noted as with *vivax* infection.

(*c*) *Dosage of pyrimethamine.*—Although the dosage of pyrimethamine, as recommended by the Malaria Institute of India, was adhered to, yet it is felt that this has got to be worked out carefully. Since pyrimethamine is a derivative of diaminopyrimidine group, which belongs to the group of folic acid antagonists, larger doses have to be given with caution from the viewpoint of producing granulocytopenia. Other workers who have given larger doses of pyrimethamine, either on the first day or subsequently spreading for five days, have not found better or remarkable response.

(*d*) Whether pyrimethamine can be used effectively for prophylactic use or as a suppressant, has yet to be proved.

CONCLUSION.

The present series are too small to draw any definite conclusion. Probably the most important criteria to a clinician in the choice of an antimalarial in the treatment of an acute attack is its clinical response. In the opinion of the author, pyrimethamine alone would not be the drug of choice, particularly in a heavy infection and more so in the *falciparum* infection, because of its delayed action. Probably its only superiority to others is its very small dosage.

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SUSCEPTIBILITY OF BLOOD-INDUCED *P. CYNOMOLGI* INFECTION TO PYRIMETHAMINE (DARAPRIM), PROGUANIL AND BROMOGUANIDE.*

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IN laboratory studies, high degree of activity of pyrimethamine has been demonstrated by various workers against several species of plasmodia ; against *P. gallinaceum* by Falco *et al.* (1951) ; Jaswant Singh, Ray and Chandrasekhar (1953) and Greenberg *et al.* (1953) ; against *P. berghei* by Falco *et al.* (*loc. cit.*) and Jaswant Singh, Krishnaswami *et al.* (1952) ; against *P. knowlesi* by Jaswant Singh, Misra *et al.* (1951) and against *P. cynomolgi* by Falco *et al.* (*loc. cit.*) and Schmidt and Genther (1953).

In the present paper, the authors report their findings on a comparative study based on quantitative evaluation of pyrimethamine, proguanil and bromoguanide against *P. cynomolgi* in *rhesus* monkeys.

MATERIALS AND METHODS.

Fifty-eight healthy *rhesus* monkeys weighing 2·5 to 5 kg. were used for the study. All these monkeys showed negative tuberculin reaction prior to these trials (Jaswant Singh, Balbir Singh *et al.*, 1951 ; Nair and Ray (In press). The methods used for testing the drugs were exactly on the same lines as reported in the earlier publication (Jaswant Singh, Nair and Ray, 1953).

The strain of *P. cynomolgi* used for infecting the monkeys was the one isolated by Sinton and Mulligan (1932). The dosage of inoculum in all cases was five million parasitized erythrocytes per kg. body weight of the recipient monkey administered by intravenous route. Thick and thin blood smears from these

*Summary of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

monkeys were collected twice daily once in the morning between 8·00 and 9·00 a.m. and in the evening between 4·30 and 5·30 p.m. up to the last day of drug administration, and subsequent to that only in the mornings. Parasite densities were estimated as the number per 10,000 red blood corpuscles.

Drug administration to the animals was commenced when 0·1 to 0·2 per cent cell infection had been established. The dosages employed were always in terms of the milligramme base of the drug per kg. body weight of the animal given once a day in the mornings for seven days. The drug was given from stock solutions containing one mg. base of either pyrimethamine or proguanil or four mg. base of bromoguanide per c.c. and kept in refrigerator. The necessary dilutions were made at the time of drug administration and 5 to 10 c.c. was administered orally using Ryle's rubber tube. The dosage regimes employed varied from 0·05 to 5 mg. base per kg. body weight of the animal. One to nine monkeys were used for testing each dosage regime, but at the minimum effective dosage levels, at least three monkeys were utilized.

The minimum dosage that cleared parasites from the peripheral blood of all monkeys used for a particular regime at least by the day following the last dose of the drug, was declared as the minimal effective dose " Class II effect of Shannon " (Wiselogle, 1946). Those that showed significant reduction in parasitæmia during the course of drug administration as compared to controls but without the ultimate clearance of parasites within 24 hours after the last dose of the drug, were considered to exhibit the " Class I effect of Shannon ". If the course of infection was not materially changed as compared to controls in spite of treatment, the drug at that particular dosage regime was taken as " inactive ". The blood smears of monkeys that became parasite free after the termination of treatment, were examined daily for recrudescence (relapse) for a period of 30 days and those that did not become positive up to that time, were subjected to splenectomy and further blood examination for another period of 30 days. Failure to detect parasites during this period was taken as complete cure of infection in these monkeys *i.e.* Class III effect.

RESULTS.

The results obtained with pyrimethamine are recorded in Table I. A dose of 0·05 mg. of this drug was not adequate in changing the course of infection in two of the monkeys while 0·1 mg. dose produced Class I effect in one out of the eight monkeys. Perhaps this is the lowest dosage in this investigation, below which no antimalarial property of the drug could be measured against this strain. The minimum dosage that indicated a Class II effect was 0·5 mg. Of the nine monkeys placed on this regime, in nearly 45 per cent of the animals, there was no response at all, while Class I effect was observed in 33 per cent. Out of the remaining 22 per cent., Class II and Class III effects were observed in equal number of monkeys. Somewhat similar results were observed with 0·6 and 0·7 mg. dosages. With 0·8, 0·9 and 1·0 mg. doses, better results were observed, but Class II effect in hundred per cent cases could be obtained only with 1·1 mg. dosage. Whereas 2·5 mg. dosage was effective in producing only 50 per cent cures, the highest dosage of 5 mg. cured the infection in both the monkeys tried.

TABLE I.

Response of P. cynomolgi *infection to treatment with pyrimethamine.*

Dosage mg. base/kg. weight.	Number of animals used.	Nature of response.				Relapse.*		Remarks.
		Inactive (number)	Class I effect (number)	Class II effect (number)	Class III effect (number)	Number.	Interval in days.†	
0·05	2	2	...	...	...	...	...	
0·1	8	7	1	...	...	...	...	
0·5	9	4	3	1	1	1	2	
0·6	2	2	...	...	...	...	...	
0·7	2	1	1	...	...	...	...	
0·8	3	...	1	2	...	2	7, 16	
0·9	3	1	...	2	...	2	12, 16	
1·0	3	...	1	2	...	2	6, 10	
1·1	3	...	...	3	...	3	13,15,18	M.E.D.
2·5	2	...	...	1	1	1	17	
5·0	2	...	...	...	2	...	...	

*Among those that showed class II effect. †From the cessation of treatment.

TABLE II.

Response of P. cynomolgi *infection to treatment with proguanil*

Dosage mg. base/kg. weight.	Number of animals used.	Nature of response.				Relapse.*		Remarks.
		Inactive (number)	Class I effect (number)	Class II effect (number)	Class III effect (number)	Number	Interval in days.†	
0·05	2	2	...	...	...	...	...	
0·1	2	2	...	...	...	...	...	
0·2	2	...	1	1	...	1	8	
0·3	2	...	2	...	...	...	...	
0·4	2	1	1	...	...	...	...	
0·5	4	...	1	3‡	...	1	5	
1·0	5	...	...	4	1	4	3,6,9,15	M.E.D.
0·7	2	...	1	1	...	1	11	
0·8	3	...	1§	2	...	2	4,5	

*Among those that showed Class II effect. †Since the cessation of treatment.

‡No relapse in two during 4 to 5 weeks observation when they died due to intercurrent disease.

§Became negative during the course of drug administration but parasites reappeared again before the cessation of treatment.

As could be seen from Table II, only two monkeys each were placed on dosages ranging from 0.05 to 0.4 mg. of proguanil. Up to 0·1 mg., the drug was ineffective in changing the course of infection. Doses of 0·2 to 0·4 mg., were, though not effective in clearing the parasites within the normal period, enough to change the course of infection to a milder one than in the controls. As compared to these, 0·5 mg. dosage controlled the infection in three out of four monkeys tried. With 1·0 mg., parasites could not be detected in the peripheral blood of any of the five monkeys tried by the day following the last dose of the drug. In one, no relapse occurred during the whole observation period.

Table III shows the results obtained with bromoguanide. Out of the three monkeys treated with 0·5 mg. dosage, one each showed Class I and Class II effect, and in the third there was no appreciable effect at all. A dose of 2·2 mg. was the lowest that was found effective in producing Class II effect.

TABLE III.

Response of P. cynomolgi *infection to treatment with bromoguanide.*

Dosage mg. base/ kg. weight.	Number of animals used.	Nature of response.				Relapse.*		Remarks.
		Inactive (number).	Class I effect (number).	Class II effect (number).	Class III effect (number).	Number.	Interval in days.†	
0·3	1	1	...	...	...	...	...	
0·5	3	1	1	1 (under observation)	...	1	8	
0·8	3	...	2	1	...	1	8	
1·0	3	...	3	...	...	...	...	
1·2	2	...	2	...	...	...	...	
1·5	3	...	1	2‡	...	1	14	
2·0	3	...	1§	2	...	2	3,5	
2·2	3	...	...	3	...	3	3,3,5	M.E.D.

*Among those that showed Class II effect.
†From the cessation of treatment.
‡One died of intercurrent disease after the cessation of treatment.
§Parasites disappeared during drug administration but reappeared before the completion of treatment.

DISCUSSION.

The minimal effective dose for Class II effect in blood-induced *P. cynomolgi* infection was found to be 1·1 mg. for pyrimethamine, 2·2 mg. for bromoguanide and 1·0 mg. for proguanil. In a previous communication, Jaswant Singh, Nair and Ray (1953) recorded the M.E.D. of quinine as 20 mg. Thus the quinine equivalent of these three drugs is in the order of 18·2 for pyrimethamine, 9·1 for bromoguanide and 20 for proguanil. Jaswant Singh, Basu and Ray (1952) reported that the quinine equivalent of proguanil in chicks infected with *P.*

gallinaceum was 16. Subsequently, Jaswant Singh, Ray and Chandrasekhar (1953) observed that the quinine equivalent of pyrimethamine against the same species was in the region of 1066. As compared to proguanil, pyrimethamine was reported to be 30 times more active in *P. cynomolgi* (Schmidt and Genther, *loc. cit.*), four times more active in a non-virulent strain of *P. knowlesi* (Jaswant Singh, Misra *et al.*, 1951) and 200 times more in *P. berghei* (Falco *et al.*, 1951). These reported results are in variance with the present observations. It is noted that actually proguanil is slightly (1·1 times) more active than pyrimethamine in blood-induced *P. cynomolgi* infection. The higher value of the minimal effective dose of pyrimethamine obtained in the present investigation is attributed to (*a*) the "flat" dose response curve, (*b*) the slow action of daraprim (Goodwin, 1952 ; Schmidt and Genther, 1953), and (*c*) the particular standard (Class II effect of Shannon) followed for the estimation of M.E.D. in the present studies.

Considering the speed with which parasites were cleared from the peripheral blood as well as the relapse rate, Jaswant Singh, Nair and Basu (1950) remarked that bromoguanide was somewhat less potent than proguanil against *P. gallinaceum*, *P. knowlesi* (non-virulent strain), *P. cynomolgi* and *P. inui*. The present investigation confirms the superiority of proguanil over bromoguanide by finding the minimal effective dose of the two drugs against blood-induced *P. cynomolgi* infection.

SUMMARY.

The comparative efficacy of pyrimethamine, bromoguanide and proguanil against blood-induced *P. cynomolgi* infection was determined using 84 *rhesus* monkeys.

The minimal effective dose of these three drgs was found to be 1·1, 2·2, and 1·0 mg., respectively. The quinine equivalent, therefore, is 20 for proguanil, 9·0 for bromoguanide and 18 for pyrimethamine.

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DEVELOPMENT OF RESISTANCE TO PYRIMETHAMINE IN *P. CYNOMOLGI.**

BY

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PYRIMETHAMINE has been assayed at this Institute against avian malaria (Jaswant Singh, Basu and Ray, 1951 ; Jaswant Singh, Ray and Chandrasekhar, 1953), rodent malaria (Jaswant Singh, Krishnaswami *et al.*, 1952), simian malaria (Jaswant Singh, Misra, Ray *et al.*, 1951) and human malaria (Jaswant Singh, Ray, Basu and Misra, 1952 : 1953 ; Jaswant Singh, Ray, Misra and Basu, 1952). Further, Jaswant Singh, Ray, Basu and Nair (1952) have reported that *P. knowlesi* which develops resistance to proguanil, is also refractory to pyrimethamine. Recently, Schmidt and Genther (1953) observed that a strain of *P. cynomolgi* which is normally sensitive to this drug, can be converted into a highly resistant strain by repeated treatment with sub-effective doses. Further, when such a resistance is built up, the strain proved refractory to proguanil also. In their studies, pyrimethamine was administered for a period of seven days.

During the present studies, similar observations have been made with *P. cynomolgi* but the course of treatment was much shorter (three days) as is often used for treatment of human malaria.

METHODS AND MATERIALS.

Young adult *rhesus* monkeys of both sexes weighing 2·5 to 5 kg. were used for these experiments. Prior to their usage, they were subjected to routine blood smear examinations and tuberculin tests (Nair and Ray, 1953).

The strain of *P. cynomolgi* used, was originally isolated by Sinton and Mulligan (1932) and ever since maintained by serial blood passages from untreated donors.

*Summary of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

The animals were in all cases infected by injecting intravenously with 5×10^6 parasitized erythrocytes per kg. body weight.

For parasite count, Ehrlichs eye-piece adjusted to count 100 erythrocytes per field by the oil immersion lens, was used. One hundred such fields were examined for estimation of the number of parasites per 10,000 R.B.C. A smear was declared negative only when no parasites could be detected in 100 fields of the thick smear. The treatment was initiated when parasitæmia reached 0·1 to 0·2 per cent cell infection.

Pyrimethamine was given in terms of mg. base per kg. body weight of the animal. It was suspended in water and given orally in 2 to 10 c.c. volume, using Ryle's tube attached to a syringe, once daily for three consecutive days.

Monkeys which showed negative blood smears for a period of three to four weeks after the cessation of drug administration, were subjected to splenectomy. If an animal remained parasite free for a period of four weeks after splenectomy, it was considered to have been cured.

KNOWN ACTIVITY OF DRUGS AGAINST THE PARENT STRAIN OF *P. CYNOMOLGI.*

0·8 to 1·1 mg. dose of pyrimethamine for seven days cleared all parasites from the peripheral blood by the day following the last dose of the drug [Class II effect of Shannon (Wiselogle, 1946)] but on occasions even a dose of 0·1 mg. for seven days produced some deceleration in the course of parasitæmia (Class I effect of Shannon).

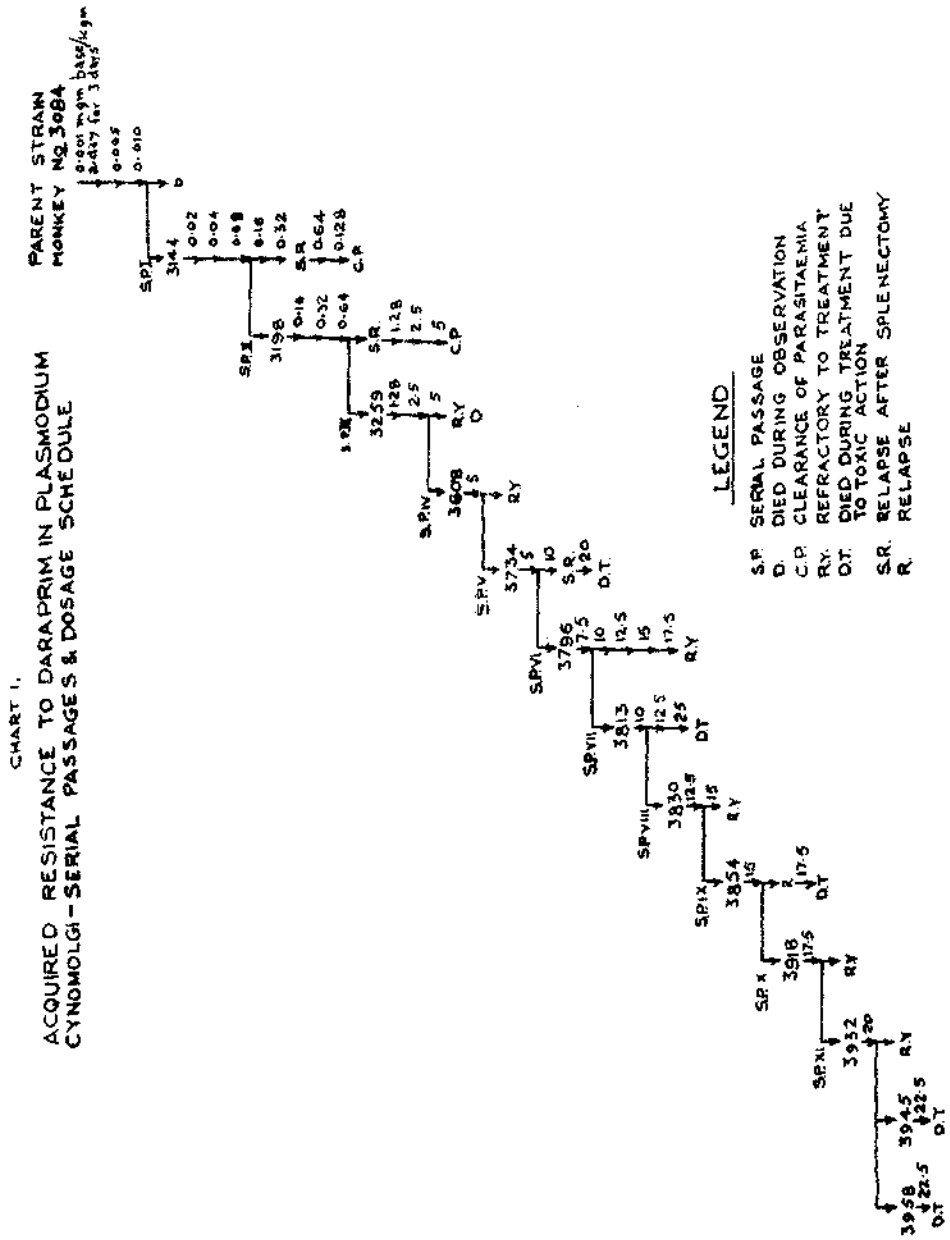
Similarly the dosages required to produce Class II and Class I effect of proguanil were 1·0 and 0·2 mg., and for bromoguanide 2·2 and 0·5 mg., respectively (Nair, Ray and Jaswant Singh, 1953).

Results of the preliminary observations in the Malaria Institute laboratories on the comparative efficacy of active proguanil metabolite and active bromoguanide metabolite (Bami, 1953), M 3349 (proguanil precursor), and sulphadiazine indicate that approximately 0·2, 0·5, 15·0 and 0·25 mg., respectively, are required for the clearance of parasites (Class II effect) in blood induced *P. cynomolgi* infection.

PROCEDURE.

Monkey 3084 was treated with a sub-effective dose of 0·001 mg. base pyrimethamine per kg. body weight a day for three days. Two days after the cessation of treatment, 0·005 mg. was given for another three days. Two days later, the dosage was again increased to 0·01 mg. After this, Monkey 3144 was sub-inoculated and treated successively with 0·02, 0·04 and 0·08 mg. of the drug. Treatment in all the cases lasted for three days and the interval between two successive treatments was one to two days. Monkey 3198 was sub-inoculated from Monkey 3144 after the latter had received 0·08 mg. dosage. Further sub-inoculations were made in this manner up to Serial Passage 4, and the recipient monkey received double the dose every time. In the fifth serial passage, 5 mg. was repeated in Monkey 3734. In the subsequent Serial Passages VI to IX, the doses were increased to 7·5, 10, 12·5, 15, 17·5 and 20 mg., respectively. Further increase to

22·5 mg. proved toxic to two monkeys treated (Numbers 3945 and 3958). After each serial passage, the donors were treated similarly with progressively increasing doses till either there was clearance of parasites or the treatment failed even when the doses reached toxic level. Details regarding serial passages and the doses employed are given in Chart I.



ASSESSMENT OF THE DEGREE OF RESISTANCE DEVELOPED.

It could be seen from Chart 1 that passage of the strain through the first five monkeys treated with successively increasing doses, made the parasite to acquire some degree of refractoriness, and during the sixth passage the recipient monkey showed continued parasitæmia in spite of a series of successive treatments with 7·5, 10, 12·5, 15 and 17·5 mg. dosage regimes.

TABLE I.

Effect of daraprim on the daraprim resistant strain of P. cynomolgi.

Number of the monkey.	Dosage mg./kg.	TOTAL PARASITE COUNT PER 10,000 R.B.C. ON DAYS FOLLOWING START OF TREATMENT.							Remarks.
		1st	2nd	3rd	4th	5th	6th	7th	
3509	0·5	16	120	240	1200	700	200	32	Infection persisted.
3523	0·5	20	40	240	220	200	340	200	Infection persisted.
3519	1·0	100	740	1600	2200	1000	40	80	Infection persisted.
3524	1·0	80	380	1200	1000	1000	700	600	Infection persisted.
3511	2·5	20	60	100	104	1000	1200	160	Infection persisted.
3525	2·5	60	100	150	125	120	120	200	Infection persisted.
3512	5	14	8	6	2	2	2	Neg.	Temporary clearance. Recrudescence 6 days after cessation of treatment.
3746	5	80	500	70	80	14	7	<1	Temporary clearance 2 days after cessation of treatment. Recrudescence 4 days after.
4011	10	30	100	800	1000	400	700	150	Died with high parasitæmia on the fourth day after the cessation of treatment.
4012	10	30	160	650	800	1000	700	1000	Died with high parasitæmia on the fourth day after the cessation of treatment.
3997	15	25	100	...	...	...	...	...	Died due to the toxic effect of the drug.
3998	15	35	400	...	...	...	...	...	Died due to the toxic effect of the drug.
3903	20	200	1000	1200	...	...	...	...	Died due to the toxic effect of the drug.

When it was observed that a 3-day course with 22·5 mg. (Chart 1) was too toxic to the animals (Monkey 3945 and 3958), thirteen normal monkeys were

sub-inoculated from Monkey 3933 (the donor in Serial Passage 12) showing persistent parasitæmia subsequent to its treatment with 20 mg. for three days. Thereafter two monkeys each in six groups were treated with varying doses of 0·5, 1, 2·5, 5, 10 and 15 mg. base for seven days. The remaining one received 20 mg. dosage. At the same time, two additional monkeys were inoculated from the parent strain of *P. cynomolgi* and treated one each with 0·1 and 0·8 mg., respectively, for the same period. The effects of treatment are shown in Tables I and II. Whereas with the parent strain, clearance of parasites could be obtained two days after the cessation of treatment with 0·1 mg., the infection persisted in all the monkeys inoculated with the resistant strain even when treated with 10 mg. dosage (Chart 2). Higher doses than this generally proved fatal.

TABLE II.

Effect of daraprim on the parent stain of P. cynomolgi.

Number of monkey	Dosage mg./kg.	TOTAL PARASITE COUNT PER 10,000 R.B.C. ON DAYS FOLLOWING START OF TREATMENT.							Remarks.
		1st	2nd	3rd	4th	5th	6th	7th	
3589	0·1	40	800	655	690	500	360	180	Negative 2 days after the cessation of treatment. Transient parasitæmia 20 days after.
4071	0·8	18	16	2	<1	...	...	...	Transient parasitaemia 7 days after cessation of treatment.

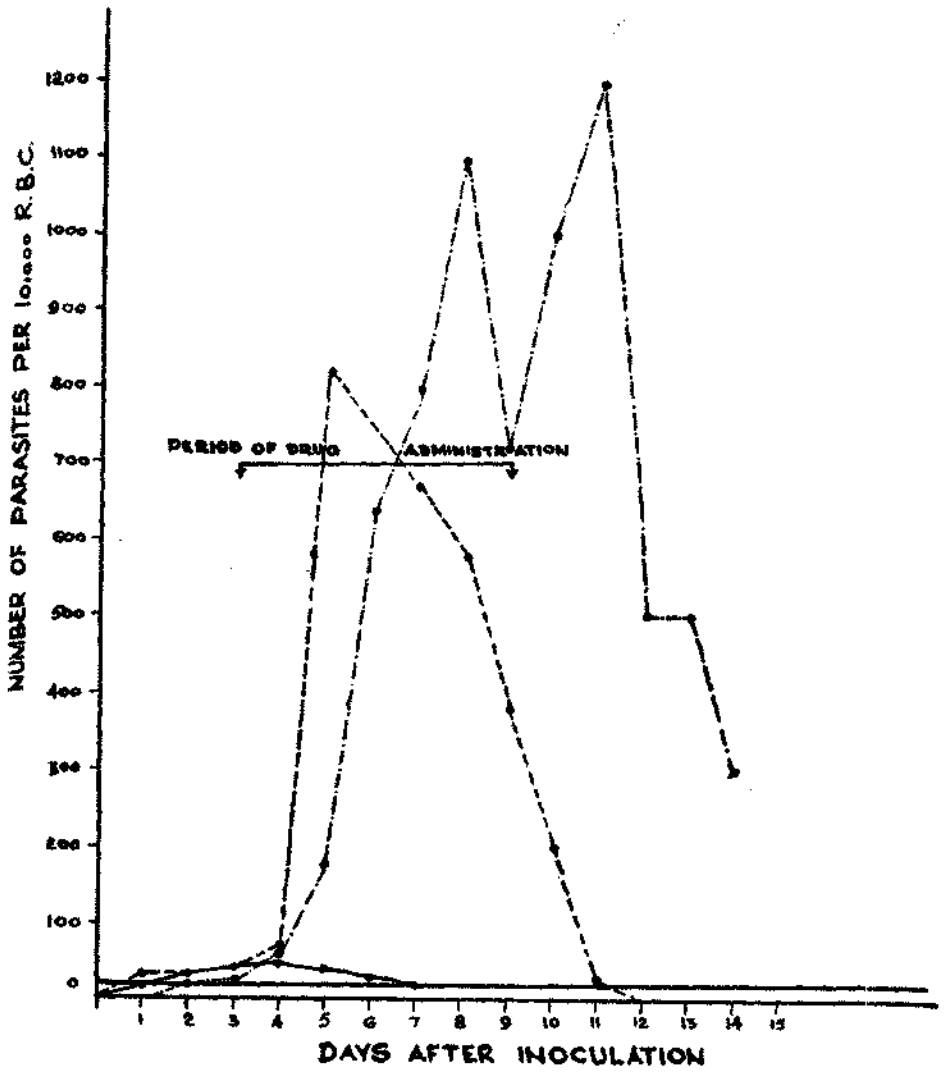
RESPONSE OF THE RESISTANT STRAIN TO OTHER DRUGS.

Two monkeys (Numbers 3996 and 4057) were infected with the resistant strain. Monkey 4057 was given 30 mg. proguanil and the other 15 mg. proguanil a day for seven days. While the former dosage proved effective, the infection persisted for several days even after treatment with 15 mg. In Chart 3, the comparative effect of the drug against the parent and resistant strains is shown.

Bromoguanide.—Response of bromoguanide against the resistant strain was tried in three monkeys (Numbers 4104, 4080 and 4058). Of these, Monkey 4058 was treated with 30 mg., 4080 with 15 mg. and 4101 with 10 mg. dose of bromoguanide for seven days. The results, except of Number 4058, are indicated in Chart 4. Monkey 4058 responded well to treatment. In Monkey 4080, there was first temporary clearance of parasites but the infection reappeared again before the cessation of treatment. The reappearance of parasites during treatment is probably an indication of the early appearance of resistance to the drug (Cooper *et al.*, 1950). In the third monkey, the infection proved insensitive to treatment with 10 mg. dosage.

CHART 2.

EFFECT OF DARAPRIM ON PARENT AND DARAPRIM RESISTANT STRAINS OF P. CYNOMOLGI

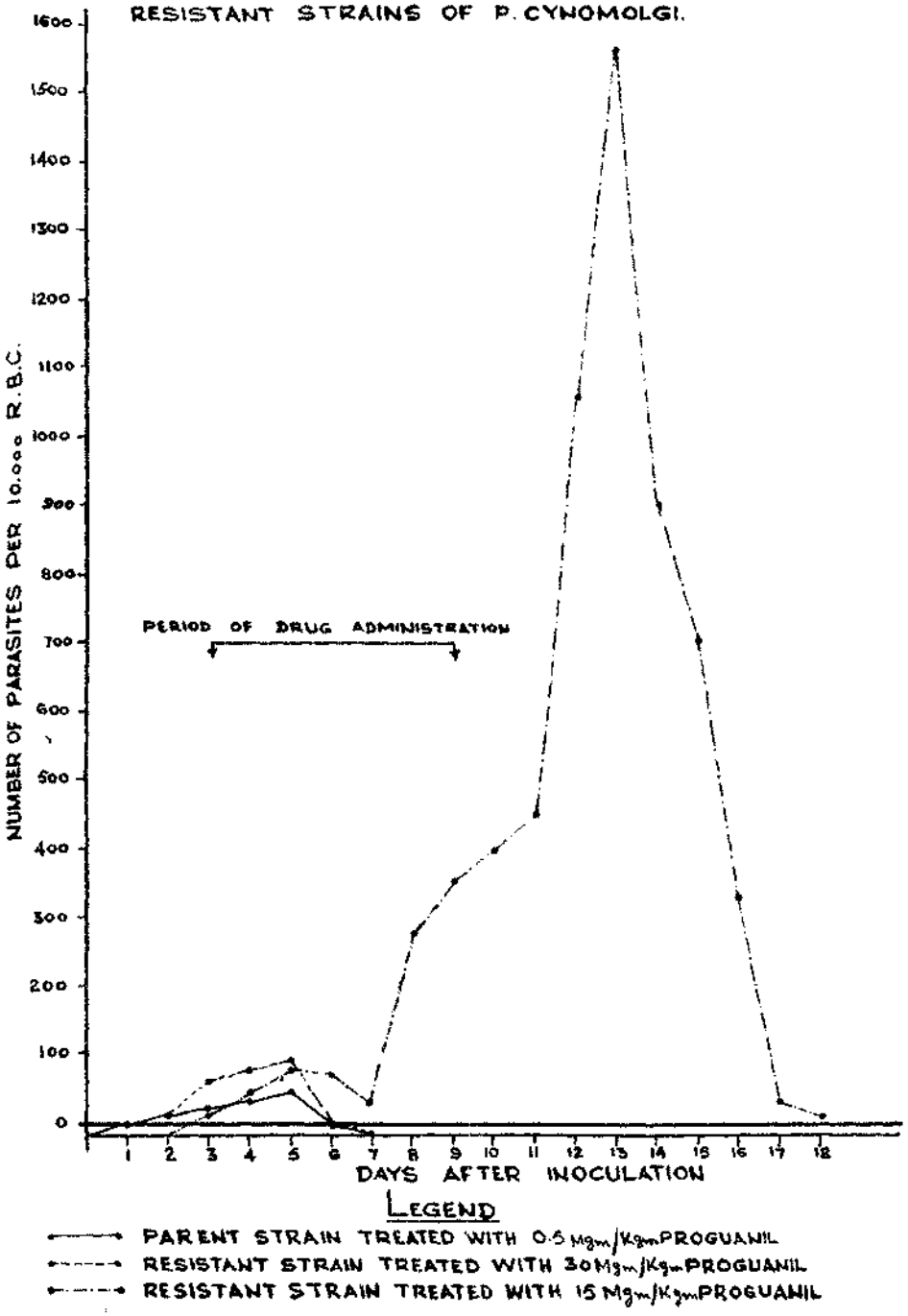


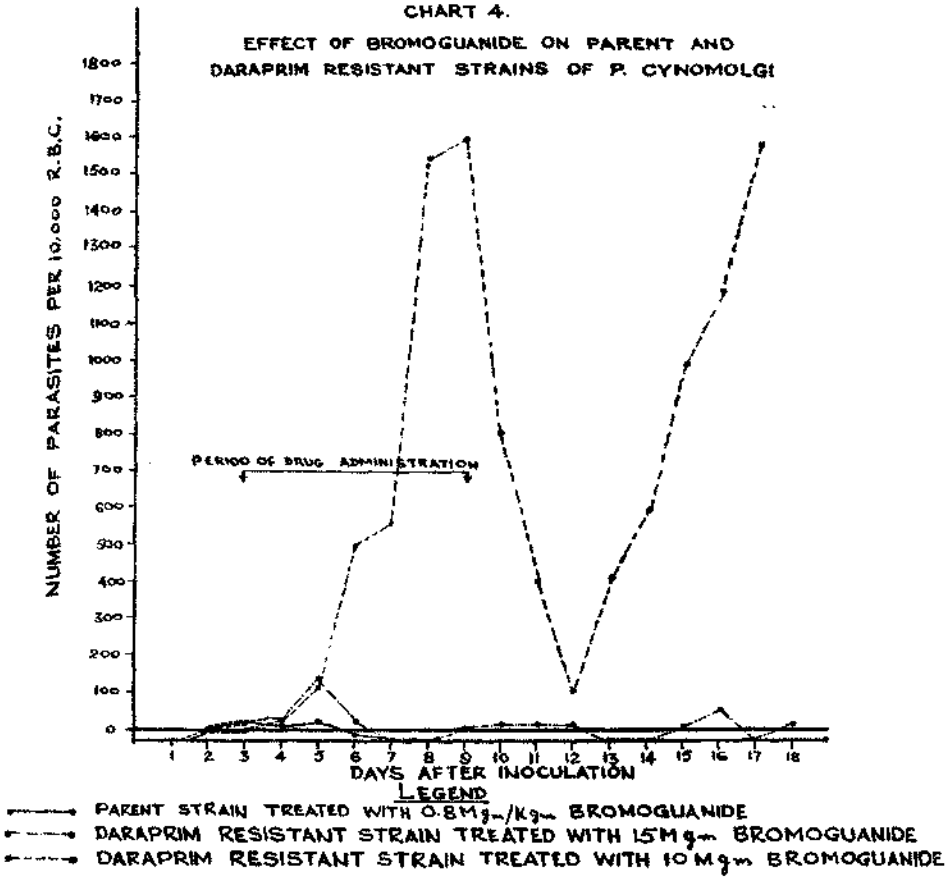
LEGEND

PARENT STRAIN TREATED WITH 0·8 Mgm./Kg. DARAPRIM.
PARENT STRAIN TREATED WITH 0·1 Mgm./Kg. DARAPRIM.
RESISTANT STRAIN TREATED WITH 10 Mgm./Kg. DARAPRIM.

CHART 3.

EFFECT OF PROGUANIL ON PARENT AND DARAPRIM RESISTANT STRAINS OF P. CYNOMOLGI.





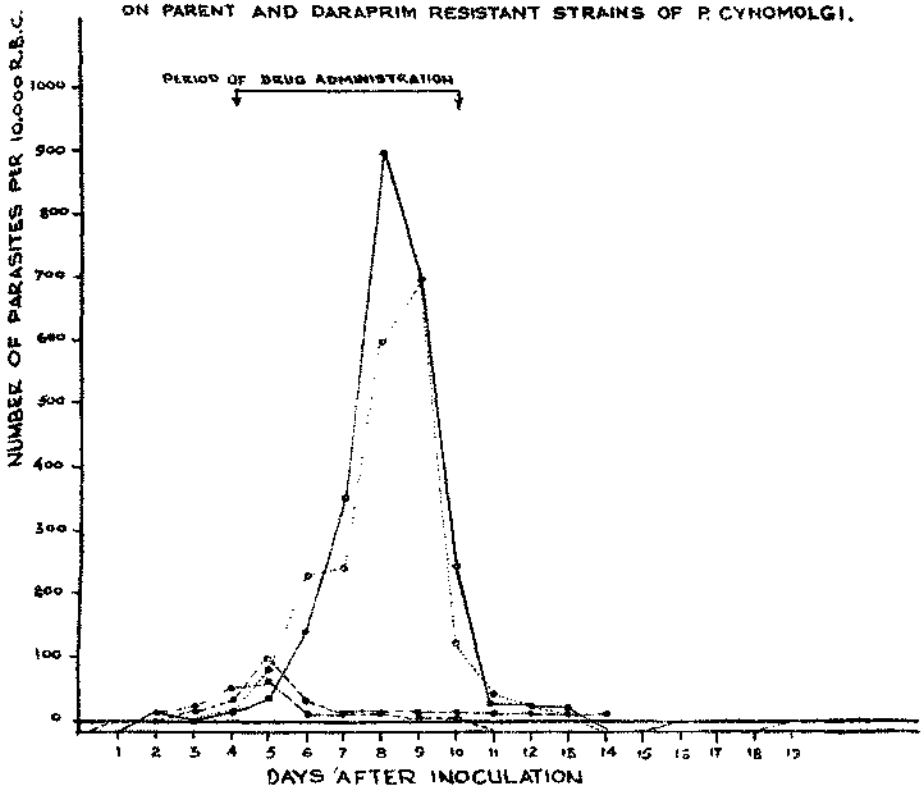
Proguanil and bromoguanide metabolites.—One monkey each was treated with 15 mg. of proguanil active metabolite-dihydrotriazine, and bromoguanide active metabolite-dihydrotriazine for seven days. In both cases, the infection was not amenable to treatment (Chart 5). Thus it would appear that resistance to pyrimethamine directly influenced the response to proguanil and bromoguanide and their metabolites.

Paludrine precursor M 3349.—This drug was administered in 15·0 mg. dosage for seven days to two monkeys, one infected with the parent strain and the second infected with the pyrimethamine resistant strain. Both the strains proved to be equally susceptible.

Sulphadiazine.—Sulphadiazine in 20, 15, 1·0, 0·5 and 0·25 mg. dosages was administered for seven days to one each inoculated with the resistant strain. The response was good in all cases and the parasites disappeared within the period of drug administration. Thus the resistant strain appears to be equally susceptible to sulphadiazine like the parent strain.

CHART 5.

EFFECT OF ACTIVE METABOLITES OF PROGUANIL AND BROMOGUANIDE ON PARENT AND DARAPRIM RESISTANT STRAINS OF P. CYNOMOLGI.



LEGEND

PARENT STRAIN TREATED WITH 0·2 Mgm./Kgm. ACTIVE METHBOLITE OF PROGUANIL

PARENT STRAIN TREATED WITH 0·2 Mgm./Kg. ACTIVE METABOLITE OF BROMOGUANIDE.

DARAPRIM RESISTANT STRAIN TREATED WITH 10 Mgm./Kgm. ACTIVE METABOLITE OF PROGUANIL.

DARAPRIM RESISTANT STRAIN TREATED WITH 10 Mgm./Kgm. ACTIVE METABOLITE OF BROMOGUANIDE.

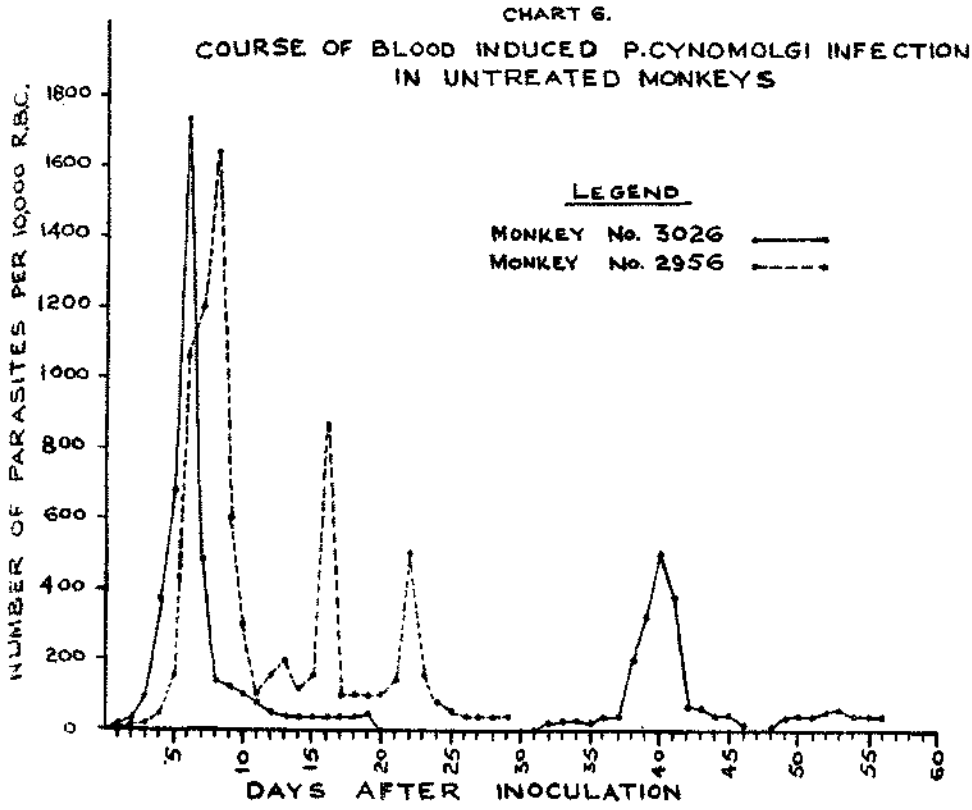
Chloroquine.—Chloroquine in 1·5 mg. base/kg. dosage which is also the minimum effective dose of the drug against the normal strain (Jaswant Singh, Nair and Ray, 1953), was tried against the resistant strain. Complete clearance of parasites was obtained within 36 hours. No change, therefore, was evident in the susceptibility of the resistant strain.

DISCUSSION.

Observations in these laboratories involving several monkeys show that the course of parasitæmia in untreated monkeys inoculated with five million parasitized

erythrocytes per kg. body weight of the parent strain, consists in the following order of sequence.

(1) Prepatent period 24 to 72 hours.
(2) Acute primary attack for about six to ten days.
(3) Parasitic crisis.
(4) Developed infection with continued low parasitæmia lasting a few days to two or three weeks, interrupted by sharp rise in parasitæmia in some cases at irregular intervals during this period (Chart 6).
(5) Latency followed by
(6) Several relapses at irregular intervals.



In some cases, the animals died due to secondary anæmia, either during the parasitic crisis or subseqeunt to that.

The procedure involved in the development of pyrimethamine resistant strain of *P. cynomolgi* in the present experiment, consisted of three days consecutive treatment of *P. cynomolgi* blood-induced infection with gradually increasing doses of daraprim and transfer of the strain, successively to fresh monkeys. These serial passages were done in all cases before the actual clearance of parasites from the

peripheral blood of the donors, which occurred either as a result of treatment or host's immunity reaction, as may be evident from the course of parasitæmia referred to above. Thus, since starting the treatment with a sub-effective dosage of 0·001 mg. base/kg. body weight of the monkey, the dosage was gradually worked out to 20 mg. Dosages higher than this proved invariably toxic and hence further attempts to build up the resistance had to be abandoned.

Definite indication of the development of resistance was evident in Serial Passage 4, at which stage, the donor Monkey 3796 consistently showed parasites in circulating blood even after treatment with five dosage regimes, consisting of 7·5, 10, 12·5, 15 and 17·5 mg. of the drug. Thus it would appear that even with a limited sub-passage, refractoriness could be produced easily.

In the final assessment of the degree of resistance developed after the eleventh serial passage, it was found that the resistant strain was refractory to a treatment with 10 mg. pyrimethamine for seven days. This is actually about ten times the amount of drug which is required to clear the infection caused by the parent strain, by the day following the treatment, and about one hundred times the amount which causes either a deceleration of parasites during treatment or clearance of parasites from peripheral blood within two or three days after the cessation of treatment.

Schmidt and Genther (1953) in a similar experiment could evoke at least a thousand fold change in response to pyrimethamine against *P. cynomolgi* by treating with successively increasing doses and passage of the plasmodia to new monkeys until a point was reached when the drug in daily dose of 2·5 mg./kg. had little or no effect on parasitæmia. Thus the degree of resistance obtained in their experiment was much more than what is recorded in this paper. But this can be easily explained by the fact that the experiments in the two laboratories were done under different methods with significant variation in the dosage of inoculum, time of treatment, etc. Moreover different criteria are adopted in the estimation of the minimum effective doses on the basis of which the degree of resistance obtained is finally calculated. On account of the "flat" dose response activity of pyrimethamine, the M.E.D. becomes much higher when the method generally followed by the authors is adopted (Nair, Ray and Jaswant Singh, 1953).

From the figures already given in the preceding paragraphs in respect of the M.E.D. of proguanil, bromoguanide and the active metabolites of these two drugs, and the dosages of these drugs found ultimately refractory to treatment against the pyrimethamine resistant strain, it is clearly evident that there is cross resistance between pyrimethamine and the above four drugs. The degree of resistance manifested was found to be fifteen times in the case of proguanil, seven times in the case of bromoguanide and approximately seventy-five and thirty times, respectively, with the metabolites of these two drugs. Schmidt and Genther (1953) also showed that pyrimethamine resistant trophozoites are equally refractory to treatment with proguanil. Jaswant Singh, Ray, Basu and Nair (1952) reported that proguanil resistant *P. knowlesi* strain needed 1600 times the M.E.D. of 50-63 (daraprim). The Malaria Institute laboratories have already got a strain of the virulent *P. knowlesi* which is highly resistant to pyrimethamine treatment. Several workers have already reported the existance of proguanil resistant strains of human plasmodia.

Recently, Hernandez *et al.* (1953) could induce in the chesson strain of *P. vivax* more than twenty-five fold resistance to pyrimethamine. Authentic reports about pyrimethamine resistant strain in human malaria, under field conditions, are however yet to be reported.

Resistance to antimalarials is generally worked out in the laboratories by following 7-day treatment regimes. But there is the disadvantage that by following this method, the results obtained cannot be interpreted with much confidence, regarding its applicability to human malaria, as under natural circumstances, people are reluctant unless supervised to take drugs over such prolonged periods. On the other hand, the present experiment with the three days treatment courses, has greater significance in the fact that it parallels to a great extent the irregularities of dosages in a population and the short term, comparatively small doses, that are generally recommended for therapeutic and suppressive treatments with pyrimethamine. The rapid way by which the drug fastness could be produced in the laboratory in this way should serve as a warning that such happenings could happen in human malaria also.

This position, together with the possibility of cross resistance between proguanil and daraprim and *vice versa*, will have to be borne in mind in their use.

SUMMARY.

A pyrimethamine resistant strain of *P. cynomolgi* was developed in the laboratory by treating the parent strain with progressively increasing doses of the drug and serially transforming the infection at different stages of the experiment to fresh monkeys. Cross resistance tests showed that this strain was highly refractory to treatment with proguanil, bromoguanide and the active metabolites of these two drugs but not to M 3349 (precursor of proguanil), sulphadiazine and chloroquine.

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STUDIES ON NURI STRAIN OF *P. KNOWLESI.*

II. Therapeutic effect of pyrimethamine, proguanil and quinine.*

BY

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A STRAIN of *P. knowlesi* originally isolated by Sinton and Mulligan (1932) had, by 1949, lost its former virulence (Jaswant Singh *et al.*, 1949*a*). Working with this strain, the same workers (1949*b*) reported that in trophozoite-induced infection, action of proguanil was tardy as compared to that of chloroquine and amodiaquin (camoquin). Subsequently, Jaswant Singh *et al.* (1951) showed that pyrimethamine was four times more active than proguanil against the same strain of *P. knowlesi.*

In the present report, results have been recorded of a similar assay of the comparative merits of pyrimethamine, proguanil and quinine against blood-induced infection of a highly virulent strain (Nuri) of *P. knowlesi* isolated early in 1953 from a 'Kra' monkey in Malaya and passaged to *S. rhesus* monkeys (Jaswant Singh, Ray and Nair, 1953 ; Edeson and Davey, 1953).

METHODS AND MATERIALS.

One hundred and two young normal monkeys weighing 2·5 to 6·0 kg. and infected with the Nuri strain of *P. knowlesi* (using a standard dose of inoculum of five million parasitized erythrocytes per kg. body weight of the animal) were utilized for these experiments. The inoculation was given by intravenous route usually soon after sporulation had occurred in the donor.

*Abstract of this paper was read at the "SYMPOSIUM ON PYRIMETHAMINE" held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

Blood smears (both thick and thin on the same slide) were prepared from these monkeys both in the morning and evening from the day following the inoculation up to the day following the cessation of treatment. Thereafter it was collected only once a day in the mornings. The smears were stained with J.S.B. stain (Jaswant Singh and Bhattacharji, 1944) and parasite count estimated in terms of the number of parasites per 10,000 R.B.C. If no parasites could be detected in 100 fields of the thick film, the smear was considered negative.

Drug administration was commenced at the stage of 0·1 to 1·0 per cent cell infection. The dosages employed were in terms of milligrammes per kilogramme body weight of the animal and were administered orally for seven days using Ryle's tube attached to a syringe.

Criteria for the assessment of results were based mainly on the disappearance of parasites from the peripheral blood by the day following the last dose—Class II effect of Shannon (Wiselogle, 1946). The minimum dose which produced such an effect was taken as the minimum effective dose (M.E.D.). Where the average daily parasitæmia and the peak were significantly lower than in the comparison series, and the animals survived for three days after the cessation of drug administration, the result was registered as Class I effect. Those animals in which a Class II effect was observed, were kept under observation for a period of four weeks. If no relapse occurred during this period, they were subjected to splenectomy and their blood smears examined daily for a further period of four weeks. If no parasites were observed in the peripheral blood during this period, the animals were considered to have been completely cured (Class III effect of Shannon).

TABLE I.

Effect of quinine on P. knowlesi (*Nuri strain*) *infection.*

Serial number.	Dose mg. base/kg.	Number of monkeys.	Activity.							Remarks.
			Ineffective.	Effective.						
				Class I.	Class II.			Class III.		
					Number.	Parasite clearance (in hours).	Recrudescence (in days).	Number.	Parasites clearance (in hours).	
I	15	1	1	...	...	...	...	...	...	...
II	20	5	1	1	2	108, 144	3, 8	1	96	...
III	22	3	...	3	...	...	...	...	...	...
IV	23	3	1	...	2	84, 144	2, 3	...	...	...
V	25	5	...	2	...	...	...	3	108, 144, 144	...
VI	27	5	...	1	4	108, 120, 144, 156	2, 2, 15, 16	...	...	...
VII	30	5	...	...	3	72, 108, 120	5,7,7	2	72, 84	M.E.D.

RESULTS.

Quinine.—From Table I, it would be observed that a dose of 15 mg./kg. was ineffective in changing the course of parasitæmia as compared to the untreated series. No consistant result was obtained with any of the dosages ranging from 20 to 27 mg. base per kg. body weight of the animal. Of the five monkeys that received 20 mg. doses, one showed Class III effect, two Class II effect and one Class I effect. In the fifth monkey, this dosage proved ineffective. With 25 mg. while Class I effect was recorded in two monkeys, the infection was completely cured in the other three. However, when a dose of 30 mg./kg. was administered, parasites were cleared from peripheral circulation in all the five monkeys within 72 to 120 hours. This dosage, therefore, represents the M.E.D. of quinine. Three monkeys relapsed within five to seven days after the completion of treatment but the others remained negative throughout the observation period.

TABLE II.

Effect of proguanil on P. knowlesi *(Nuri strain) infection.*

Serial number.	Dosage mg. base/kg.	Number of monkeys.	Activity.							Remarks.
			Ineffective.	Effective.						
				Class I.	Class II.			Class III.		
					Number.	Parasite clearance (in hours).	Recrudescence (in days).	Number.	Parasite clearance (in hours).	
I	0·001	2	2	...	...	...	...	...	...	...
II	0·01	2	2	...	...	...	...	...	...	...
III	0·03	4	2	2	...	...	...	...	...	...
IV	0·05	5	...	2	3	120,132, 132	1(all)	...	...	...
V	0·06	3	1	2	...	...	...	...	...	...
VI	0·08	3	1	1	1	96	1	...	...	...
VII	0·1	6	...	3	3	48,48,60	3,4,17	...	...	...
VIII	0·15	3	...	3	...	...	...	...	...	...
IX	0·17	3	...	2	1	84	3	...	...	...
X	0·2	5	...	...	5	48,48,60, 96, 120	2,3,3,5,12	...	...	M.E.D.
XI	0·3	2	...	...	2	60,72	6*	...	...	*One died of intercurrent disease.
XII	0·5	3	...	...	...	...	...	3	48,72,72	
XIII	1·0	2	...	...	1	72	7	1	72	...

Proguanil.—Results obtained after treatment with different doses of proguanil are shown in Table II. None of the monkyes treated with 0·001 or 0·01 mg. doses responded to treatment but succumbed to infection. With 0·03 mg. dose, Class I effect was obtained in two out of four monkeys, and in the rest there was no effect at all. The results obtained with 0·05 to 0·17 mg./kg. doses were also variable. While Class II effect was attained in some, in others complete clearance of parasites from peripheral circulation was not observed. On the other hand, parasite clearance was obtained within 48 to 120 hours in all monkeys treated with 0·2 mg. or above. Thus the minimum dose which was able to produce Class II effect was 0·2 mg./kg. and this was taken as the M.E.D. of proguanil. While recrudescence was common in those treated with 0·2 or 0·3 mg./kg., results attained with 0·5 mg. dose showed Class III effect in all the three monkeys. Parasite clearance in these cases occurred with 48 to 72 hours.

TABLE III.

Effect of pyrimethamine on P. knowlesi (*Nuri strain*) *infection.*

Serial number.	Dose mg. base/kg.	Number of monkeys.	Activity.							Remarks.
			Ineffective.	Effective.						
				Class I.	Class II.			Class III.		
					Number.	Parasite clearance (in hours).	Recrudescence (in days).	Number.	Parasites clearance (in hours).	
I	0·00001	5	5	...	...	...	...	...	...	...
II	0·0001	3	1	...	2	120,132	1,2	...	...	...
III	0·0005	3	1	...	1	96,168	3,3	...	...	...
IV	0·001	4	1	2	...	...	...	1	72	...
V	0·01	5	4	1	...	...	...	...	...	...
VI	0·03	3	...	1	2	60,84	2,3	...	...	...
VII	0·04	3	...	2	1	144	5	...	...	...
VIII	0·05	3	...	...	3	84 (all)	1,2*	...	...	M.E.D.
IX	0·1	3	...	...	3	60,72,72	4,4,5	...	...	

*On died of intercurrent disease.

Pyrimethamine.—The details of the results in the series treated with pyrimethamine are recorded in Table III. A dose of 0·00001 mg./kg. did not have any effect on the course of parasitæmia in any of the monkeys treated. The results obtained in the series treated with 0·0001 to 0·04 mg. were varied. In some cases, the drug proved wholly ineffective whereas in others it showed either Class I or

Class II effect. In one animal, even Class III effect could be detected with a dose of 0·001 mg./kg. But in none of the series, complete clearance of parasites in all the animals, could be obtained by the day following the last dose of the drug (Class II effect) until the dose was increased to 0·05 mg./kg. or above. Thus the M.E.D. was established as 0·05 mg./kg. In the latter series, the clearance occurred between 60 and 84 hours.

DISCUSSION.

Jaswant Singh, Nair, Ramakrishnan and Ray (1953) recorded the course of untreated infection caused by the Nuri strain of *P. knowlesi* in healthy *S. rhesus* monkeys. Death occurred in these animals within five to six days from the first appearance of parasites in the peripheral blood with a peak parasitæmia of more than 90 per cent. For the sake of brevity, details like average daily parasitæmia, peak parasitæmia, day of death etc., in some of the treated monkeys in this investigation are not included in this report, but it may be said that the infection in most of the animals not affected by treatment, ran more or less a similar course as in the untreated controls.

In all quantitative therapeutic studies against *P. cynomolgi* and the non-virulent strain of *P. knowlesi*, it has been the practice to initiate treatment when infection reached 0·1 to 0·2 per cent cell infection. But during trials against the present strain (Nuri) of *P. knowlesi*, it was experienced that it was hardly possible to get as close as to 0·1 to 0·2 per cent cell infection, on account of extremely rapid multiplication of the parasites. Therefore, treatment was commenced when parasitæmia reached between the range of 0·1 to 1·0 per cent cell infection.

The minimum effective doses (M.E.D.) of quinine, proguanil and pyrimethamine have been found to be 30, 0·2 and 0·05 mg./kg., respectively. Thus the quinine equivalent (Q.E.) of proguanil is 150 and that of pyrimethamine 600. Against *P. gallinaceum* in 7-day old chicks, it was earlier observed that the M.E.D. of quinine, proguanil and pyrimethamine were 1·6 mg./50 gm. (32 mg./kg.), 0·1 mg./50 gm. (2·0 mg./kg.) and 0·0015 mg./50 gm. (0·03 mg./kg.) respectively (Jaswant Singh, Basu and Ray, 1952 ; Jaswant Singh, Ray and Chandrasekhar, 1953). Thus the Q.E. of proguanil and pyrimethamine were calculated to be 16 and 1066 *i.e.*, pyrimethamine is 66 times more active than proguanil against *P. gallinaceum*. On the other hand against *P. knowlesi* (Nuri), it was found to be only four times more effective and this ratio is actually the same as recorded by Jaswant Singh, Misra *et al.* (1951) in the case of a non-virulent strain of *P. knowlesi*, notwithstanding the different results attained by Nair *et al.* (1953) against *P. cynomolgi* where it was observed that proguanil was slightly (1·1 times) more active than pyrimethamine. Working with the latter species (*P. cynomolgi*), Schmidt and Genther (1953) showed that pyrimethamine was more potent than proguanil. Even in the absence of a strain difference, such a reversal in the comparative efficacy of the two drugs is perhaps possible when it is considered that pyrimethamine has shown a 'flat' dose response curve much more than proguanil.

There is one other point which needs some consideration. It was observed that during treatment with proguanil, some monkeys receiving 0·1 to 0·17 mg./kg. showed a temporary clearance or marked reduction of parasitæmia initially, but later parasites either reappeared or increased in number before the actual cessation

of treatment. Such reappearance of parasites is generally believed to be an indication for development of acquired resistance to a drug (Jaswant Singh, Nair, Ray and Misra, 1953). It may also be noted that such results were obtained only in dosage ranges approximating the minimum effective dose of the drug. Similar results were also observed in a few cases treated with pyrimethamine. It is now a well established fact that different species of parasites become refractory to these two antimalarials. In the face of this evidence, one is apt to speculate on the possibility during the search for potential antimalarial drugs of early detection, either during screening or at subsequent stages, of newer antimalarials readily producing resistant strains of plasmodia.

SUMMARY.

Quinine, proguanil and pyrimethamine were tested in 102 *S. rhesus* monkeys infected with the blood forms of the highly virulent *P. knowlesi* (Nuri strain) parasites following the usual standard techniques adopted in the Malaria Institute laboratories.

The minimum effective dose of quinine was found to be 30, of proguanil 0·2 and of pyrimethamine 0·05 mg. base per kg. body weight of the animal. The quinine equivalent, therefore, of proguanil is 150 and of pyrimethamine 600.

It was observed that in some monkeys treated with proguanil and pyrimethamine in doses approximating to the M.E.D. of the respective drugs, there was a temporary clearance of parasites followed by their reappearance before the cessation of treatment.

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SYMPOSIUM ON PYRIMETHAMINE (DARAPRIM).

Critical Review*.

BY

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(November 21, 1953.)

PYRIMETHAMINE (Daraprim), the latest of the series in synthetic antimalarials, has been developed after the last World War. Though search for effective pyrimidines had commenced much earlier, Curd *et al.* (1945) and Curd and Rose (1946*a* : 1946*b* : 1946*c*) synthesized series of compounds like M-2665 and M-3349. While M-3349 was the turning point from pyrimidine derivatives to proguanil, Hitchings *et al.* (1948) observed that some pyrimidine compounds were antagonistic to PGA (Pteroylglutamic acid) in the same way as proguanil. This led finally to the development of several substituted 2 : 4-diminopyrimidines, and pyrimethamine has been found to be the most effective of that series.

After hearing the present discussions, I am prompted to classify the work carried out into (1) Laboratory studies, and (2) Clinical and field studies. They include prophylactic, suppressive, therapeutic, curative, biochemical and toxicological aspects in the field of chemotherapy in addition to studies related to potentiation tests and the development of acquired resistance in plasmodia.

CAUSAL PROPHYLACTIC ACTION.

Pyrimethamine was found to be effective as a causal prophylactic against *P. gallinaceum* in fowls (Jaswant Singh, Basu and Ray, 1952) and chicks (Greenberg, Coatney and Trembley, 1953), but Schmidt and Genther (1953) report that against *P. cynomolgi* in *rhesus* monkeys, it is a suppressant.

In human malaria, Goodwin (1952) and Covell *et al.* (1953) have shown that not unlike proguanil, pyrimethamine is effective as a causal prophylactic against *P. falciparum*. Coatney *et al.* (1953) observed that in sporozoite-induced *P. vivax* infection " the drug is not true causal prophylactic though evidently the prepatent period is lengthened to a great extent ".

*This paper was read at the " SYMPOSIUM ON PYRIMETHAMINE " held on November 21, 1953, at G. R. Medical College, Gwalior, under the auspices of Indian Council of Medical Research.

ACTION AGAINST ASEXUAL ERYTHROCYTIC FORMS.

High degree of activity of pyrimethamine in small doses against *P. gallinaceum* has been observed by Jaswant Singh, Ray and Chandrasekhar (1953) and *P. berghei* by Jaswant Singh, Krishnaswami *et al.* (1952). It has also been said that it is four times more active than proguanil against *P. knowlesi* (Jaswant Singh, Misra *et al.*, 1951). Falco *et al.* (1951) and Schmidt and Genther (1953) report that it is many times more active than proguanil against *P. cynomolgi* infection, while Nair (1953) reports that the M.E.D. of pyrimethamine for Class II effect is higher than proguanil.

Against *P. vivax* infection, opinions are more or less unanimous that majority of cases react well with as small a dose as 25 to 50 mg.

From the reports of Chakravarty and Chaudhuri (1953), Srivastava (1953) Srivastava *et al.* (1953) and our published work (Jaswant Singh, Ray, Misra and Basu, 1952), it would be evident that action of pyrimethamine in *P. falciparum* infection is comparatively slower than any of the 4-aminoquinolines. Moreover some cases are either refractory from the beginning or recrudescence occurs frequently. On the other hand, Schneider *et al.* (1952) and McGregor and Smith (1952) had reported excellent results against *P. falciparum* infection.

From Dr. Laha's observation (Laha *et al.*, 1953*a* : 1953*b*), it would appear that a single dose of 3 ×25 mg. is better than with 2 ×25 mg., but they state that some untoward side effects developed in this dosage. With 4 ×25 mg., the clinical response was in no way better than 3 ×25 mg.

It would seem that at least against certain strains of *P. falciparum*, the drug, in doses of 25 to 50 mg., is not as effective in all cases.

SUPPRESSIVE TREATMENT.

From reports available from this country and abroad, it would appear that it is indeed an effective suppressant in as small a dose as 25 mg. a week. In this dosage, no report of any toxic manifestation has been received so far.

Although we are not aware of the correct price structure, yet it is likely that in view of the comparatively small doses required, it would be available at lower costs than other antimalarials.

At this stage, we have to consider two aspects. Firstly, there is an increasing evidence that pyrimethamine is likely to give rise to resistant strains of plasmodia (*see* below). Secondly, in view of the large scale malaria control project as envisaged under the National Malaria Control Programme, need of large scale use of any antimalarial does not arise.

RADICAL CURE.

Coatney *et al.* (1953) reported that in *P. vivax* infection, if the initial treatment is followed up by a weekly dose of 25 mg. pyrimethamine for eight weeks, a radical cure is effected. This has been termed by them as " suppressive cure ".

In order to determine whether such a regime is really effective under field conditions, similar studies have been taken up at eight centres in India. In addition to pyrimethamine, some cases have been placed under primaquine for comparative studies.

Preliminary reports appear to be encouraging.

TOXICOLOGY.

From laboratory investigations it would appear that pyrimethamine is four times more toxic than proguanil (Jaswant Singh, Misra *et al.*, 1951) and when the drug is administered daily in comparatively large doses, there is considerable damage to the liver and kidneys (Jaswant Singh, Ray, Misra and Basu, 1953).

Hitchings (1952) reported that in prolonged, uninterrupted daily dosage (large) of pyrimethamine, toxic manifestations were seen in rats which are analogous to dietary deficiency syndrome observed after deprivation of folic acid and its congeners. Ramalingaswami and Sriramachari (1953) are now studying the effect of vitamin B_{12} on the toxicity of pyrimethamine in *rhesus* monkeys.

During trials against human plasmodia, no toxic manifestations have so far been recorded in 25 to 50 mg. dosage. However, Laha *et al.* (1953*a*) observed several cases of untowad side effects attributed to pyrimethamine in 75 to 100 mg. single dosage. In some, the condition was severe enough for prompt medical attention.

ACQUIRED RESISTANCE.

Although sufficient evidence relating to development of acquired resistance to pyrimethamine in human malaria is not available at the moment, laboratory studies have shown that high degree of acquired resistance can be built up in *P. gallinaceum* (Wilson *et al.*, 1952), *P. cynomolgi* and *P. knowlesi*, and that a strain resistant to pyrimethamine is cross resistant to proguanil and proguanil analogues and *vice versa* (Jaswant Singh, Ray, Basu and Nair, 1952; Schmidt and Genther, 1953; Jaswant Singh, Ray, Misra and Nair, 1953). Further, it has been shown that a strain of *P. gallinaceum* which has developed resistance to pyrimethamine, retains its character even after mosquito passage (Jaswant Singh, Ramakrishnan *et al.*, 1952).

SYNERGISTIC ACTION.

The observations recently made by Jaswant Singh, Ray, Misra and Nair (1953) and Ray *et al.* (1953) in *P. gallinaceum* and *P. falciparum* that quinine and pyrimethamine potentiate the action of each other, are interesting and open newer lines of studies.

SUMMARY.

To sum up, therefore,

(*a*) the drug has causal prophylactic action against some species of plasmodia like *P. gallinaceum* and *P. falciparum.*

(*b*) It is a good suppressant and is effective for suppressive treatment in small weekly doses of 25-50 mg.

(*c*) It is effective in small doses in most cases of acute infection due to any of the three human plasmodia but its action is somewhat slower than the 4-aminoquinolines. A few cases of *P. falciparum* have been found to be refractory, and early recrudescence has been observed in a few others. It is yet to be seen how far pyrimethamine can be regarded as a suitable drug against Indian strains of *P. falciparum.*

(*d*) Some workers have labelled pyrimethamine as having the potentialities of being a dangerous drug. But majority of the investigators failed to disclose any toxic manifestations attributable to pyrimethamine administered in 25 to 50 mg. dosage. It has also been reported that pyrimethamine has antirelapse properties. Large scale investigations are now in progress in the country, and definite opinion will have to await their completion.

(*e*) Development of acquired resistance to the drug in a few plasmodia has been observed in the laboratory, and this is no doubt disturbing. But it must be said that the process has taken some time after the hosts had been treated with sub-effective doses for a prolonged period. It would, therefore, be interesting to know whether under natural conditions similar resistance could be developed in human cases.

(*f*) Some new light has been thrown regarding the action of quinine and pyrimethamine when administered concurrently, and further studies should be undertaken on these lines.

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