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Hepatocystis semnopitheci in Rhesus Monkey Populations from Northern Pakistan

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Seventy *Macaca mulatta villosa* were examined for the presence of malaria parasites in the blood and gametocytes of *Hepatocystis semnopitheci* were detected in 31 examples. This is apparently the first record of the parasite in rhesus monkeys. The enzootic locality lay in the foothills of the Himalayas at altitudes of 2000-3000 m. *Hepatocystis* spp. have not been found in any species of primate at such altitudes before.

Introduction

Studies on the ecology and behavior of the rhesus monkey, *Macaca mulatta*, have recently been initiated at an area near Dunga Gali in the Murree Hills region of Northern Pakistan. The elevation of the site ranges between 2000-3000 m, and the habitat is a dense forest composed mainly of mixed coniferous trees with interspersed areas of deciduous vegetation. The climate of the Murree Hills is characterized by well below freezing temperatures in winter with accumulated snowfall up to two meters. In the summer, temperatures may exceed 30°C and high rainfall occurs during the July-August monsoon season.

During the process of trapping monkeys for various studies, blood smears were prepared and stained with Giemsa for parasitological examination from some of the animals captured between Oct. 1978 and Aug. 1979. A protozoon, *Hepatocystis semnopitheci* (Knowles, 1919) was found in the peripheral blood of 31/70 monkeys examined (Table 1).

Although the data indicate a seasonal variation in parasitemia, the rates shown may not be representative of the actual prevalence rate for each group. Most of the members in each group usually were trapped, but blood smears were not prepared from all of these captives. The parasitemia rates for the two groups sampled in August indicate that the infection was similar for different groups at any particular point in time. This might be expected since the movements of all groups examined overlapped considerably within the study area. Sex differences in parasitemia rates based on the total rates or on the combined rates for the Kong and Shadow groups, in which all of the members captured were examined except for infants, were not significant. Age was not subdivided into smaller intervals because too few individuals were available in the younger age groups to make the comparison meaningful. A single infant examined from the Shadow group was negative for *H. semnopitheci*, while its mother was parasitemic. No obvious debilitating effect of the infection was observed in any of the animals whether they were captive or free in the forest.

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Table 1.—*Hepatocystis semnopitheci* Infection Rates by Data Captured, Age and Sex for Rhesus Monkey Groups at Dunga Gali, Pakistan

Group	Date captured	Infection rate					
		Adults*		1-5 years		Total	
		Male	Female	Male	Female	Male	Female
Backtail	Oct.-Nov. 1978	(a) 2/9	(a) 1/7	(a) 0/8	(a) 1/4	(a) 2/17	(a) 2/11
		(b) (22.2)	(b) (14.3)	(b) (0 0.0)	(b) (25.0)	(b) (11.8)	(b) (18.2)
Corner Meadow	June 1979	(a) 2/2	(a) 3/3	(a) 5/5	(a) 2/4	(a) 7/7	(a) 5/7
		(b) (100)	(b) (100)	(b) (100)	(b) (50.0)	(b) (100)	(b) (71.4)
Kong	Aug. 1979	(a) 3/4	(a) 1/1	(a) 2/3	(a) 1/4	(a) 2/4	(a) (4/8)
		(b) (75.0)	(b) (100)	(b) (66.7)	(b) (25.0)	(b) (5.00)	(b) (50.0)
Shadow	Aug. 1979	(a) 1/2	(a) 5/7	(a) 2/5	(a) 1/2	(a) 3/7	(a) 6/9
		(b) (50.0)	(b) (71.4)	(b) (40.0)	(b) (50.0)	(b) (42.9)	(b) (66.7)
Total		(a) 5/14	(a) 12/21	(a) 9/21	(a) 5/14	(a) 15/35	(a) 16/35
		(b) (35.7	(b) (57.1)	(b) (42.9)	(b) (35.7)	(b) (42.9)	(b) (45.7)

*Adults were ⩾ 5½ yrs.

Note : (a) No. infected/total examined.
(b) Figures in parentheses indicate % positive.

Morphology of Parasite in Blood

Gametocytes only were present in the blood of infected monkeys; the asexual stages (merocysts) are confined to the parenchyma cells of the liver. In most instances, macrogametocytes were present in greater numbers than microgametocytes; usually the parasites were mature forms and as in all species of *Hepatocystis*, no trace of the infected erythrocyte could be seen. Immature forms were infrequent and were either visible as rings or as larger forms with amoeboid processes and with fine dots of malaria pigment in the cytoplasm.

The macrogametocyte was slightly larger than the size of an uninfected corpuscle and measured between 7.5-8.2 μm in diameter. They were uninucleate, spherical objects, which stained a steely blue color with the red nucleus, placed almost invariably on the periphery. The nucleus (about 2 μm) was oval or round and comprised a lighter outer zone and an inner darker clump, often consisting of 4 darkly staining granules strung out in a line. The cytoplasm was slightly vacuolated.

The microgametocyte was of similar size and also apparently free of the corpuscular envelope. The usual striking color differentiation between the two sexes aided identification; the cytoplasm of the male was much less dense and was very pale. The tiny, light brown pigment granules were thus much more easily seen. The nucleus was considerably larger in the male and occupied one-third or more of the parasite; it measured about 7 × 3 μm and had a pale pink color. The contour of the male tended to be more irregular than that of the female.

Discussion

Hepatocystis semnopitheci has a wide distribution in Southeast Asia and was first described

as a new species of *Plasmodium*, by Knowles (1919) from a langur monkey (*Presbytis entellus*) in Assam. Garnham (1951) showed that the parasite was not a true species of *Plasmodium* and removed it to the genus *Hepatocystis*. The parasite was subsequently found in *Macaca* spp. as well as in other species of langurs, and undoubtedly is frequently present at the rates ranging from 24 to 43% as reported from Asia, and 13% in macaques imported into England (Eyles and Warren, 1963; Garnham, 1966, 1977).

In spite of the number of different species of monkeys found infected with *H. semnopitheci*, no records have hitherto been found of the presence of the parasite in *Macaca mulatta* (subspecies *villosa*). These were captured at an unusually high altitude in the foothills of the Himalayas at altitudes of between 2000-3000 m.

The morphology of the parasite in the blood appears to be identical to that of earlier descriptions, though no semilunar forms were seen in the growing parasite as were noted by Knowles and other workers in the past. However, the appearance of gametocytes has little significance in regard to the taxonomic position which can only be confirmed by the characteristic formation of the merocyst in the liver. The finger-like projections from the body of the merocyst are unmistakable. Unfortunately no material from liver was available in the present investigation.

Acknowledgement

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A Technique of Preparing Thick Blood Films on Soft Slides

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The blood is taken on a plastic transparent film and stained as usual. After staining, the pieces of about 5×5 mm are clipped out, mounted on the glass slide using a transparent scotch tape, and examined under oil immersion. The technique gives a possibility to multiply specimens which seem to be interesting for teaching purposes. Sets of many almost identical slides may be easily produced from a single finger-prick, the similarity of slides being essential in many cases of group training.

Introduction

Frequently malariologists and others have prepared blood films on transparent materials other than standard glass slides. To the author's knowledge, however, these materials were never used in a systematic way, but only in emergency cases when glass slides were not available.

In 1965, O. L. Losev and the writer tried to use washed photographic film for mass blood investigation in Congo. The main purpose was to reduce the weight of the collection, since in some locations included in the itinerary, access was obtained by foot. After completing the journey the transparencies were stained and examined in the traditional way. However, during examination difficulties experienced with focussing due to warping resulted in the technique being abandoned for the time being.

Later, it became clear to the writer that the difficulties with warping might be overcome, if only a small piece of the initial transparency was used for each examination. A small piece might be easily mounted on a glass supporting slide.

In practical work in malariology, only a portion of about 1 to 2% of the area of a standard blood film is examined (the area of a film 2 cm in diameter is about 300 mm^2; the surface corresponding to 100 microscopic fields is about 4 mm).

Despite the simplicity of this idea, the new approach gives unique possibilities in creation of teaching collections of malaria parasites and the technique is described below.

Material and Methods

The equipment needed is the standard equipment used for collection and staining of thick blood films as recommended by WHO (1961). Additional items are :

(a) transparent film for blood collection, e.g. films for overhead projectors. Washed photographic film seems less appropriate, since washing leads to scratches. The film is cut into pieces which might be easily taken by their edges between the thump and the index. They may be of a standard size of 25 × 75 mm, as well.

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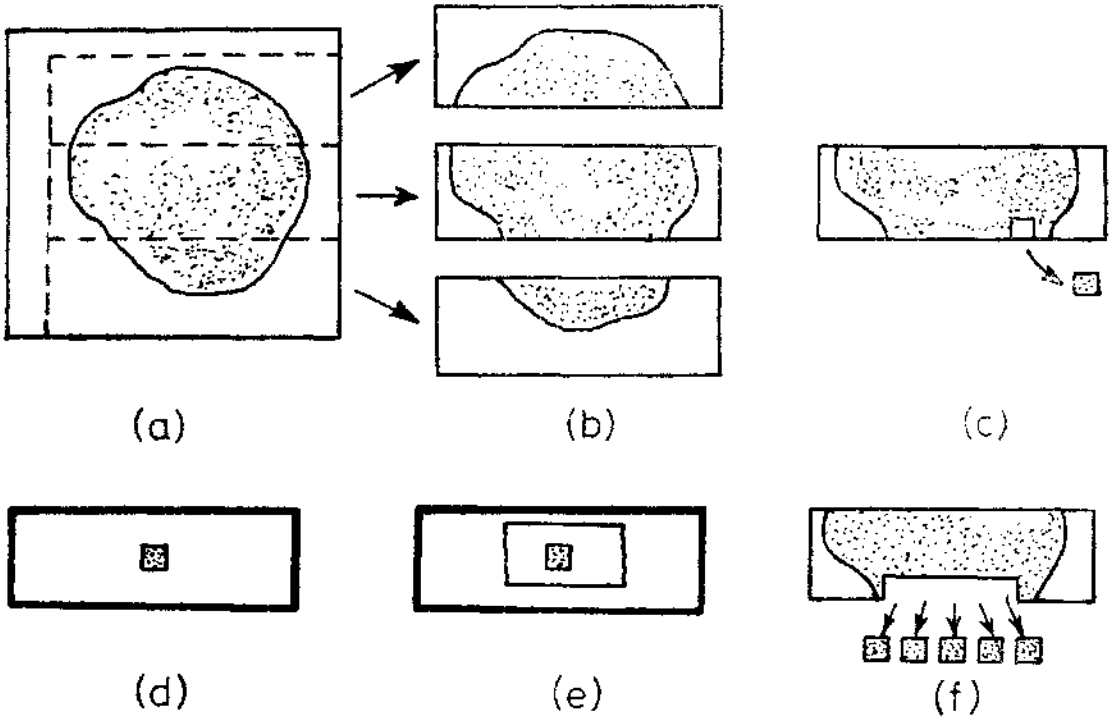

Fig. 1 — Slides of the film (half of the natural size): (a) blood is taken on to a big piece of transparent film; (b) the film is cut into 25×75 mm pieces for staining; (c) a 5×5 mm piece is clipped out from the master slide; (d) the piece is put on a glass slide; (e) covered with adhesive tape and examined under microscope; and (f) new slides are prepared in the same way.

(b) Adhesive colorless transparent tape, e.g. scotch transparent tape

(c) Scissors

(d) Forceps

Blood is taken from a fingerprick and distributed on the transparent film. When the aim is to make teaching preparations for a large group of trainees, as much blood as may be obtained from a fingerprick is used, and the blood is distributed over a big sheet of soft film (Fig. 1a). If only few slides are to be prepared from the specimen, the drop of a normal size is taken, and a standard 25 × 75mm piece of soft film is used. The film is then labelled.

For staining, slides are cut into pieces approximately equal to a normal glass slide, i.e. 25 × 75 mm (Fig 1b) (not necessary if standard 25 × 75 mm slides are used). They are next placed in a standard staining trough with empty glass slides inserted as support. Staining is performed in the usual manner (WHO, 1961).

After drying, a piece of about 15-20 mm^2 is clipped out (Fig. 1c), put on a standard glass slide, the blood being on its upper side (Fig. 1d), and covered with adhesive tape (Fig. 1e). Some care must be taken to reduce the number of air bubbles to minimum which may be trapped between the blood film and the tape.

Slides are examined in a usual way. During focussing, when the oil immersion lens is being lifted after a contact with the slide, the lower part of the transparent film appears first. It contains only few particles of dust and stain.

When continuing the upward motion of the lens, the upper surface of the slide becomes visible. Air bubbles are a good indicator that the upper surface of the slide is being examined.

If, after examination, the microscopic picture seems to be interesting for teaching purposes, new pieces may be clipped out of the master slide and mounted in the same way (Fig. 1f). Usually, not less than 20 good slides may be prepared from a fingerprick. If the parasites demonstrated on the collecting transparency are of no interest, the film may be destroyed.

The covering tape does not react with immersion oil or anisol. After examination, immersion oil may be removed using dry cotton wool, since the surface of blood film is protected. Benzene and xylene may also be used for cleaning, if necessary, since they do not spoil the adhesive covering.

Results

The first preparations were taken on washed photographic film in December 1965. They were stained and examined and then kept as record. In 1975 some of them were mounted using scotch tape as the adhesive cover, results indicated that they were still quite good for examination and continued so until the last examination in September 1981.

From 1975, this technique has been used for preparing slides for teaching purposes especially when it was desirable to demonstrate the same microscopic picture to a large group of students (up to 35-40 persons). This was extremely useful during the initial stages of training and in cases when unusual morphological elements which might be present in rare specimens were to be studied.

The slides could be used for several years and stand repeated examinations even by unskilled microscopists.

Discussion

The following merits of this technique seem to be important (in order of priority) :

(a) A multiplication of specimens which seem to be interesting is possible. The decision to prepare many slides from a particular case may be made after the patient was treated.

(b) Sets of almost identical slides may be easily produced, the similarity of slides being essential in many cases of group training.

(c) Long life of slides being used for training

(d) No need to use benzene or xylene for cleaning slides after examination.

(e) It becomes possible to prepare arrays of different specimens on the same slides, for easy comparison.

(f) Low weight of the master specimen, which (or parts of which) may be easily sent by ordinary letters.

(g) The master slide is unbreakable.

The demerit is that it is impossible to avoid air bubbles. However, they do not cause much trouble since they are met only in few microscopic fields.

The merits of the new technique listed under (a) to (e) offer many advantages when training is concerned. However, for routine blood examinations, the old technique seems superior. The use of the new technique for this purpose is justified only in rare occasions, when the transportation of specimens from the field becomes a problem.

Acknowledgement

The author expresses his gratitude to Mr W. Rooney, Laboratory Specialist, WHO/SEARO, for helpful discussion.

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Pyrimethamine in Combination with Sulfadoxine or Sulfalene in Vivax Malaria in India

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In an endemic area of Arsikere, Hassan District (Karnataka) the effects of three drug regimes were tried on *P. vivax* i.e., sulfadoxine 1000 mg base + pyrimethamine 50 mg base, sulfalene 1000 mg base + pyrimethamine 50 mg base, and chloroquine 600 mg base (all single adult dose). Even this low dosage of chloroquine produced hundred per cent clearance of both sexual and asexual parasites by Day 7, whereas the other two regimes had a failure rate of 11.1 and 23.1% respectively.

In Rajpur, Kanpur District (Uttar Pradesh) results of chloroquine 1500 mg base were compared with two dose levels of sulfalene + pyrimethamine combination (i.e., 1000+50 and 1500+75). There was complete clearance of all stages on Day 6 with all the three regimes.

Introduction

The use of long-acting sulfa drugs like sulfadoxine or sulfalene with pyrimethamine as a single dose therapy has gained popularity in the treatment of malaria in the recent past. Though these drug combinations are of special importance in chloroquine resistant *P. falciparum*, these drugs are widely used for the treatment of other species also. *P. vivax* constitutes about 90% of all malaria cases in India (Pattanayak and Roy, 1980). Results of a trial in two states to find out the comparative efficacy of these two drug combinations in relation to chloroquine are presented in this paper.

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Material and Methods

The study was started with a mass blood survey in two widely separated states i.e., Rajpur (Kanpur District of Uttar Pradesh) and Arsikere (Hassan District of Karnataka). In Rajpur, the study area comprised of six villages with 31 567 population and in Arsikere, it comprised of 5 villages with 21 900 population. In both the areas, *P. vivax* was practically the sole plasmodial species and chloroquine resistance had not so far been reported in this species. Patients with similar age group from indigenous populations were taken for the experiment. The areas being endemic, it was presumed that the patients were semi-immune to malaria. In Rajpur, two different doses of sulfalene and pyrimethamine, i.e., sulphalene 1000 mg base plus pyrimethamine 50 mg base (SL 1000+P 50) and sulfalene 1500 mg base plus pyrimethamine 75mg base (SL 1500+P 75) were compared with chloroquine 1500 mg base which was administered over a period of three days (600 mg base on Day 0 and Day 1

and 300 mg base on Day 2). In Arsikere, sulfadoxine 1000 mg base with pyrimethamine 50 mg base (SD 1000+P 50) as well as sulfalene 1000 mg base with pyrimethamine 50 mg base (SL 1000+P 50) were compared to the effects of chloroquine 600 mg base.

Each SDP and SLP tablet consisted of 500 mg base sulfadoxine or sulfalene in combination with 25 mg base pyrimethamine. Dose was regulated according to age of the patient, i.e., 1-4 years, 5-8 years, 9-14 years, and 15 years and above receiving one-fourth, half, three-fourth, and full dose (two or three tablets), respectively. SDP and SLP in different dose levels were administered as a single dose.

One-hundred standard thick smear fields were examined. Parasite count per microlitre was expressed on the basis of count against 300 leucocytes by taking 7 500 leucocytes as normal count per microlitre of blood. In Arsikere, both asexual and sexual stage count were noted separately, but in Rajpur, all stages were counted together.

Dill and Glazko test (Lelijveld and Kortmann, 1970) was employed to find out the presence of 4-aminoquinolines in the urine and Lignin test for detecting the presence of sulfonamides in the urine, on Day 0 before giving the drug (s) and one day after (Day 1).

Primaquine was not administered with either chloroquine or SDP or SLP. Immediate response was the main objective of the study and the impact on relapse phenomenon was not studied.

Results

Results are given in Table 1. In Arsikere, both SDP and SLP (1000+50) had a failure rate of 11.1 and 23.1% respectively though the parasite density in the unsuccessful cases was extremely low in SLP. With SDP, the reduction of asexual stage was slightly more pronounced than on sexual stage but with chloroquine, both the sexual and asexual stages were cleared by Day 7.

In Rajpur, total parasite clearance was noted in SLP (both the doses) as well as in chloroquine. With SL 1000+P 50 and SL 1500+P 75, the clearance was earlier to chloroquine by one day i.e., Day 5 and Day 4 against Day 6 in chloroquine. No untoward reaction was observed in any group, and the drug (s) was accepted well.

Discussion

When used alone, sulfonamides and sulfones have little, if any, action, on *P. vivax* (WHO, 1973). Laing (1968) in Malaysia carried out a trial with chloroquine, sulfadoxine and combination of sulfadoxine and pyrimethamine, and found that with chloroquine the response was quick in all 13 cases. With only sulfadoxine, there were 5 failures out of 14 and the action was slow in the remaining 9 cases and gametocytes persisted. With SDP in varying combinations, there were two failures and gametocytes persisted. In the same species of malaria, a study was carried out in Thailand (Doberstyn *et al.*, 1979) on the relative efficacy of pyrimethamine 50 mg base versus sulfadoxine in combination with pyrimethamine. The latter combination was used at two dose levels, i.e., 1000+50 and 1500+75. It was found that pyrimethamine 50 mg base was able to clear the parasites in only one-third of the cases by Day 7. With SDP, due to "sequential block" type of action, better results were obtained and three-fifths of the cases showed parasite clearance on Day 7 but with the higher dose, all the vivax cases showed clearance. They also observed that the mean parasite clearance time was relatively long (90 hr) even with the higher dose and concluded that chloroquine remained the drug of choice in the treatment of vivax malaria.

Table 1- Results of Treatment with Sulfadoxine-Pyrimethamine, Sulfalene-Perimethamine and Chloroquine in *P. vivax* Susceptible to Chloroquine: July-November, 1979

Drug (s) mg base (adult)		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 14	Day 21
(A) Arsikere (Hassan District, Karnataka)											
SDP (1000+50)	(a)	9/9	6/8	4/9	—	3/9	—	—	1/9		
	(b)	3305	10007	526	—	379		—	181		
	(c)	53	1207	2321	—	88	—	—	236		
Chloroquine (600)	(a)	15/15	4/15	4/15	—	3/15	—	—	0/15		
	(b)	2134	142	33	—	4	—	—	0		
	(c)	1435	110	0	—	180	—	—	0		
SLP (1000+50)	(a)	13/13	10/12	7/12	—	6/13		—	3/13		
	(b)	3348	713	315	—	28	—	—	3		
	(c)	1323	957	541	—	66	—	—	2		
(B) Rajpur (Kanpur District, U.P.)											
SDP (1000+50)	(a)	12/12	12/12	12/12	4/12	3/12	0/12	0/12	0/12	0/12	0/12
	(b)	256.3	64.3	22.5	2.5	1.3	0	0	0	0	0
SLP (1500+75)	(a)	11/11	11/11	7/11	3/11	0/11	0/11	0/11	0/11	0/11	0/11
	(b)	304	86	25	9	0	0	0	0	0	0
Chloroquine (1500)	(a)	15/15	15/15	10/15	5/15	3/15	1/15	0/15	0/15	0/15	0/15
	(b)	208	59	19	6	3	1	0	0	0	0

Note : (a) Number positive for asexual parasite/number examined.
(b) Asexual parasite density per ml.
(c) Sexual parasite density per ml.

In the present study, pyrimethamine effect without adding any other drug was not studied, as the main objective was to find out the relative merit of SDP and SLP compared to chloroquine. In Arsikere, SDP (1000+50) showed a clearance rate of 89.9% against 100% success with 600 mg base of chloroquine. SLP (1000+50) gave different results in Arsikere and Rajpur. In the former area, on Day 7, the failure rate was 23.1% but in the latter there was no failure at all. When compared to chloroquine 1500 mg base even the lower dose of SLP 1000 + 50) was slightly superior as complete parasite clearance was noted on Day 5 against Day 6 in chloroquine group. Higher dose of SLP (1500+75) was slightly superior to the lower dose SLP (1000+50) by one day.

Within the short period of observation (21 days) in Rajpur, there was no relapse in any case, though no primaquine was administered. Reasons for the differential response in Arsikere and Rajpur are not clear. It could be due to the difference in the immunity status or strain difference or any other factor like the

susceptibility of the vivax parasites to pyrimethamine. Some people are known to be rapid acetylators of sulfa drug and whether such persons were more in Arsikere group is not known. Since the number of persons treated is small, further studies are desirable in different areas of India. In the mean while, indiscriminate use of SDP or SLP in the treatment of all types of malaria should be discouraged and these drugs should be reserved for chloroquine resistant cases.

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Indirect Haemagglutination and Indirect Fluorescent Antibody Tests for Human Malaria Using *Plasmodium knowlesi* Antigen

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Plasmodium knowlesi antigens preserved in liquid nitrogen (−196°C) have been successfully employed in the indirect haemagglutination (IHA) and indirect fluorescent antibody (IFA) tests for serodiagnosis of human malaria infections. Using glutaraldehyde-fixed sheep erythrocytes, tanned and sensitised with soluble *P. knowlesi* antigen, positivity of 75, 8.82, 4.23 and 4% was obtained among patients of malaria with characteristic splenomegaly or hepatosplenomegaly (60 cases), pyrexia of varied origin (34 cases), random hospital patients (133 cases), and healthy subjects (50 cases), respectively. The IFA test gave positive diagnosis in 89.47% of the malaria cases and 11.76% cases of pyrexia of varied origin. None of the random hospital patients or the healthy subjects gave a positive IFA test. This study strongly suggests the possible use of *P. knowlesi* antigen for serodiagnosis of human malaria. The IFA test has special advantage that it can be used for screening of small number of sera.

Introduction

Serologic tests for malaria have been reported to be useful for studying malarial endemicity rates, patterns of malaria transmission, and to detect foci of malaria in the epidemiological surveys (Kagan, 1972). Indirect haemagglutination (IHA) and indirect fluorescent antibody (IFA) tests have been widely applied for the serodiagnosis of human malaria (Farshy and Kagan, 1972; Wilson *et al.*, 1975). Use of homologous antigens is reported to yield more positive results (Meuwissen *et al.*, 1974; Sulzer, *et al.*, 1973). However, due to non-availability of the owl-monkey, *Aotus trivirgatus*, the experimental host for *Plasmodium falciparum*, and long delay expected in large scale *in vitro* culture of *P. falciparum* to produce malaria antigens for serodiagnostic work in our country, it was desired by the Indian Council of Medical Research to standardise *P. knowlesi* antigen for the serodiagnosis of human malaria (Task-Force Meeting on Serology of Malaria held in Oct. 1980, ICMR Report). In the present communication, we report our results on the utility of liquid nitrogen preserved and unlyophilised *P. knowlesi* antigen in the IHA and IFA tests for the serodiagnosis of human malaria.

Material and Methods

Sera — Serum samples were obtained from the four groups of subjects : Group I included 60 patients of malaria positive for *Plasmodium vivax* by slide test. They gave a history of characteristic periodic high fever with chills. Most of these cases also had splenomegaly or hepatosplenomegaly. Group II comprised of 34 patients of pyrexia of varied origin; Group III included 133 random hospital patients with no signs/symptoms or history of malaria; and

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Group IV consisted of 50 medical students apparently in good health and having no history of malaria. All sera were stored at −20°C and inactivated at 56°C for 30 min prior to use.

Parasite — *Plasmodium knowlesi* was routinely maintained in the healthy, tuberculin negative rhesus monkeys (*Macaca mulatta*) at this Institute (Banyal *et al.*, 1981). Monkeys showing parasitaemia ⩾ 50% were bled at schizont stage by cardiac puncture. The blood collected in the acid-citrate dextrose solution was centrifuged at 1500 rpm for 10 min. The schizont infected erythrocytes sedimented as a distinct chocolate brown layer just beneath the "buffy coat". The 'buffy coat' was carefully removed and discarded. The brown layer was aspirated and layered on a Ficoll-conray 420 density gradient (density equivalent to 1.07) and centrifuged at 400 g for 20 min at 20°C. The schizont infected erythrocytes appeared as a distinct band at the interphase and were aspirated and washed thrice with chilled normal saline in a refrigerated centrifuge. After the final washing, the sediment was used as source of antigen. It was divided into small aliquots and stored at −196°C in the liquid nitrogen.

IHA Test — On the day of use, an aliquot of the washed schizont infected erythrocytes was removed from the liquid nitrogen, diluted ten times with phosphate buffered saline, (*p*H 7.5, 0.15 *M*) and subjected to ultrasonication, 20Kc per sec, for 10 sec at 4°C. The ultrasonicate was centrifuged at 4000 g for 1 hr at 4°C. The sediment which largely contained pigment granules was discarded while the supernatant was used as antigen in the IHA test.

Determination of optimal sensitising dilution of antigen, and sensitisation of the glutaraldehyde fixed and tanned sheep erythrocytes were carried out as recommended by WHO (1974).

The IHA test was performed in the 96 wells (U-shaped) polystyrene microtitre plates (Laxbro, Pune) as described elsewhere (Sharma *et al.*, 1980).

IFA test — The schizont rich brown layer of parasitised cells were washed thrice with phosphate buffered saline (*p*H 7.2, 0.15 *M*) by centrifugation at 4°C. After the final washing the parasitised red cells were suspended to the original blood volume in the phosphate buffered saline, *p*H 7.2. Eight drops of this suspension were placed on each of the clean, grease free, microscope slides and allowed to dry at room temperature. The dry antigen slides were wrapped individually in the filter paper and stored at −196°C in the liquid nitrogen. Fluorescein isothiocyanate tagged anti-human (heavy and light chains) IgG produced in goat obtained from the Cappel Laboratories, Cochranville, USA was used for IFA test. A preliminary study indicated that 1:40 dilution of the original fluorescent conjugate was the optimal dilution for IFA studies.

The test was performed essentially as described by Collins and Skinner (1973) except that the antigen coated slides were fixed in dry acetone for 30 min at −20°C. The slides were examined using HBO 200 W as the light source in the "Fluorolume" fluorescent microscope (American Optical, Inc.) with a combination of appropriate excitor and barrier filters.

Reactions were graded as − to + + + + depending upon the intensity of fluorescence and the last dilution of a serum showing + + reaction was considered the end point (Voller and O'Neil, 1971).

Results

Results of the IHA test are presented in Table 1. Analysis of the data indicates that IHA titre of 1 in 32 may be taken as the cut-off point for the specific diagnosis of human malaria. At this titre, 75% of the

Table I -- Reciprocal IHA Titres in Different Groups Using *P. knowlesi* Antigen

Group		No.	GMRT	No. of cases showing reciprocal titre											
				4096	⩾2048	⩾1024	⩾512	⩾256	⩾128	⩾64	⩾32	⩾16	⩾8	⩾4	⩽2
I.	Malaria patients (*P. vivax* infection)	60	70.790	3 (5)	5 (8.33)	8 (13.33)	9 (15)	12 (20)	17 (28.33)	36 (60)	45 (75)	55 (91.66)	59 (98.33)	60 (100)	Nil
II.	Pyrexia of varied origin	34	4.898	Nil	Nil	Nil	Nil	Nil	Nil	1 (2.94)	3 (8.82)	5 (14.70)	10 (29.41)	25 (73.53)	9 (26.47)
III.	Random hospital patients	118	4.571	Nil	Nil	Nil	Nil	Nil	Nil	2 (1.69)	5 (4.23)	20 (16.94)	39 (33.05)	75 (63.55)	43 (36.45)
IV.	Healthy subjects	50	3.548	Nil	Nil	Nil	Nil	Nil	Nil	Nil	2 (4)	6 (12)	13 (25)	21 (42)	29 (58)

Note : GMRT = Geometric Mean Reciprocal Titre.

Figures in parentheses indicate cumulative percentages.

Table 2 — Reciprocal IFA Titres in Different Groups Using *P. knowlesi* Antigen

Group	No.	GMRT	No. of cases showing reciprocal IFA titre								
			512	⩾256	⩾128	⩾64	⩾32	⩾16	⩾8	⩾4	⩽2
I. Malaria patients (*P. vivax* infection)	57	66.07	6 (10.52)	12 (21.05)	26 (45.61)	39 (68.42)	42 (73.68)	52 (89.47)	54 (94.73)	57 (100)	Nil
II. Pyrexia of varied origin	34	3.631	Nil	Nil	Nil	Nil	1 (2.94)	4 (11.76)	8 (23.52)	16 (47.05)	18 (52.95)
III Random hospital patients	133	2.089	Nil	Nil	Nil	Nil	Nil	Nil	Nil	8 (6.01)	125 (93.99)
IV. Healthy subjects	50	2.18	Nil	Nil	Nil	Nil	Nil	Nil	Nil	7 (14)	43 (86)

Note : GMRT = Geometric Mean Reciprocal Titre.
Figures in parentheses indicate cumulative percentages.

malaria patients (Group I) yielded a positive reaction. However, 8.82, 4.23 and 4% of patients of pyrexia of varied origin (Group II), random hospital patients (Group III), and healthy subjects (Group IV), respectively, also gave positive IHA result. Highest titres were obtained in Group I, geometric mean reciprocal titre of this group being 70.79 as compared to 4.898, 4.571 and 3.548 of groups II, III, IV respectively.

Data presented in Table 2 indicate that at an IFA titre of 1 in 8, none of the random hospital patients (Group III) and healthy subjects (Group IV) gave a positive reaction. However, an IFA titre of 1 in 16 was taken as the cut-off point in order to impart maximum specificity to the test. At this titre, 89.47% malaria patients (Group I) and 11.76% of the patients of pyrexia of varied origin (Group II) yielded a positive IFA reaction. The geometric mean reciprocal IFA titres of Groups I to IV were 66.07, 3.631, 2.089 and 2.180 respectively.

Discussion

Results of the present study clearly indicate that antigens prepared from *P. knowlesi* can be successfully employed in the IHA and IFA tests for serodiagnosis of malaria. Seventy-five per cent positivity with the IHA test and nearly 90% positivity with the IFA test among the cases of malaria recorded in the present study are in broad agreement with the results reported by others using heterologous antigen (Lobel *et al.*, 1973; Sulzer *et al.*, 1969). IFA test with *P. knowlesi* antigen has shown better diagnostic value than the IHA test as the former could detect nearly 90% of the infections, while the latter only 75% of the infections. The high degree of specificity resided in both the tests as is evident from the very low positivity (nearly 4%), obtainedamong random hospital patients (Group II) and healthy subjects (Group IV). An IFA positivity of 11.76% and IHA positivity of 8.82% among the patients of pyrexia of varied origin (Group II) suggest that a small number of these cases may indeed be suffering from malaria.

Our IHA results are, however, at variance with those reported by Chandanani *et al.* (1981). Using a cut-off titre of 1 in 8, these authors obtained very low IHA positivity (47.2%) among the malaria patients using *P. knowlesi* antigen. Apart from the fact that Chandanani *et al.* (1981) were studying cases of early infections, differences in the method for preparations of *P. knowlesi* antigen and storage conditions may explain low titres reported by these workers. Using intact schizont, antigen stored at −196°C, we could employ the cut-off titre of 1 in 32 for diagnosis of malaria. In fact WHO (1974) has also recommended storage of parasitised erythrocytes rather than their lysate at −70°C and the ultrasonic disruption of the parasitised erythrocytes to get the soluble antigen on the day of use.

In our opinion, storage of malaria antigen at −196°C gives best results in the IHA and IFA tests.

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New Antimalarials : Synthesis and Study of 4,6-Diamino-1,2-dihydro-1-(haloalkoxyphenyl)-2,2-dialkyl (or aryl)-*s*-triazines as Possible Antimalarial Agents-I

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In the course of efforts to develop new chemotherapeutic agents, a series of novel 1-aryl-4,6-diamino-1,2-dihydro-*s*-triazines were prepared from various dihalo- and monohalo-alkoxy anilines and antimalarial activity of these synthetic agents was evaluated using Rane's rapid screen technique.

Introduction

4,6-Diamino-1,2-dihydro-*s*-triazines, a class of compounds possessing a wide spectrum of medicinal properties have been studied for activity against the common malarial plasmodia of mammals and birds (Capps *et al.*, 1968; Thompson and Werbel, 1972); pathogenic and nonpathogenic bacteria (Modest *et al.*, 1952; Capps *et. al.*, 1968); coccidia (Schalit and Cutler, 1959); toxoplasma (Winter and Foley, 1956); intestinal helminths (Schalit and Cutler, 1959; Roth *et al.*, 1963; Capps *et al.*, 1968); and against experimental tumors (Rosowsky *et al.*, 1973). Many dihydrotriazines are also good enzyme inhibitors in microbiological systems (Modest *et al.*, 1952; Baker, 1967). In the past few years cycloguanil pamoate (I) and other dihydrotriazine salts that exhibit remarkable antimalarial properties in experimental animals have been reported. Dihydrotriazines prepared by Capps *et al.* (1968) were found to possess activity against various types of pathogens in biological screening.

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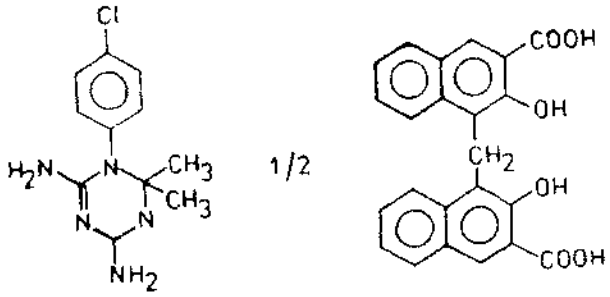
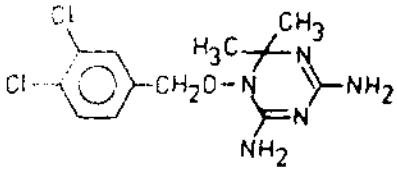
Very recently, the synthesis of clociguanil i.e., WR-38,839 (II) and WR-99,210 (III) which are DHFR inhibitors and are closely related to cycloguanil have been reported to exhibit remarkable antimalarial activity against both pyrimethamine sensitive and resistant strains of *Plasmodium berghei* in the mouse, and active against chloroquine sensitive and resistant or pyrimethamine sensitive and resistant *P. falciparum* in the owl monkey (WHO, 1973).

In man, single intramuscular dose of cycloguanil pamoate has the unusual capacity to protect man for many months against challenges with susceptible strains of *P. vivax, P. falciparum, P. malariae* and *P. ovale* (Elslager, 1967).

Considering the promising activity exhibited by cycloguanil and the special interest shown by WHO in cycloguanil analogous dihydrotriazines for their antimalarial programme, a variety of 1-haloalkoxyphenyl-4, 6-diamino-1,2-dihydro-*s*-triazine derivatives were synthesised from various 3,5-dihalo- and monohalo alkoxy anilines according to the scheme outlined in Chart-I. These aniline derivatives have produced several active biological agents in various fields of medicinal chemistry in our laboratory,



(I)



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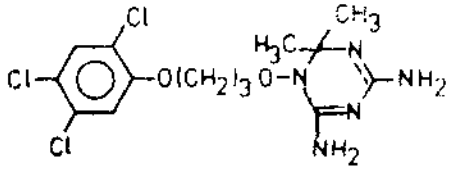


(III)

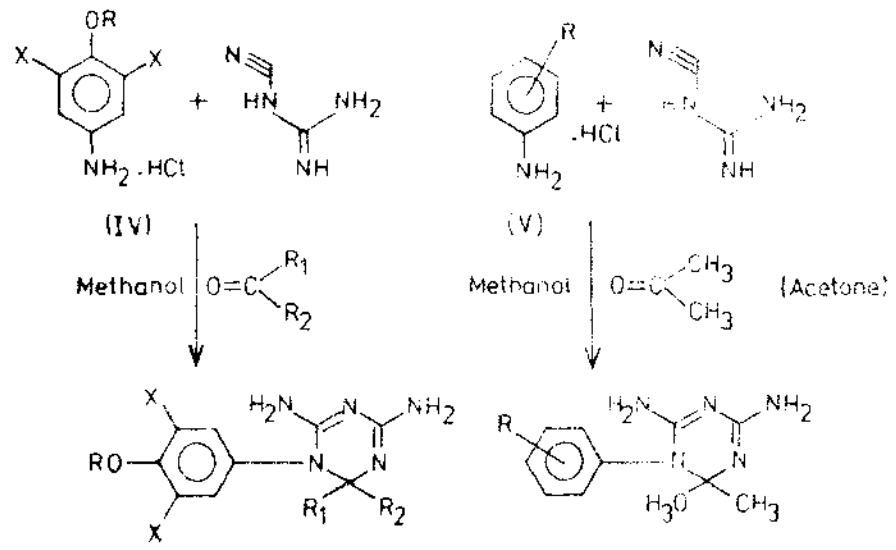
e.g., compounds derived from these anilines have been found to possess anthelmintic (Khadse *et al.*, 1976) and anticancer (Shekawat *et al.*, 1972) activities in experimental test system in mice. Very recently pyrimidine compounds obtained from these haloalkoxy anilines have also shown significant antimalarial activity against *P. berghei yoelii* in mice (Sanghavi *et al.*, 1980).

Chemistry

These aryldihydrotriazines (VI) (Tables 1 & 2) were synthesised by the condensation in one step of haloalkoxyaniline hydrochloride (or arylamine and an equivalent mole of hydrochloric acid), dicyandiamide and a ketone or an aldehyde in presence of absolute methanol at reflux temperature from 2 to 24 hours, preferably for 17 hr by three component synthesis (Capps *et al.*, 1968). Alternatively, the required dihalo- (IV) and monohalo-alkoxy aniline (V) were prepared according to the reported methods (Shekawat *et al.*, 1972).

In alkali, or upon heating to the melting point, 1-aryl-4, 6-diamino-1,2-dihydro-*s*-triazines undergo a characteristic and irreversible rearrangement to their 6-arylamine isomers. This rearrangement is accompanied by characteristic changes in the ultraviolet absorbance (Roth *et al.*, 1963; Capps *et al.*, 1968).

Most of the dihydrotriazines (VI) (Tables 1 & 2) described in this work were checked for their appropriate spectral changes upon heating in 0.5*N* sodium hydroxide to confirm that the indicated isomers were obtained. In this work, practically all of the diamino dihydrotriazines have λ_{max} between 234-242 μm in agreement with the UV spectra of the similar dihydro-*s*-triazines were reported earlier (Roth *et al.*, 1963; Capps *et al.*, 1968; Thompson and Werbel, 1972). Representative members of each series were studied for infra-red spectra and nuclear magnetic resonance spectrum. All the compounds have IR spectra compatible with their assigned structure.



(VI)

Where, R = H, CH_3, C_2H_5, C_4H_9, etc
R_1 = H, CH_3;
R_2 = CH_3, C_2H_5, C_3H_7, C_6H_5, etc
X = Br, Cl, I, etc.

Where, R = 4-X-3-OCH_3 ; X = Br, Cl, I.
5-Br-2-OCH_3 ;
5-Br-2-OC_2H_5 ;
5-Br-2-OH;
3-Br-4-OC_3H_7, etc

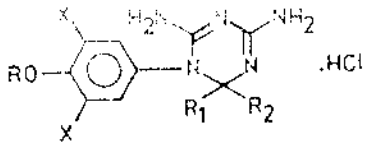
CHART-I

Experimental Procedure

4,6-Diamino-1-(3',5'-diiodo-4'-methoxyphenyl)-2-ethyl-2-methyl-1,2-dihydro-s-triazine hydrochloride (19, Table 1) — A mixture of 0.412g (0.001 mole) of 3,5-diiodo-4-methoxy aniline hydrochloride, 0.089g (0.00107 mole) of cyanoguanidine, 3.0 ml of ethyl methyl ketone (2-butanone), and 0.2 ml of methanol when stirred at 35-40°C over a period of 1 hr, preferably under anhydrous condition, became a clear amber solution, and crystalline product began to be thrown out shortly thereafter. After few minutes, the reaction was stopped, the product was collected by filtration and washed with ethyl methyl ketone (I). The washings and the filtrate were transferred to the flask and the solution was heated to 30-40°C with stirring for 24½ hr. The reaction mixture was concentrated, refrigerated overnight and the crystalline material thus deposited was collected and washed again with ethyl methyl ketone and ether (II). Recrystallisation of the combined products (I and II) from water afforded the title compound (monohydrate) as colourless prisms [Yield : 0.363 g (64.0%); m.p. 221-222°C (uncorrected); Elemental analysis for $C_{13}H_{17}I_2N_5O.HCl.H_2O$: Calc. : C 27.49, H 3.52, N 12.34%; Found : C 27.21, H 3.27, N 12.19%].

4,6-Diamino-1-(3',5'-dichloro-4'-methoxyphenyl)-2-phenyl-1, 2-dihydro-s-triazine hydrochloride (4, Table 1) — A mixture of 0.457 g (0.002 mole) of 3,5-dichloro-4-ethoxyaniline hydrochloride,

Table 1 — 4,6-Diamino-1,2-dihydro-1-(3',5'-dihalo-4-alkoxyphenyl)-2,2-dialkyl (or aryl)-s-triazine hydrochlorides (VI)



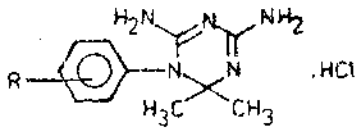
Compound	X	R	R_1	R_2	Yield %	m.p. °C (uncorrected)	Mol. formula†
1	Cl	H	CH_3	CH_3	61	225-226	$C_{11}H_{13}Cl_2N_5O.HCl.H_2O$
2	Cl	CH_3	CH_3	CH_3	48	219-221	$C_{12}H_{15}Cl_2N_5O.HCl.H_2O$
3	Cl	CH_3	CH_3	C_2H_5	51	195-196	$C_{13}H_{17}Cl_2N_5O.HCl$
4	Cl	CH_3	H	C_6H_5	70	218-219	$C_{16}H_{15}Cl_2N_5O.HCl.H_2O$
5	Cl	C_2H_5	CH_3	CH_3	55	223-225	$C_{13}H_{17}Cl_2N_5O.HCl$
6	Cl	C_2H_5	CH_3	C_2H_5	60	196-198	$C_{14}H_{19}Cl_2N_5O.HCl$
7	Cl	C_2H_5	H	C_6H_5	78	237 (d)	$C_{17}H_{17}Cl_2N_5O.HCl$
8	Br	H	CH_3	CH_3	65.5	204-206	$C_{11}H_{13}Br_2N_5O.HCl.H_2O$
9	Br	H	CH_3	C_2H_5	54	215 (d)	$C_{12}H_{17}Br_2N_5O.HCl.H_2O$
10	Br	CH_3	CH_3	CH_3	64	215-217	$C_{12}H_{15}Br_2N_5O.HCl$
11	Br	CH_3	CH_3	C_2H_5	60	210-212	$C_{13}H_{17}Br_2N_5O.HCl$
12	Br	CH_3	H	C_6H_5	42	214-215	$C_{16}H_{15}Br_2N_5O.HCl.H_2O$
13	Br	C_2H_5	CH_3	CH_3	90	204-205	$C_{13}H_{17}Br_2N_5O.HCl$
14	Br	C_2H_5	CH_3	C_2H_5	68	220-222	$C_{14}H_{19}Br_2N_5O.HCl$
15	Br	C_2H_5	H	C_6H_5	76	215	$C_{17}H_{17}Br_2N_5O.HCl$
16	Br	C_3H_9	CH_3	CH_3	42	183-184	$C_{15}H_{21}Br_2N_5O.HCl$
17	I	H	CH_3	CH_3	59	198-200	$C_{11}H_{13}I_2N_5O.HCl.H_2O$
18	I	CH_3	CH_3	CH_3	59	220	$C_{12}H_{15}I_2N_5O.HCl$
19	I	CH_3	CH_3	C_2H_5	64	221-222	$C_{13}H_{17}I_2N_5O.HCl.H_2O$
20	I	C_2H_5	CH_3	CH_3	58	215-216(d)	$C_{13}H_{17}I_2N_5O.HCl$

* Capillary melting points.

† Analysed correctly for C, H and N within ± 0.45%.

0.175 g (0.00208 mole) of dicyandiamide, 0.320 g (0.003 mole) of benzaldehyde, and 2 ml of 95% ethanol was refluxed for 6 hr. It became a clear solution. Shortly thereafter crystalline product was deposited. Reaction was stopped after 7 hr and the reaction mixture was cooled. After thorough cooling, the product was collected, washed with acetone and ether, dried in vacuum desiccator over calcium chloride for 12 hr. This afforded light brown prisms of the title compound of analytical purity [Yield : 0.586 g (70.0%); m.p. 218-219°C (uncorrected); Elemental analysis for $C_{17}H_{15}Cl_2N_5O.HCl.H_2O$: Calc. : C 45.88, H 4.30, N 16.73%; Found : C 46.28, H 4.19 N 17.19%].

Table 2 — 4,6-Diamino-1,2-dihydro-1-(monohaloalkoxyphenyl)-2,2-dimethyl-*s*-triazine hydrochlorides (VI)



Compound	R	Yield (%)	m.p. °C (uncorrected)*	Mol. formula†
21	3,6-Br, OH	58	233-235	$C_{13}H_{14}BrN_5O.HCl$
22	3,6-Br, OMe	85	217-218	$C_{12}H_{16}BrN_5O.HCl.H_2O$
23	3,6-Br, OEt	60	213-214	$C_{13}H_{18}BrN_5O.HCl.H_2O$
24	4,5-Br, OMe	81	216 (d)	$C_{12}H_{16}BrN_5O.HCl$
25	4,5-Cl, OMe	75	209	$C_{12}H_{16}ClN_5O.HCl$
26	4,5-OPr, Br	52	210 (d)	$C_{14}H_{20}BrN_5O.HCl.H_2O$

* Capillary melting points.

† Analysed correctly for C, H and N within ±0.45%.

Biological Activity

Evaluation of antimalarial activity by the assessment of survival time (primary screening) — The antimalarial activity of compounds was evaluated using *P. berghei yoelii* mouse model. The Rane's blood schizonticidal test described by Osdene *et al.* (1967) was used for screening the compounds. This is a virulent strain and produced death of infected mice in an average period of 8.5±0.5 days. Extension of the mean survival time of treated albino mice (MSTT) is interpreted as evidence of antimalarial activity. The tests were based upon the relative response of *P. berghei yoelii* malaria in mice to each of the administered compounds as expressed by the mean survival time of treated animals (MSTT) and mean survival time of controls (MSTC).

Albino mice of H.I. strain approximately weighing 20 g received a standard i.p. inoculum of approx. 1×10^6 infected donor erythrocytes suspended in citrated saline. Test compounds were dissolved in distilled water or suspended in Tween-80 and injected once s.c. 72 hr after the mice were infected with trophozoites of *P. berghei yoelii*. In each experiment, test compounds were administered in six graded doses of 160, 80, 40, 20, 10 and 5 mg/kg. A minimum of five animals per dose were used. If the drug proved to be toxic, lower dosages were tried. Five mice were used as controls. The control mice were not administered any drug. Besides, a group of five mice was treated with pyrimethamine and another group of five mice received cycloguanil hydrochloride. These drugs were used as standard drugs at doses 4.5 mg/kg and 25 mg/kg respectively and were included in all trials for comparison.

Results and Discussion

Untreated animals died between 8-9 days and had a mean survival time (MSTC) of 8.5±0.5 days. Treated animals were kept under observation for 40 days. Compounds having double the survival time as compared to controls were considered to be active (A) when they produced at least 100% increase in MST of treated mice. Animals which survived for

Table 3 — Antimalarial Activity Against *P. berghei yoelii* in Mice by Rane's Method

Compound*	Dosages (mg/kg)					
	5	10	20	40	80	160
1	—	7.4	—	7.4	—	7.9
5	—	—	—	10.8 (A)	—	4.0 (T)
6	—	—	9.4	—	8.0	3.6
7	—	5.7	—	4.0 (C)	—	4.0 (C)
8	—	—	8.8	—	7.5	—
10	—	4.7	—	2.0	—	3.0 (T)
13	—	5.7	—	9.5	—	8.8
16	—	—	3.8	10.0 (A)	—	10.0 (A)
18	2.6	—	2.0 (T)	—	4.0	—
19	—	7.5	9.5	2.0	3.0 (T)	4.0 (T)
20	23.5 (A)	10.5	1.2	4.0 (T)	5.0 (T)	—
23	2.8	—	7.7	—	1.0 (T)	—
24	—	—	—	21.4 (A)	23.4 (A)	—
25	—	—	8.6	19.5 (A)	—	2.0 (T)

* Compounds refer to those from Table 1 and 2. Hydrochlorides of these compounds were screened.

Note : (—) represents no test being done. In each run pyrimethamine and cycloguanil hydrochloride were included as standard drugs for comparison.

more than 40 days and showed no parasitaemia were classed as cured (C).

Deaths prior to MSTC were regarded as due to toxicity (T) to mice. The dose at which double the survival time is attained is considered as the minimum effective dose (MED). All drugs showing a survival time of 14-18 days in mice were examined for presence of parasites and parasite counts were recorded.

In all, 26 dihydrotriazines were synthesised and 14 representative members (Table 3) were screened for their possible *in vivo* antimalarial activity by Rane's test (Walter Reed Army Institute of Research, Personal Communication, 1973). Activity determined vs. *P. berghei yoelii* is presented in Table 3. Out of these, nine compounds have shown activity in doses ranging from 5.0 to 160 mg/kg. The compounds **7**, **16**, **20**, **24** and **25** have shown some promise for further investigations. Compounds **3** and **4** were active and non-toxic in the doses of 40, 80 and 160 mg/kg. **7** Gave four cures out of five animals treated at both dose levels of 40 and 160 mg/kg. While, the chloro analogue (**25**) of compound **24**, though suppressive at 40 mg/kg, was found to be toxic at 160 mg/kg. Further compounds **5**, **6**, **8** and **13** exhibited activity in doses from 20 to 160 mg/kg. The other compounds were found to be inactive.

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Plasmodium berghei : Studies on Host Metabolism Using *Mastomys natalensis* as Experimental Model

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Sequential changes in some biochemical parameters pertaining to host metabolism during malaria infection were studied in *Mastomys natalensis*, a rodent host susceptible to *Plasmodium berghei*, with gradual rise in parasitaemia and longer survival period. The effect on carbohydrate metabolism was indicated by gradual depletion of liver glycogen corresponding to increasing parasitaemia, marked fall in blood glucose level at late stage of infection (parasitaemia > 25%), and consequent rise in lactic acid content of blood. There were significant rise in SGOT and SGPT and enzymes-glycogen synthetase and glycogen phosphorylase signifying marked tissue damage, defective synthesis and rapid degradation of glycogen respectively. As regards the protein metabolism, significant decrease in liver protein values observed indicate extensive proteolysis with increased availability of amino acids pool for proliferation of the parasites. Gradual increase in the production of lipid peroxides proportional to intensity of parasitaemia in the liver, spleen and lung were observed suggesting oxidative damage caused to these organs. Observed fall in phospholipid level indicates disturbance in the membrane structure.

Introduction

Eversince the discovery of malarial parasite in human blood by Laveran in 1880, extensive research on various aspects of malaria have been carried out, but even today comparatively little is known about the sequential changes that take place in different biochemical parameters of the host during the course of malaria infection. This is probably due to the lack of a model host-parasite system wherein gradual changes would be observed. Albino rat, though susceptible to *Plasmodium berghei*, quite often develops sterile immunity. On the other hand, albino mouse dies with a parasitaemia not high enough solely to cause death. The cause of death is attributable to immuno-pathological lesions consequent to *P. berghei* infection, as evidenced by much higher parasitaemia without death with concurrent corticosteroid treatment. Recent studies conducted in this Institute have shown that the rodent *Mastomys natalensis* is not only very susceptible to *P. berghei* infection but the infection runs a gradual course in this host with increasing parasitaemia to a level as high as 70-80%, ultimately causing death (Sen *et al.*, 1980). Further, this host has a much longer survival time than mouse when infected with *P. berghei*. It was thought that this species would be a suitable host to provide better information regarding the sequential changes in some main biochemical parameters during the course of *P. berghei* infection. This paper reports the results of such studies.

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Material and Methods

Parasitic material — The strain of *P. berghei* used in the present study was originally obtained from National Institute of Communicable Diseases,

Delhi and is being routinely maintained in albino mice and *M. natalensis* by serial syringe passage.

Animals and Infection — Six week old *M. natalensis*, originally obtained from Institut Für Parasitologie, Giessen through the kind courtesy of late Prof. (Dr) G. Lammler, now drawn from the colony of Central Drug Research Institute (CDRI) animal house, was used as experimental host. The animals had free access to standard pellet diet (Hindusthan Lever, Bombay) and water. These were inoculated (i.p.) with one million parasitised red blood cells suspended in sodium citrate (3.8%). For determining the percentage of parasitised red cells, thin blood smears made from tail blood were stained with 5% Giemsa and examined under oil immersion microscope. The animals were sacrificed at different levels of parasitaemia by cervical dislocation after starving them for 24 hr and blood/serum/tissues were analysed for various biochemical constituents.

Enzyme assays—Liver was homogenised with ice cold KCl (150 m*M*) centrifuged at 800 g (15 min) for removing cell debris and nuclear fraction and the supernatant employed for the assay of various enzymes according to standard procedures viz., glycogen phosphorylase (EC 2.4.1.1), Rall *et al.* (1957); glycogen synthetase (EC 2.4.1.11), Villar-Palasi *et al.* (1966); glutamate oxalo-acetate transaminase (EC 2.6.1.1); and glutamate pyruvate transaminase (EC 2.6.1.2), Reitman and Frankel (1957).

Chemical estimations — Glycogen was isolated according to Good *et al.* (1933) and assayed according to Montogomery (1957). Glucose content of blood was estimated by glucose oxidase method of Bergmeyer and Benut (1963), while lactic acid content of serum was assayed according to Barker and Summerson (1941). Protein content was measured colorimetrically according to Lowry *et al.* (1951) using bovine serum albumin as standard. Lipids were extracted according to Folch *et al.* (1957) and phosphorus content was determined by the procedure of Wagner *et al.* (1962). The non-enzymatic lipid peroxidation was assayed by the method of Utley *et al.* (1967).

Units of serum glutamate oxalo-acetate (SGOT) and glutamate pyruvate (SGPT) transaminases have been expressed in terms of Reitman and Frankel units/ml serum. Glycogen phosphorylase unit is expressed as μ moles Pi liberated/min/g wet tissue. Activity of glycogen synthetase is expressed as counts incorporated/min/g wet weight. The results of lipid peroxidation have been expressed as nmoles of malonyldialdehyde (MDA) formed/g wet tissue/3 hr. An extinction coefficient of 1.56×10^5 at 535 nM was used to calculate MDA equivalents.

Results

Table 1 shows the weight of liver and spleen, the most affected organs during the course of infection. Liver of infected group showed, 18, 36 and 50% increase in wet weight over the

Table 1—Weight of Liver and Spleen of *M. natalensis* during *P. berghei* Infection

Group	Wet weight (g)		Dry weight of Liver (g/g wet tissue)
	Liver	Spleen	
Control	1.83±0.23	0.23±0.08	0.32±0.01
Infected (parasitaemia %)			
12-15	2.16±0.38	0.73±0.23	0.27±0.02
22-25	2.49±0.25	1.36±0.13	0.28±0.01
35-40	2.76±0.36	1.59±0.10	ND

Note : Values are mean ± SE.
Dry and wet weight represent the average of 10 animals.
ND = Not done.

Table 2—Protein, Lipid Phosphorus and Phospholipid Content of Liver of *M. natalensis* during *P. berghei* Infection

Group	Protein (mg/g) whole homogenate	2000 rpm supernatant	Lipid phosphorus (mg lipid Pi/g)	Phospholipid*
Control	221.44±20.72	151.30±20.90	0.89±0.16	22.45±4.00
Infected (Parasitaemia%)				
12-15	206.49±29.37	126.50±13.30	0.65±0.20	16.17±5.00
22-25	165.05±26.97	102.03±3.46	0.58±0.06	14.70±0.30
35-40	150.00±15.10	93.80±6.00	0.50±0.50	11.80±0.20

Note : Values are mean ± SE.

Protein determination is average of 10 animals in each group.

Number of determinations for lipid phosphorus and phospholipid were 8.

* The phospholipid content was obtained by multiplying the lipid Pi value by 25.

control group during different degree of parasitaemia while spleen showed three, six and seven fold increase at 12-15, 22-25 and 35-40% parasitaemia respectively. The dry weight of liver recorded slight alteration, though the decrease in weight was significant.

The total protein content of liver registered 6.7, 25.3 and 32% decrease during the development of parasitaemia (Table 2) and TCA precipitable proteins of cell-free extract exhibited 16.5, 32 and 38% fall at the above levels of parasitaemia. Blood glucose level remained unaltered at lower level of infection (2.5-4%). However, the sugar content of blood started declining with progressive increase of parasitaemia (Fig. 1). Hepatic glycogen showed 42.5% fall at 12-15% parasitaemia, however, at 22-25% infection the fall was increased upto 80% (Table 3). Considerable amount of lactic acid, the metabolic end product of glucose utilised by the parasite, was released into the circulation of the host. The increase in serum lactic acid concentration was 20 and 63% at 12-15% and 22-25% parasitaemia (Table 3).

Table 2 represents the phospholipid content of liver at various level of parasitaemia. A gradual

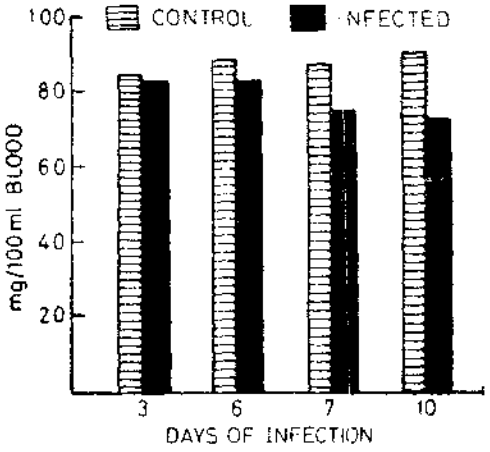

Fig. 1—Levels of Blood Glucose during *P. berghei* Infection in *M. natalensis*. The Parasitaemia during Different days was as follows: 3rd day, 2.5-4%; 6th day, 11-12%; 7th day, 12-18%; and 10th day, 22-25%

decline of the phospholipid i.e. (27,35 and 44%) was noticed during the development of infection. The lipid peroxide content of liver, spleen, lung and brain of *M. natalensis* during *P. berghei* infection has been summarised in Table 4. Significant increase in the formation of lipid peroxides due to oxidative damage of liver, spleen and lung tissue was noticed. Maximum concentration of lipid peroxides was observed in hepatic tissue where two to three fold increase was

recorded in animals having 12-15 and 22-25% infection. In spleen the increase was one and two folds only.

The activities of two transaminases viz., SGOT and SGPT showed elevation in serum of *M. natalensis* during the course of *P. berghei* infection (Table 5). The increase in the activity of these two enzymes could be observed as early as third day after infection having a parasite count of 2-4%. SGPT/SGOT ratio, an index of liver damage, also recorded significant increase over the control groups during malarial infection, the increase being 96, 82 and 88% at 2-4, 12-15 and 22-25% parasitaemia.

Table 3 — Glycogen and Serum Lactic Acid Concentration of *M. natalensis* during *P. berghei* Infection

Group	Glycogen (mg/g liver)	Serum lactic acid (mg/100 ml serum)
Control	62.5±3.5	140.0±5.0
Infected (Parasitaemia%)		
12-15	35.9±5.0	170.8±6.8
22-25	12.5±1.5	240.2±7.8

Note : Values are mean ± SE.
Number of determinations were six in each case.

Table 4 — Lipid Peroxide Concentration of Various Tissues of *M. natalensis* during *P. berghei* Infection

Tissue	Control	Infected (Parasitaemia%) 12-15	22-25
Liver	156.5±4.3(5)	338.0±96.4(5)	444.6±55.9(5)
Spleen	112.0±4.3(3)	198.0±15.3(4)	215.3±7.6(5)
Lungs	116.8±4.0(3)	193.8±10.0(3)	151.8±4.0(3)
Brain	315.1±15.0(3)	347.0±20.3(3)	349.0±8.0(3)

Note : The values are mean ± SE.
Figures in parenthesis represent the number of determinations.
Unit of liquid peroxide = n moles MDA/g tissue/3 hr.
Five animals were used in each set of experiments.

Table 6 shows the activities of enzymes responsible for the breakdown and synthesis of glycogen reserves of hepatic tissue. The increase in glycogen phosphorylase was 48% at 12-15% parasitaemia while at 22-25% infection, the percentage of increase was 33%, the glycogen synthetase showed 28 and 16% decrease at 12-15 and 22-25% infection.

Discussion

Parasites derived their nutrition from host materials and liberate metabolic end products in the host system. Upto a certain limit no deleterious effects become manifest in the host though host immune system is stimulated. When the balance breaks down ill effects of parasitism consequent to factors mentioned above in the form of pathological lesions, nutritional deficiencies and significant changes in the biochemical parameters become apparent.

The erythrocytic stages of *P. berghei* are dependent upon continuous supply of glucose derived

Table 5—Aminotransferase Activity of the Serum of *M. natalensis* during *P. berghei* Infection

Enzyme	Group of animals Control	Infected (2.5-4%)	Control	Infected (12-15%)	Control	Infected (22-25%)
SGOT	45.39±5.23	62.34±5.52	46.15±6.28	63.95±12.37	36.18±1.90	40.41±4.95
SGPT	19.04±0.00	51.42±5.71	16.60±6.28	41.90±4.95	15.99±0.76	33.50±7.80
SGPT/SGOT	0.42±0.00	0.82±0.10	0.36±0.00	0.65±0.40	0.40±0.40	0.83±0.15

Note : The values are mean ± SE.
Figures in parenthesis denotes the percentage of parasitaemia.
Number of determinations were ten in each case.

from host's plasma for their metabolism. They do not store glycogen or other polysaccharides. The metabolic end product of glucose metabolism in *P. berghei* is lactate (Moulder, 1948). All these processess would effect host metabolism and would be reflected in alternations in biochemical parameters. In the present study it would be seen that there was no change in blood glucose and glycogen level at 2.5-4.0% parasitaemia. However, the decrease in glycogen level at 12-15 and 22-25% parasitaemia was 42.5 and 80% respectively. The blood sugar level started declining with progressive increase in the level of parasitaemia registering 6.5, 15 and 20% fall at 11-12, 12-15 and 22-25% infection. Serum lactic acid also registered 20 and 62.5% increase at these levels of infection. The above results would indicate that the levels of glucose, glycogen and lactic acid are not initially affected during infection. With the increase in number of parasites more glucose is utilised and in order to maintain normal blood glucose level, liver glycogen store is depleted. Consequently lactic acid, the end product of glucose metabolism also starts accumulating in the blood. With increasing parasitaemia a stage is reached when the liver glycogen falls so low that normal blood glucose level can not be maintained. Ultimately blood glucose level also falls.

Table 6 — Levels of the Enzymes of Glycogen Degradation and Synthesis in the Liver of *M. natalensis*

Enzyme	Control	Infected (Parasitaemia %)	
		12-15	22-25
Glycogen phosphorylase (units)	4.53 ± 0.07	6.80 ± 0.05	6.08 ± 0.06
Glycogen synthetase (units × 10^3)	8.02 ± 2.00	5.78 ± 1.35	6.78 ± 2.17

Note : Values are mean ± SE.

Number of determinations were five in each case.

Units of glycogen phosphorylase = μ moles Pi liberated/min/g wet tissue.

Units of glycogen synthetase = radioactive counts incorporated/min/g wet tissue.

It would be clear from Table 6 that the excessive breakdown of glycogen needed for energy requirement was primarily due to increase activity of polysaccharide degrading enzyme viz., glycogen phosphorylase and also upto some extent due to defective synthesising machinery i.e., glycogen synthetase.

A review of concerned literature would show that isolated studies have been conducted by other authors as well, but there is no report of continued studies on sequential changes. Thus Fulton (1939) reported lowering of blood glucose during *P. knowlesi* infection of monkeys. Singh *et al.* (1956) reported that fall in blood glucose level is inversely proportional to the per cent infection with nuri strain. Fall in blood glucose level at terminal stage of infection, viz., *P. lophurae* in ducks (Marvin and Rigdon, 1945) and *P. berghei* infection of rats (Mercado, 1952) and mice (Sadun *et al.*, 1965) have been reported. The decrease was more significant when the parasitaemia was above 40%.

The decrease in glycogen level is also reported during malarial infection in *M. mulatta.* (Mercado and Von Brand, 1954) and in albino rat (Chatterjee and Sen Gupta, 1957). Mercado and Von Brand (1954) histochemically demonstrated lowering of glycogen synthesis in heavy infection with *P. berghei* in white rats.

As the malarial parasite utilises glucose by glycolytic pathway, the end product i.e., lactic acid, starts accumulating in the blood of the host. At the initial phase the increase in concentration of blood lactate level is not so harmful to the host but as the concentration increases with the parasitaemia, it causes toxicity and other tissue damage. In Mastomys where this increase is proportional to the intensity of parasitaemia, it indicates that the mechanism of synthesis of glucose from lactate is disturbed in the infected host.

The decrease in protein level of liver during *P. berghei* infection in Mastomys points towards

the extensive proteolysis resulting in the availability of free amino acid pool needed for the rapid proliferation of the parasite. The phospholipids are essential for the maintenance of membrane structure, the extent of tissue damage with gradual rise in parasitaemia is thus reflected in decrease in phospholipid content.

With rise in parasitaemia the various tissues of the host are damaged to varying degrees. A close relationship exists between cellular injury, peroxidation of membrane lipids and oxidative damage to cells (Sharma, 1976). In the present study, liver, lung and spleen were found to produce higher amounts of lipid peroxides, which is due to increased susceptibility of these tissues to oxidative damage under the stress of malarial infection. The reason for higher production of lipid peroxides may be due to increased concentration of ascorbic acid. This is well supported by the findings of Sharma *et al.* (1979) in mice during *P. berghei* infection.

Levels of transaminases in serum are considered as the indicator of tissue damage during various stress processes. The elevation in the activity of two transaminases (SGOT and SGPT) in Mastomys during *P. berghei* infection reflects the state of tissue damage in the host. Similar findings are reported by Sadun *et al.* (1965) in mice. The increase in transaminase may probably be related to the destruction of erythrocytes, anoxia and liver damage.

It can thus be inferred from the study that *P. berghei-M. natalensis* system is a good host parasite model for studying the sequential biochemical changes in malaria and significant information about the state of disease process can be obtained and prognosis of the case assessed from changes observed in the biochemical parameters.

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Isolation of *P. falciparum* in *in vitro* Continuous Culture from a Patient Treated with Chloroquine 24 hr before

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A strain of *Plasmodium falciparum* isolated from a patient, who had taken orally 600 mg of chloroquine (diphosphate) 24 hr before was successfully adapted to continuous *in vitro* culture. The parasites appeared degenerated/injured and by Day 7 of the culture the parasitaemia was reduced to undetectable levels; yet it was continued with blind subcultures. It took more than 4 weeks before the parasite was fully adapted to *in vitro* conditions.

Introduction

Following the successful continuous *in vitro* cultivation of *P. falciparum* (Trager and Jensen, 1976) many laboratories in the world are now able to cultivate this parasite. All available reports show that primary cultures were initiated by selecting an infected donor with no immediate history of antimalarial therapy presumably to avoid working with drug injured parasite and consequent failure.

The present communication describes isolation and successful adaptation to *in vitro* culture system of a strain of *P. falciparum* from a patient drugged with chloroquine.

Material and Methods

A malaria patient with history of recrudescence and patent *P. falciparum* parasitaemia reported to this laboratory for advice. For the current episode he had taken orally 600 mg of chloroquine (diphosphate) 24 hr before. A sample of his urine was collected for Haskin's test to detect presence of chloroquine (WHO, 1965). A thin blood smear was made and stained by JSB technique. Only 0.6 ml of blood could be obtained asceptically for the culture and this was mixed in ACD solution and processed following the modified technique of Rai Chowdhuri *et al.* (1979).

The infected blood after three cycles of washing and removal of the buffy coat yielded a packed cell volume of 0.15 ml. It was then divided into two equal parts and two separate lines of cultures were set up immediately; one with 10% sheep serum and the other with 10% human serum. Media were changed at 24 hr intervals in all the vials. Subcultures were done on Day 4, 10, 23 and 26. In the first three subcultures, parasitised and normal erythrocytes were mixed in equal volumes, whereas in the fourth, the ratio of parasitised to normal erythrocytes was 1 : 4. The progress of the culture was monitored daily from smears stained with JSB technique. Parasitaemia expressed as number of parasites per 100 erythrocytes, was calculated from a count of 10^4 erythrocytes per slide.

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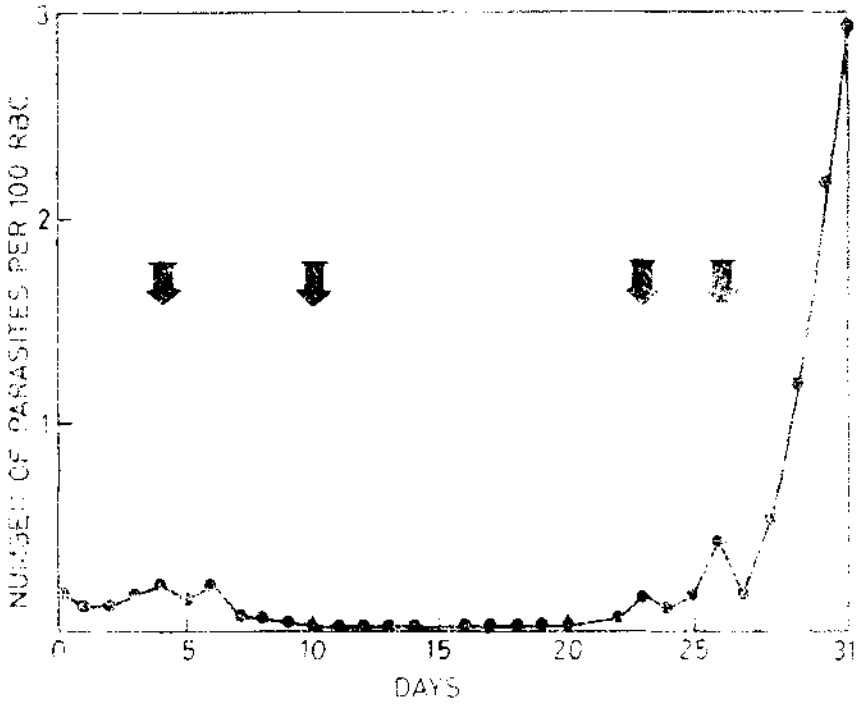

Fig. 1 — Parasitaemia Profile of *P. falciparum* (FDN-1) *in vitro* Culture from Day 0-31. Arrows Indicate Day of Subcultures

Results

The patient's urine was positive for chloroquine, confirming the history of oral intake of the drug.

The infected donor's blood showed typical rings and early trophozoites of *P. falciparum* with a parasite count of 0.8% of which 33% had abnormal morphology, such as pycnosis and vacuolation of cytoplasm. The input parasitaemia on Day 0 was 0.14 and 0.12% for human and sheep serum lines respectively.

The parasitaemia profile of the human serum line from Day 0 to 31 is shown in the Fig. 1. Although, there was only a marginal increase of parasitaemia, a subculture was made on Day 4, with 0.1% input parasitaemia. Two days later the parasitaemia reached 0.18%, but there was a sudden decline in the parasite number by Day 7. During the period between Day 10 to Day 20 no parasite could be detected despite examination of 50 000 to 1 00 000 erythrocytes. However, from Day 22 onwards parasite multiplication became evident and reached a level of 2.96% by Day 31, whereas the line set up with 10% sheep serum showed no parasites on scanning 2×10^6 erythrocytes. However, it is being continued with blind subcultures.

The differential counts of the parasites during the adaptation period is given in Table 1. Morphologically a proportion of parasites showed varying degrees of degeneration from Day 1 onwards, and the number of such affected parasites increased by Day 7. The changes observed were pycnosis of the parasite with increased basophilia, complete loss of cytoplasm leaving only chromatin dots, vacuolation of the

Table 1 — Growth of *P. falciparum* (FDN-1) *in vitro* Culture

Day of culture	Number of parasites per 10 000 erythrocytes				parasitaemia %
	Rings	Trops.	Schi.	Total	
0	12	2	0	14	0.14
2	6	1	5	12	0.12
4	2	6	12	20	0.20
First subculture on Day 4					
6	7	10	1	18	0.18
8	0	2	1	3	0.03
Second subculture on Day 10					
21	0	1	0	1	0.01
23	6	5	6	17	0.17
From Day 10 to 20 no parasite was seen					
Third subculture on Day 23					
24	5	2	2	9	0.09
26	24	15	9	48	0.48
Fourth subculture on Day 26					
27	6	7	1	14	0.14
29	51	35	16	102	1.02
31	80	103	113	296	2.96

cytoplasm with margination of fragmented chromatin material and degeneration of the parasites leaving behind ghosts in eosinophilic erythrocytes. The increase in such degenerated parasites, was followed by a sudden decline in parasitaemia. However, from Day 23 onwards, the parasites presented healthy morphology followed by steady increase in parasitaemia.

The strain (FDN-1)* has undergone 17 subcultures in a period of 11 weeks and the parasitaemia is consistently between 5 and 10%.

Discussion

The therapeutic history of the patient having taken 600mg of chloroquine 24 hr prior to the withdrawal of blood was confirmed by the positive Haskin's test. The parasites with abnormal morphology in the patient's blood and in the first few days in culture suggested drug induced injury. An initial elevation of parasite number, though marginal, indicated growth and multiplication. A sudden drop in parasite number reducing to undetectable level for an extended period though not properly understood may suggest emergence of a competent population fit to survive and multiply under *in vitro* culture conditions.

A very small quantity of blood used in this successful adaptation would suggest that the method of handling and the technique followed for cultivation are more important than the quantity available for initiation of a culture. In our opinion the success in adapting this new strain to *in vitro* culture system could be ascribed to daily change of medium and blind subcultures. The experience gained in this case is similar to the one reported by Phuc Nguyen and Trager (1980) but unlike those we had earlier in this laboratory with three other strains of *P. falciparum*. Besides the fact that this strain was isolated from a donor treated with antimalarials, and initially the parasites appeared degenerated, makes this experience noteworthy.

Acknowledgement

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* F=falciparum, D=Delhi, N=National Institute of Communicable Diseases, 1=Strain No. 1.

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Geographic Variation in Life Table Attributes of Four Populations of *Anopheles stephensi* Liston from India

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Studies on the four populations of *A. stephensi* originated from widely different geographic areas and colonised in the laboratory showed variations in their life table attributes. Pondicherry type showed significant variation from the other types. Arthala and Okhla populations were homogeneous. *A. stephensi* var. *mysorensis* were similar to Arthala and Okhla type. This study did not give any evidence of two races of *A. stephensi*.

Introduction

A. stephensi is an important vector of malaria in India. Two races of this species, *A. stephensi* type form and *A. stephensi* var. *mysorensis*, have been recognised by Sweet and Rao (1937) and Rao *et al.* (1938), based on the differences in the number of ridges in the egg float. Puri (1949) gave subspecific status to these forms which was later accepted by Stone *et al.* (1959). Rutledge *et al.* (1970), based on hybridization studies on this species, consider that *A. stephensi* type form and *mysorensis* are sympatric species and the subspecies status is inappropriate. Since life tables have been used to study the intraspecific and subspecific difference in *Aedes aegypti* (Crovello and Hacker, 1972; Schlosser and Buffington, 1977), *Culex quinquefasciatus* (Gomez *et al.*, 1975), and *Culex tritaeniorhynchus* (Reisen *et al.*, 1979); experiments were carried out to study the life table attributes of *A. stephensi* type form and *A. stephensi* var. *mysorensis*.

Material and Methods

Three geographic strains of *A. stephensi* type form and one strain of *A. stephensi* var. *mysorensis* were utilised in this study. The type forms were collected from Pondicherry, Okhla (Delhi), Arthala (UP), and the var. *mysorensis* from Bhuj (Gujarat). All the strains were established from wild caught mosquitoes and are well adapted to the insectary environment. They have undergone many generations in the laboratory. Rearing was done as per the methods developed by Ansari *et al.* (1978).

Cohorts of 100 first instar larvae, six replicates of each strain, were used to study the larval attributes. Median pupation (P_{50}) and emergence (E_{50}) times were calculated by fitting regressions of the form probit $(P) = a+b \ln X$ where P = cumulative proportion pupating or emerging in a given day (X) transformed to probits. The P_{50} and E_{50} values were then calculated by solving the equation for $P=50\%$.

To study the adult attributes, cohorts of 25 males and 25 females of each species were kept in 30 cm^2 cages within 12 hr of emergence. Six replicates were used. They were continuously provided glucose solution on a cotton pad. Each night a chicken was offered as blood meal allowing the females to feed on the same night after oviposition. Every morning the dead mosquitoes were removed

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and recorded. This was continued till the last mosquito died.

The calculations used to study the life table attributes of adult mosquitoes are summarised below :

Age specific survivorship l_x = the number of adults alive on each day x, corrected so as to commence with a fixed number 1000 (Deevey, 1947).

Age specific life expectancy,

$$e_x = \frac{\int_x^w l_x \, dx}{l_x}$$

where d_x is deaths at a given age and w is the day the last individual died (Service, 1977).

Mean constant daily survival S_r = back transformed slope of the fitted regression function of the log l_x.

Net reproductive rate,

$$R_o = a \sum_{x=1}^{w} l_x \, m_x$$

where a = the proportion of female that survived from egg through adult emergence and $m_x = E_x S$, where E_x is the mean number of larvae produced per female per age interval, and S = the proportion of the offspring that were females (Crovello and Hacker, 1972). Experimentally determined sex ratio was used.

Age of the mean cohort reproduction,

$$T_o = a \sum_{x=1}^{w} l_x \, m_x \, x \frac{x}{R_o}$$

Capacity for increase,

$$r_e = \ln_e \frac{R_o}{T_o}$$

Innate rate of increase,

$$r_m = a \sum_{x=1}^{w} l_x \, m_x \, e^{-r_m (x+d)} = 1$$

where e = the base of the natural logarithm and d = length of time from oviposition in the present generation to first oviposition in the offspring generation (Dobzhansky *et al.*, 1964).

Table 1 - - Larval Life Table Attributes of *A. stephensi* of Different Strains ($\bar{x} \pm$ Confidence Interval $N=6$)

Attribute	*A. stephensi* var. *mysorensis* (Bhuj)	*A. stephensi* type form (Arthala)	*A. stephensi* type form (Okhla)	*A. stephensi* type form (Pondicherry)
Survivorship L to P	0.658 ± 0.139^a	0.690 ± 0.042^a	0.790 ± 0.037^b	0.841 ± 0.056^b
P_{50} (days)	9.18 ± 0.900^a	9.615 ± 0.406^a	9.432 ± 0.238^a	7.150 ± 0.510^b
Survivorship P to A	0.936 ± 0.055^a	0.839 ± 0.044^b	0.907 ± 0.078^a	0.803 ± 0.149^b
E_{50} Female (days)	10.300 ± 1.740^a	10.630 ± 1.078^a	10.027 ± 1.218^a	8.850 ± 0.770^b
E_{50} Male (days)	10.110 ± 1.770^a	10.000 ± 0.989^a	10.705 ± 1.163^a	8.770 ± 1.040^b
Total survivorship	0.614 ± 0.078^a	0.576 ± 0.201^a	0.730 ± 0.160^b	0.693 ± 0.146^b
Sex ratio (male/total)	0.488 ± 0.021^a	0.632 ± 0.103^b	0.538 ± 0.115^b	0.480 ± 0.032^a

Abbreviations : L = Larvae, P = Pupae, A = Adults, P_{50} = Median pupation time, and E_{50} = Median emergence time.
Note : Means followed by the same letter were not significantly different ($P \leqslant 0.05$) by Student-Newmen-Keuls multiple range test.

Table 2 — Adult Life Table Attributes of *A. stephensi* of Different Strains ($\bar{x} \pm$ Confidence Interval $N=6$)

Attribute	*A. stephensi* var. *mysorensis* (Bhuj)	*A. stephensi* type form (Arthala)	*A. stephensi* type form (Okhla)	*A. stephensi* type form (Pondicherry)
e_o Female	11.66 ± 0.486^b	15.13 ± 2.35^a	14.89 ± 3.46^a	8.92 ± 1.55^c
e^{σ} Male	7.15 ± 1.20^a	6.95 ± 1.04^a	6.87 ± 2.35^a	6.39 ± 1.20^a
s_r Female	0.884 ± 0.01^{ab}	0.909 ± 0.02^{ab}	0.916 ± 0.01^b	0.872 ± 0.03^a
s_r Male	0.850 ± 0.02^a	0.796 ± 0.03^a	0.798 ± 0.10^a	0.790 ± 0.18^a
R_o	72.75 ± 4.94^a	89.46 ± 2.41^a	119.04 ± 2.78^b	67.40 ± 6.51^a
T_o	9.08 ± 0.24^b	11.78 ± 1.18^a	11.34 ± 0.82^a	7.65 ± 1.04^b
r_c	0.472 ± 0.01^b	0.382 ± 0.03^a	0.421 ± 0.02^a	0.550 ± 0.05^c
r_m	0.348 ± 0.01^a	0.356 ± 0.01^a	0.398 ± 0.01^a	0.382 ± 0.01^a
G	12.32 ± 0.01^a	12.62 ± 0.01^a	12.01 ± 0.01^a	11.02 ± 0.02^a
b	1.354 ± 0.08^a	1.337 ± 0.08^a	1.339 ± 0.03^a	1.487 ± 0.10^a
d	1.006 ± 0.09^a	0.981 ± 0.09^a	0.941 ± 0.02^a	1.105 ± 0.09^a
λ	1.416 ± 0.01^a	1.428 ± 0.02^a	1.489 ± 0.01^a	1.465 ± 0.03^a

Abbreviations : e_o = Mean life expectancy at emergence in days; S_r = Mean survival; R_o = Net reproductive rate in living female progeny per female per generation; T_o = Age in days of mean cohort reproduction; r_c = Capacity for increase; r_m = Innate rate of increase; G = Mean generation time in days; b = Birth rate; d = Death rate assuming a stable distribution; and λ = Finite rate of increase.

Note : Means followed by the same letter were not significantly different ($P \leqslant 0.05$) by Student-Newmen-Keuls multiple range test.

Mean generation time,

$$G = \ln \frac{R_o}{m}$$

Birth rate,

$$b = \ln (1 + \beta) \text{ and } 1/\beta = l_x e^{-r_m(x-a)}$$

(Reisen *et al.*, 1979).

Death rate,

$$d = b - r_m$$

The finite rate of increase,

$$\lambda = e^{r_m}$$

Comparisons between strains were made by analysis of variance and posteriori Student-Newmen-Keuls multiple range test (Sokal and Rohlf, 1969) and among life table attributes by correlation analysis.

Results and Discussion

The larval life table attributes of the four populations of *A. stephensi* studied, are shown in Table 1. Immature survivorship was highest for Okhla and Pondicherry type and lowest for Arthala and Bhuj type. In the larval attributes studied, Pondicherry strain showed significant difference from other strains. *A. stephensi* var. *mysorensis* did not show significant difference from Arthala and Okhla strain with respect to its survival, pupation and emergence characteristics.

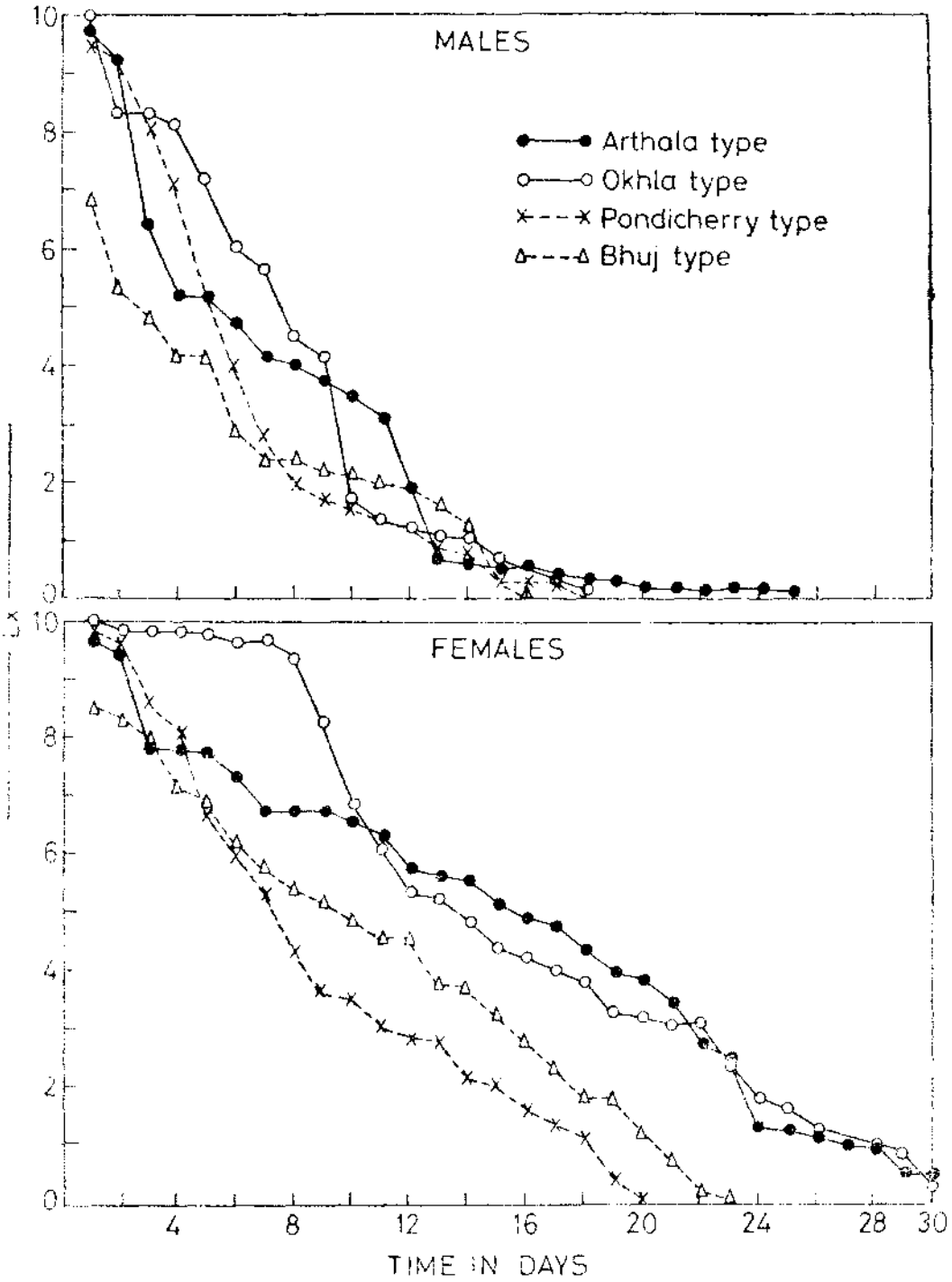

Fig. 1 -- Age Specific Survivorship per Individual (l_x) for Males and Females of *A. stephensi* Type Forms and *A. stephensi* var. *mysorensis*

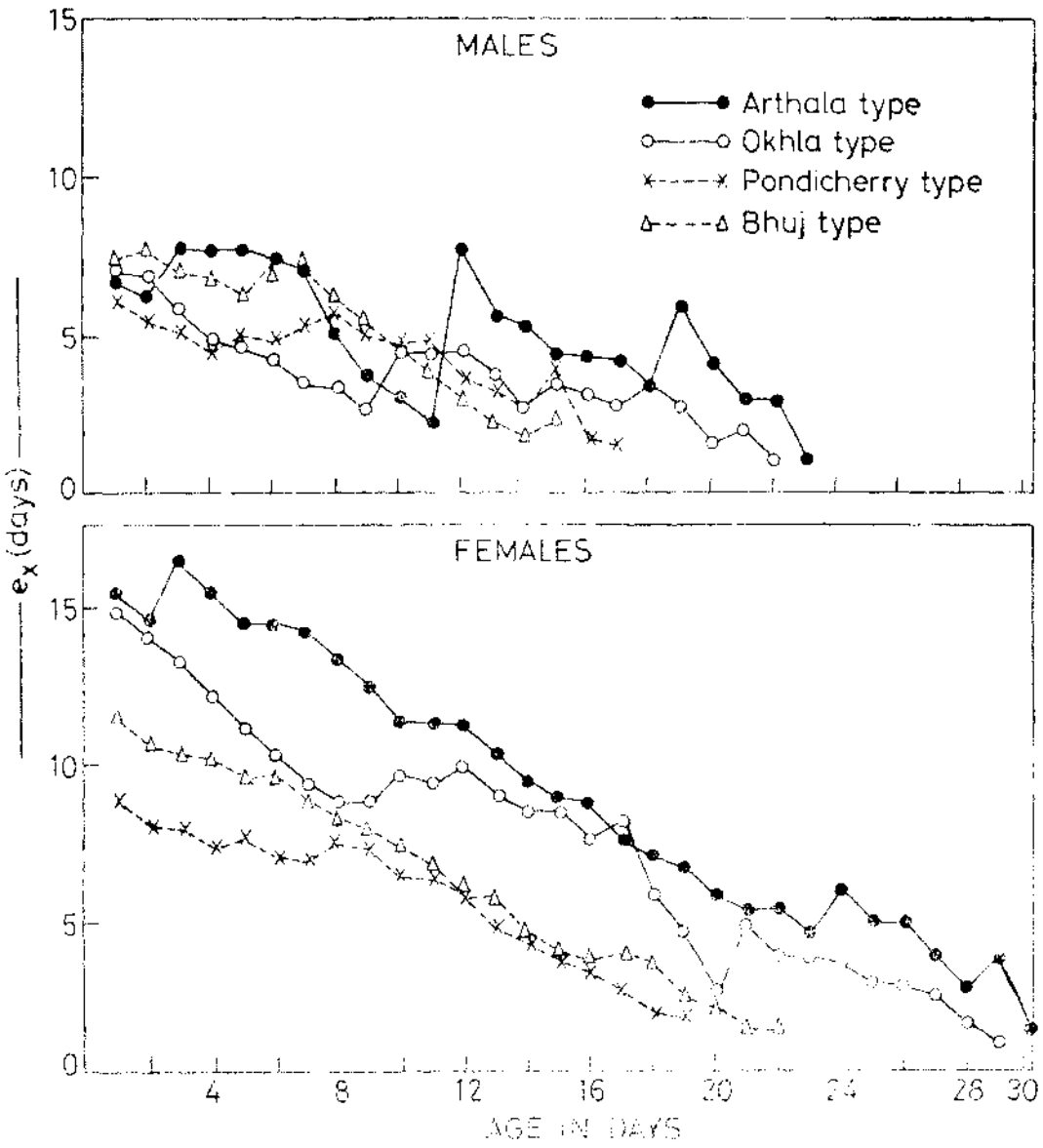

Fig. 2 — Age Specific Life Expectancy (e_x) for Males and Females of *A. stephensi* Type Forms and *A. stephensi* var. *mysorensis*

Age specific survivorship curve for males and females of the four populations studied is shown in Fig. 1. More than 50% of the males of *A. stephensi* var. *mysorensis* died on 3rd day. The male mortality was less in other strains. The female mortality was very less in the early days of life. The lowest survival was observed for Pondicherry type and the highest for Okhla type. Expectancy of life at different age intervals for males and females of the different strains is depicted in Fig. 2. The female life expectancy was higher for all the strains than

male life expectancy, and was highest for Arthala type and lowest for Pondicherry type. Net reproductive rate (R_o) was highest for Okhla type and lowest for Pondicherry type (Table 2). Pondicherry, Arthala and Bhuj types did not show significant differences in R_o. R_o was significantly correlated with e_x at emergence ($r=0.803$, $P<0.01$). The age of the mean cohort reproduction (T_o) was highest for Arthala and Okhla types and lowest for Pondicherry type, and this was positively correlated with R_o ($r=0.786$, $P<0.05$). The capacity for increase (r_c) was highest for Pondicherry type and showed significant difference from other types. The innate rate of increase (r_m) was more or less same for all the strains studied. The r_c was not significantly correlated with r_m as the time from parent oviposition to offspring oviposition varied for different strains. The r_m was positively correlated with R_o ($r=0.583$, $P<0.1$) and G ($r=0.772$, $P<0.05$). Mean generation time (G) also did not show any significant differences in the four types. The birth rate (b), death rate (d), and finite rate of increase (λ) were homogeneous for all the four types studied.

Many investigators have used life tables to study the intraspecific and subspecific variation for reproductive characteristics among insect species. Significant variation in the life table phenomena among subspecies and within subspecies of *Aedes aegypti* was observed by Crovello and Hacker (1972). They found significant differences in net reproductive rate (R_o) at the subspecies level. Birch *et al.* (1963) found subspecific differences in r_m in *Drosophila serrata*. Dobzhansky *et al.* (1964) calculated r_m for ten populations of *Drosophila pseudoobscura* to study the success of chromosomal type at different temperatures. They found that polymorphic replicates had a higher r_m and that r_m increased significantly in the next year due to increased fecundity. The life table attributes of *A. stephensi* var. *mysorensis* did not show significant variation with respect to reproductive and survivorship characteristics from other population of *A. stephensi* studied. Only Pondicherry type showed significant variation from the other types in many of its larval and adult life table attributes, probably due to its origin from a widely isolated region. Arthala and Okhla populations were homogeneous in many of their reproductive attributes as they were from the same geographic region. The life table attributes of *A. stephensi* var. *mysorensis* were very much similar to Arthala and Okhla type forms except for a few characters which might be strain variation. It was concluded that comparative studies of various parameters of life tables were not helpful in providing any evidence of two races in *A. stephensi*.

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Linkage Relationship between Three Autosomal Mutants and Functional Relationship between Two Eye Colour Mutants in *Anopheles stephensi*

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Linkage relationship between three autosomal mutants — *colorless-eye*, *green-larva* and *greenish brown-larva* has been established in *Anopheles stephensi*. *Green-larva* and *greenish brown-larva* showed linkage while *colorless-eye* showed independent assortment with both the larval body colour mutants. *Colorless-eye* was found to be epistatic to sex-linked recessive mutant *red-eye*.

Introduction

Anopheles stephensi is one of the major malaria vectors in Indo-Pakistan subcontinent and Middle East. Very few genetic studies have been reported in this species. So far, only seven morphological mutants viz., *black-larva* (Mason and Davison, 1966); *white-eye* (Aslamkhan, 1973); *stripe* (Sakai *et al*., 1974); *colorless-eye* (Sharma *et al*., 1977); *green-larva* (Subbarao and Adak, 1978); and *greenish brown-larva* and *red-eye* (Sharma *et al*., 1979) and their inheritance pattern have been reported. Sakai *et al*. (1974) have established the linkage relationship between the *stripe* mutant and two enzyme systems, alcohol dehydrogenase and acid phosphatase. Subbarao and Adak (1978) demonstrated that *green-larva* and *colorless eye* belong to different linkage groups.

This paper describes the linkage relationship between three autosomal mutants, *colorless-eye*, *greenish brown-larva* and *green-larva* and functional relationship between two eye colour mutants, *red-eye* and *colorless-eye*.

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Material and Methods

The following strains of *Anopheles stephensi* were used in the experiments:

Colorless-eye (*c*): Sharma *et al*. (1977) — It expresses white eyes in larval, pupal and adult stages, and also reduces the intensity of body colour in larvae and pupae and are thus easily distinguishable from wild type.

Green-larva (*g*): Subbarao and Adak (1978) — It expresses its phenotype in early instars as dark coloured larvae but in late III and early IV instars green colour is clearly expressed. Pupae are green in colour but adults are indistinguishable from the wild type.

Greenish brown-larva (*gb*): Sharma *et al*. (1979)— It expresses in early instars as dark coloured larvae same as *green-larva* but in late III and early IV instars greenish brown abdomen with a clear green thorax is seen. This mutant is easily distinguishable from *green-larva* in IV instar.

Red-eye (*r*): Sharma *et al*. (1979)—It expresses its phenotype as red eyes in larval, pupal and adult stages. Larval and pupal body colour appears to be slightly lighter than the wild type. It is a sex-linked mutant expressing in a

Table 1 — Linkage Relationship between Autosomal Mutants — *green-larva, colorless-eye* and *greenish brown-larva*, in *Anopheles stephensi*

Cross No	Parental genotypes/ phenotypes Female		Male	F_2 Progeny phenotypes: Wild type	White eyes	Greenish brown	Green	White eyes + green thorax	Total progeny	Ratio	χ^2
1	$\frac{c}{c}$; $\frac{gb^+}{gb^+}$	×	$\frac{c^+}{c^+}$; $\frac{gb}{gb}$	541 (543.9)	174 (181.3)	197 (181.3)	—	55 (60.4)	967	9:3:3:1	1.95 n.s.
2	$\frac{c^+}{c^+}$; $\frac{gb}{gb}$	×	$\frac{c}{c}$; $\frac{gb^+}{gb^+}$	247 (275.4)	99 (91.8)	114 (91.8)	—	30 (30.6)	490	9:3:3:1	8.85 $P<.05$
3	$\frac{g\ gb^+}{g\ gb}$	×	$\frac{g^+gb}{g^+gb}$	241 (220.5)		110 (110.25)	90 (110.25)		441	2:1:1	5.66 n.s.
4	$\frac{g^+gb}{g^+gb}$	×	$\frac{g\ gb^+}{g\ gb^+}$	480 (462.5)	—	218 (231.5)	227 (231.5)		925	2:1:1	1.52 n.s.
5	F_2 green (cross 3)	×	$\frac{g^+gb}{g^+gb}$	389	—	33	—	—	422		
6	$\frac{g^+gb}{g^+gb}$	×	F_2 green (cross 3)	42	—	—	Nil	—	42		
7	F_2 greenish brown (cross 3)	×	$\frac{g\ gb^+}{g\ gb^+}$	258	—	—	26	—	274		
8	$\frac{g\ gb^+}{g\ gb^+}$	×	F_2 greenish brown (cross3)	203	—	Nil	—	—	203		
9	F_2 greenish brown (cross 4)	×	$\frac{g\ gb^+}{g\ gb^+}$	279	—	—	Nil	—	279		
10	$\frac{g\ gb^+}{g\ gb^+}$	×	F_2 greenish brown (cross 4)	314	—	—	27	—	341		
11	F_2 green (cross 4)	×	$\frac{g^+gb}{g^+gb}$	74	—	Nil	—	—	74		

Note : Figures within parentheses show the expected numbers.

homozygous state in females and in a hemizygous state in males.

Genetic Crosses

Experiment I — To study the linkage relationship mosquitoes from *gb* and *c* strains were reciprocally crossed and F_1 were inbred to obtain F_2 progeny. F_2 Progeny were screened for different phenotypes.

Experiment II — To study the linkage relationship between *g* and *gb*, mosquitoes from both the stocks were reciprocally crossed and F_2 progeny were screened for different mutant phenotypes. Since double homozygote stock was not available to study the crossing over, F_2 mutant phenotypes, green and greenish brown were back crossed to greenish brown and green respectively. Presence of mutant phenotype in these crosses would indicate the occurrence of crossing over between the two mutants.

Experiment III — To study the functional relationship between two eye colour mutants, *r* and *c* were reciprocally crossed and the F_1 progeny were inbred to obtain F_2 offspring. F_2 Offspring were screened for different eye colour phenotypic categories. Further, F_2 red and white phenotypic categories of both the crosses were inbred among their own group to examine the genetic constitution of each category.

Results

Results of crosses between *greenish brown-larva*, *colorless-eye* and *green-larva* are presented in Table 1 (crosses 1-4). Reciprocal crosses of *gb* and *c* (crosses 1 and 2) produced wild type F_1 progeny. Among the F_2 progeny, four phenotypes viz., (i) wild type; (ii) greenish brown; (iii) white eyes; and a new category (iv) white eyes, green thorax with a larval abdomen, much lighter in colour than the wild type were observed in a ratio of 9:3:3:1 respectively. The new category resembled *c* parental type in having white eyes but resembled neither parental type in body colour. To establish its genotype, it was inbred and back crossed to the *gb* parental type. The inbred cross bred true to its phenotype and the back cross progeny were identical to greenish brown phenotype.

F_1 Progeny of reciprocal crosses between *g* and *gb* strains (cross 3 and 4) were wild type. Among the F_2 progeny three phenotypic categories viz., (i) wild type; (ii) greenish brown; and (iii) green, in a ratio of 2:1:1 respectively, were observed. Interestingly, phenotype representing the double homozygote, $\frac{g\ gb}{g\ gb}$ was not seen.

Results of F_2 phenotype back crosses are shown in Table 1. In crosses 5, 7 and 10, both wild type and mutant offspring were observed, while in rest of the crosses only wild type offspring was observed. Pertaining to cross 5, 20 females were individually tubed for egg laying and out of them only three laid eggs. Out of these cultures two had wild type and mutant offspring in 1:1 ratio while the third had all wild type offspring. Percentage crossing over observed in crosses 5, 7 and 10 are 7.1 ± 1.25, 7.9 ± 1.62, and 9.1 ± 1.56 respectively.

Results of reciprocal crosses between *r* and *c* strains are given in Table 2. In cross 1 where females were red eyed, F_1 females were all wild type and males were all red eyed. In the reciprocal cross all were wild type. However, among all the F_2 progeny from the reciprocal crosses, three phenotypes viz., (i) wild type; (ii) red eyes; and (iii) white eyes were observed in varying proportions. In cross 1, phenotypic ratio was 3:3:2 among both the sexes while in cross 2 the ratio in males was the same as in cross 1 but in females it was 6:0:2 as red females were totally absent. In neither cross double recessives, *r/r*; *c/c* and *r/-*; *c/c* were seen.

Table 2 — Functional Relationship between Two Eye Colour Mutants, *red-eye* and *colorless-eye* in *Anopheles stephensi*

Cross No.	Parental genotypes Female	Male	F_2 Progeny phenotypes: Wild type Female	Wild type Male	Red eye Female	Red eye Male	White eye Female	White eye Male	Total Female	Total Male	χ^2
1	$\frac{r}{r}$; $\frac{c^+}{c^+}$	$\frac{r^+}{-}$; $\frac{c}{c}$	59 (54)	43 (48.75)	48 (54)	47 (48.75)	37 (36)	40 (32.50)	144	130	7.95 $P<0.05$
2	$\frac{r^+}{r^+}$; $\frac{c}{c}$	$\frac{r}{-}$; $\frac{c^+}{c^+}$	140 (150)	75 (63.75)	0	51 (63.75)	60 (50)	42 (42.5)	200	168	3.60 n.s.

Note : Figures within parentheses show the expected numbers.

Red group inbreeding of cross 1 produced 5.5% white eyed progeny and the rest were red eyed. Among the white eyed progeny, two males had red spots on white eyes. In cross 2, since F_2 red females were absent, red females from cross 1 were used for inbreeding and among the progeny 9.5% were white eyed and the rest were red eyed. White eye group inbreeding of both the crosses produced only white eyed progeny.

Discussion

Appearance of four phenotypic categories among the F_2 progeny of cross 1 and 2 (Table 1), in a ratio of 9:3:3:1 indicates that *c* and *gb* are not linked. The fact that the double homozygote category did not resemble the greenish brown phenotype eventhough it had *gb/gb* genotype, suggests that the intensity of greenish brown pigment might have been reduced by *c/c* genotype. The authors have already reported that *c* gene independently assorts with *g* gene and a similar observation was made that *c/c* genotype reduces the intensity of pigmentation of a double homozygote, *c/c*; *g/g* (Subbarao and Adak, 1978). Phenotypic interaction of *c/c* genotype with *g/g* and *gb/gb* genotypes suggests that c^+ gene in some way controls the larval pigmentation.

The fact that *c* is not linked with *g* and *gb* suggests that *g* and *gb* may be linked. Reciprocal crosses between *g* and *gb* (cross 3 and 4 of Table 1) supported this fact where wild, greenish brown, and green phenotypes in 2:1:1 ratio were found in F_2 generation. Further, the wild type expression of F_1 offspring indicates that *g* and *gb* are not functionally allelic.

Presence of mutant progeny in three of the F_2 phenotype back crosses suggests the possibility of occurrence of crossing over between *g* and *gb*. The 1:1 ratio of wild type and mutant progeny in two single female cultures of cross 5 confirms that the crossing over occurs between these two mutants. Lack of mutant phenotype in other crosses may be due to small numbers. Map distance between *g* and *gb* appears to be 8.29 ± 0.86 map units.

Due to the sex-linked recessive nature of the *r* gene, F_1 males of cross 1 were red eyed and F_2 phenotypic ratio varied in two sexes of crosss 2 (Table 2). Absence of a new phenotypic class representing double homozygotes, *r/r*; *c/c* and *r/-* ; *c/c*, among the F_2 progeny of both the crosses suggests that this group was either lethal or was being classified under one of the three phenotypes. Phenotypic ratio of F_2 progeny suggests that double homozygotes were being classified under white phenotype. This was confirmed by the absence of red eyed offspring from F_2 white eyed inbred crosses. White eyed phenotype observed among the offspring of inbred F_2 red groups are apparently double homozygotes. These results indicate that *c* is epistatic to *r*. Similar situation was observed by Sakai *et al.* (1976) in *Culex tritaeniorhynchus*, where *w w* (white eye); *bw/bw* (brown eye) genotype produced white eye phenotype. Observation of red spot on white eyes of double homozygotes indicates the failure of expression of *c* gene in those cells.

As suggested in the case of *C. tritaeniorhynchus*, a product under the control of c^+ gene may be necessary for the synthesis of red eye pigment in *Anopheles stephensi*. However, larval body pigmentation appears to be only partly under the control of c^+ gene as *c/c* genotype does not inhibit, but only reduces the pigmentation when combined with *g/g* and *gb/gb* genotypes.

Several groups of scientists are working with *Anopheles stephensi* and are assigning the markers available to them to different linkage groups. A standard genetic map of all the available markers in this species is necessary. Till such time *c* has been tentatively assigned to linkage group II, *g* and *gb* to linkage group III, and *r* gene to X-chromosome of linkags group I.

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Salivary Gland Chromosomes of *Anopheles annularis*

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Specimens of *Anopheles annularis* were collected from North India (village Burj, Chandigarh) for the preparation of their salivary gland chromosomes. A comparison of the polytene chromosomes of *A. annularis* with those in the published maps of *A. stephensi* and *A. philippinensis* revealed more homologies with *A. stephensi* than with *A. philippinensis*. Morphologically, within the group *Neocellia* (subgenus *Cellia*) *A. annularis* has more similarities with *A. philippinensis* than with *A. stephensi*.

Introduction

Anopheles annularis is a vector of considerable importance in Orissa and Vishakapatnam area. In many other regions it is a recognised vector of secondary importance (Venkatrao, 1954). It is widely distributed in the country throughout the plains and has also been recorded from hills upto 250m. In recent years *A. annularis* has assumed greater importance in view of its increasing role in malaria transmission in parts of coastal Orissa, Nepal (Indo-Nepal border), and certain other areas. In subgenus *Cellia*, polytene chromosome maps of at least 12 species have been published (Kitzmiller, 1976) whereas *A. annularis* has not been studied cytologically. Therefore a map of the salivary polytenes of *A. annularis* was prepared (Fig. 1) so that studies on the evolution, natural polymorphism and sibling species complexes, etc. may be initiated in this important vector of malaria.

Material and Methods

Fully fed females of *A. annularis* were collected from cattlesheds of Burj village, 25 km NE of Chandigarh in the foothills of Shivalik range. Mosquitoes were brought to the laboratory and held in 30 ×30×30 cm cages at 28±1°C and 78-80% RH for egg laying. During the holding period, adults were offered 1% glucose on cotton pads. Eggs were collected from the cages and floated on water in enamel pans. The immatures were reared upto fourth instar following the routine laboratory procedures. Salivary glands from the fourth instar larvae were dissected in normal saline, fixed in 45% acetic acid for one min, and then immersed in a drop of lacto-aceto-orcein stain for 5-7 min. The glands were then squashed with thumb (French *et al.*, 1962) and 24 hr later sealed with nail polish.

Measurement of chromosomes and location of major bands were made with the help of photographs taken at 700x magnification and enlarged 4x. Details of banding pattern were filled by direct observations at 1000x. Banding pattern reported in this paper is based on a sample size of about 300 temporary slides, and described following the traditional division and numbering systems (Frizzi, 1947) i.e., X-chromosome contains zones 1-6; 2R, 7-19; 2L, 20-28; 3R, 29-37; and 3L, 38-46.

Results and Discussion

The salivary gland nuclei of *A. annularis* showed three pairs of polytene chromosomes — a short

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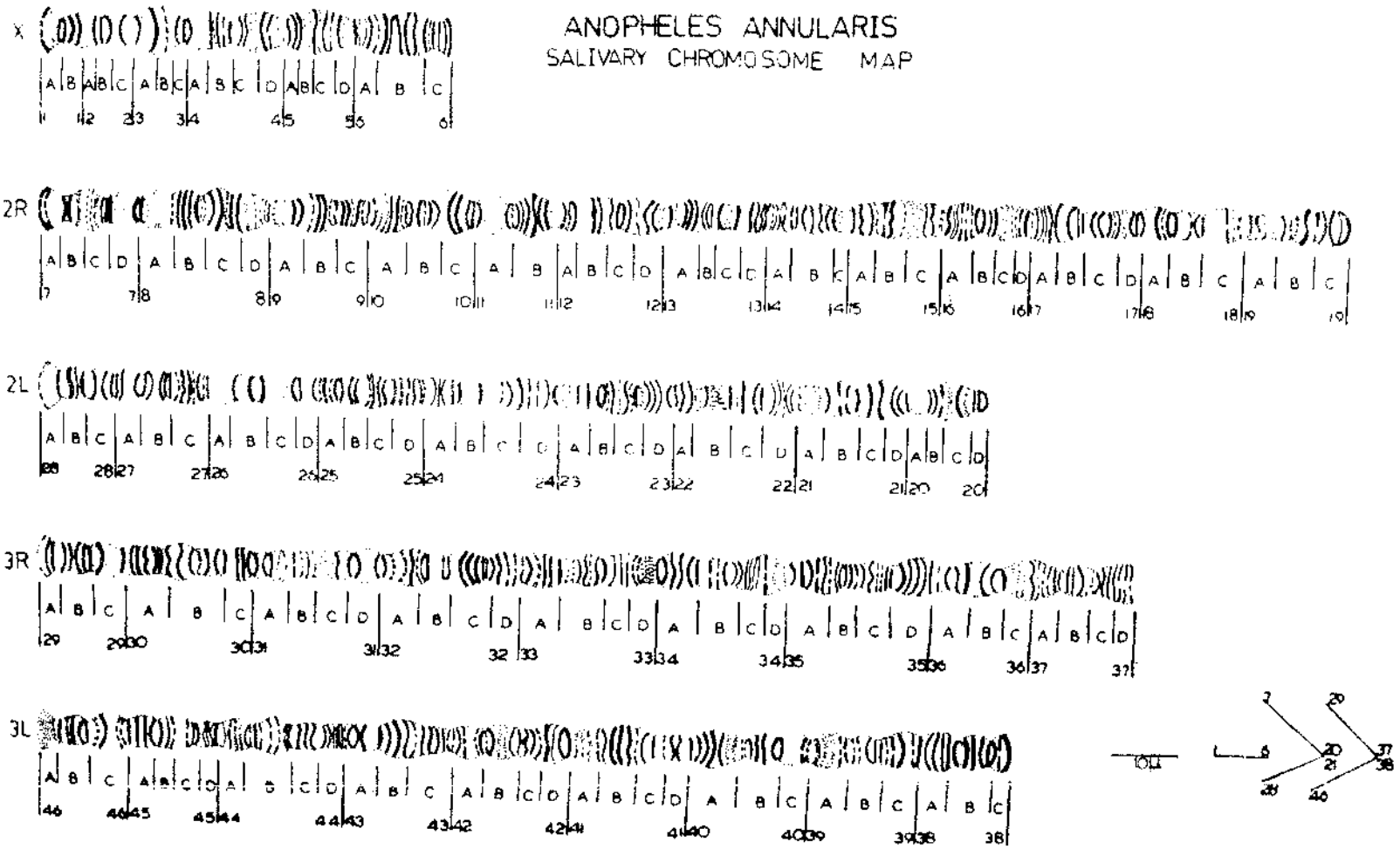

Fig. 1 — Polytene Chromosomes from Larval Salivary Glands of *Anopheles annularis*

Fig. 2 — Salivary Chromosome Complement of *Anopheles annularis*. C=Centromere; X=X-Chromosome; 2R & 2L =Right and Left Arms of Chromosome 2; and 3R & 3L=Right and Left Arms of Chromosome 3

X-chromosome and two long autosomes with the characteristic subgeneric banding pattern (Fig. 2). The chromocentre was not observed in any preparation. Some degree of asynapsis was observed at the centromeric tips in all chromosomal arms. Average measurements for each of the chromosomal arm was as follows: X—75 micra; 2R—280 micra; 2L—190 micra; 3R—250 micra; and 3L—105 micra.

Description of the Chromosomes

X-Chromosome — This chromosome was recognised by its short length. The free end, which contained many weak bands, was moderately flared and had a dotted dark band at the tip. This was followed by two pairs of dark bands in 1B. Zone 2 was slightly diffused with three dark bands in 2B and one dark band in 2C. Zone 3 had a large puff with five dark bands, all separated by few weak dotted bands. This was followed by two moderate sized puffs in 4AB and in 4CD. These puffs contained few dark, smooth and dotted bands. Zone 5 had a diagnostic enlarged puff with a pattern of 3-2-1-1 of smooth and dotted dark bands, including a few weakly stained dotted bands in 5B and 5D.

The centromeric end was recognised as asynaptic in zone 6 which partly involved 6B and 6C (not shown in the map). Zone 6 contained 12 sharp dark bands.

Chromosome 2R — This was the longest arm with many identifying landmarks. Free end was flared with two dotted dark bands at the tip. The most prominent was the diagnostic terminal puff in 7BC. It consisted of smooth dark bands at its proximities and a pair of dotted bands in the centre. Zone 7D-8A was indistinct and was followed by two characteristic moderate puffs. There were four sharp dark bands in 8B and 8C each. In 9B a small puff with one dotted and two sharp dark bands is a

convenient landmark followed by three solid and dotted dark bands in 9C. Two frequently occurring and diagnostic puffs are found in 10A and 10BC, followed by a diffused light area in 11A. A large and characteristic puff with six prominent dark bands was present in 11B. Zone 12 was identifiable with 3-2-4-1-2 patterns of dark bands well placed in the puffs of 12AB and 12 CD respectively. These were followed by a small puff in 13 AB. In this zone, there were two sets of three dark bands. Zones 14 and 15 were without any distinct landmark. The next three puffs in 16AB, 16CD and 17AB were diagnostic. These were characterised by the presence of a series of dark bands of different intensities.

The centromeric end was recognised by the presence of two small puffs in 18A and 18C each, having three heavy dark bands in 18A and a single dark band in 18C. Zone 19 contained a series of weak dotted bands in the first half while 19C was studded with four sharp dark bands.

Chromosome 2L — The free end, characteristically devoid of any dark band, was followed by a series of seven dark bands in 28BC which served as an excellent landmark. Zone 27 had a series of three small puffs which consisted mostly of sharp dark bands. Zone 26 was indistinct but with three dark bands in 26B. A small puff in 25B consisted of four dark bands which preceded a series of dotted and smooth dark bands from 25D to 23A. This was followed by three small puffs in 23BC, 23D and in 22A which consisted of characteristic sharp dark bands. A small puff in 22C with four dark bands in 22C was followed by another light puff consisting of four dark bands of low intensity.

The three diagnostic puffs in 21B, 21CD and 20BC marked the centromeric end of the arm. These puffs included many sharp dark and a few dull bands. Three characteristic bands at the tip were an excellent identification mark in 20D.

Chromosome 3R — The free end began with a few diffused bands followed by one pair of dark bands in 29A. A series of five sharp and prominent dark bands was the first landmark of the arm in 29B. Zone 29C consisted of a small puff with two well spaced dark bands. Zone 30 was characterised by the presence of a series of sixteen sharp dark bands. Zone 31 was an indistinct area with mostly light dotted bands preceding a small puff in 31D with one characteristic pair of dark bands on both the extremities. Next four consecutive puffs in 32A, 32C, 32D and 33A were characteristically studded with dark bands. Six prominent dark bands in a small puff in 34A preceded a series of ten dark bands with different intensities from 34B and 34D.

The centromeric end was characteristic because of the presence of a series of dark bands from 35A to 36B. A medium sized puff was well marked in 36C to 37A with three light bands in the beginning followed by a series of dark bands of different shapes and sizes. The centromeric tip was characteristically asynaptic.

Chromosome 3L — Free end was characteristically devoid of any dark band at the tip. Zone 46B had one dark and one dotted band, followed by three dark bands. This was followed by three moderate puffs in 46C, 45A and 45BC which were studded with a series of dark bands. A small puff with two dotted dark bands in the centre and one pair of dark band at the ends, in 44B was characteristically bounded by four light bands in 44A, and three dark bands in 44C. A large diagnostic puff in 43BC consisted of nine dark bands followed by a puff in 42CD with eight dark bands. Characteristic puffs in 41BC had three heavy dark bands in the centre along with four other dark bands. A series of five sharp dark bands in the puff in 40A preceded a pair of light bands and another

set of five dark bands in 40B. Zone 40C-39B was indistinct.

An enlarged diagnostic puff consisting series of nine dark bands in 39C-38A was observed at the centromeric end. A small puff in 38B had five dark bands while a single heavy dark band was present at the tip of 38C.

A. annularis is morphologically more similar to *A. philippinensis* than to *A. stephensi* within the group *Neocellia* of the subgenus *Cellia* (Christopher, 1933). A histogram based on the percentage of homosequential bands was prepared (Fig. 3). A band to band comparison of the polytene chromosomes of *A. annularis* with those of *A. stephensi* (Sharma *et al*., 1969) and *A. philippinensis* (Sharma, 1977) was also made (Table 1). These observations revealed that in *A. philippinensis* about 2/3 of the bands in autosomes were homologous with *A. annularis*, the remaining 1/3 had no similarity. The sex chromosome had bands which were species specific. The distal and proximal parts of right arm of chromosome 2 were almost identical but central segment had tenuous homology. Left arm of chromosome 2 was conservative as only region 28-27 and 22-21 had displayed some similarity. In

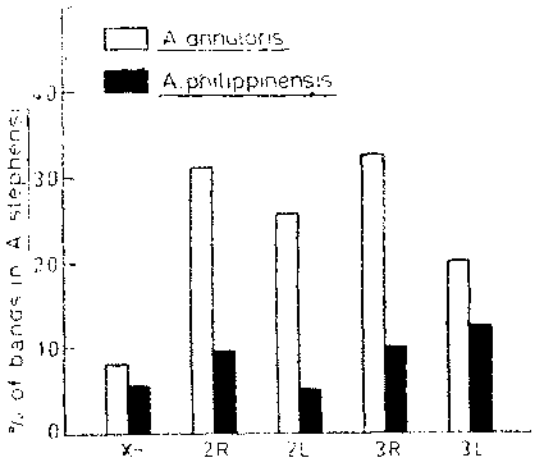

Fig. 3 — Histogram Showing Per Cent of each Arm of *A. annularis* and *A. philippinensis* Homosequential with *A. stephensi*

Table 1—A Band to Band Comparison of the Maps of *A. annularis*, *A. stephensi* and *A. philippinensis*

Chromosome	*A. annularis*	*A. stephensi* bands homosequential with		*A. philippinensis*
		A. annularis	*A. philippinensis*	
X-	3A-3C	2A-2C	3B	
	4B-3D	3B-3D	3D	4A-4B3A
	6B	6B	—	—
2R	7A-7C	7A-7D	—	—
	11B-12D	11C-12C	—	—
	17C-18C	18C-17A (inverted)	18B-18C	18D-19A
2L	20D	22A	—	—
	20B-21A	22C-21C	—	—
	21B-22A	21D-20D (inverted)	19A	19A
3R	33A-34D	33C-34C	32B	32C
	36A-37D	36B-37C	34B	34C
3L	39A-38F	38C-39A (inverted)	40D 38D	40D 38F

chromosome 3 homologies were quite clear in the right arm. In the left arm there was no similarity except in the regions 46A-46C and 39C-40A which had little similarity, and this arm was also distinct like X-chromosome.

In *A. stephensi* in the 2R chromosome, free and centromeric ends are identical with 2R of *A. annularis*. There were 2 paracentric inversions in zone 8B-9B and 16A-17B. Rest of the banding pattern was found identical. in 2L chromosome of *A annularis* only zone 22A-20D is similar to *A. stephensi*, if inverted. In 3R of *A. annularis* zones 33A-34D and 36A-37D had the banding pattern of *A. stephensi* if inverted and there was no other similarity. Chromosome 3L had no similarity except zone 38-39 which had some similarity, if inverted. In *A. annularis* chromosome 3 was found to be a resultant of an inter-chromosomal arm exchange between zones 31-32D of 3R and 41A-40C of 3L in *A. stephensi*.

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Pyrimethamine in Combination with Sulfadoxine or Sulfalene in *P. falciparum* Infected Cases in India

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Pyrimethamine combined with sulfadoxine or sulfalene was tried in *P. falciparum* cases, which were groups of (1) Chloroquine susceptible, (2) Chloroquine resistant, and (3) Unknown status to chloroquine susceptibility in chloroquine resistant areas. In chloroquine sensitive areas of Arsikere (Karnataka), patients given SDP (1000 + 50) mg base in single adult dose, asexual parasites of *P. falciparum* were cleared from all the 35 cases by Day 7. In patients given SLP (1000 + 50) mg base as single adult dose, two out of 34 did not show the clearance. At Rajpur (Uttar Pradesh), the above dose of SLP combination given to 16 cases, was as effective as chloroquine 1500 mg base, in fully clearing asexual parasites by Day 7. In Manja PHC (Assam) where chloroquine resistant cases were selected by an immediately preceding 7-day *in vivo* test, SLP (1000 + 50) mg base cleared the asexual parasites from all 16 cases by Day 7, but 4 were again positive by Day 28. With the same dose of SLP one out of 18 by Day 7 and 9 out of 18 by Day 28, did not show clearance to the asexual parasite. Results were poor with both repeat chloroquine and repeat amodiaquine, thereby showing failure by Day 21 in 19 out of 22 cases and in 12 out of 18 cases respectively. In areas of Bokajam (Assam); Bagmara (Meghalaya); and Gumagarh (Orissa) where both chloroquine resistant and susceptible strains were found, these two drug combinations were administered without prior determination of chloroquine susceptibility status. One out of seven cases showed asexual parasitaemia on Day 28 with the SLP combination at Bagmara.

Introduction

Chloroquine resistance in *P. falciparum* was first recorded in 1973 and subsequently in some other states of India (Sehgal *et al.*, 1973; Pattanayak *et al.*, 1979 a, b; De *et al.*, 1979; Das *et al.*, 1979; Guha *et al.*, 1979). Both for individual treatment and for a rational use of chemotherapeutic measures in the National Malaria Eradication Programme, information about the efficacy of the alternative drugs has therefore become vital.

Sehgal *et al.* (1974, 1973) found quinine-pyrimethamine combination quite effective for the chloroquine resistant strains in Assam, but its 5-day course of treatment deprives it of the advantages offered by the single dose treatment with long-acting sulfonamide in combination with pyrimethamine. Two such combinations i.e., sulfadoxine-pyrimethamine (SDP) and sulfalene-pyrimethamine (SLP) are in wide use in other countries for the treatment of chloroquine resistant cases. The present paper

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[2] Regional Office for Health and Family Welfare, Bhubaneswar.
[3] Regional Office for Health and Family Welfare, Lucknow.
[4] National Malaria Eradication Programme, Delhi.

Table 1 Result of Treatment with Sulfadoxine (SD) + Pyrimethamine (P), Sulfalene (SL) + Pyrimethamine (P), and Chloroquine in two areas having Chloroquine Susceptible *P. falciparum* : July-November 1979

(A) Arsikere, Hassan District, Karnataka

Drug (s) mg base (adult dose)		Day 0	Day 1	Day 2	Day 4	Day 7
SD 1000 + P 50	(a)	35/35	17/35	4/34	4/35	0/35
	(b)	3221	1541	44	57	0
Chloroquine	(a)	17/17	7/17	0/17	0/17	0/17
600	(b)	2924	779	0	0	0
SL 1000 + P 50	(a)	35/35	16/34	2/34	3/34	2/34*
	(b)	3231	995	31	55	29

(B) Rajpur, Kanpur District, Uttar Pradesh

		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 14	Day 21
SL 1000 + P 50	(a)	16/16	16/16	16/16	12/16	4/16	1/16	0/16	0/16	0/16	0/16
	(b)	388	122	33	8	5	0.2	0	0	0	0
SL 1500 + P 75	(a)	3/3	3/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	(b)	178	55	25	0	0	0	0	0	0	0
Chloroquine	(a)	10/10	10/10	0/10	7/10	1/10	1/10	0/10	0/10	0/10	0/10
1500	(b)	233	118	30	12	3	1	0	0	0	0

Note : (a) Number positive for asexual parasites/number examined.

(b) Asexual parasite density per µl.

* Both belonged to the age group 8-14 years.

reports the findings of treatment with SDP and SLP in areas where strains of *P. falciparum* were : (1) sensitive to chloroquine, (2) resistant to chloroquine, and (3) mixed populations consisting of chloroquine sensitive and resistant strain.

Material and Methods

This field study was started with a mass blood survey in localities endemic for malaria to select asexual parasite positive *P. falciparum* cases. Seriously ill patients, those with scanty or mixed infections were excluded. Many cases had to be discarded because of the presence of 4-aminoquinolines in their urine. Drop-out rate was heavy during the follow-up period, because of the inconvenience caused to the patients by repeated finger pricking.

The study on chloroquine sensitive cases was carried out in : (1) Arsikere (Hassan district of Karnataka), and (2) Rajpur (Kanpur district of Uttar Pradesh). The status of pyrimethamine resistance in these areas was not known (Table 1).

In Manja Primary Health Centre (PHC) of Karbi-Anglong district (Assam), the Annual Parasite Incidence (API) in 1979 was 66.8. Chloroquine resistant cases were selected from 6 villages through the 7-day *in-vivo* 1500 mg base adult dose test (WHO, 1973) and confirmed RII cases were taken for SDP/SLP treatment. Pyrimethamine resistance to the extent of 38.6% was recorded in the adjoining PHC of Bokajam of the same district (Das *et al.*, 1980).

For the study of the mixed populations of chloroquine resistant and susceptible cases, three areas were chosen. (a) Dillai of Bokajam PHC (Karbi-Anglong district) : This PHC had an 24.8 API in 1979 and 33.3% of *P. falciparum* cases had chloroquine resistance (RII); (b) Bagmara PHC of West Garo Hills (Meghalaya State) : This had an 49.7 API in 1979 and 40.0% of *P. falciparum* cases were chloroquine resistant (RII); and (c) Gumagarh PHC of Phulbani district (Orissa) : In this area only RI cases were suspected. This block had an API of 40.97 during 1979. Pyrimethamine resistance in 38.6% *P. falciparum* cases were noted in Bokajam PHC (Das *et al.*, 1979). In Bagmara and Gumagarh, pyrimethamine resistance was not studied.

In all the three areas, comparison was made with chloroquine and effect of repeat dose of chloroquine, amodiaquine was also studied in Manja PHC. All the study areas were endemic for malaria and the cases were taken as semi-immune from the view of immune status.

One hundred thick blood smear fields were examined for declaring a slide negative. Parasite count per µl was expressed on the basis of count made against 300 leucocytes and then multiplying it by 25 (i. e., 7 500 WBC taken as standard count per µl), where actual total WBC count was made, the parasite count was based accordingly.

Dill and Glazko test, (Lelijveld and Kortmann, 1970) was employed for the presence of 4-aminoquinolines in urine and Lignin test was used for sulfonamides before giving the drug and also one day after the drug administration.

Each SDP and SLP tablet consisted of 500 mg base of sulfadoxine or sulfalene respectively in combination with 25 mg base pyrimethamine. Two adult dose levels i.e., SD or SL 1000 mg + P 50 mg, or SD or SL 1500 mg +P 75 mg were tried*. Children were given the drug as per their age i.e., under 1, 1-4 years, 5-8 years,

* This was considered necessary as in some countries (e.g., Thailand) with chloroquine resistance SD 1500 + P 75 is being used due to poor response of SD 1000 + P 50.

Table 2 — Results of Treatment with Sulfadoxine + Pyrimethamine, Sulfalene + Pyrimethamine, Repeat Chloroquine and Amodiaquine on Proven Chloroquine Resistant *P. falciparum* in Manja PHC, Karbi-Anglong District, Assam : June-July 1979

Drug (s) mg base (adult dose)		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28
SD 1000 + P 50	(a)	16/16	15/15	2/12	0/13	0/9	0/12	0/6	0/16	0/15	0/14	0/14	1/13	1/12	4/16
	(b)	3138	2469	6	0	0	0	0	0	0	0	0	38	38	80
SL 1000 + P 50	(a)	18/18	17/18	5/14	1/14	1/11	1/14	1/10	1/18	1/16	1/16	1/14	2/16	2/16	9/18
	(b)	6515	2153	34	2	82	116	200	417	469	469	539	539	4879	2660
Repeat chloroquine 1500	(a)	23/23	12/14	8/11	4/8	4/17	2/10	—	7/19	11/21	12/18	17/22	19/22	—	—
	(b)	5851	580	927	103	715	18	—	145	504	746	832	866	—	—
Amodiaquine (repeat 4-amino)	(a)	18/18	—	5/17	—	4/15	—	—	3/18	5/18	7/13	10/16	12/18	13/18	14/18
	(b)	827	—	94	—	57	—	—	180	4444	5536	4634	4308	4388	4388

Note : (a) Number positive for asexual parasite/number examined.

(b) Asexual parasite density per µl.

and 9-14 years received 1/8, 1/4, 1/2 and 3/4 of the adult dose respectively. Those of 14 and above years were given the adult dose. To observe the effects of SDP and SLP, total WBC count and hemoglobin estimation were also done on some cases in Manja PHC. No attempt was made to study the prophylactic or suppressive action and only the curative property was the subject of the investigation.

Results

(1) *Chloroquine sensitive cases* (Table 1) — In Arsikere, there was complete clearance of asexual parasites with SD 1000 + P 50 and chloroquine 600 mg base by Day 7, but with SL 1000 + P 50, 5.9% of the cases remained positive for this stage on Day 7. Sexual stage persisted with both SDP and SLP upto Day 7, the last day of the follow up.

In Rajpur, SL 1000+P 50, SL 1500+P 75, and chloroquine 1500 mg base showed clearance of asexual stage by Day 6 which was maintained upto Day 21, the last day of follow up.

(2) *Chloroquine resistant cases (RII) in Manja PHC* (Table 2) — With SD 1000+P 50, all the 16 got cleared of asexual stage by Day 7 but by Day 28, 25.0% were again positive. With SL 1000+P 50, 5.5% did not show clearance of asexual stage on Day 7 and by Day 28 50% became again positive. With repeat chloroquine 1500 mg base 36.8% still showed asexual parasitaemia on Day 7 and by Day 21, this rose upto 86.4%. With repeat course of amodiaquine 1500 mg, 16.6% was positive for asexual parasitaemia on Day 7 and this rose upto 77.7% by Day 28.

Total WBC count and hemoglobin estimation made on 11 cases under SD 1000+P 50 and 10 cases under SL 1000+P 50 showed that there was a mild reduction of WBC in both the groups. In the former the pre-treatment mean WBC count of 5 782 per μl came down to 5 118, 5 392 and 5 347 on Days 7, 17 and 28 respectively. In the latter, the pre-treatment mean level of 6 810 came to 5 380, 6 010 and 5 250 on Days 7, 17 and 28 respectively. Hemoglobin (14.30 g=100%) which was at 70.5% level before SD 1000 + P 50 therapy rose to 82.0% on Day 28; with SL 1000+P 50, it rose from 63.4% pre-treatment level to 71.5% on Day 28.

(3) *Chloroquine resistant and susceptible cases-co-existing* (Table 3) — With chloroquine 1500 mg base therapy in Dillai, RII resistance was noted in 33.3% by Day 7, and asexual stage rose to 63.2% by Day 28. With SD 1000 + P 50 and SD 1500+P 75, there was complete clearance of asexual stage by Day 4 and Day 2 respectively, which was maintained upto Day 28. Crescent density per μl showed a rise from 11.3% on Day 0 to 94.3% on Day 7. In Gumagarh, there was complete clearance of asexual stages by Day 7 which was maintained upto Day 28. In Bagmara with 40% cases of chloroquine resistance at RII level, both SDP and SLP (1000+50) group showed no asexual parasitaemia by Day 2. This was maintained upto Day 28 except one case under SLP which was positive on the last day.

Discussion

Sulfadoxine-pyrimethamine — Sulfadoxine is rapidly absorbed and one of the most long-acting sulfonamides. Plasma half life of 1g dose of this drug is about 200 hr (Brooks *et al.*, 1969). Due to sequential action in the same metabolic pathway, this drug combination having high antimalarial property, has been taken up for extensive study by different workers for the treatment of both sensitive and chloroquine resistant *P. falciparum* cases.

The finding were consistent and quite satisfactory and the treatment was acceptable to children also. Single dose treatment with SD 1000+P 50 or SD 1500 +P 75 was found to be effective against both chloroquine sensitive and chloroquine resistant cases (cure rate 90-100%) with toxic manifestations seldom noted.

Table 3 : Results of Treatment with Sulfadoxine + Pyrimethamine, Sulfalene + Pyrimethamine, and Chloroquine in Bokajan PHC, Karbi-Anglong District of Assam, Bagmara PHC, West Garo Hills of Meghalaya, and Gumagarh PHC of Phulbani District of Orissa : August-September, 1979

(A) Dillai, Bokajan PHC, Karbi-Anglong District, Assam

Drugs (s) mg base (adult dose)		Day 0	Day 2	Day 4	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28
Chloroquine	(a)	20/20	12/20	7/16	6/18	6/18	9/19	10/19	10/19	10/19	12/19
1500	(b)	3595	463	442	686	746	836	842	1042	1250	1768
SD 1000+	(a)	22/22	8/21	0/20	0/22	0/18	0/22	0/20	0/15	0/14	0/22
P 50	(b)	1780	62	0	0	0	0	0	0	0	0
SD 1500+	(a)	6/6	0/5	0/5	0/6	0/6	0/5	0/6	0/6	0/3	0/6
P 75	(b)	933	0	0	0	0	0	0	0	0	0
SD 1000+	(a)	22/22	8/20	1/16	0/22	0/20	0/14	0/18	0/16	0/12	0/22
P 50	(b)	2830	1083	5	0	0	0	0	0	0	0
SL 1500+	(a)	7/7	1/7	0/4	0/7	0/7	0/7	0/5	0/7	- -	0/6
P 75	(b)	9604	29	0	0	0	0	0	0		0

(B) Gumagarh PHC, Phulbani District, Orissa

		Day 0	Day 2	Day 4	Day 7	Day 10	Day 14	Day 21	Day 28
SL 1000+	(a)	8/8	8/8	0/8	0/8	—	0/3	0/3	0/3
P 50	(b)	923	0	0	0	—	0	0	0

(C) Bagmara PHC, West Garo Hills, Meghalaya (November-December 1979)

Chloroquine	(a)	5/5	4/5	2/5	2/5	. . . . (Study closed, one left the place and other took SDP)			
1500	(b)	10025	570	81	44				
SD 1000+	(a)	7/7	0/3	0/4	0/5		0/5	0/5	0/7
P 50	(b)	1407	0	0	0	—	0	0	0
SL 1000+	(a)	7/7	0/4	0/5	0/7	0/4	0/5	0/7	1/7
P 50	(b)	16019	0	0	0	0	0	0	0

Note : (a) Number positive (asexual) number examined.
(b) Asexual parasite density per µl.

In the present study with SDP, results were good in chloroquine sensitive and mixed group of sensitive and resistant cases. But in the chloroquine resistant cases by Day 28, 25% were again showing asexual parasitaemia. How many of them are due to reinfection or to abnormal metabolisers of sulfa drug was not known. Pyrimethamine resistance to the extent of 38.6% was noted in the nearby Bokajam PHC of Karbi-Anglong district and all probability existed in Manja PHC also. This might also have contributed to this high failure rate.

Sulfalene-pyrimethamine — Like sulfadoxine, sulfalene has some action against *P. falciparum* which gets potentiated by the addition of pyrimethamine and therefore has the advantage of single dose treatment like SDP.

In the present study with SL 1000 + P 50 mg base, there was good response in the chloroquine sensitive cases, but in the chloroquine resistant cases of Manja, there was an initial failure rate of 5.5% on Day 7 and 50.0% of the cases showed asexual parasitaemia by Day 28. How many cases were due to reinfection or to abnormal metabolism of sulfa drugs by the hosts is not known. It is also worthnoting that with SDP, it was 25% against 50.0% noted with SLP.

Repeat course of chloroquine or amodiaquine— In the chloroquine resistant cases, repeat treatment with another 1500 mg base was not found to be useful as 86.4 and 77.7% cases were again positive by Day 28. In such cases, hardly any reliance can be placed on repeat 4-aminoquinoline therapy and alternative drug (s) may be used.

The use of SDP or SLP in the areas having both chloroquine resistant/susceptible *P. falciparum* showed promising results in the present study, even in areas of Bokajam and Bagmara where 33.3 and 40.0% of *P. falciparam* cases showed chloroquine resistance (RII). As it is not easy to carry out prior *in vitro* or *in vivo* tests before drug administration, the use of SDP or SLP has operational convenience in areas from where chloroquine resistance has been reported.

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Short Notes

Experiments to Test Paris Green Emulsion to Control Mosquito Breeding

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Usefulness of paris green in the control of mosquito breeding was first demonstrated by Barber and Hayne (1921). Soper and Wilson (1943) used it successfully in the control of *A. gambiae* in Brazil. Paris green is a stomach poison and resistance to arsenicals in malaria control programmes has not been reported so far. Organophosphorus compounds such as temephos "Abate" or fenthion "Baytex" may prove more effective larvicides than paris green but these insecticides result in the development of resistant strains, a problem which has already become difficult to tackle. This has reawakened our interest in paris green as larvicide for the control of mosquito breeding. Paris green was used as 1 or 2% dust or mixed in kerosene or diesel oil. Transportation of the bulk quantities of dusts was operationally difficult and mixing in kerosene required constant agitation. Therefore, attempts were made to improve its formulation by adding an emulsifier and surfactant for its wider acceptability in the malaria control programmes. This formulation was tested in the laboratory and in the field. Results of this study are reported below:

Paris green emulsion was prepared in bottles containing 60 ml kerosene or diesel. Thirty grams of paris green (90% commercial grade containing 57 ± 2% arsenious oxide) and 1.5 g polysorbate-20 (emulsifier and surfactant supplied by HICO Products Private Limited, Mahim, Bombay 16 DD) was then added. The bottles were shaken thoroughly and diluted to 500 ml volume by adding water. This stock solution was diluted 20 × in water before application.

Bioassay tests were carried out in white circular enamel basins (35 cm diameter). Third and fourth instar anopheline larvae consisting of *A. subpictus* and *A. culicifacies* occurring in nature in a ratio of approximately 3:1, were collected from the field. Fifty larvae were put in each basin in the dechlorinated water. The larvae were fed with yeast powder and left overnight for treatment on the following day. Dosages calculated to produce ½ kg, 1 kg, 2 kg, and 4kg per hectare were applied on the water surface of each basin. Controls consisted of kerosene and diesel oil. Each test was replicated 4 times. Larval mortality was recorded after 24 hr and larvae that pupated were eliminated from the tests.

Results of the experiments revealed that while mortality in the controls i.e., kerosene and diesel averaged 18.6% (range: 17.8 to 22.2%) and 20.6% (range: 17.5 to 25.2%) respectively, mortalities at 4 levels of application in the paris green emulsion in kerosene and diesel averaged 87.5% (range: 86.4 to 88.7%) and 75.6% (range: 66.2 to 81.0%) respectively. There was no difference in the percentage mortality at the 4 levels of application. A dose of 0.5 kg/hectare was found to give optimum kill in the laboratory tests.

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In field trials, paris green emulsion formulation was tested at double the minimum dose i.e., 1 kg/hectare. This was done to compensate insecticide loss due to vegetation and organic matter. The application was made at weekly intervals in 63 villages covering a population of 84 000 in Jawhar and Thane District of Maharashtra. Larviciding was carried out from February to June 1979 with an untreated area held for comparison. During this period adult anopheline densities were measured in the experimental and control villages. In experimental villages mean per hour densities from February to June were 26.4, 23.7, 19.1, 17.3 and 13.9 respectively. In comparison villages, the mean per hour densities of the anophelines for the same months were 29.8, 46.5, 41.8 and 42.0 respectively. There was, therefore, a decreasing trend in adult densities in experimental villages reducing the anopheline populations to about half over a six month period. A reverse trend was observed in the comparison villages and the populations doubled during the same period. The field experiment clearly demonstrated the usefulness of emulsion formulation of paris green in the control of mosquito breeding.

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Sensitivity Status of *P. falciparum* to Chloroquine in Some Parts of Andhra Pradesh and Madhya Pradesh, India

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In Andhra Pradesh, 28-day extended *in vivo* field tests were carried out in the districts of Warangal, Khammam, East Godavari and Vishakapatnam in 1979 and 1980. Only in the last district, one case out of 12 showed RII resistance. *in vitro* Test (macro) showed two cases of schizont maturation at 1.25 nmol chloroquine concentration or above, one each in Khammam and Vishakapatnam districts.

Similar *in vivo* tests were carried out in Bastar and Raipur districts (Madhya Pradesh). Only in Bastar, RII resistance was detected in 6 out of 47 in the test done in Nov. 1980, though RI was suspected on earlier occasions.

Chloroquine resistant strains of *P. falciparum* have been reported from some states of India from 1973-79 : in Assam (Sehgal *et al.*, 1973 and 1974; Pattanayak *et al.*, 1979a); Meghalaya (Chakraborty *et al.*, 1979); Nagaland (Das *et al.*, 1979); Arunachal Pradesh (Pattanayak *et al.*, 1979b); Orissa (Guha *et al.*, 1979); Maharashtra (De *et al.*, 1979; Chowdury *et al.*, in press); and in Uttar Pradesh (Dwivedi *et al.*, 1981). Studies were conducted in some parts of Andhra Pradesh and Madhya Pradesh in 1979 and 1980 for detection of resistance in *P. falciparum* and the findings are presented in this note.

The test procedure and interpretation of results were as per the recommendations of WHO (1973). Initially, a mass blood survey was carried out in different Primary Health Centres (PHC) for selecting suitable cases. Persons having asexual parasitaemia of *P. falciparum* were taken for either *in vitro* (macro) or *in vivo* studies. Chloroquine base was administered on Day 0, Day 1 and Day 2 at the rate of 10 mg, 10 mg and 5 mg per kg respectively, i.e. a total dose of 25 mg/g body weight spread over three days. Urine was examined for the presence of chloroquine as per the technique of Wilson and Edison (1954). Only those who were negative initially for chloroquine in their urine were selected for the study. Parasites were counted against 300 leucocytes.

Results of *in vivo* tests in both Andhra Pradesh and Madhya Pradesh are presented in Table 1 and that for the *in vitro* tests for Andhra Pradesh in Table 2. These show that *P. falciparum* was found to be sensitive (*in vivo* tests) in the districts of Warangal, Khammam and East Godavari. Only in Downuru PHC of Vishakapatnam district, out of 12 cases, one RII and one RI were detected. *in vitro* Test in Downuru PHC confirmed resistance by showing schizont maturation at 2.00 nmol chloroquine in one case. Though *in vivo* test did not show any case of resistance in Khammam district, one case out of 15 showed schizont maturation at

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Table 1—Result of *in vivo* Tests for Determining Chloroquine Sensitivity in *P. falciparum* in Andhra Pradesh and Madhya Pradesh (1979-80)
28-Day Field Test (1500 mg base)

District (period)	PHC	Day 0	Day 2	Day 4	Day 6	Day 7	Day 10	Day 14	Day 18	Day 21	Day 24	Day 28	Remarks
Andhra Pradesh													
Warangal (March 1979)	Mangapet	10/10	1/10	0/10	0/10	0/10	ND	0/5	ND	0/5	ND	0/5	S
Khammam (July 1979)	Satyanarayanapuram	16/16	5/16	0/16	0/16	0/16	ND	0/16	ND	0/16	ND	0/16	S
E. Godavari (Sept. 1979)	Buttayagudem	32/32	4/32	0/32	0/32	0/32	ND	0/31	ND	0/32	ND	0/32	S
Vishakapatnam (July 1980)	Downuru	12/12	6/12	1/12	1/11	1/12	0/10	1/10	0/9	0/9	0/9	0/9	RI—1 RII—1 S—Rest
Madhya Pradesh													
Bastar (Jan. 1979)	Pakhanjore Dandakaranya project (DNK)	15/15	1/15	0/15	0/15	0/15			Study Closed				S or RI
Bastar (Aug. 1979)	Bhopalapatnam	32/32	8/32	0/32	0/32	0/32	ND	0/32	ND	0/32	ND	0/32	S
Bastar (Feb./May 80)	Parakote zone DNK project, Pakhanjore	28/28	27/28	26/28	0/28	0/28	8/28	18/28	20/28	22/28	22/28	24/28	S or RI
Bastar (June 1980)	Bhanupratap pur	34/34	10/34	0/3	20/34	1/34	0/34	0/34	0/34	1/34	0/34	0/32	RI—2 S—Rest
Bastar (Nov. 80)	Pakhanjore Dandakaranya project (DNK)	48/48	21/48	6/48	6/47	6/46	7/43	6/42	4/40	4/37	6/40	7/40	S—31 RIII—2 RII—4 RI—11
Raipur (Aug. 1979)	Bagicha	31/31	5/31	1/31	0/31	ND	0/31	ND	0/29	ND	ND	0/30	S

Note : ND—Not done; Numerator indicates number +ve for asexual stage and Denominator indicates number examined.

Table 2—Results of *in vitro* Tests for Determining Chloroquine Resistance in *P. falciparum* in Khammam and Vishakapatnam Districts of Andhra Pradesh (1980)

Sl. No.	Age and Sex	Asexual parasite/ml of blood large vial %	Control	Schizont count per 300 WBC on exposure to various chloroquine concentration (nmol) expressed as % of control								Remarks
				0.25	0.50	0.75	1.00	1.25	1.50	2.00	3.00	
				(A) District Khammam, Nellipaka PHC								
1	5F	40000 (58)	60	23.23	8.33	0	0	ND	0	0	ND	S
2	8M	17300 (44)	5	0	0	0	0	0	0	0	ND	S
3	25M	8850 (74)	188	5.32	2.66	1.60	1.66	ND	0	0	0	R?
4	5M	41000 (95)	29	20.69	10.34	6.90	0	ND	0	ND	ND	S
5	8F	20160 (86)	98	55.10	12.24	5.10	2.04	ND	0	0	ND	R?
6	10M	21850 (94)	303	11.55	3.63	0.99	0.66	ND	0	0	ND	R?
7	20F	21650 (80)	23	ND	0	ND	0	ND	ND	ND	ND	S
8	26M	1000 (40)	66	7.58	4.55	4.55	0	0	ND	ND	ND	S
9	16M	10800 (67)	317	0.63	0	0	0	0	0	0	0	S
10	18M	10375 (62)	29	13.79	0	0	0	0	ND	0	0	S
11	48M	20775 (85)	539	13 36	2.78	0.93	0.37	ND	0	ND	ND	R?
12	25F	10875 (54)	5	40	40	20.0	0	0	0	0	0	S
13	25M	15825 (53)	324	4.63	3.09	1.23	0.93	0.62	0.93	1.23	ND	R
14	16M	26725 (50)	5	0	0	0	0	0	0	0	ND	S
15	22F	10425 (69)	224	0.89	1.34	0	0	ND	0	ND	ND	S
				(B) District Vishakapatnam, Downuru PHC								
1	14F	5975 (76)	482	60.16	74.68	63.27	48.75	21.16	7.26	0.62	ND	R
2	16F	875 (45)	4	0	0	0	0	0	0	ND	ND	S
3	5M	2450 (44)	12	41.66	0	0	0	0	0	0	ND	S
4	8M	1100 (55)	8	37.5	0	0	0	0	0	0	ND	S
5	18F	8750 (41)	5	0	0	0	0	0	0	0	ND	S
6	13M	12050 (68)	62	32.25	0	0	0	0	0	0	ND	S

Note : Figures within parentheses show the large vial per cent; ND Not done; S—Sensitive; R-Resistant; R?—Doubtful resistant; Control—Mean of 2 tablets.

2.00 nmol in the *in vitro* test. Four more cases showed schizont maturation at 1.00 nmol, which, though within the discriminatory dose of 1.25 nmol, is on the higher side.

In Bagicha PHC of Raipur district (Madhya Pradesh), all the 31 tested were sensitive to chloroquine. In Bastar district where tests were done on five occasions, chloroquine resistance was noted in Bhanupratap pur PHC (one RI, reappearance on Day 7) and Dandakaranya project (two RIII, four RII and ten either RI or S in 1980 test).

Vishakapatnam and East Godavari districts (Andhra Pradesh) and Bastar district (Madhya Pradesh) form a contiguous area. Chandrapur district of Maharashtra which is showing chloroquine resistance adjoins Bastar district on the west. In Orissa State two districts (Phulbani and Keonihar) which lie on the east of this Chandrapur - Baster - East Godavari-Vishakapatnam complex (Guha *et al.*, 1979) have also shown chloroquine resistance. This hilly belt forms a hard core area for malaria with high *P. falciparum* infection. Tribals constitute the major section of the population in this area except DNK project which is the resettlement centre for the refugees from East Bengal (Bangladesh). This therefore, constitutes another major belt of chloroquine resistance outside the North-Eastern part of India.

To meet the situation two pronged attacks has been envisaged: (1) to control the transmission of malaria, insecticidal operations have been intensified with better supervision and epidemiological services under the *P. falciparum* Containment Programme (Ray, 1979) and (2) drugs like sulfalene + pyrimethamine and amodiaquine have been made available for passive and active case detection services respectively. Quinine has also been made available for serious cases and when other drugs fail.

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Bioassay Tests on the Effectiveness of Malathion Spraying on *Anopheles culicifacies* Resting on Different Wall Surfaces in Aurangabad Town, Maharashtra

M. VITTAL[1] and M. R. BHATE[2]

In Maharashtra, *Anopheles culicifacies* resistant to DDT and HCH resulted in the adoption of malathion spraying in 1969. The first signs of resistance to malathion in *A. culicifacies* in Maharashtra were noticed in the year 1973 in Dahanu Tehsil of Thane district (unpublished record). Rajagopal later reported malathion resistance in *A. culicifacies* populations in neighbouring Gujarat State in 1977. Malathion is still used in Maharashtra State to control *A. culicifacies* populations in areas showing epidemiological response to spraying. Tests were therefore carried out on the effectiveness of malathion spraying on *A. culicifacies* that may rest on commonly found wall surfaces. Such a study would be of operational importance in planning the intervals between two rounds of spraying in certain regions of Maharashtra.

The objective of the study was to examine the effectiveness of malathion spraying on different wall surfaces in triple resistant (DDT, HCH and malathion) population of *A. culicifacies*. The experiments were carried out in Aurangabad town from *A. culicifacies* collections made from nearby Chitegaon village.

Before starting biossay tests, wild caught female adults were collected from structures and tested for their susceptibility status to malathion. Adult blood fed females were exposed to 5% malathion impregnated papers for one hour and held in recovery cages for 24 hr before recording mortality (WHO, 1970). There was on an average 60.6% mortality in the pre-test collections. Malathion spraying was carried out in Aurangabad town in July 1980 at the rate of 2 g/m^2 with 50 w.d.p. on the different types of surfaces. The effectiveness of malathion spray deposits was then monitored following the procedure recommended by WHO (1975). Plastic cones were attached to cement, wood and mud wall surfaces. Ten to fifteen adult females were introduced in each cone for 30 minutes. Each test was replicated 3 to 5 times for a period of 6 weeks. After exposure, adults were removed and held in recovery cages. The temperature and relative humidity varied from 25 to 29°C and 85 to 93% respectively. Mortality was recorded 24 hr after the recovery period. Results of this study are given in Table 1.

Results revealed that malathion was effective in the control of *A. culicifacies* on all wall surfaces tested. On cement walls, there was a sudden drop in effectiveness from 81.8% to 63.0% in the first week and then it stayed at that level for another 3 weeks. For the remaining two weeks its effectiveness dropped to 44.4% and 41.5%. In comparison, wood surface gave a better initial kill (87.2%) and effectiveness also lasted for a longer period i.e. 58.4% mortality in the 5th week against 44.4% on cement or 31.2% on the mud. On mud wall the initial kill was highest (91.1%) but then there was a sudden

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Table 1—Bioassay Tests of *A. culicifacies* on Different Wall Surfaces Sprayed with Malathion

Week	Control	Mortality % of *A culicifacies* on surfaces of		
		Cement	Wood	Mud
1	0 (35)	81.8 (44)	87.2 (39)	91.1 (45)
2	8.7 (46)	63.9 (70)	86.9 (61)	60.9 (64)
3	2.8 (36)	67.7 (65)	56.7 (67)	66.7 (60)
4	3.3 (36)	64.3 (70)	54.3 (70)	62.9 (62)
5	3.3 (30)	44.4 (63)	58.4 (65)	31.2 (64)
6	0 (33)	41.5 (65)	44.9 (69)	37.5 (64)

Note: Figures in brackets represent the total number of *A. culicifacies* tested.

drop in effectiveness in the following week (60.9%) and the level of effectiveness remained at about the same level for 3 weeks and then suddenly dropped to 31.2% in the 5th week. Malathion spraying was found more effective on wood (5 weeks) whereas on cement and mud walls the period of effectiveness was 4 weeks. Since the proportion of wood surface in the villages is negligible, the residual toxicity of malathion lasts for 4 weeks under conditions prevailing in this region.

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Further Studies on Chloroquine Resistance in *P. falciparum* in Karnataka State India

G. RAMAN[1], C. ACHUTHAN[1], H. V. PRASAD[1], R. G. ROY[2] and S. PATTANAYAK[2]

Twentyeight-day extended *in vivo* field test (1500 mg base) for determining the chloroquine sensitivity status of *P. falciparum* as recommended by WHO (1973) was carried out in the districts of Chickmagalur (Hirenellur PHC), Tumkur (Nonavinkere and C. N. Halli PHC), and Chitradurga (Jagalur PHC) in 1979. The prevalent strain was found sensitive. The alternative test (600 mg base; 7-day observation) carried out in the districts of Bangalore (Kanaswadi PHC) and Bellary (Arsikere) also showed that the strain was sensitive.

Soon after Sehgal *et al.* (1973) reported chloroquine resistance from Diphu, Assam State. Studies conducted in some districts of Karnataka State to find out the sensitivity status of prevalent *P. falciparum* to chloroquine (Roy *et al.*, 1975; Roy and Prasad 1979; and Roy *et al.*, 1979) by employing the *in vivo* technique (WHO, 1973), and no evidence of chloroquine resistance was found by them. The present note reports the findings of further studies conducted between June 1979 to December 1979. The procedure adopted was the same as recommended by WHO (1973).

The studies were conducted in Chickmagalur, Tumkur, Chitradurga, Bellary, and Bangalore districts. While 28-day extended field test was carried out in the first three districts with 1500 mg base (adult dose), the alternative test in the last two districts was conducted with 600 mg base. Persons of 0-1, 2-4, 5-8, 9-14 and 14 and above years of age respectively received 1/8, 1/4, 1/2, 3/4 and full adult dose of chloroquine. Urine was examined both on Day 0 before the drugging and on Day 1 and Day 2 after the drug administration for detecting the presence of chloroquine in the urine by Dill and Glazko technique (Lelijveld and Kortmann,

Table I -Results of the Mass Blood Surveys in Six PHC's of Five Districts, Karnataka State: June-Dec. 1979

Name of the PHC (district)	Villages/ localities surveyed	Total persons examined	No. positive (all species & all stages)	No. positive *P. falciparum* (asexual stages)
Hirenellur (Chickmagalur)	29	4595	114	20
Nonavinkere (Tumkur)	47	3180	238	27
Jagalur (Chitradurga)	22	7723	440	89
C. N Halli (Tumkur)	20	4875	63	14
Kanaswadi (Bangalore)	9	364	36	19
Arsikere Bellary	3	280	148	96

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[2] National Malaria Eradication Programme, Delhi 110 054.

Table 2—*in vivo* Tests in Six PHCs of Five Districts of Karnataka State : June-December 1979

PHC (district)	Days of follow-up										
	0	2	4	6	7	9	14	16	20	23	28
	(A) 28 Day field test (1500 mg base adult)										
Hirenellur (Chickmagalur)	17/17	4/17	1/16	0/17	0/13	0/15	0/16	0/17	0/17	0/17	0/17
Nonavinkere (Tumkur)	21/21	1/21	0/20	0/20	0/20	0/19	0/16	0/17	0/17	0/17	0/17
Jagalur (Chitradurga)	32/32	11/32	1/32	0/28	0/26	0/21	0/20	0/18	0/19	0/19	0/21
	(B) 7 Day test (1500 mg base adult)										
C.N. Halli (Tumkur)	14/14	0/14	0/14	0/14	0/14	—	—	—	—	—	—
	(C) 7 Day alternative test (600 mg base adult)										
Kanaswadi (Bangalore)	19/16	4/19	0/9	0/17	0/12						
Arsikere (Bellary)	17/17	0/17	2/17	ND	0/17						

Note: Numerator indicates number positive for asexual stage, denominator indicates number examined.
ND= Not done.

1970). Two hundred thick smear fields were examined before declaring a slide negative.

The results of mass blood survey and the *in vivo* tests are presented in Table 1 and 2 respectively. Area wise number of positive persons for *P. falciparum* infection are given in Table 1. The number of subjects included in the *in vivo* test and the number successfully followed up are shown in Table 2. The relatively low incidence of *P. falciparum* did not permit bigger study groups. By Day 6, there was complete clearance of all the asexual stage parasites from both the 1500 mg base and 600 mg base groups and there was no reappearance during the follow-up period and in all the study areas, *P. falciparum* was found to be sensitive.

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OBITUARY

Professor R. N. Chaudhuri
(1.2.1901-6.8.1981)

Professor R. N. Chaudhuri was born on 1st February, 1901 at Kanthalgoria village (District Hooghly, West Bengal). He obtained his post-graduate qualifications from Edinburgh (MRCP later FRCP) and Wales (TDD) and worked at the School of Tropical Medicine (STM), Calcutta (1927-1970) in various capacities including as Professor of Tropical Medicine (1945-1966), also Director (1950-1966) and Emeritus Medical Scientist, ICMR (1966-1970). During his tenure as Director, School of Tropical Medicine, Calcutta, there has been an all round improvement and expansion of the School including creation of new departments of Virology, Biophysics, Nutrition and Metabolic Diseases, Pathology (separated from Bacteriology), Mycology (separated from Dermatology), and Parasitology Division. Professorial chairs were created in Virology, Haematology, Helminthology, Biochemistry and Leprology. Constructions of a new building, a floor on the School and Hospital building each, expanded much needed laboratory and hospital accommodation. Space for a Museum and extension of the Library were also provided with establishment of a Canteen for staff and students.

He is well known in India and abroad, particularly in the field of Tropical Medicine. His important research contributions are on Malaria, Amoebiasis, Tropical Eosinophilia, Epidemic Dropsy, Cholera, Clinical Malnutrition and Food Toxicosis. More than 400 articles, research papers and Editorials in Indian and Foreign Journals are in his credit. In the field of malaria, he contributed mainly on single and combination dose treatment, chemoprophylaxis, etc., and many of these were published in this journal. He contributed chapters in authoritative books on Tropical Medicine. However, recent contributions are mainly on Cholera. He was a recipient of National and International awards, medals, prizes and fellowships including Rockefeller Fellowship and 'Padma Bhushan' awarded by the President of India (1960).

He had been associated with many National and International bodies viz., Indian Council of Medical Research, WHO Expert Committee, Indian National Science Academy, National Academy of Medical Sciences, Indian Science Congress, Indian Association of Pathologists and Microbiologists, Royal Society of Tropical Medicine and Hygiene, London (Vide-President, 1973-1975 and presently was the Local Secretary), Honorary Member, American Society of Tropical Medicine and Hygiene, Member, Indian Public Health Association, Asiatic Society, Indian Medical Association, etc. He was closely associated with ICMR Governing Body, Scientific

Advisory Body and Clinical Research and other Committees, Chairman—Cholera Section, International Congress of Tropical Medicine and Malaria in Lisbon (1958), in Rio de Janeiro (1963) and in Teheran (1968), President — Medical and Veterinary Section, Indian Science Congress (1952), and President, Calcutta Medical Club (1974-1976).

He served as WHO Consultant on Cholera in several countries including Philippines, Indonesia, Burma, Afghanistan and Maldive Island. He toured China, USSR, USA, Africa, Singapore, South America and West Europe and delivered many lectures and orations. He was Founder-Editor, *Bull. Calcutta School Trop. Med.*, and Member, Editorial Board, *Indian J Med. Res.*, *Tropical Doctor* (London), *International Review of Tropical Medicine*, and the *Journal of International Medical Research*, (London).

He died at his Calcutta residence—77, Jodhpur Park, Calcutta 700 068 on August 6, 1981 and survived by his wife, a daughter, a son and a large number of students and admirers

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