Correction.

The isolates of Plasmodium which were thought to have derived from animals collected near Brazzaville have been subsequently found to have derived from animals captured near Bangui in the Central African Republic. This error does not apply, however, to isolate 194ZZ which is correctly listed as originating from Brazzaville.

This correction should be born in mind on pages 11, 12, 25, 26, 44, 45, 46 and 51.

ENZYME VARIATION IN MALARIA PARASITES

R. Carter, B.Sc.

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Institute of Animal Genetics

University of Edinburgh

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SUMMARY

1) A study has been made of electrophoretic forms of enzymes in strains of the rodent malaria parasites Plasmodium berghei and Plasmodium vinckei and their host tissues. The mouse, the African tree rat, the white rat and the golden hamster were used as vertebrate hosts. Anopheles stephensi was used as the mosquito host.

2) The technique of starch gel electropheresis was used to separate electrophoretic forms of enzymes. Regions of enzyme activity were identified by colour linked reaction in which MTT-tetrazolium was generally used as the depositing dye.

3) A survey of the activities and electrophoretic forms of about twenty enzymes was made in the blood parasite and the red blood cells of the rodent hosts. Most of the enzymes examined in this survey were associated with the pathways of aerobic and anaerobic respiration. The activities of the enzymes were compared on the basis of intensity of staining and length of time of incubation of the starch gel electrophoretogram. The relative activities of the enzymes were discussed in terms of the metabolic priorities of the host and parasite cells.

4) Six of the enzymes surveyed were selected for a study of comparative electrophoretic mobility in strains of malaria parasites in the blood stage. Unique electrophoretic forms of several enzymes were found to characterize different species and subspecies of parasite. It was thus demonstrated that the electrophoretic forms of these enzymes could be used to distinguish between species and subspecies of parasite.

5) For two enzymes - glucose phosphate isomerase (GPI) and 6-phosphogluconate dehydrogenase (6PGD) - variation was found among strains of single subspecies. Some strains were found to be characterized by two variants of GPI. By subinoculation of diluted blood parasitized by such a strain it was shown possible that lines of parasites could be produced in which one or other variant only was present.

6) A theoretical model is proposed to enable the analysis of the distribution of variants among strains of a single population of parasites. It is shown that values for some of the parameters governing the distribution of variant alleles in a population of malaria parasites can be estimated from the application to the model of their distribution among strains of parasites.

7) The presence of three enzymes - GPI, 6PGD and NAD-dependent malate dehydrogenase (MDH) - was detected in preparations of mosquito mid-gut tissue heavily infected with oocysts and shown to have distinct electrophoretic mobility from the enzymes of the mosquito tissue. By comparing the oocyst enzymes with those of the blood parasite it was shown that GPI and probably 6PGD were identical in both stages of the parasite life cycle. The mobility of MDH was found to differ in the two stages.

8) It was shown that differences in the electrophoretic mobilities could be used to distinguish the enzymes of the host and parasite. Different enzyme reactions occurring during incubation of the same electrophoretogram were also identified in this way.

9) Studies on genetic hybridization were carried out using two strains of P. berghei yoelii characterized by different variants of GPI, one strain being resistant and the other sensitive in the blood stages to the antimalarial drug pyrimethamine. Preliminary results indicated that reassortment of characters had taken place after simultaneous transmission of the two strains through mosquitoes.

INTRODUCTION

The development of resistance of human malarias to chemotherapy has become in recent years, one of the most serious problems facing the effort to control malaria (Bruce-Chwatt 1967, Peters 1969). The search for effective new drugs, therefore, is one of continuous endeavour. In addition, however, to the attempts to combat resistance by the use of new drugs, there is also an obvious need to study, in the parasite itself, the mechanisms of resistance to the drugs in current use, and the mechanisms of acquisition and transfer of resistance among parasites.

Morphological and biochemical aspects of drug action on the malaria parasite in sensitive strains, and of resistance mechanisms in resistant strains, have been given special attention since the extent of the problem was recognized in the mid 1960's (WHO 1965). However, with the exception of an investigation by Yoeli et al. (1969), the mechanisms of transfer of resistance among malaria parasites have not been considered. Yoeli produced evidence of transfer of drug resistance during concurrent infection of mice with a pyrimethamine resistant strain of Plasmodium vinckei and a drug sensitive strain of Plasmodium berghei. As a mechanism for the apparent transfer of resistance he proposed that cells of the two species, lying adjacent to each other in a single red cell, exchanged segments of their genomes. Events occurring by such a mechanism are, however, probably rare. A more likely basis for the rapid spread of resistance among malaria parasites is by cross fertilization of gametes of resistant and sensitive parasites. In attempting to understand the transfer of drug resistance, therefore, it is obviously important to study the conventional genetics of the malaria parasite.

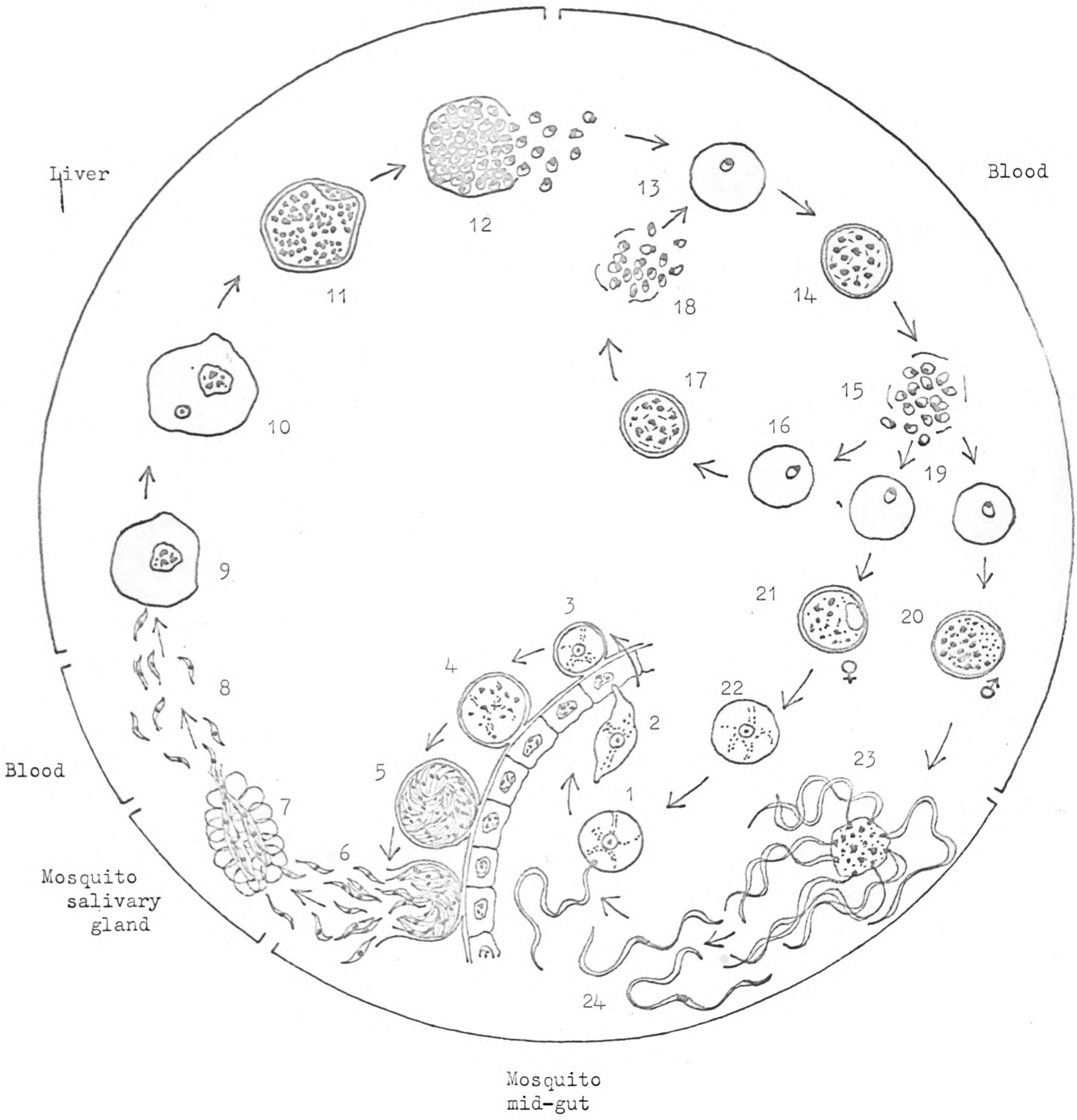


FIGURE 1 Life cyle of a mammalian malaria parasite.

Key to Figure 1

1. Zygote formation in the lumen of the mosquito mid-gut by fertilization of a macrogamete by a single microgamete.

2. Ookinete, motile form of the zygote, penetrates the epithelial lining of the mosquito mid-gut.

3. Early oocyst formed from the ookinete after penetration of the mosquito mid-gut. It lies under the elastic membrane on the outer surface of the mosquito mid-gut.

4. and 5. The oocyst develops with production of sporozoites.

6. The mature oocyst ruptures and sporozoites are released into the haemocoel.

7. Sporozoites collect in the salivary gland of the mosquito.

8. When the mosquito bites sporozoites from the salivary gland enter the blood of the vertebrate host.

9. Sporozoites migrate to the liver and enter the parenchyma cells.

10. An early exoerythrocytic parasite.

11. The exoerythrocytic schizont develops.

12. The fully developed exoerythrocytic schizont ruptures and releases merozoites into the circulating blood.

13. The merozoites enter circulating red blood cells.

14. The erythrocytic schizont develops.

15. The mature erythrocytic schizont ruptures with release of merozoites.

16., 17 and 18. The blood parasite is maintained by recurring cycles of erythrocytic schizogony.

19. Merozoites destined to form gametocytes enter red blood cells.

20. and 21. The male and female gametocytes develop to maturity.

22. The macrogamete develops from the macrogametocyte in the mosquito mid-gut gollowing a blood meal by the mosquito.

23. Exflagellating microgametocyte produces microgametes in the mosquito mid-gut.

24. Microgametes.

Until now, genetic studies on malaria parasites have been rarely attempted. In the early 1950's, Greenberg and Trembly attempted to cross strains of the bird malaria Plasmodium gallinaceum (Greenberg and Trembley 1954, Trembley and Greenberg 1954, Greenberg 1956). However, evidence that hydridization had occurred was inconclusive. The techniques of manipulation of the organism and the character differences between the strains of P. gallinaceum used in this work, were not adequate for making a satisfactory genetic analysis.

Unlike most of the free living protozoa used in genetic research, the complexities of the life cycle and the environment in which the malaria parasite develops present many obstacles to work involving the manipulation and cultivation of cells. In all stages of development the parasite has either an intracellular existance or is otherwise closely associated with its host tissue. Moreover the parasite passes through a number of transformations in the course of its development in the vertebrate and invertebrate host.

The life-history of a typical mammalian malaria parasite is shown in Figure 1. Following a blood meal by a mosquito on an infected mammal host, male and female gametes are released into the lumen of the mosquito gut where fertilization takes place. The zygote (ookinete) penetrates the gut wall and develops into an oocyst on the outside wall of the mid-gut. Although very little is known of nuclear events in the malaria parasite, it seems probable that a reduction division occurs shortly after zygote formation (Canning and Anwar 1968). Within the oocyst a series of nuclear divisions results in the formation of several thousand uninucleate sporozoites. Sporogony is completed by release of the sporozoites into the haemocoel. The sporozoites migrate to the salivary glands of the mosquito and are injected into the blood of the mammal host when the mosquito bites.

In the blood the sporozoites are carried to the liver where they enter parenchyma cells and undergo a process of division known as schizogony. The products of schizogony are uninucleate merozoites. These are released into the blood stream where they invade red blood cells. Here the parasites undergo further schizogony and merozoites released from the blood schizonts invade fresh red blood cells. Asexual development of the blood parasites is thereafter continued by repeated cycles of schizogony in red blood cells. Some merozoites, however, produce not schizonts but gamete forming cells or gametocytes. The mature gametocytes, male and female, when ingested by the mosquito during a blood meal complete their development into active gametes and break out of the red cell membrane. Fertilization takes place and the life cycle is completed.

In the precise but changing environments in which the malaria parasite develops considerable difficulties arise in manipulating and growing single cells. Satisfactory genetic analyses can be made, however, only if, at certain stages single cells can be handled and cultured. For example, to examine the progeny of individual fertilizations, individual oocysts, derived from individual zygotes, must be isolated and cultured to maturity. The analysis of the progeny of crossing experiments and the creation of "true breeding" lines prior to fertilization necessitates the isolation of single cells at other stages of the life cycle. Separation of male and female gametes is highly desirable but presents special difficulties of separation.

The organism thus presents major technical obstacles to genetic studies. These obstacles, moreover, remained to be overcome at the outset of the work presented here. Nevertheless, in co-ordination with other workers studying

these problems, the main object of this investigation was to assist in a project to study the genetics of the African rodent malaria parasite Plasmodium berghei, by identifying variation suitable for use as genetic markers, among different strains of the parasite.

While the study of the inheritance by malaria parasites of resistance to drug treatment may be considered to be the primary aim of such work it is important to have available as large a selection of genetic markers as possible. The result of work with other biological systems has shown that electropheretic mobility of proteins is frequently a rich source of genetic variation (Lewontin and Hubby 1966, Harris et al. 1968, Selander and Yang 1969). The technique of starch gel electrophoresis has been found to be ideally suited to such studies and is amenable to use with small quantities of material. By identifying enzymes by specific colour linked reaction mixtures it is possible to examine single gene products. This fact alone lends the technique a unique attraction as a means of studying genetic variation. There are, moreover, about fifty different enzyme reactions which have been satisfactorily linked to a colour forming reaction. The number of possible loci which can be examined, therefore, is probably considerably greater than fifty, according to current genetic theory. The use of electrophoretically variant enzymes as genetic markers has other attractions. Such variants are not normally either dominant or recessive but are equally expressed and identifiable in diploid cells. Genetic analysis of hybrid generations is, therefore, at its simplest. In the case of enzymes consisting of two or more subunits derived from the same locus, chemical hybridization may occur in diploid, heterozygous cells. Such heterozygotes may then be

readily identified among cells homozygous for either variant, by their distinctive pattern of electrophoretic mobility.

In addition to identifying variation between strains of parasite, it was also hoped to use the technique of starch gel electrophoresis to examine the enzymic relationships between different stages of the parasite life cycle. Because of the difficulty in obtaining material from other stages, however, most of the work concerned with the study of variation between strains was carried out using the blood parasites. The mature cocyst was the only other stage examined.

The stimulus to make this study was the search for markers for genetic studies. Many of the difficulties of manipulation of the experimental organism, however, continued to evade solution during the course of the present work. The application to genetic hybridization studies of such markers as were identified was limited, therefore. The investigation was, however, extended to cover the full range of species and subspecies of African murine rodent plasmodia at present known. An attempt was thus made to clarify the relationship between rodent plasmodia from different regions of Africa in terms of the distribution of electrophoretic forms of enzymes among them, and to understand the mechanisms of distribution of genotypes among wild populations of parasites. The opportunity was also taken to make a comparative study of the activities of enzymes of parasite and host cell on starch gel electrophoretograms.

It is hoped, however, that the results of the present study will ultimately help to establish an understanding of genetic mechanisms in malaria parasites.

MATERIALS AND METHODS

1) Isolates, strains and lines of Plasmodium (Vinckeia) berghei Vincke and Lips 1948, and Plasmodium (Vinckeia) vinckei Rodhain 1952

a) Definition of terms

For the purposes of the present work the following definitions of terms are used.

Isolate:- The term "isolate" is applied to parasites derived from a single wild host specimen on a unique occasion. A single isolate may contain malaria parasites of more than one species; e.g. isolates collected from the Central African Republic frequently contain both Plasmodium berghei yoelii and Plasmodium vinckei chabaudi.

Strain:- The term "strain" is applied to all parasites of a single subspecies present in an isolate. Thus more than one strain may be present in a single isolate.

Most isolates studied in the present work were from blood specimens collected from the rodent host. Strains derived from such isolates, therefore, comprise all parasites of a single species present in a single blood sample. Consequences deriving from this definition are discussed in section 2) of the Discussion.

Stabilate:- The term "stabilate" was defined by Lumsden and Hardy (1965) in the following way. "A 'stabilate' may be defined as a population of an organism preserved in viable condition on a unique occasion. There will be only as many examples of a stabilate as there were individual samples of it laid down on the unique occasion. It will therefore be possible to designate each stabilate by a unique code letter or number".

Line:- The term "line" is applied to parasites derived from a single strain on a specified occasion.

By strict definition every laboratory manipulation of a strain involves the creation of a new line of parasites. Thus each time blood is passaged from one infected animal to another a new line is formed. In general, however, parasites are referred to as a line only after a strain has been subjected to a special form of manipulation or treatment. For instance, it is common practice to refer to a "drug resistant line" of parasites, such a line having been derived from a drug sensitive strain; parasites derived from a single sample of a deep frozen stabilate are usually referred to as a "line".

A line is sometimes accorded a unique code letter to distinguish it from the parent strain. Thus strain "Keyberg 173" is derived from an isolate collected in Katanga in 1948. The line derived from this strain and used in the present work, is a line of parasites which has lost its ability to produce gametocytes; it is designated line "N".

b) Origins of isolates, strains and lines

Table 1 lists the isolates and strains of Plasmodium berghei and Plasmodium vinckei studied in the present work. A single strain was studied from each isolate. The strains labelled*, are from isolates derived from wild African tree rats (Thamnomys rutilans) delivered to this laboratory. The remaining strains were received from other laboratories as stabilates frozen in liquid nitrogen or solid CO_2, or as parasitaemias in live laboratory mice.

Sub-species of parasite	Isolate	Strain	Locality of capture of wild host	Species of wild host
P. berghei berghei	Anka	Anka	Katanga, River Kasapa	Anopheles dureni
	RLL	RLL	" River Kisanga	" "
	NK65	NK65	" " "	" "
	Keyberg 173	Keyberg 173		
		Line N	" Keyberg	" "
P. berghei yoelii	17X	17X	C.A.R., nr. Bangui	Thamnomys rutilans
	32X	8L	" " "	" "
	33X	3L	" " "	" "
	55X	6L	" " "	" "
	86X	11L	" " "	" "
	146X	146X	" " "	" "
	5BY*	2AR	" " "	" "
	10BY*	4AF	" " "	" "
	29BY*	6AD	" " "	" "
	151BY*	2AK	" " "	" "
	157BY*	4AE	" " "	" "
	206BY*	2BE	" " "	" "
	350BY*	4AZ	" " "	" "
	877*	2BG	" " "	" "
P. berghei Killicki	194ZZ	194ZZ	R.C., nr. Brazzaville	" "
P. berghei yoelii	BR*	2BR	C.A.R., nr. Bangui	" "
	CF*	2CF	" " "	" "
	CL*	2CL	" " "	" "
	CN*	2CN	" " "	" "
	CU*	2CU	" " "	" "
	CX*	2CX	" " "	" "
"P. berghei-like"	N67	N67	Nigeria, nr. Ilobi	" "

TABLE 1a Origin of isolates and strains of malaria parasites - Plasmodium berghei.

The isolates labelled * were derived from tree rats delivered to this laboratory.

C.A.R. = Central African Republic.
R.C. = Republic of Congo.

Sub-species of parasite	Isolate	Strain	Locality of capture of wild host	Species of wild host
P. vinckei vinckei	vinckei-52	vinckei-52	Katanga, River Kinga	Anopheles dureni
P. vinckei chabaudi	20BY*	3AC	C.A.R., nr. Bangui	Thamnomys rutilans
	87BY*	4AT	" " "	" "
	166BY*	3AJ	" " "	" "
	288BY*	1AQ	" " "	" "
	309BY*	1BC	" " "	" "
	326BY*	1AL	" " "	" "
	344BY*	14BJ	" " "	" "
	387BY*	1BK	" " "	" "
	399BY*	1AS	" " "	" "
P. vinckei ~~lentum~~ chabaudi	BQ*	2BQ	~~R.C., nr. Brazzaville~~ C.A.R., nr. Bangui	" "
	BZ*	2BZ	" " "	" "
	CB*	2CB	" " "	" "
	CE*	2CE	" " "	" "
	CP*	2CP	" " "	" "
	CQ*	2CQ	" " "	" "
	CR*	2CR	" " "	" "
	CW*	2CW	" " "	" "
"P. vinckei-like"	1-69	1-69	Nigeria, nr. Ilobi	" "

TABLE 1b Origin of isolates and strains of malaria parasites - Plasmodium vinckei.

c) Classification of strains

The classification of strains received from other laboratories was accepted as that accorded to them by the authorities from whom they were received.

The strains derived in this laboratory were identified at species level from the morphology of the parasites on Giemsa stained blood smears.

P. berghei and P. vinckei from different regions of Africa have been accorded subspecies classification on the basis of the morphology and the developmental characteristics of complete life cycles (Landau and Killick-Kendrick 1966, Landau et al. 1968, Landau et al. 1970). Thus P. berghei from the Central African Republic is classified as P. berghei yoelii, while that from Brazzaville is classified as P. berghei killicki; P. vinckei from the Central African Republic is classified as P. vinckei chabaudi, while that from Brazzaville is classified as P. vinckei lentum.

It was not a practical possibility in the present work to study the complete life cycle of each strain derived in the laboratory. In making a complete classification, therefore, such strains were allocated subspecies classification according to their area of collection in Africa.

d) Strains of single subspecies from isolates derived in the laboratory

Before using isolates for further investigation, strains of single subspecies were derived from the isolates. Where they were found to contain only one subspecies, isolates were used without further refinement. When an isolate was found to contain two species of parasite, however, it was necessary to derive strains of a single subspecies before it was used in further study.

All infected specimens of T. rutilans from the Central African Republic were found to contain P.v. chabaudi. P.b. yoelii was found in isolates derived from about half of these animals. P.v. chabaudi is not infective to laboratory rats (Garnham 1966). Strains of P.b. yoelii were, therefore, freed from contamination with P.v. chabaudi by inoculating infected blood into rats. Such strains were used in subsequent examination of enzymes of P.b. yoelii. In examining enzymes of P.v. chabaudi, only isolates in which it had not been possible to detect P.b. yoelii were used.

The isolates derived from specimens of T. rutilans captured at Brazzaville contained either P.b. killicki or P.v. lentum. Thus each of these isolates represented a strain of a single parasite subspecies.

2) Host species used in the laboratory

Table 2 lists the rodent and insect host species used in the present study. Mice, both inbred and outbred strains, and tree rats were used in most experiments. White rats and golden hamsters were used in special circumstances.

3) Maintenance of malaria parasites in the laboratory

a) Preservation in liquid nitrogen

Stabilates of infected blood were stored in sealed capillary tubes kept in liquid nitrogen, following the method of Lumsden et al. 1966. Blood infections were derived from frozen stabilates by intraperitoneal inoculation of the thawed contents of a capillary tube into the rodent host.

	Host species		Source
Vertebrate	Tree rat	(*Grammomys surdaster*)	laboratory colony
	Mouse	(*Mus musculus*)	commercial suppliers
	White rat	(*Rattus norvegicus*)	" "
	Golden hamster	(*Mesocricetus auratus*)	" "
Invertebrate	Mosquito	(*Anopheles stephensi*)	laboratory colony

TABLE 2 Vertebrate and invertebrate host species used in the laboratory.

b) Blood passage of P. berghei and P. vinckei in laboratory rodents

Strains of P. berghei and P. vinckei were maintained by inoculation of blood from an infected rodent to uninfected rodents. Blood from the infected animal was diluted in citrate saline (0.9% NaCl, 1.5% Na citrate adjusted to pH 7.2) prior to inoculation. About 0.1 ml of the diluted blood was inoculated into each animal. Such an operation is referred to as a "blood passage".

Infected animals were routinely supplied with drinking water supplemented with para-aminobenzoic acid (PABA) to promote high parasitaemia (Garnham 1966).

c) Cyclical transmission of P. berghei

Only strains of P.b. yoelii and strain N67 of the "P. berghei - like" parasite from Nigeria were cyclically transmitted through the mosquito host.

Parasitized blood from an infection not more than five blood passages from a sporozoite induced infection was inoculated into a mouse or tree rat. On the fourth day after inoculation the animal was examined for parasitaemia and exflagellation. It was then anaesthetized with ether or nembutal and placed in a cage of unfed seven to fourteen day old Anopheles stephensi. When the mosquitoes had stopped feeding the animal was removed from the cage. Subsequently a 10% solution of glucose supplemented with PABA was provided as a food source. The mosquitoes were kept in a room maintained at 25°C and 90% humidity, with an alternating 12 hour sequence of light and dark.

On the tenth day after feeding, when ripe sporozoites had reached their glands, mosquitoes were disected and the crushed glands suspended in "199" tissue culture medium. Aliquots of about 0.1 mls. of suspension containing the crushed glands of about ten infected mosquitoes were inoculated into the tail vein of a mouse or tree rat. Patent blood infections could usually be detected on the fifth or sixth day after inoculation.

4) Preparation of parasites and host tissues

a) Blood parasites

Two types of preparation of the blood parasite were used in this work,

i) preparations of parasites freed from the host blood by immune lysis,

ii) preparations of concentrated parasitized blood cells.

i) parasites freed by immune lysis.

To obtain a preparation of a single strain of parasites freed from the host cell, five to ten mice, twenty to thirty days old, were used. The mice were inoculated intraperitoneally with diluted parasitized blood. When parasitaemias had reached a maximum, between 5% and 80% of red blood cells parasitized depending upon the strain, the mice were etherized and bled from the brachial vessels. The blood was transferred immediately into an equal volume of citrate saline. When all the blood had been collected, a volume of 4% $CaCl_2$ was added equal to one tenth the volume of citrate saline. The preparation was then shaken manually with 3mm. glass beads for ten minutes until the beads had clumped with precipitated fibrin. This procedure removed platelets from the blood. Each defibrinated sample was filtered through glass wool and centrifuged at 1000 g for five minutes, yeilding a brown layer of parasitized cells on top of uninfected erythrocytes.

The brown layer was resuspended in up to 10 mls. of phosphate saline, pH 7.4 (0.67% NaCl, 0.34% KCl, 0.029% $MgSO_4$ $7H_2O$, 0.045M phosphate, Krebs and Eggleston 1940) and recentrifuged at 1,000 g for 5 minutes. The final parasitized layer was resuspended in phosphate saline (10% v/v).

The parasites were freed from the erythrocytes by immune lysis using the method of Bowman et al. (1960) with specific anti-mouse erythrocyte serum and preserved guinea pig serum as a source of complement. After lysis of red cells parasites were freed from the red cell stroma by vigorous manual treatment with a teflon pestle. One drop of antiserum was added and the samples were allowed to stand for about 10 minutes until the stroma had floculated. Each sample was then centrifuged for 30 seconds at 300 g. The supernatants, containing most of the freed parasites, were retained and another drop of antiserum added to each. The sediments were suspended in about 5 mls. phosphate saline and again broken up with the teflon pestle, treated with antiserum and centrifuged as before. The combined supernatants, to which a further drop of antiserum was added, were allowed to stand for about 10 minutes and again centrifuged at 500 g for 30 seconds. The final supernatant was centrifuged at 5,000 g for 10 minutes, yielding a hard packed, dark brown pellet of parasites.

By this method parasite preparations entirely freed from contamination with platelets, and with very little red blood cell contamination, were obtained. No precautions were taken against contamination by white cells, but these were estimated to be less than 10% of total cell mass by examination of preparations under a phase contrast microscope.

The final pellet was resuspended in about 5 volumes of phosphate saline and kept at 0° to $5^{\circ}C$ until required.

ii) concentrated parasitized blood cells

When it was not required to use preparations of parasites "purified" by the above procedure much smaller quantities of blood could be used. In such cases preparations of concentrated parasitized blood cells were made. A single well infected animal, i.e. one having at least 5% of blood cells parasitized, provided sufficient parasite material on which to carry out electrophoresis. The method was suitable for use when rodent hosts other than mice were used e.g. tree rats, hamsters or white rats, for which it was not convenient to prepare the corresponding anti-erythrocyte serum. The procedure was much faster than that of the "immune lysis" method and allowed preparations of as many as ten strains of parasite to be made and electrophoresis carried out on the same day.

Blood from the infected animals was taken from the branchial vessels and transferred into 10 volumes of citrate saline. The suspension of parasitized blood was then centrifuged at 1,000 g for 5 minutes yielding a brown layer of concentrated parasitized cells on top of uninfected erythrocytes. The brown layer was resuspended in a further 10 mls. of citrate saline and recentrifuged at 1,000 g for 5 minutes. The final parasitized layer was removed and resuspended in one or two volumes of citrate saline; this was the final parasitized red cell preparation and will be subsequently referred to as a "brown layer" preparation.

Brown layers could not be produced from blood infected with P.v. chabaudi. In this case, therefore, preparations of whole parasitized blood from highly infected animals were used. Such preparations were adequate for the study of some parasite enzymes, but not for all.

b) Host blood cells

Whole blood from uninfected host specimens was suspended in 10 volumes of citrate saline and spun for 5 minutes at 500 g. The sedimented cells, which consisted almost entirely of mature erythrocytes, were resuspended in two or three volumes of phosphate saline as the final preparation of host blood cells.

c) Oocysts and mosquito tissue

The only sporogonic stage examined in this work was the late oocyst.

Mosquitoes fed on infected rodents were examined for oocyst infections on the sixth or seventh day after feeding. If a satisfactory proportion were found to be adequately infected, i.e. more than 100 oocysts on the mid-gut, a selection of mosquitoes was dissected on the eighth day. The mid-guts of about thirty such heavily infected mosquitoes were removed into about 0.1 mls. of "199" tissue culture medium and used as the final preparation of oocyst material.

As controls, thirty mid-guts from uninfected mosquitoes were prepared in a similar way.

5) Preservation of preparations

Whenever possible preparations of parasites and host tissues were used on the same day as that on which they were prepared. In such cases preparations were kept on ice until used.

When a large number of samples was to be examined preparations were freeze dried as whole cells. The dried material was redissolved in distilled water and spun at high speed in a refrigerated centrifuge to

remove all particulate matter. The supernatants were then freeze dried and stored under vacuum at -20°C. In this form samples were found to maintain activity of most enzymes for a period of at least one month.

6) Starch gel electrophoresis (Smith 1968)

a) Preparation of gels

A system of horizontal starch gel electrophoresis was used. All gels for electrophoresis were prepared with 9.4 g hydrolysed potato starch per 100 mls. of buffer solution. The suspension of starch granules in buffer was heated gently in a 250 mls. evacuating flask over an open bunsen flame until a clear easily poured liquid was produced. Bubbles were removed by attaching the flask to a vacuum pump. The liquid gel was then poured into a plate glass mould. Once poured the gels were allowed to cool for about half an hour and were then transferred to a cold room at 0° - 5°C until required. Gels were always used on the same day as they were prepared.

The dimensions of the gels were varied according to the number of samples. A gel 15 cms. wide by 25 cms. long was capable of running 12 samples. The sides of the mould were formed by four strips of glass about 1 cm. wide by 0.5 cms. deep, sealed onto a glass plate with vacuum grease.

b) Preparation of samples for electrophoresis

When preparations of freeze dried material were to be run, samples of 2 to 3 mg. of the dried powder were dissolved in 0.02 mls. of distilled water. Such samples were then ready for immediate electrophoresis.

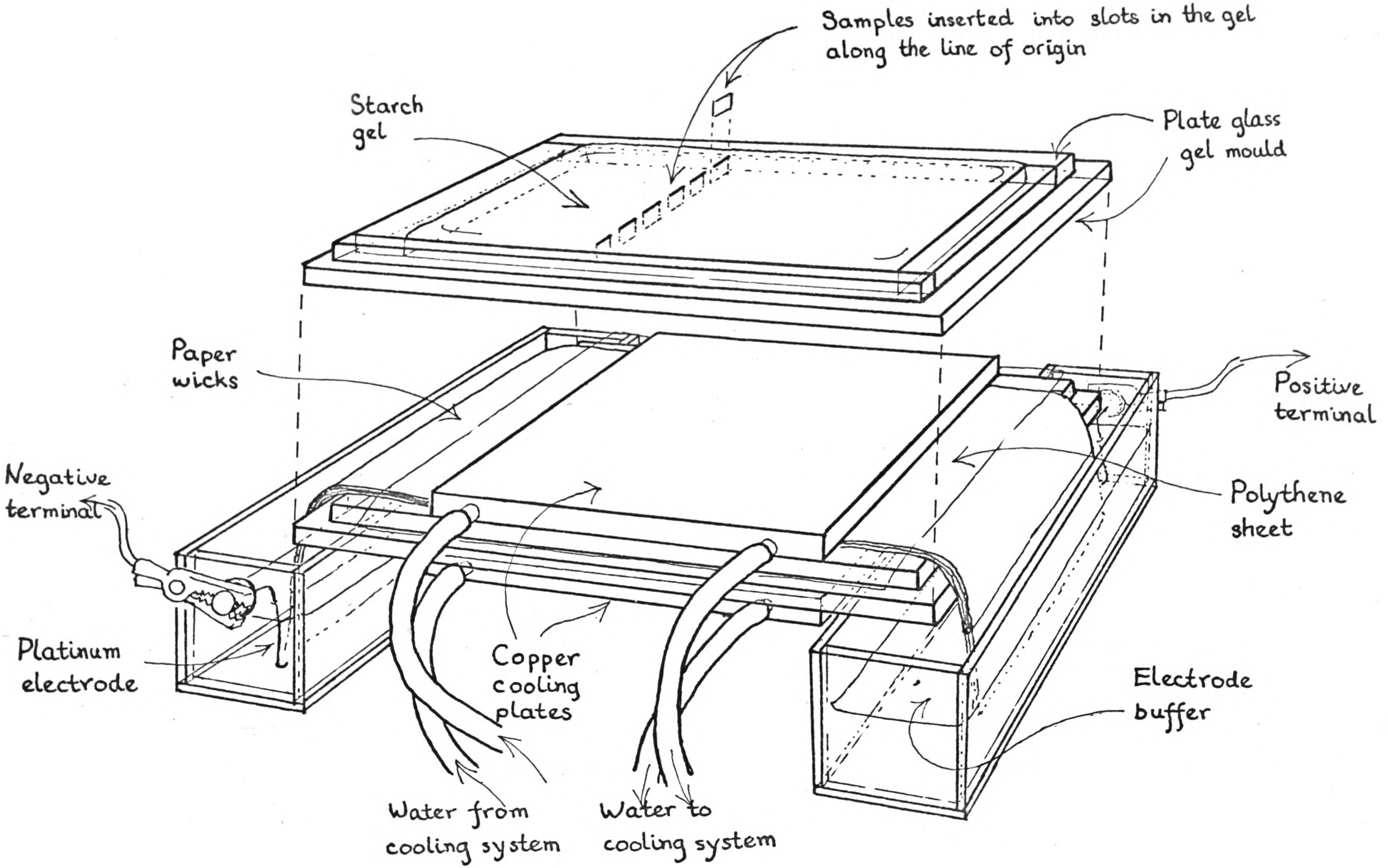


FIGURE 2 Apparatus for starch gel electropnoresis.

Preparations of whole cell suspensions or unfractured tissues were broken up to release the cell contents before electrophoresis. Lysis of blood parasite preparations and of unparasitized blood cells, was carried out immediately prior to electrophoresis by adding 5% Triton X (detergent) to a final concentration of 1% in the cell suspension. Lysis occurred almost instantaneously on agitation of the suspension. Insoluble debris was sedimented by centrifuging at 5,000 g and the supernatant used for electrophoresis.

Preparations of infected and uninfected mosquito mid-guts were treated in a similar way. 0.1 ml. of 5% Triton X was added to each batch of material followed by rapid repeated passage of the samples in and out of a 1 ml. plastic syringe fitted with a 28 g needle.

After the final preparation of samples electrophoresis was performed immediately.

c) Electrophoresis (Figure 2)

Prior to electrophoresis a line of origin on the gel was chosen, 3 cms. to 10 cms. from the cathodal end, the position varying according to the conditions of electrophoresis being used. Samples were soaked onto strips of Whatman filter paper, about 7 mm. x 3 mm., and inserted along the line of origin in slots formed in the gel with a broken razor blade. Paper wicks were made from four layers of filter paper about 8 cms. long and cut to a width equal to that of the gel. These were soaked in the electrode buffers and laid along the edges of the gel to form a bridge for the current at the cathodal and anodal ends. The gel and wicks were then overlaid

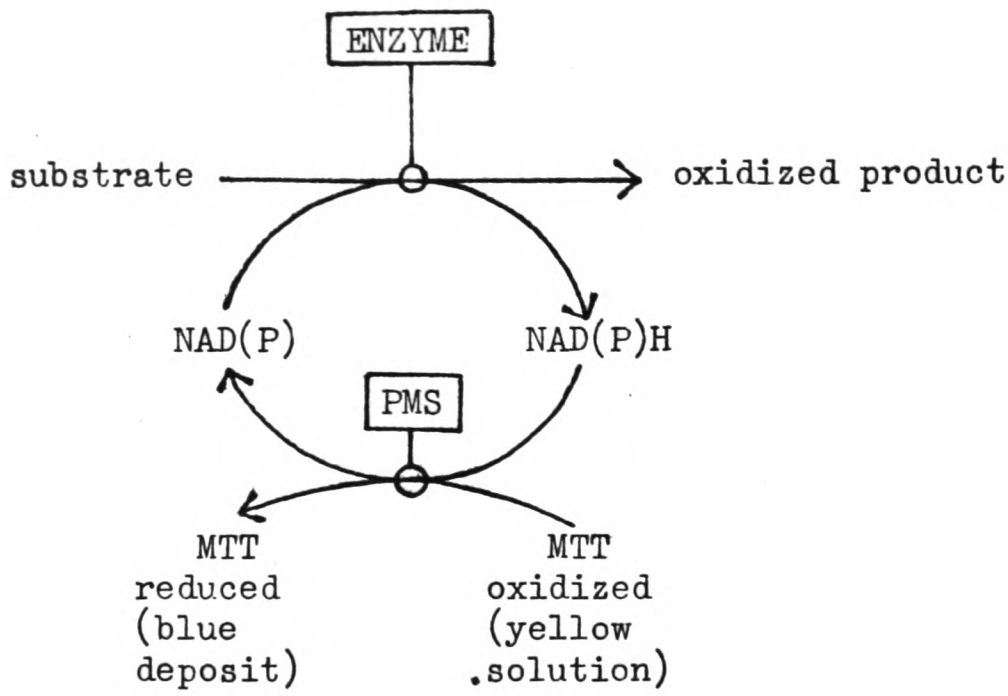


FIGURE 3 Scheme linking enzyme reaction to deposition of dye during enzyme on starch gel.

with a sheet of polythene. During electrophoresis in the open laboratory the gel, overlaid with polythene, was placed between two copper cooling plates attached to a cooling system. Alternatively, during electrophoresis in a cold room, the gel was cooled by placing a metal box containing ice and water on top of the polythene covered gel.

A source of e.m.f. was supplied by Roband Power packs. The current, voltage and length of time of run varied according to the system of buffers being used, the size of gel and the enzymes under study.

The buffer systems used are presented in Table 3.

After electrophoresis gels were sliced horizontally to give top and bottom sections of equal thickness; both were usually developed for enzyme activity.

7) Enzyme assay systems and development of gels for enzyme activity

The reaction components for each enzyme studied are shown in Table 4.

Regions of enzyme activity on starch gel sections were developed by overlaying the sections with a solution containing the specific reaction components of the enzyme under study.

For all enzymes except the α-naphthyl acetate esterases the reaction was linked directly, or indirectly through the mediation of a second enzyme system to the reduction of either NAD or NADP. The reduced pyrimidine nucleotide, in turn, reduced the soluble yellow oxidized form of the dye MTT-tetrazolium to the insoluble blue precipitate of the reduced form, by mediation of the catalyst, phenazine methosulphate (PMS). The regions of enzyme activity were thus made visible by the deposit of blue staining reduced MTT-tetrazolium (Figure 3).

System	Buffers	
	Gel	Electrode
Phosphate-citrate, pH 7.0	0.01M phosphate, 0.0014M citrate, pH=7.0	0.05M phosphate, 0.007M citrate, pH=7.0
Tris-HCl, pH 8.0	0.01M tris-HCl, pH=8.0	0.05M tris-HCl, pH=8.0
Tris-citrate, pH 6.0, 13.0mM	13.0mM tris, 5.5mM citrate, pH=6.0	0.378M tris, 0.165M citrate, pH=6.0
Tris-citrate, pH 6.0, 0.05M	0.050M tris 0.022M citrate, pH=6.0	0.378M tris, 0.165M citrate, pH=6.0
Poulik "discontinuous system"	0.075M tris 0.005M citrate pH=8.9	0.3M borate-NaOH, pH=8.9

TABLE 3 Buffer systems for electrophoresis.

Enzyme	Substrate	Coenzymes	Other additions	Buffer
Hexokinase	glucose, 500 mg.	NADP, 5mg. ATP, 100mg.	G6PD, 6i.u. $MgCl_2$, 20mg. KCN, 5mg. MTT, 6mg. PMS, 0.5mg	0.1M tris-HCl, pH=7.4, 30mls.
Glucose phosphate isomerase	fructose-6-phosphate, 50mg.	NADP, 5mg.	G6PD, 2i.u. $MgCl_2$, 20mg. MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=8.0, 30mls.
Aldolase	fructose-1, 6-diphosphate, 100mg.	NAD, 7mg.	G3PD, 1mg. di-sodium arsenate, 30mg. MTT, 6mg. PMS, 0.5mg.	0.03M pyrophosphate, pH=8.5, 30 mls.
Glyceraldehyde-3-phosphate dehydrogenase	fructose-1, 6-diphosphate, 100mg.	NAD, 7mg.	Aldolase, 1mg. di-sodium arsenate, 30mg. MTT, 6mg. PMS, 0.5mg.	0.03M pyrophosphate, pH=8.5, 30mls.
Lactate dehydrogenase	lithium lactate, 300mg.	NAD, 7mg.	MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=8.5, 30mls.
α-Glycerol phosphate dehydrogenase	α-glycerol phosphate, 200mg.	NAD, 7mg.	MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=8.5, 30mls.

TABLE 4 Enzyme assay systems on starch gel electrophoretograms. For abbreviations see section 9) of Materials and Methods.

Enzymes	Substrate	Coenzymes	Other additions	Buffer
Glucose-6-phosphate dehydrogenase	glucose-6-phosphate, 200mg.	NADP, 5mg.	$MgCl_2$, 40mg. MTT, 6mg. PMS, 0.5mg.	0.1m tris-HCl, pH=7.0, 30mls.
6-phospho-gluconate dehydrogenase	6-phospho-gluconate, 100mg.	NADP, 5mg.	MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl pH=7.0, 30mls.
Malate dehydrogenase (NADP-dependent)	sodium malate, 1g.	NADP, 5mg.	$MnCl_2$, 40mg. MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=7.4, 30mls.
β-Hydroxybutyrate dehydrogenase	solium-β-hydroxy-butyrate, 0.5g.	NAD, 7mg.	MTT, 6mg. PMS, 0.5mg.	0.1M sodium phosphate, pH=7.4, 30mls.
Glutamate dehydrogenase	sodium glutamate, 0.5mg.	NAD, 7mg.	MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=8.5, 30mls.
Isocitrate dehydrogenase	sodium isocitrate, 50mg.	NADP, 5mg.	$MnCl_2$, 10mg. MTT, 6mg. PMS, 0.5mls.	0.1M tris-HCl, pH=8.0, 30mls.
Succinate dehydrogenase	sodium succinate,	FAD (enzyme bound) ATP, 15mg.	MTT, 6mg. PMS, 20mg.	0.5M potassium phosphate, pH=7.0, 30mls.
Fumarase	sodium fumarate, 150mg.	NAD, 7mg.	MDH, 40i.u. MTT, 6mg. PMS, 0.5mg.	0.1M sodium phosphate, pH=7.4, 30mls.

TABLE 4 (contd.) Enzyme assay systems on starch gel electrophoretograms.

Enzyme	Substrate	Coenzymes	Other additions	Buffer
Malate dehydrogenase (NAD-dependent)	sodium malate, 1g.	NAD, 7mg.	MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl, pH=8.5, 30mls.
Diaphorase (NADH-dependent)		NAD, 20mg.	MTT, 6mg.	0.1M tris-HCl, pH=8.0, 30mls.
Diaphorase (NADPH-dependent)		NADP, 20mg.	MTT, 6mg.	0.1M tris-HCl, pH=8.0, 30mls.
Adenylate kinase	glucose, 40mg.	NADP, 5mg. ADP, 20mg.	G6PD, 6i.u. HK, 20i.u. $MgCl_2$, 10mg. MTT, 6mg. PMS, 0.5mg.	0.1M tris-HCl pH=7.4, 30mls.
α-Naphthyl acetate esterases	sodium α-naphthyl acetate, 30mg.		Fast Blue RR, 30mg.	0.1M sodium phosphate, pH=7.4, 30mls.

TABLE 4 (concld.) Enzymes assay systems on starch gel electrophoretograms.

The products of the α-naphthyl acetate esterase reaction reacted with the soluble dye, Fast Blue RR (4'-amino-2, 5-dimethyl benzanilide) to form an insoluble brown precipitate in the regions of enzyme activity.

All enzyme reactions linked to MTT-tetrazolium were incubated at 37°C for between 30 minutes and 3 hours, or overnight, according to the enzyme under study. The gels being developed for esterase activity were incubated for 30 minutes to 1 hour at 25°C.

8) Recording results

Records were made of the regions of enzyme activity on starch gels either by photographing gels using Panatomic X film, or by overlaying the gel with a sheet of polythene and tracing the regions of dye deposit in acetone soluble black marker. The latter method was used by routine.

9) Abbreviations

The following abbreviations for the names of enzymes and enzyme co-factors, are used throughout the text:

Enzymes

hexokinase	HK
glucose phosphate isomerase	GPI
aldolase	Ald
glyceraldehyde-3-phosphate dehydrogenase	G3PD
lactate dehydrogesase	LDH
α-glycerol phosphate dehydrogenase	α-GPD
glucose-6-phosphate dehydrogenase	G6PD
6-phosphogluconate dehydrogenase	6PGD

malic enzyme (NADP-dependent malate dehydrogenase)	ME
β-hydroxybutyrate dehydrogenase	β-OHBD
glutamate dehydrogenase	GDH
NADP-dependent isocitrate dehydrogenase	ICDH
succinate dehydrogenase	SDH
fumarase	Fum
NAD-dependent malate dehydrogenase	MDH
NADH-dependent diaphorase	Diaph (NADH)
NADPH-dependent diaphorase	Diaph (NADPH)
adenylate kinase	AK
α-naphthyl acetate esterases	α-NAE's

Coenzymes

nicotinamide adenine dinucleotide	NAD
nicotinamide adenine dinucleotide phosphate	NADP
adenosine triphosphate	ATP
adenosine diphosphate	ADP

Reagents

MTT-tetrazolium	MTT
phenasine methosulphate	PMS
4'-amino-2, 5-dimethyl benzanilide	Fast Blue

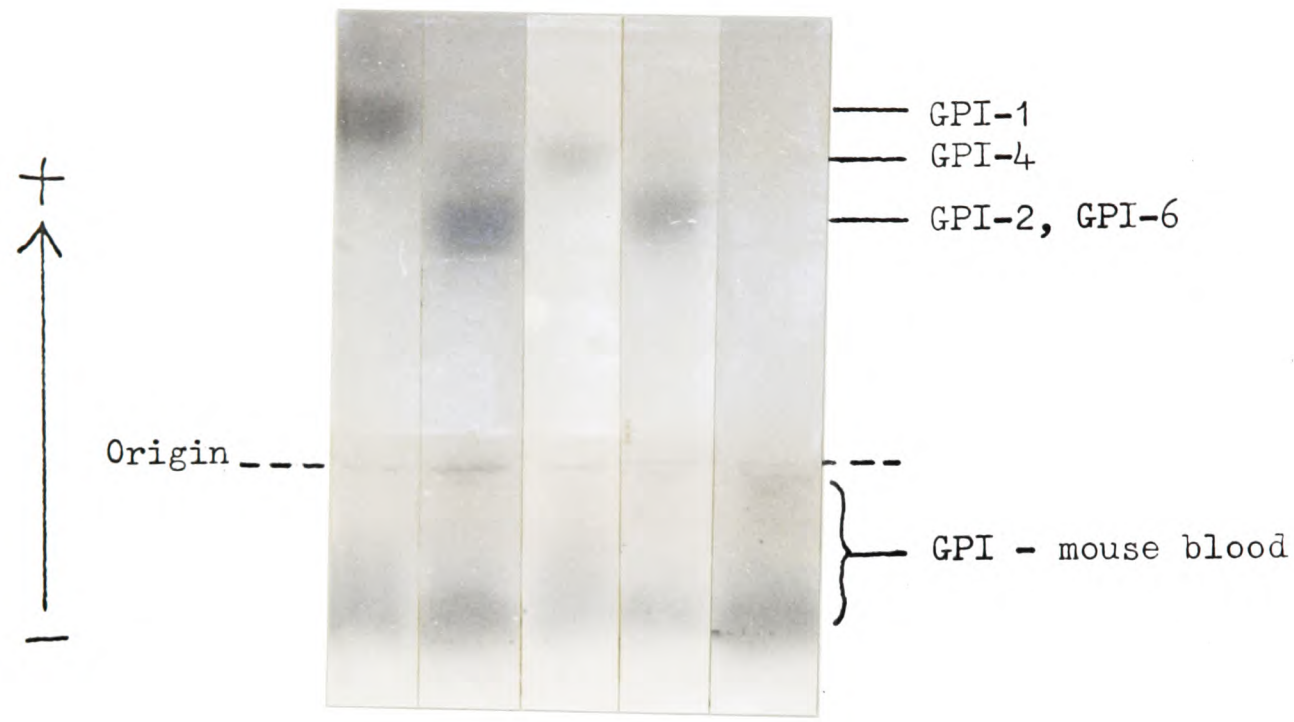


PLATE 1 Electrophoretic forms of GPI in samples of mouse blood and parasitized mouse blood on the Tris-HCl pH 8.0 system.

RESULTS

1) Enzyme variation in blood parasites, among strains of rodent plasmodia

A preliminary survey of about twenty enzymes in the blood parasites was made using starch gel electrophoresis. It was found that several of the enzymes assayed in this way showed no activity, or only traces of activity. Of the enzymes for which activity was demonstrated, many were found to be unsuitable, for technical reasons, for use in studies of comparative electrophoretic mobility. The following were finally chosen for inclusion in the survey:

glucose phosphate isomerase

6-phosphogluconate dehydrogenase

NAD-dependent malate dehydrogenase

adenylate kinase

hexokinase

glucose-6-phosphate dehydrogenase

lactate dehydrogenase

α-naphthyl acetate esterases

Due to practical difficulties involved in the examination of each of the other enzymes only GPI and 6PGD were studied in all the isolates available.

In preliminary work preparations of parasites released from the host red blood cells by immune lysis were used. In most of the subsequent work, however, it was found that satisfactory results could be achieved using the brown layer of parasitized blood cells formed by centrifuging infected blood. For consistency in diagrammatic presentation, only results obtained

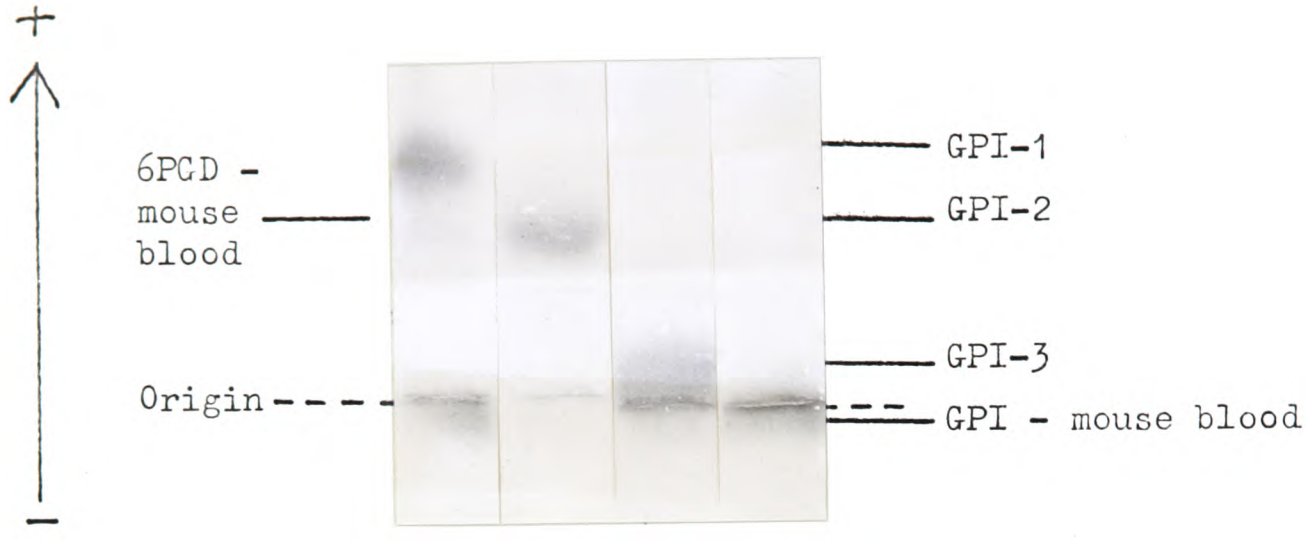


PLATE 2 Electrophoretic forms of GPI in samples of mouse blood and parasitized mouse blood on the Tris-citrate pH 6.0, 0.05M system.

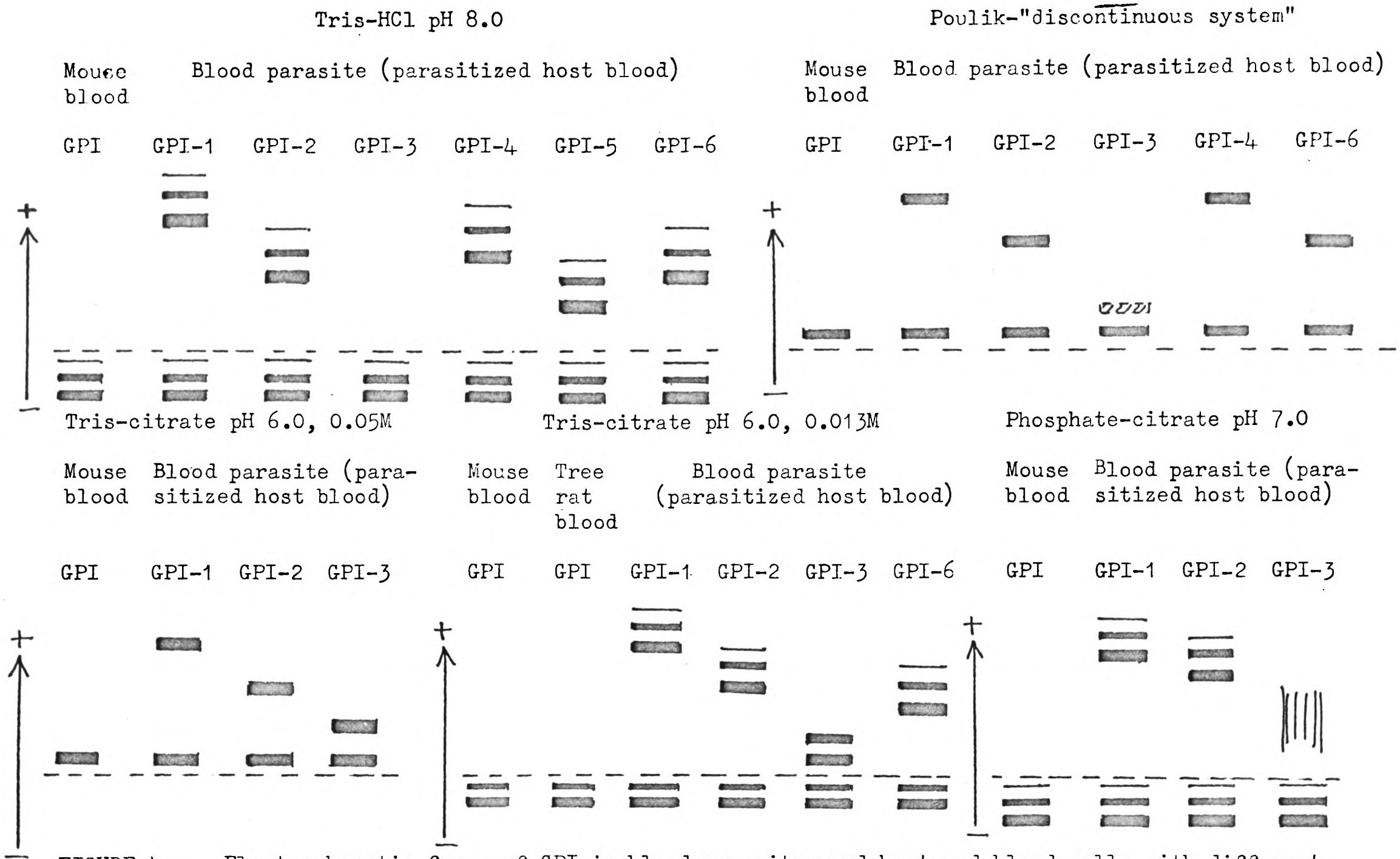


FIGURE 4a Electrophoretic forms of GPI in blood parasites and host red blood cells with different systems of electrophoresis.

using brown layer preparations and host blood cell controls will be shown.

a) Electrophoretic forms of enzymes of the blood parasites and the host blood cell

The electrophoretic forms of each enzyme examined in the parasite and host blood cell are presented diagrammatically in Figure 4. Each electrophoretic form of the parasite enzymes is given a separate annotation. Results using several different systems of electrophoresis are given.

Several different strains of mouse were used as host. No variation between them was found for any of the red blood cell enzymes examined. In some instances, however, the African tree rat, G. surdaster, was used as host. This rodent differs from the mouse in the electrophoretic mobility of certain blood cell enzymes. For this reason the mobilities of the enzymes of G. surdaster are represented, where relevant, as well as those of the mouse. A more detailed analysis of the distinction between host cell and parasite enzymes, and other complications involved in identifying enzyme variants in the parasite, are dealt with in section 3 of the results.

b) Distribution of electrophoretic forms of enzymes among strains

The parasite strains, together with the electrophoretic forms of enzymes by which each strain is characterized are presented in Table 5. The strains are grouped by their taxonomic classification.

	Sub-species of parasite	Enzyme	GPI	6PGD	MDHa	MDHc	AK	HK	G6PD	α-NAE's	LDHa	LDHc
		Strain										
Group 1	P.b. yoelii	17X	1	4	1	1	1	1	1	+	+	-
		8L	1	4	1	1	1	1	1	+	+	-
		3L	2	4	1	1	1	1	1	+	+	-
		6L	1	4	1	1	1	1	1	+	+	-
		11L	1	4	1	1	1	1	1	+	+	-
		146X	1	4	1	1	1	1	1	+	+	-
		2AR	2	4								
		4AF	1	4								
		6AD	1+2	4								
		2AK	1	4								
		4AE	1	4								
		2BE	1	4								
		4AZ	1	4								
		2BG	1	4								
	P.b. killicki	194ZZ	1	4	4	4	4	4	4	+	+	-
	P.b. yoelii	2BR	2	4								
		2CF	2	4								
		2CL	1	4								
		2CN	1	4								
		2CU	1	4								
		2CX	1	4								
	"P. berghei-like"	N67	2	4	1	1	1	1	1	+	+	-
Group 2	P.b. berghei	Anka	3	1	2	1	2	2	1	+	+	-
		RLL	3	1	2	1	2	2	1	+	+	-
		NK65	3	1	2	1	2	2	1	+	+	-
		Keyberg 173	3	1	2	1	2	2	1	+	+	-

TABLE 5a Distribution of electrophoretic forms of enzymes among strains of P. berghei.
The digits are those designated to the electrophoretic forms of enzymes shown in Figure 2.4.
+ represents the presence of enzyme activity but no clear differences being found in electrophoretic mobility.
- represents the absence of enzyme activity.

i) glucose phosphate isomerase

The distribution of variants of GPI divides the strains into three groups.

Group 1, characterized by variants GPI-1 and GPI-2, comprises the strains of P.b. yoelii, from the Central African Republic, of P.b. killicki from Congo, Brazzaville, and the strain of P. berghei from Nigeria. For convenience this group of strains will be referred to as the "P.b. yoelii group".

Group 2, characterized by variant GPI-3, comprises the strains of P.b. berghei. These strains are all derived from host specimens collected in Kantanga.

Group 3, characterized by GPI-4 and GPI-5, comprises the strains of P.v. chabaudi from the Congo, Brazzaville and the strain of P.v. vinckei from Katanga. Included in this group, for reasons given below, is the strain of P. vinckei from Nigeria, characterized by GPI-6.

In groups 1 and 3, in which enzyme polymorphism was found among strains, individual strains were found to be characterized either by one or other variant of GPI typifying the group, or else by the presence of both variants. In such dimorphic isolates the two variants were always present at similar levels of activity, as judged by the relative intensities of staining of each variant on electrophoretograms. Thus, among dimorphic strains, none was found in which the intensity of one variant appeared to be significantly greater than that of the other.

The distinction between the mobilities of variants of GPI was not demonstrated by all systems of electrophoresis used. Thus on the Poulik - "discontinuous" system GPI-1 and GPI-4 become indistinguishable,

Sub-species of parasite	Enzyme	GPI	6PGD	LDHa	LDHc
	Strain				
Group 3					
P.v. vinckei	vinckei-52	4	3	+	+
P.v. chabaudi	3AC	4	2		
	4AT	4	3	+	-
	3AJ	5	3	+	-
	1AQ	4	3	+	-
	1BC	4	3	+	+
	1AL	4	2	+	+
	14BJ	4+5	2		
	1BK	4			
	1AS	4	2		
P.v. ~~lontum~~ chabaudi	2BQ	5	5	+	-
	2BZ	5	5		
	2CB	4+5	3	+	-
	2CE	4+5	3	+	-
	2CP	4	2		
	2CQ	4	3	+	-
	2CR	5	5	+	-
	2CW	4	3	+	-
"P. vinckei-like"	1-69	6	3		

TABLE 5b Distribution of electrophoretic forms among strains of P. vinckei.

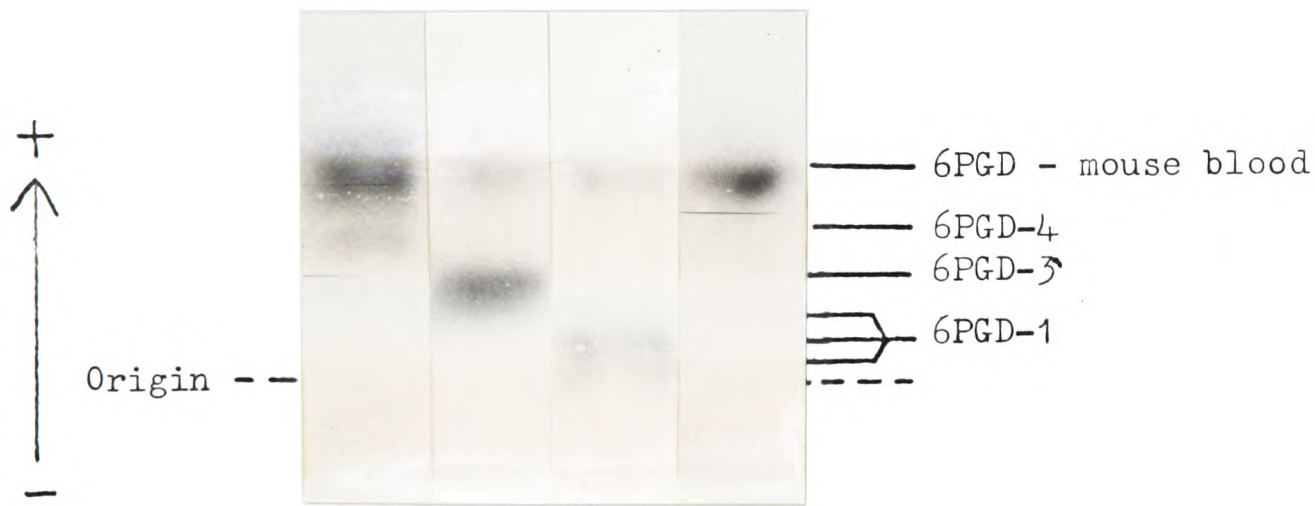


PLATE 3 Electrophoretic forms of 6PGD in samples of mouse blood and parasitized mouse blood on the Tris-citrate pH 6.0, 0.05M system.

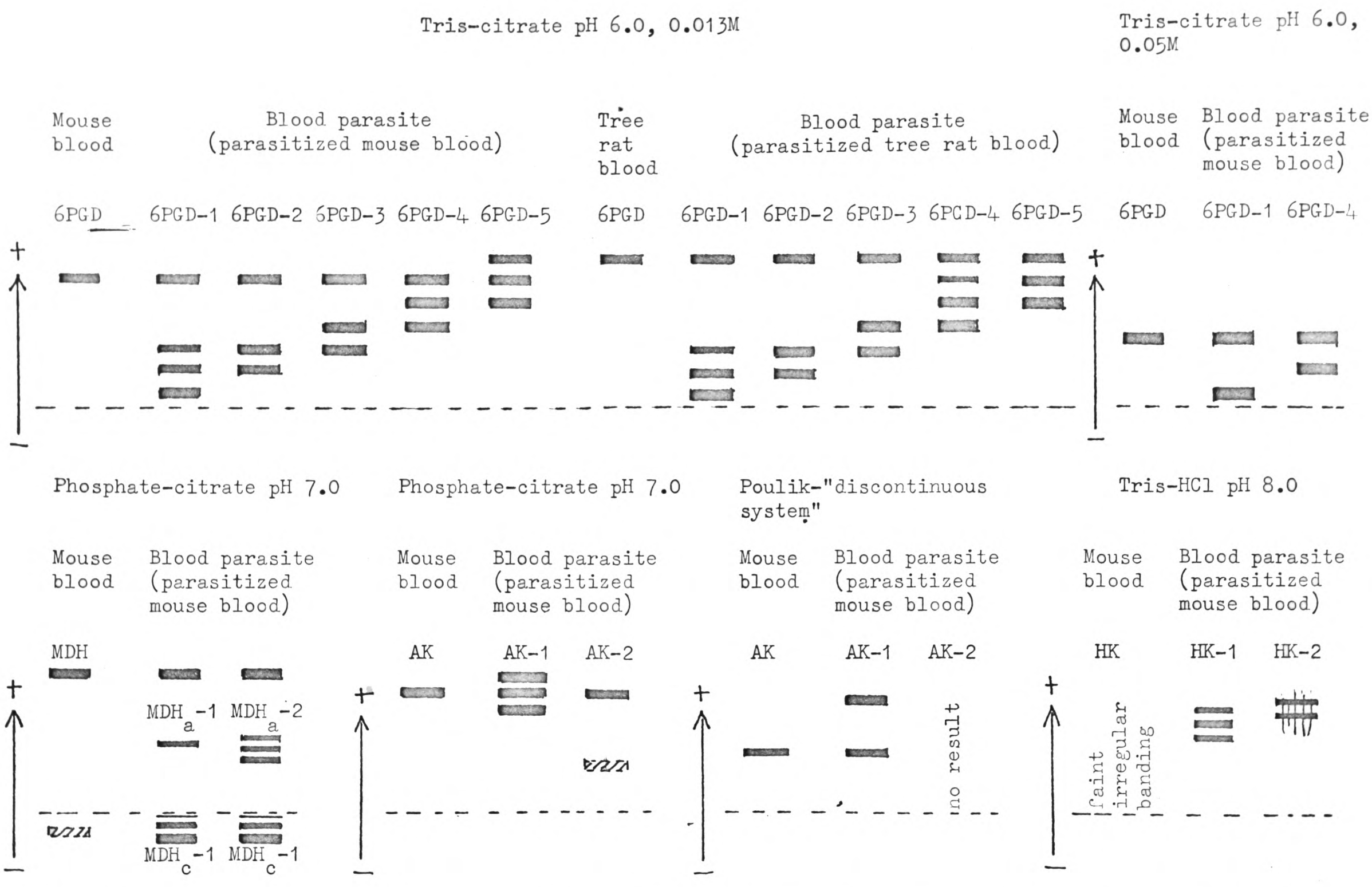


FIGURE 4b Electrophoretic forms of 6PGD, MDH, AK and HK in blood parasites and host red blood cells with different systems of electrophoresis.

as do GPI-2 and GPI-5. The Tris-HCl, pH 8.0 system was found to give the most satisfactory resolution between these variants. Variant GPI-6 represents a case of doubtful classification. On most systems GPI-6 migrates to the same position as GPI-5, but corresponds in mobility to GPI-2 on Tris-HCl, pH 8.0.

ii) 6-phosphogluconate dehydrogenase

The distribution of variants of 6PGD divides the strains into the same three groups as does the distribution of GPI variants. Thus group 1, comprising the "P.b. yoelii group" is characterized by 6PGD-4; group 2, comprising the strains of P.b. berghei, is characterised by 6PGD-1.

Group 3, comprising the strains of P.v. chabaudi, P.v. lentum, P.v. vinckei and the isolate of P. vinckei from Nigeria, is characterized by 6PGD-2, 6PGD-3 and 6PGD-5. Thus a high degree of variation of 6PGD occurs in this group. The strains of P.v. chabaudi and P.v. lentum are not, however, characterized by the same combination of variants. All three group 3 variants of 6PGD were found among strains of P.v. lentum while only 6PGD-2 and 6PGD-3 were found among the strains of P.v. chabaudi. The strain of P.v. vinckei and the Nigerian strain of P. vinckei were each characterized by 6PGD-3.

In contrast to the situation found for the variants of GPI, no strains were identified as possessing more than one variant of 6PGD. However, because of the spatial relationship of variants of 6PGD on electrophoretograms, and the occasionally irregular sub-banding of variants, it is possible that the simultaneous presence of two variants in a strain could be obscured or misinterpreted. The resolution between variants of 6PGD,

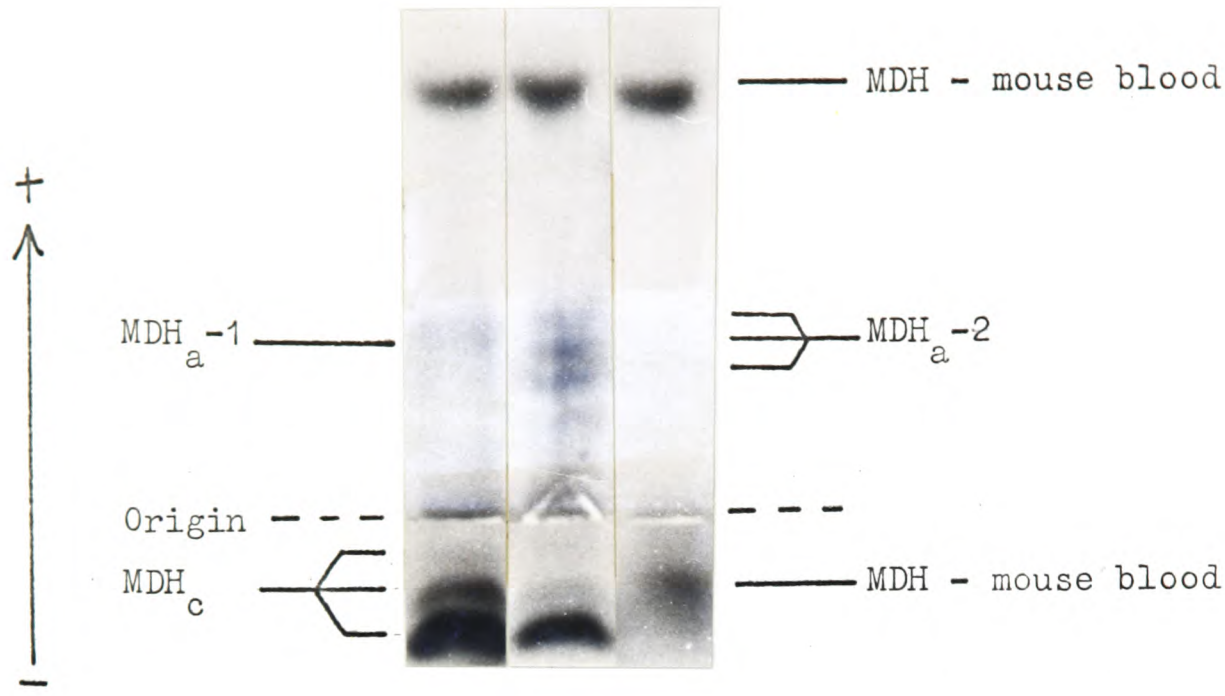


PLATE 4 Electrophoretic forms of MDH in samples of mouse blood and parasitized mouse blood on the Phosphate-citrate pH 7.0 system.

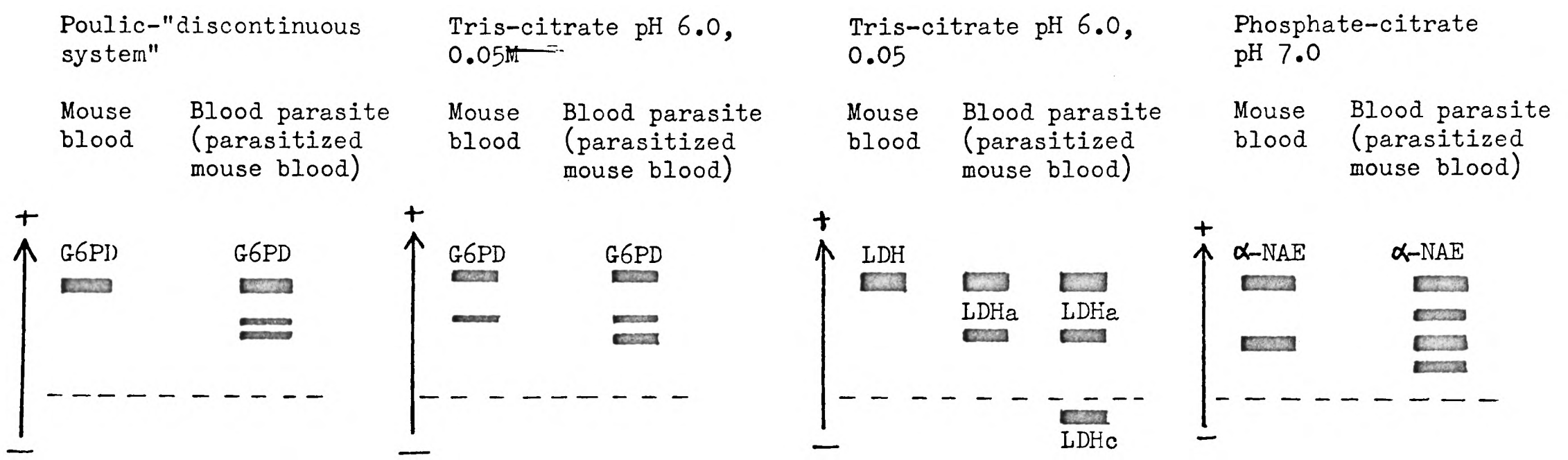


FIGURE 4c Electrophoretic forms of G6PD, LDH and -NAE in blood parasites and host red blood cells with different systems of electrophoresis.

spatially close to each other on electrophoresis, is thus in general less clear than that between variants of GPI.

iii) NAD-dependent malate dehydrogenase

MDH was examined only among strains of the "P.b. yoelii group" and among strains of P.b. berghei. Two distinct regions of parasite MDH activity were identified, one migrating towards the anode, designated MDHa, the other migrating towards the cathode, designated MDHc. The cathodal form of the parasite MDH coincided in position with that of a cathodally migrating band of red cell MDH. The blood and parasite enzymes were distinguished as described in section 3 of the results.

No variation in the position of MDHc was observed between strains examined. The division of strains into groups 1 and 2 was again confirmed, however, by the distribution of variants of MDHa. Thus all eight strains of the "P.b. yoelii group" were characterized by MDHa-1, while the three strains of P.b. berghei were each characterized by MDHa-2.

The occurrence of variation between strains for MDHa but not for MDHc may be interpreted to indicate that at least two separate gene loci are involved in the control of MDHa and MDHc.

iv) adenylate kinase and hexokinase

AK and HK were examined in the same strains for which MDH was examined. The division of strains into groups 1 and 2 was again confirmed Thus the strains of the "P.b. yoelii group" were all characterized by AK-1 and HK-1 while those of P.b. berghei were characterized by AK-2 and HK-2.

v) glucose-6-phosphate dehydrogenase and the α-naphthyl acetate esterases

G6PD and the α-NAE's were examined only in a selection of strains from

the "P.b. yoelii group" and in strains of P.b. berghei. No repeatable differences in the electrophoretic mobility of either of these enzymes could be demonstrated between strains.

The α-NAE's constitute a variety of proteins which catalyse the hydrolysis of the artificial substrate α-naphthyl acetate. A large number of bands was thus found in both parasite and red blood cell preparations. The bands varied in intensity and number from one preparation to another. The presence of a large number of bands of variable reproducibility made the identification of true variation between strains very difficult. Only major regions of activity could be identified with certainty and distinguished between blood and parasite preparations. The major regions were not, however, variable between different strains of parasites.

vi) lactate dehydrogenase

LDH was examined in a selection of strains from groups 1, 2 and 3. Unfortunately it has proved difficult to devise a system of electrophoresis which gives consistent results for this enzyme. The most satisfactory system, the Tris-citrate pH 6.0, 0.05M system, gave considerable variation in the position of the bands of the parasite enzyme in most strains. In general, therefore, it was found impossible to identify a unique mobility for LDH's of the strains examined. However it was found that the parasite LDH occurred either as an anodal region of activity or as a cathodal region. The distinction between these two forms was quite repeatable. In most strains only the anodal region of activity was found. Two strains of P.v. chabaudi, however, were found to possess the cathodal form in addition to the anodal form.

2) Analysis of a dimorphic strain by dilution of infected blood

In the course of this work several strains were found to be characterized by two variants of GPI. Two possible explanations can be proposed to account for this situation. Either the two variants occur together in each parasite cell or the strain represents two "lines" of cells each represented by a single variant of GPI. In order to determine which explanation is correct an attempt was made to derive from a dimorphic strain, lines of parasite cells characterized by one variant only.

A mouse, inoculated with the dimorphic strain of P.b. yoelii, 6AD, was checked for parasitaemia on the fifth day after inoculation. About half the infected red blood cells contained single parasites; most of the remaining parasitized blood cells contained not more than three parasites. Blood was collected from the mouse and diluted in serum Ringer (1 volume calf serum: 1 volume Ringers solution (Diggens 1970)). A level of dilution was chosen such that there was, on average, one parasitized blood cell per 0.2 mls. of diluted blood.

Fifty two mice were inoculated intravenously with 0.1 ml. aliquots of the diluted blood. The mice were examined on the seventh day after inoculation. Eleven ~~Ten~~ of the mice showed parasitaemias of 1% or more. The remaining forty two mice failed to show parasites in the blood up to a fortnight after inoculation. It was concluded that only the mice which showed parasitaemias by the seventh day had received infective parasites on inoculation.

In preliminary experiments groups of mice had been inoculated with aliquots of different dilutions of blood infected with a mixture of two strains of P.b. yoelii 17X and 3L, characterized by GPI-1 and GPI-2 respectively.

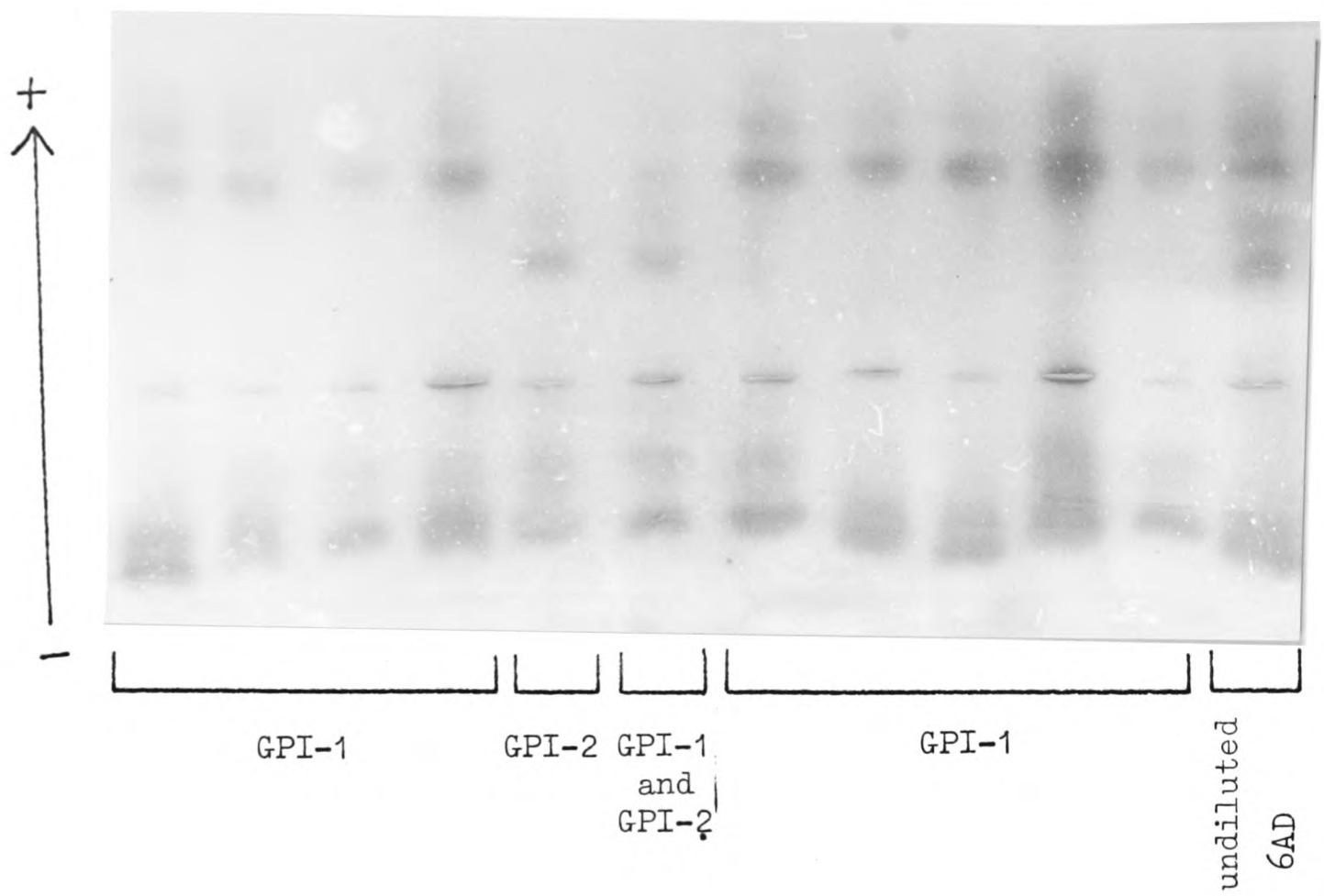


PLATE 5 Distribution of variants of GPI in lines of blood parasites derived by dilution of the dimorphic strain 6AD. The Tris-HCl pH 8.0 system of electrophoresis was used.

The proportions of mice becoming infected and the distribution of GPI variants among the infected mice were recorded. These were shown to be consistent with the values predicted from the numbers of parasitized blood cells in the diluted inocula, by the Poisson distribution.

By applying the predictions of the Poisson distribution in the present experiment it was estimated that about 80% of mice becoming infected contained parasites derived from single parasitized blood cells. Thus most of the ~~ten~~ eleven mice which become infected probably contained either single clones of parasites or parasites derived from not more than two or three parasite cells.

On examination on starch gel of blood from each of the infected mice, ~~eight~~ nine were characterized exclusively by GPI-1, one exclusively by GPI-2 and one by both GPI-1 and GPI-2 in similar intensity (Plate 5).

It was concluded, therefore, that cells characterized exclusively by GPI-1 and cells characterized exclusively by GPI-2 are represented in strain 6AD. The occurrence of parasitaemias characterized by GPI-1 and GPI-2 in a small percentage of mice inoculated, is predicted from statistical considerations of the method. It is not possible, therefore, to conclude that lines of cells characterized by both GPI-1 and GPI-2 are present in this strain.

3) Analysis of enzyme components on starch gel electrophoretograms

The material used in this study presented special problems in the analysis of the regions of enzyme activity on starch gels. The problems primarily concerned the origins of the activity found at different regions

of a gel. Two questions could be asked in this respect.

i) Was the activity at a particular region of a gel due to a parasite enzyme or to a host enzyme or to the presence of both?

ii) Were the regions of activity on a gel the result of the activities of more than one enzyme reaction?

Situations in which these questions arose either separately or together, were encountered in the course of the present work.

a) Identification of parasite and host cell glucose phosphate isomerase

For most strains of parasites examined the parasite and host forms of GPI could be clearly distinguished on starch gel electrophoresis. A special problem arose, however, in the examination of strains of P.b. berghei for GPI activity.

For most purposes the Tris-HCl pH 8.0 system of electrophoresis had been found to be the only entirely satisfactory system for the study of GPI. On this system, however, the only activity of GPI found in strains of P.b. berghei lies in the cathodal region corresponding to that of the host red cell enzyme.

On other systems of electrophoresis the GPI activity of the P.b. berghei strains was clearly distinguishable from that of the host enzyme. Moreover, when parasites freed from the host cell by immune lysis, were examined on these systems the activity in the region of the host enzyme was greatly reduced or eliminated. When such a preparation was examined on the Tris-HCl pH 8.0 system, however, the activity in the region of the host enzyme remained intense.

The GPI of strains of P.b. berghei, when examined on the Tris HCl pH 8.0 system of electrophoresis, was thus shown to migrate to the same region as the blood enzyme.

b) Identification of parasite and host cell NAD-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase

For most enzymes and strains of parasites studied the electrophoretic mobilities of the enzymes of the parasite, in preparations of parasitized red cells, were clearly different from those of the uninfected red cell. Moreover, when preparations of parasites, which had been freed from the red blood cells by immune lysis, were used, the activity in the regions due to the host enzyme was usually eliminated or markedly reduced. Thus the regions of activity of the parasite and host cell enzymes could generally be clearly distinguished. On examining MDH and G6PD in preparations of P. berghei freed from the red blood cells by immune lysis, however, it was found that certain anodal regions of activity corresponding in position to the host enzymes remained very intense. When, however, more rigorous precautions were taken to remove blood contamination during the preparation of parasites, the activities of the bands in the regions of the host enzymes were substantially reduced. It was therefore concluded that these bands derived from contamination by the host enzymes in parasite preparations.

The first observations had suggested either that the parasite enzymes had regions of activity which migrated to positions identical to those of the host enzymes, or that the enzymes of the host were still present

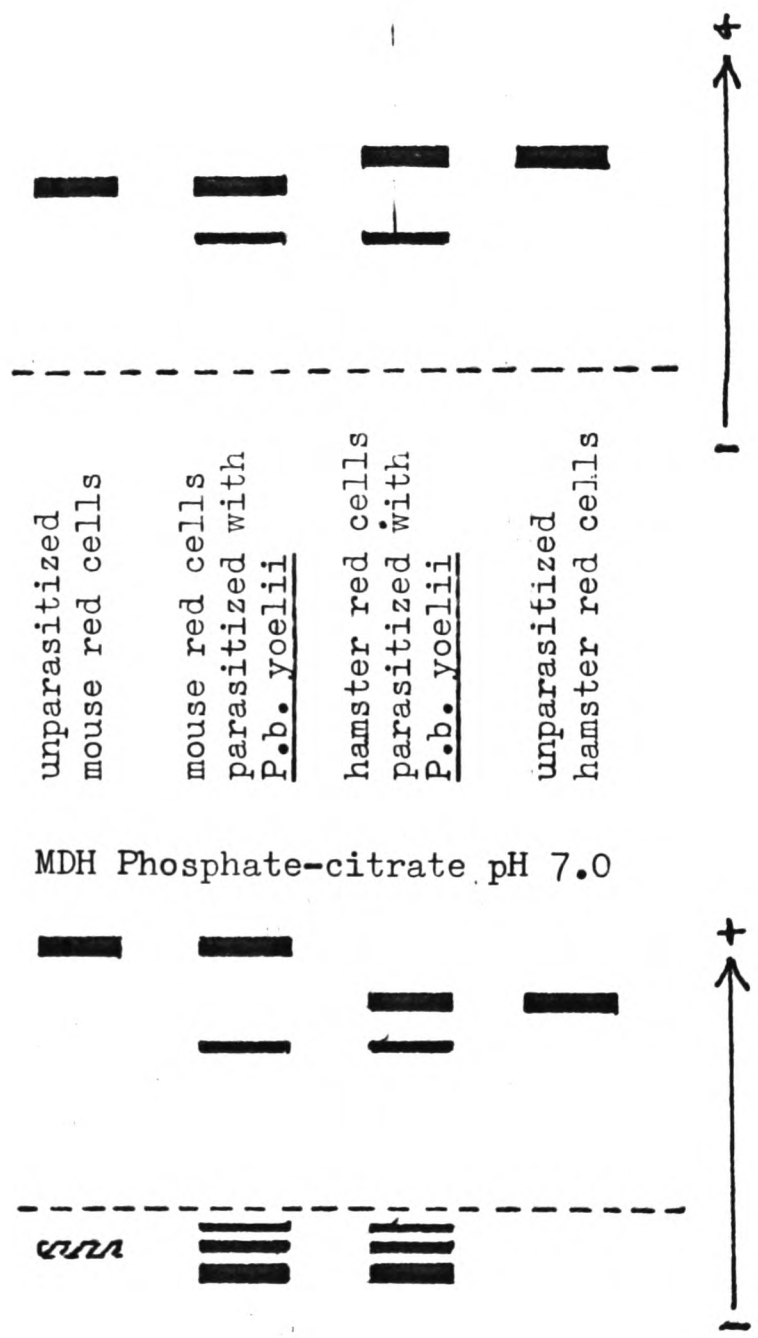


FIGURE 5 Electrophoretic mobilities of MDH and G6PD in parasitized unparasitized red blood cells of mouse and hamster.

in parasite preparations apparently freed from the red blood cells. The latter possibility had further suggested that the MDH and G6PD of the host blood might have been assimilated by the parasite. Although this possibility was subsequently eliminated by the use of more carefully "purified" preparations of parasites the following experiment was first carried out to distinguish between the alternative possibilities first postulated. The technique employed is considered to be worth recording, particularly as misinterpretations regarding the apparent incorporation of host components by a parasite have been recorded elsewhere in the literature (see Discussion section 3).

Preparations of red blood cells from a number of potential rodent hosts of P. berghei, were examined for the electrophoretic mobility of MDH and G6PD. It was found that the electrophoretic properties of both these enzymes differed between the blood cells of the mouse and the golden hamster. MDH and G6PD were thereafter examined in preparations of parasitized blood from both these rodents. In such preparations activity was found in the regions previously unequivocally identified as deriving from the parasite. The only other regions of activity found were those corresponding to the enzymes of the uninfected blood cells of mouse and hamster (Figure 5).

It was thus demonstrated in parasite preparations, that the regions of activity of MDH and G6PD which migrated to the position of the host enzymes were indeed due to host enzyme activity. This result had encouraged the idea that the parasite may assimilate these enzymes from the host cell. The results of the examination of more carefully purified parasite preparations, however, made such a hypothesis untenable.

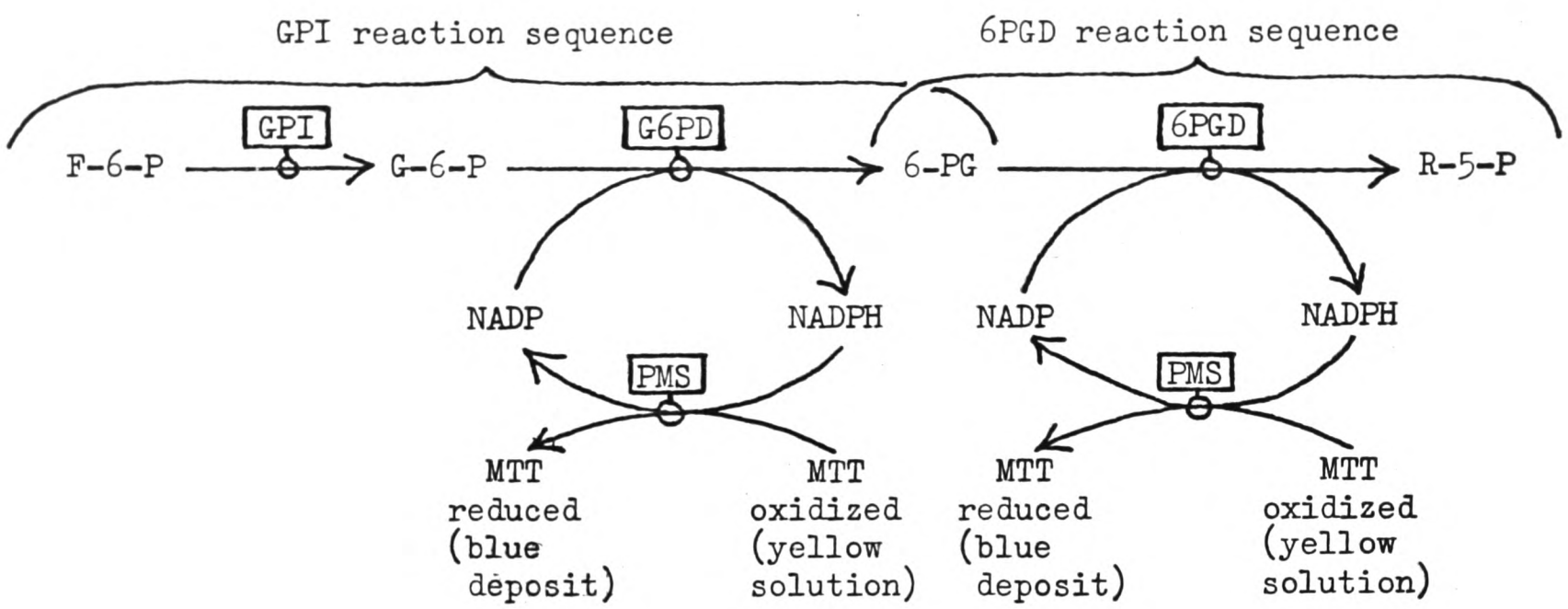


FIGURE 6 Scheme linking the GPI and 6PGD reactions as assayed on starch gel electrophoretograms.

F-6-P = fructose-6-phosphate
G-6-P = glucose-6-phosphate
6-PG = 6-phosphogluconate
R-5-P = ribulose-5-phosphate

A lesser problem arose in the identification of the origin of activity of the cathodal bands of MDH in parasite preparations. This region of activity migrated to a position very close to that of a cathodal region of MDH in the red blood cells of the mouse. The cathodal MDH of the blood, however, showed considerably weaker activity than that of the parasite preparations freed by immune lysis. It was therefore clear that the cathodal region of MDH in parasite preparations could be ascribed to the parasite itself.

c) Identification of biochemically distinct enzyme reactions on starch gel electrophoretograms

When incubating a gel for the presence of a specific enzyme it is important to know whether the bands of dye deposited arise solely from the activity of the enzyme itself or whether other enzyme reactions are catalysing the deposition of dye on the same gel. This problem was investigated during the study of GPI.

A number of anomalous results had been obtained during the examination of differences in the electrophoretic mobility of GPI among isolates of P. berghei. These anomalies had given rise to the suspicion that 6PGD was showing activity during incubation for the GPI reaction. 6PGD had been implicated as the probable contaminating enzyme on the following basis. The final product formed in the reaction used to demonstrate activity of GPI is 6-phosphogluconate (see Figure 6). This substance is the substrate of the 6PGD reaction. Since all the other components necessary for dye linked 6PGD reaction were already present, it seemed likely that regions

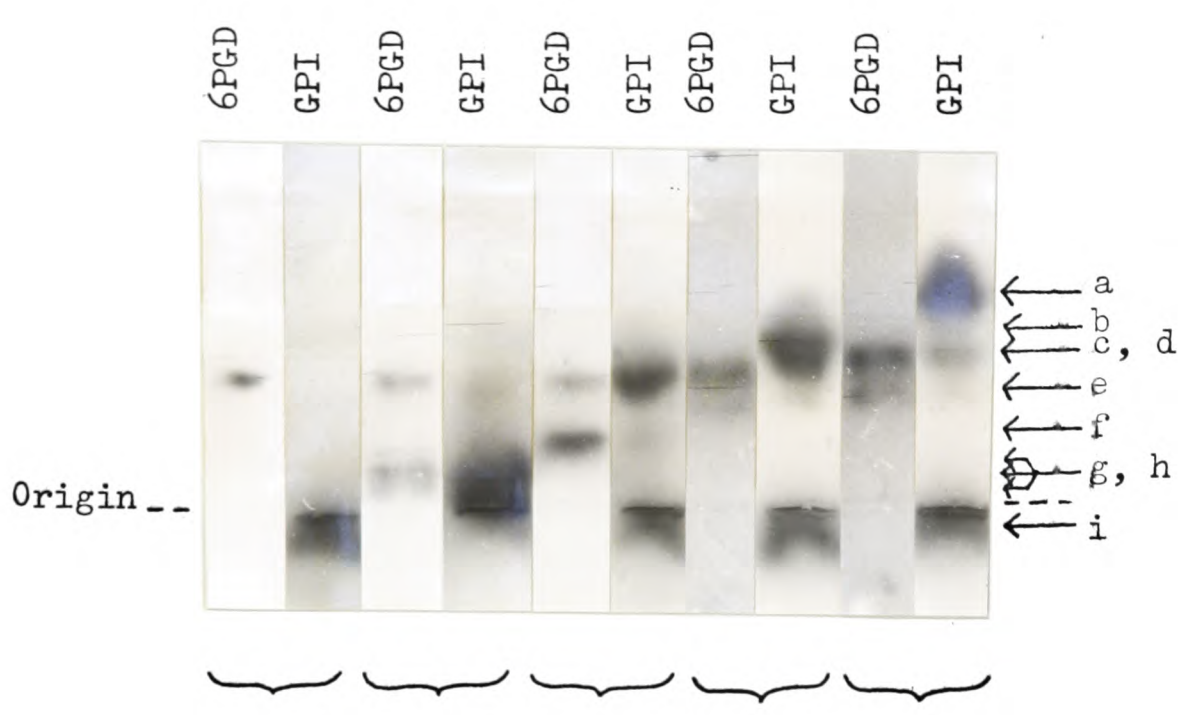
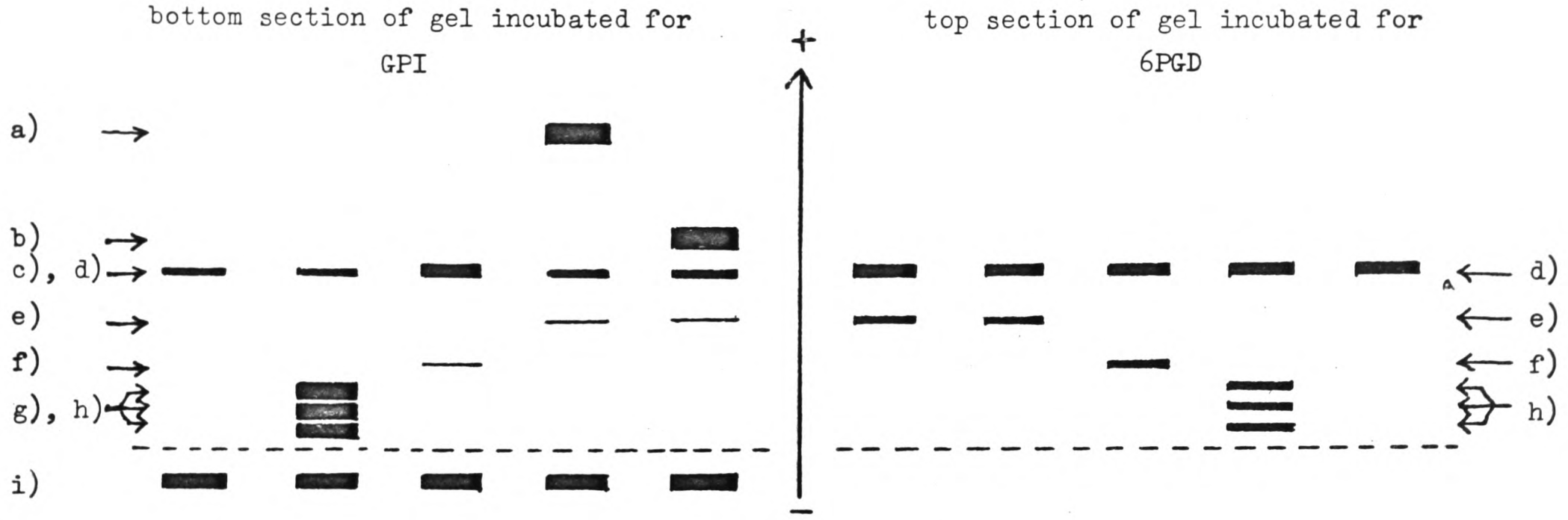


PLATE 6 Demonstration of 6PGD activity on a gel incubated for GPI. The Tris-HCl pH 6.0, 0.013M system of electrophoresis was used. (see Figure 7 for key)



a) P.b. yoelii (2AK) GPI activity
b) P. berghei (N67) GPI activity
c) P. vinckei (1-69) GPI activity
d) mouse red cell 6PGD activity
e) P.b. yoelii (2AK), P. berghei 6PGD activity
f) P. vinckei (1-69) 6PGD activity
g) P.b. berghei (Anka) GPI activity
h) P.b. berghei (Anka) 6PGD activity
i) mouse red cell GPI activity

FIGURE 7 Demonstration of 6PGD activity on gels incubated for GPI using the Tris-citrate pH 6.0, 0.013 M system of electrophoresis. The gel was sliced horizontally and the top and bottom sections incubated for different enzyme reactions.

of 6PGD activity might deposit dye during incubation for GPI.

To test this hypothesis a sample of mouse blood cells and a selection of strains of parasite characterized by different variants of 6PGD, were subjected to electrophoresis on the Tris-citrate pH 6.0, 0.05M system. The gel was sliced horizontally and the top section incubated for 6PGD, while the bottom section was incubated for GPI. Any bands due to 6PGD activity arising on the section stained for GPI would therefore form a mirror image to the regions of 6PGD activity found on the top slice.

It was found that for each sample, a region of activity occurred on the section stained for GPI which corresponded exactly in its position to the position of 6PGD for that sample (Figure 7). The presence of 6PGD activity was thus demonstrated in the section incubated for GPI.

In the analysis of previous results using the Tris-citrate pH 6.0, 0.05M system, confusion had arisen particularly in the identification of the presence of GPI-2 in parasite preparations. This was now shown to be due to the presence of mouse blood 6PGD which migrates to an almost identical position to that of GPI-2 on this system (Plate 6).

Using the Tris-HCl pH 8.0 system, however, it was found that all 6PGD activity migrated to regions anodal to the regions of GPI activity, thus eliminating the possibility of confusion. The Tris-HCl pH 8.0 system was, therefore, used in subsequent analysis of GPI.

4) Comparison of the electrophoretic forms of enzymes of the blood parasite and of the oocyst

Most of the strains studied in the course of this work were examined for the electrophoretic forms of enzymes in the blood parasite only.

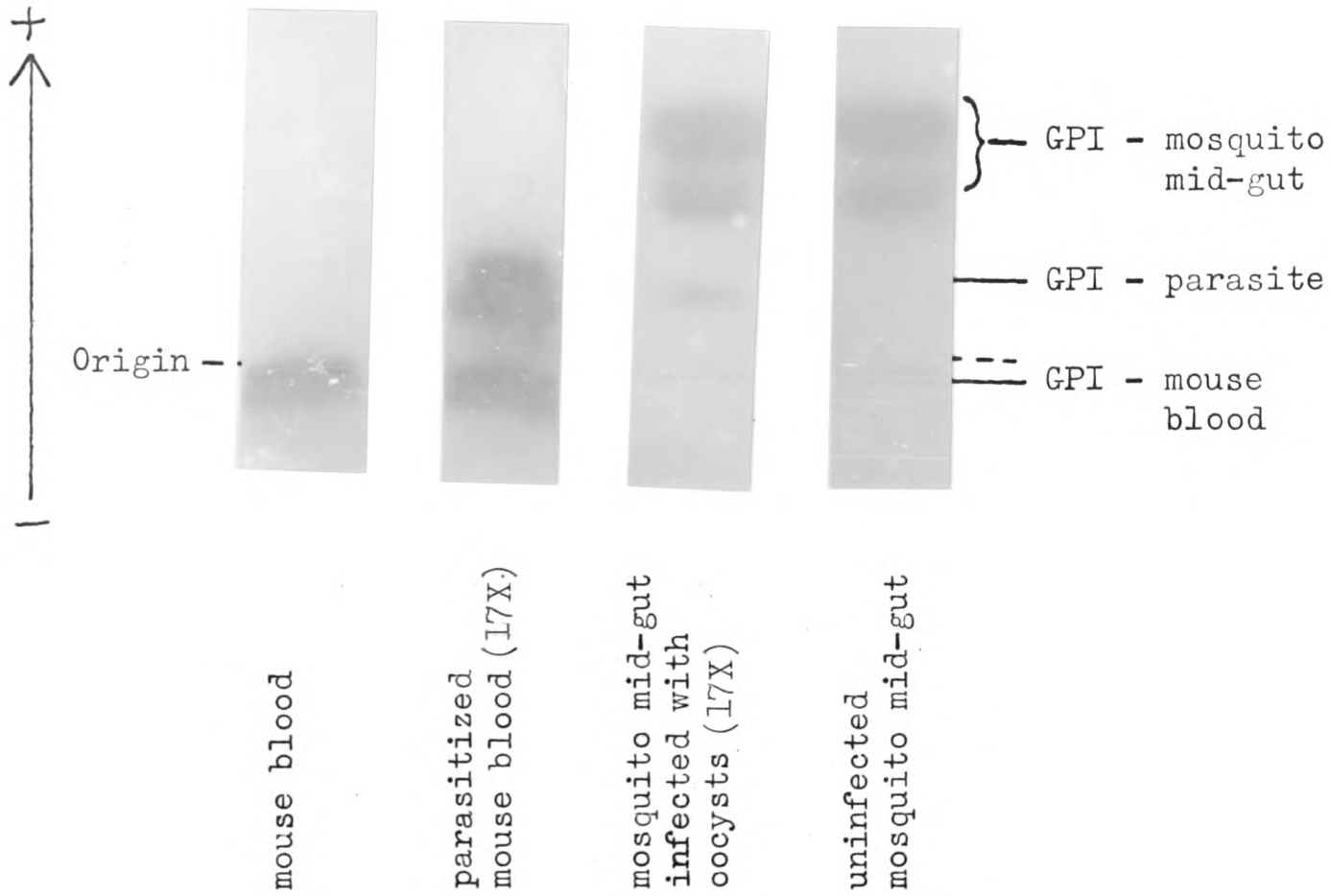


PLATE 7 GPI activity in mouse blood, parasitized mouse blood, mosquito mid-gut heavily infected with oocysts, and uninfected mosquito mid-gut. The presence of a band of activity specific to the oocyst-infected mid-gut is demonstrated. This band is shown to migrate to a position identical to that of the enzyme of the blood parasite. The Phosphate-citrate p^H 7.0 system of electrophoresis was used.

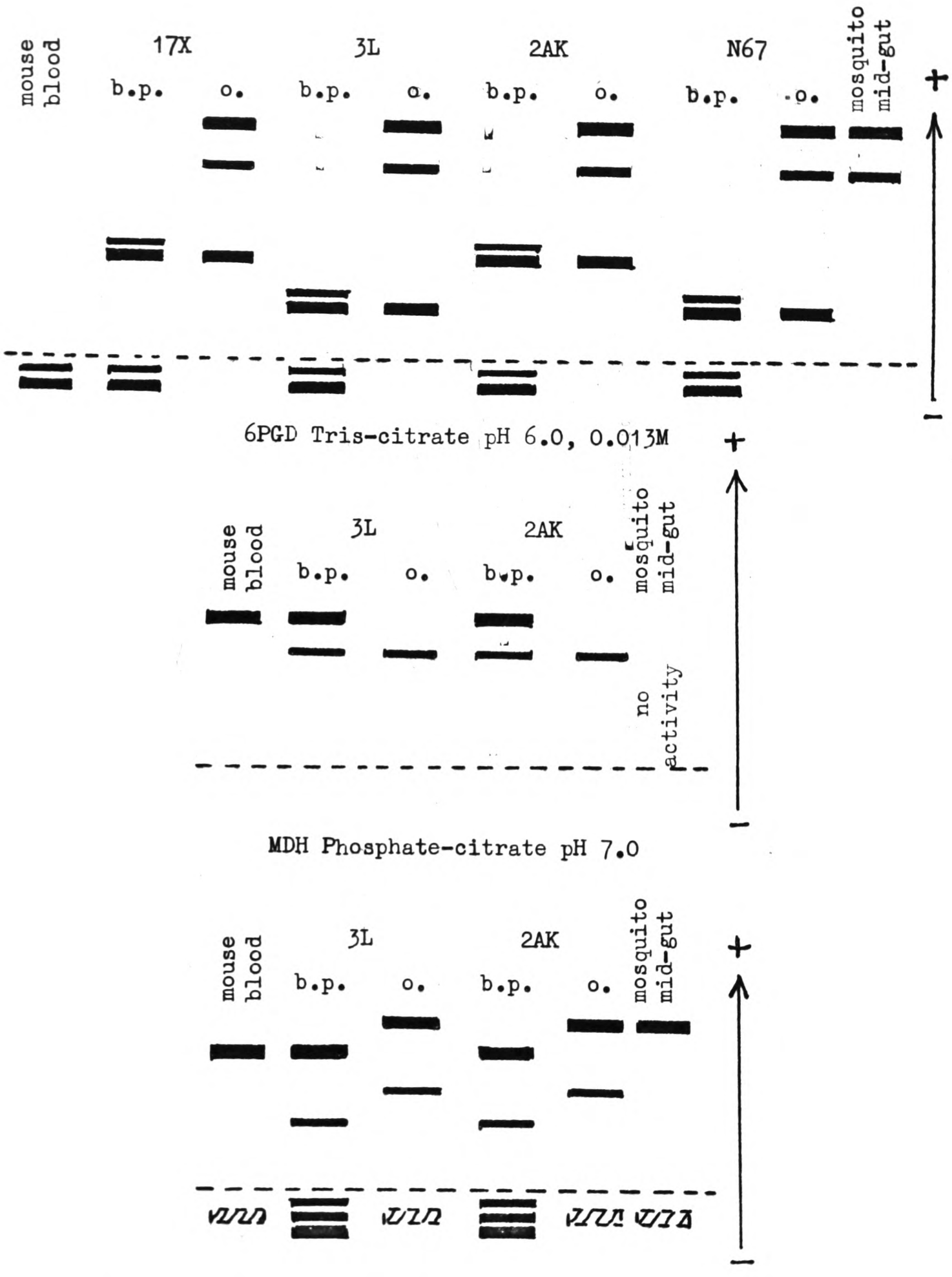


FIGURE 8 Comparison of electrophoretic forms of enzymes of the oocyst and blood parasites and their host tissues.
b.p. = parasitized red blood cells.
o. = mosquito mid-guts infected with oocysts.

Some strains, however, were examined for enzymes in the oocyst stage. Although assays were attempted for several other enzymes in the oocyst, only GPI, MDH and 6PGD showed activity. However the quantities of oocyst material which could be obtained for each assay were very small, not more than 5,000 oocysts suspended in 0.05 mls. of fluid. The results cannot, therefore, be taken to reflect the absence from the oocyst of the enzymes for which assays were attempted but for which no activity was found. Table 6 presents a list of the enzymes and strains for which assays were attempte Activity of certain enzymes was recorded in some strains but not in others. This again, can only be taken as a reflection of the different amounts of material which it had been possible to prepare on different occasions.

GPI activity was found in oocysts of strains 17X, 3L, 2AK and N67; MDH and 6PGD activity was found in oocysts of strains 3L and 2AK. A comparison of these enzymes in the blood parasites and in the oocysts is presented in Figure 8.

Each of the strains examined for GPI showed identical mobility of this enzyme in the blood stage and mosquito stage parasites. The variation found between these strains in the blood parasites is thus also exhibited in the mosquito stage. It is concluded, therefore, that the same structural gene locus probably controls the GPI protein in both the blood and the mosquito stages.

The observation of GPI activity in oocysts of strains of P. berghei has been repeated a number of times. Activity for 6PGD and MDH was found in oocysts of strains 3L and 2AK on a single occasion only. The inability to repeat these observations can be ascribed to the small amounts of material generally available for each assay. The problem did not arise

Sub-species	*P.b. yoelii*			"*P. berghei*-like"	*P.b. berghei*
Strain	17X	3L	2AK	N67	NK65
Enzyme					
GPI	+	+	+	+	+
6PGD		+	+		
MDH	-	+	+	-	-
HK	-		-	-	-
LDH	-	-	-		
ICDH	-	-	-	-	-
GDH	-			-	-
β-OHBD				-	
α-NAE's	-			-	-

TABLE 6 Enzymes assayed in oocyst preparations in strains of *P. berghei*.

\+ = enzyme activity detected in oocysts.

\- = no enzyme activity detected in oocysts.

in the study of GPI presumably because of the apparent high activity of this enzyme in the oocyst as well as in the blood parasite.

Like GPI the 6PGD of the oocyst showed apparently identical mobility to that of the blood parasite. However variation in mobility of 6PGD in the blood parasites was absent between the two strains examined. It was not possible, therfore, to confirm the identity of the oocyst and blood parasite forms of 6PGD as it had been for GPI.

The two strains, 3L and 2AK, had shown identical forms of MDH in both the cathodal and anodal regions when examined in the blood parasite. On examination of oocyst preparations of these strains only an anodal band was identified. While this band showed identical mobility in the oocyst for both strains, it was found to differ in mobility from the anodal band of the blood parasite. It was concluded, therefore, that different structural gene loci probably control the proteins catalysing the MDH reaction in the blood stage and mosquito stage parasites.

5) Survey of the comparative activities of enzymes in the blood parasite forms of P. berghei and its host red blood cell

In the course of looking for suitable enzymes with which to study variation between isolates of P. berghei about twenty different enzymes were investigated in both blood cell and parasite preparations. A list of all enzymes for which assays were attempted is given in Table 7 . For several of the enzymes listed either the blood cells or the parasite showed no activity. In some cases neither showed activity.

Enzyme	Level of enzyme activity	
	Blood parasites released by immune lysis	Mouse red blood cells
HK	+	±
GPI	+++	++
Ald	+	±
G3PD	+	±
LDH	++++	+++
α-GPD	-	±
G6PD	+	++
6PGD	++	++
ME	-	-
β-OHBD	-	-
GDH	±	-
ICDH	±	-
SDH	-	-
Fum	-	-
MDH anodal	+	+++
MDH cathodal	+++	+
Diaph (NADH)	-	-
Diaph (NADPH	-	-
AK	++	+
α-NAE's	+++	+++

TABLE 7 Levels of enzyme activity in P. berghei blood parasites and in mouse red blood cells.

- = no staining after 24 hours incubation at 37°C.
± = faint staining after 4 to 8 hours incubation at 37°C.
+ = moderate staining after 4 to 8 hours incubation at 37°C.
++ = moderate staining after 1/2 to 1 hour incubation at 37°C.
+++ = moderate staining after 10 minutes incubation to heavy staining after 1 hour incubation at 37°C.
++++ = heavy staining after 10 minutes incubation at 37°C.

Enzymes for which very light deposits of stain formed after 24 hours incubation were scored as inactive as non-specific deposition of dye can occur after this period.

In Table 7 the relative activities of each enzyme in the blood and in the parasite are shown. The intensity of staining for different enzymes and for the enzymes of the host and parasite, varied from heavy deposition of dye after ten minutes incubation to very light deposition after twenty four hours. A distinction could, therefore, be made between the activities of different enzymes from host and parasite. (For categorization of levels of enzyme activity see Table 7.) The distinctions made, moreover, were consistently observed when preparations of similar cell masses were used. The relative intensity of staining due to different enzymes was not found to vary according to the system of electrophoresis used.

In the case of all enzymes other than the α-naphthyl acetate esterases the amounts of dye deposited were directly comparable. This is because the same redox dye, MTT tetrazolium was linked stoichiometrically to the quantity of product formed for every enzyme reaction studied except that of the α-NAE's (see Materials and Methods section, Figure 3). Thus the quantity of dye deposited is a direct measure of the extent of the enzyme reaction. The concentration of substrates and co-factors for each enzymes reaction were present in excess or approaching excess concentrations. The rates of reaction and the amounts of dye deposited, therefore, represented activities approaching the maximum of each enzyme under the conditions of study. Host cell and parasite enzymes were examined on the same gels under identical conditions. The results are, therefore, considered to be representative of the relative activities of the enzymes of the host cell and the parasite as found after starch gel electrophoresis.

How well the results represent the situation in the living cell systems depends on how efficiently each enzyme is extracted onto the the starch gel and how well its activity is preserved under the conditions of the experiments.

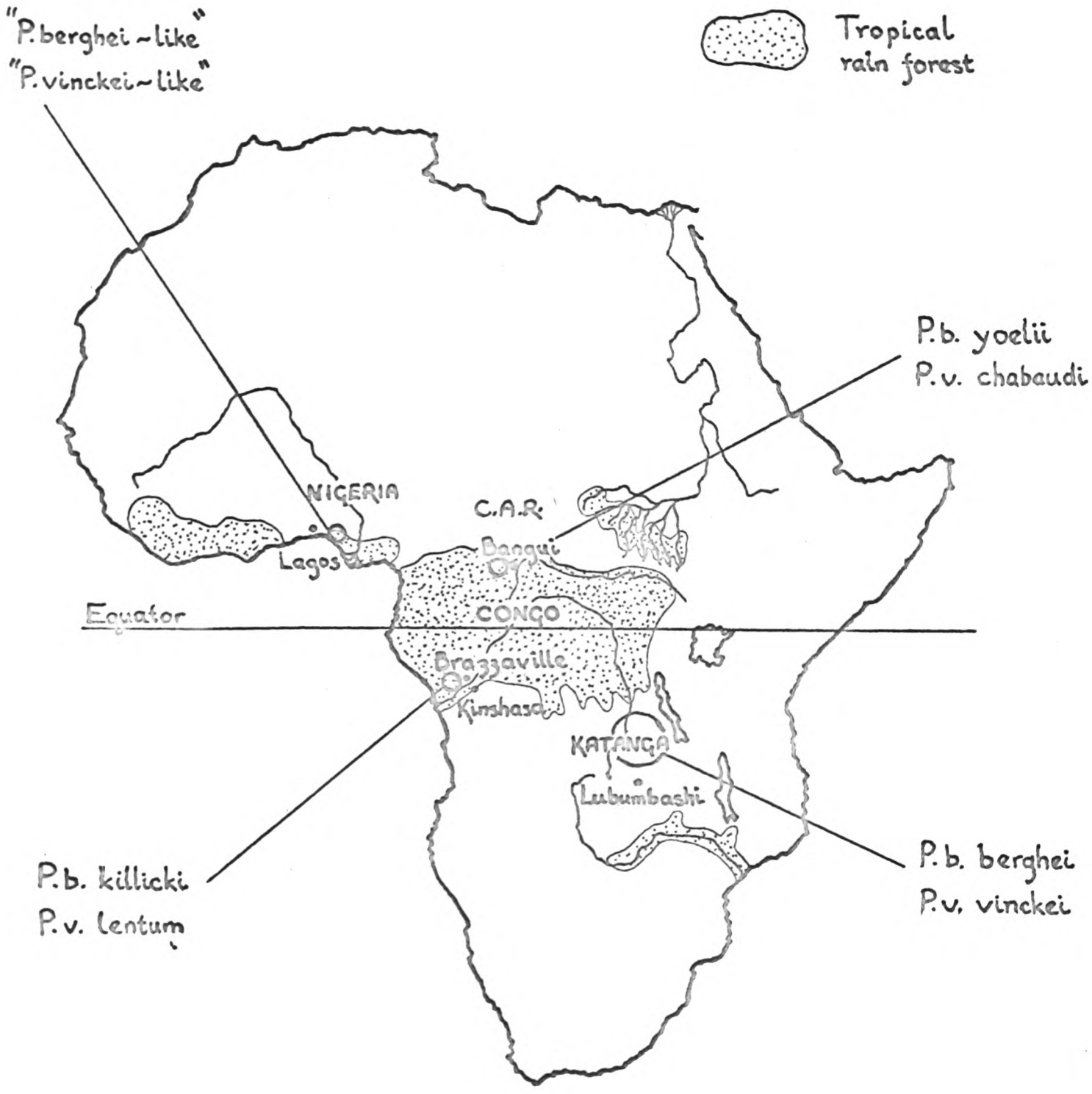


FIGURE 9 Distribution of rodent malaria parasites in Africa.

DISCUSSION

1) Enzyme variation and the classification of rodent malaria parasites

The first description of an African rodent malaria parasite was given in 1948 when Vincke and Lips isolated Plasmodium berghei from the blood of a specimen of Grammomys surdaster captured near Lubumbashi in Katanga (Vinke and Lips 1948). In 1952 a parasite infective to G. surdaster was isolated from a specimen of Anopheles dureni, the insect vector of P. berghei, captured on the banks of the Kinga River in Katanga (Rodhain 1952). The morphology and biology of this parasite was considered to be sufficiently different from that of P. berghei to warrant its classification as a new species. It was named Plasmodium vinckei.

Since the time of these isolations the search for murine rodent malaria parasites has been extended to other regions of tropical Africa. It was not until 1965, however, that general success was met with outside Katanga. In that year Landau described two species of plasmodium isolated from specimens of Thamnomys rutilans captured in the vicinity of Bangui in the Central African Republic (Landau 1965a, Landau 1965b, Landau and Chabaud 1965). One of the species resembling P. berghei was named Plasmodium berghei yoelii (Landau and Killick-Kendrick 1966). The other species originally named Plasmodium chabaudi (Landau 1965), is now classified as a subspecies of P. vinckei under the name of Plasmodium vinckei chabaudi (Bafort 1968).

In 1966 Adam, Landau and Chabaud described a P. berghei-like parasite and a P. vinckei-like parasite which they had isolated from specimens of T. rutilans captured near Brazzaville (Adam et al. 1966). These parasites have since been classified under the names of Plasmodium berghei killicki

(Landau et al. 1968) and Plasmodium vinckei lentum (Landau et al. 1970) respectively.

In 1967 Killick-Kendrick found malaria parasites in the blood of specimens of Thamnomys captured in the Ilobi Forest in Nigeria (Killick-Kendrick et al 1968). In most of the isolates the parasites resembled P. vinckei but in one isolate a P. berghei-like parasite was found. Neither of these species has yet been given a full classification.

Since its discovery P. berghei has been re-isolated in Katanga on numerous occasions from different localities. All such isolations have been grouped under the subspecific name of Plasmodium berghei berghei. The isolate of P. vinckei collected in 1952 has been given the subspecific name of Plasmodium vinckei vinckei. P.v. vinckei has been isolated in Katanga on only one occasion since this time (Bafort 1967). In the Central African Republic P.b. yoelii and P.v. chabaudi, and in the vicinity of Brazzaville P.b. killicki and P.v. lentum have all been isolated a number of times. The P. berghei collected by Killick-Kendrick in 1967, represents the only isolation of this species from Nigeria. In addition to the strains collected by Killick-Kendrick in 1967, P. vinckei was isolated in Nigeria in 1969*.

*While both P. berghei and P. vinckei have been found in all regions of Africa in which isolations of rodent malaria parasites have been made, the relative prevalence of the two species appears to differ markedly between regions. Thus virtually all specimens of T. rutilans captured in the Central African Republic were infected with P.v. chabaudi. From only about half of these was it found possible to isolate P.b. yoelii. During the present work P.b. killicki and P.v. lentum were isolated with similar frequency from specimens of Thamnomys captured in Brazzaville and delivered to this laboratory. In Katanga, on the other hand, the rodents and mosquito vectors captured appeared to be infected almost exclusively by P.b. berghei, P.v. vinckei having been isolated on only two occasions. In Nigeria P. vinckei appears to be the more common parasite having been isolated six times by Killick-Kendrick while only one isolation of P. berghei was made.

The validity of the classification of P. vinckei and P. berghei as separate species is generally accepted. The validity of the separation of subspecies of P. berghei has, however, been questioned. Bafort (1968) pointed out the considerable variation and overlap of morphological and developmental characters among strains of P. berghei from different regions of Africa. Peters et al. (1970) also expressed doubt of the validity of the separation of subspecies on the basis of the criteria currently invoked. They suggested that variation could arise from the possibility that strains consist of "mixed populations of parasites within which are individuals possessing a wide range of characters" Landau et al. (1970), on the other hand, consider that the current division of P. berghei and P. vinckei each into three subspecies is clearly justified on the available evidence. They further suggest that there is a case for raising the subspecies to the rank of species.

I believe that the results of the examination of enzyme variation among the strains studied in the present work help to clarify the relationship between the species and subspecies of rodent malaria parasites from different regions of Africa. The present study demonstrates clearly marked and repeatable differences in the electrophoretic mobility of enzymes of different strains of plasmodia to which the criticisms cited above cannot be applied. The strains examined can be separated unequivocally into classes on the basis of the distribution of enzyme variants by applying the following criteria.

1) When variation between strains occurs in two or more different enzymes, strains may be grouped together as a class when a specific variant or set of variants of one enzyme is associated with a specific

variant or set of variants of another enzyme.

Example:

The strains characterized by GPI-1 and GPI-2 are always associated with 6PGD-4; these strains are therefore recognized by criterion 1) as a single class, the "P.b. yoelii group". The strains characterized by GPI-3 are always associated with 6PGD-1 and therefore represent a class distinct from that of the "P.b. yoelii group"; this class comprises the strains of P.b. berghei.

If two such classes of strains are identified among host specimens collected in the same locality it can be concluded that exchange of genetic material does not normally occur between the two classes.

2) Strains from two different regions may be placed in separate classes

a) when strains are characterized for one enzyme by a variant or set of variants unique to each region,

Example:

In the present work all classes of strains identified by criterion 2a) are also identified by criterion 1). Thus by criterion 2a) the strains of P.b. yoelii, characterized by GPI-1 and GPI-2, are distinguished as a class from the strains of P.b. berghei, characterized by GPI-3. It is possible, however, that the strains from two different regions may be indistinguishable in all but one enzyme examined. In such a case two classes can be distinguished by criterion 2a) only.

b) when strains are characterized for one enzyme by a set of variants of which one or more, but not all, are unique to one region,

Example:

Among the strains of P. vinckei those from Brazzaville are characterized by 6PGD-2, 6PGD-3 and 6PGD-5, while those from the Central African Republic are characterized by 6PGD-2 and 6PGD-3 only. The strains from the two regions are, therefore, identified as separate classes by criterion 2b).

c) when strains are characterized for an enzyme by the same combination of variants, but the variants occur in different frequencies in each region.

Example:

To be able to distinguish between strains from different regions on the basis of criterion 2c) it is essential to have examined a large number of strains from each region. This was not possible in the present work. With larger samples, however, it is conceivable that such a distinction could be made between, for instance, the strains of P. vinckei from Brazzaville and from the Central African Republic, on the basis of the relative frequency of GPI-4 and GPI-5 in the two regions.

Among the malaria parasites examined in this work are strains of P. berghei and P. vinckei collected from each of the regions of Africa in which these parasites have been found. The strains thus comprise examples of all the currently recognized subspecies of rodent plasmodia. Table 8 summarizes the results of the survey of enzyme variation among these strains.

The strains of P.b. yoelii, P.b. killicki and the "P. berghei-like" parasite from Nigeria comprise a homogeneous class by criteria 1) and 2a) for all enzymes examined. These strains I have collectively termed the "P.b. yoelii group". The strains of P.b. berghei form a single class by

Sub-species	Region of origin	Enzyme	GPI			6PGD		MDHa	MDHc	AK	HK	G6PD
P.b. berghei	Katanga	No. of strains examined	4			4		3	3	3	3	3
		Designation of variant	3			1		2	1	2	2	1
P.b. yoelii	Central African Republic	No. of strains examined	~~11~~ 15	~~2~~ 4	1	~~14~~ 20		6	6	10	6	10
		Designation of variant(s)	1	2	1+2	4		1	1	1	1	1
P.b. killicki	Brazzaville	No. of strains examined	~~5~~ 1	~~2~~		~~7~~ 1		1	1	1	1	1
		Designation of variant	1	~~2~~		4		1	1	1	1	1
"P. berghei-like"	Nigeria	No. of strains examined	1			1		1	1	1	1	1
		Designation of variant	2			4		1	1	1	1	1
P.v. vinckei	Katanga	No. of strains examined	1			1		0	0	0	0	0
		Designation of variant	4			3						
P.v. chabaudi	Central African Republic	No. of strains examined	7	1	1	4	4	0	0	0	0	0
		Designation of variant(s)	4	5	4+5	2	3					
P.v. ~~lentum~~ chabaudi	~~Brazzaville~~	No. of strains examined	3	3	2	1	4 3	0	0	0	0	0
		Designation of variant(s)	4	5	4+5	2	3 5					
"P. vinkei-like"	Nigeria	No. of strains examined	1			1		0	0	0	0	0
		Designation of variant	6			3						

TABLE 8 Distribution of enzyme forms in the blood parasites among sub-species of P. berghei and P. vinckei from different regions of Africa. LDH and the α-NAE's are not included as clear results were not obtained for these enzymes.

the same criteria and are distinguished from the "P.b. yoelii group" for five of the six enzymes examined.

The strains of P. vinckei from Katanga, the Central African Republic and Brazzaville form a single class by criteria 1) and 2a) in that GPI-4 and 6PGD-3 are found together in strains from each region. The strain of P vinckei from Nigeria, however, can only be placed in the same class as strains of P. vinckei from other regions, by criterion 2a). The presence of GPI-6, not found among strains of P. vinckei from other regions, precludes the association of the strain from Nigeria with other strains of P. vinckei, by criterion 1). The strains of P. vinckei from Brazzaville and from the Central African Republic are distinguished on the basis of the distribution of variants of 6PGD by the application of criterion 2b).

The strains of P. vinckei from all four regions of Africa are distinguished by both criterion 1) and 2a) from either of the two classes of P. berghei. Moreover the application of criterion 1) indicates that P. vinckei and P. berghei occurring in the same locality form genetically isolated groups.

On the basis of the above analysis, it is proposed that the strains of P. berghei from Katanga be recognized as representing an organism clearly distinct from the specimens of P. berghei isolated from other regions of Africa so far. The strains of P. berghei from the Central African Republic, Brazzaville and Nigeria, on the other hand, form a homogenous class on the basis of the criteria applied to the distribution of enzyme variants. It is proposed that these be recognized as at least a very closely related group of organisms.

The strains of P. vinckei from all regions of Africa, with the possible exception of Nigeria, appear to form a closely related group of organisms. There may, however, be some grounds for distinguishing between the strains of P. vinckei from Brazzaville, and those from the Central African Republic. In the absence of other strains of P. vinckei from Nigeria, it would be premature to judge the status of this parasite on the basis of the present results.

Considered as a whole, the strains of P. vinckei form a class clearly distinguishable from either of the two classes of P. berghei.

2) Enzyme polymorphism in populations of rodent malaria parasites

The electrophoretic variation of enzymes among strains of malaria parasite was discussed in the preceeding section in terms of the taxonomic relationship between parasites isolated from different regions of Africa. In this section the discussion concerns the mechanism of distribution of enzyme variants among strains of single species collected in a single locality.

Protein polymorphism in populations of organisms has been widely studied in recent years (e.g. Harris 1966, Lewontin and Hubby 1966, Selander and Yang 1969). In these studies the electrophoretic mobility of enzymes and other proteins was examined in the tissues of individuals from natural metazoan populations. Electrophoretically variant proteins are not normally dominant or recessive; the frequencies of alleles at variant loci and the proportion of heterozygous individuals in a population could, therefore, be obtained directly from the distribution of phenotypes.

The analysis of enzyme variation among strains of malaria parasites, however, involves complications not met with in other published studies of protein polymorphism in natural populations. Individual strains of malaria parasites obviously cannot be regarded as specimens comprising cells of identical genetic origin. In order to estimate the frequencies of variant alleles at a polymorphic locus in a population of malaria parasites, it is necessary to take into account the distribution of clones of parasites among strains. At the time at which the present work was carried out a reliable technique for producing clones from blood parasites had not been perfected in our laboratory. It was thus only possible to devise an indirect method for analysing the genetic composition of strains. A theoretical model, which provided a means of analysing the distribution of variant alleles in a population of malaria parasites, was formulated.

Before describing the model it is necessary to give a meaning, in terms of the life cycle of the parasite, for what I shall term an "individual genotype"; this entity can be considered analagous to the individual animal in, for instance, a population of mice. The "individual genotype" thus comprises a clone, or line of cells of identical origin. Although the point in the life cycle at which the reduction division occurs is not known with certainty, it is generally considered to take place during sporogony in the first nuclear divisions after fertilization (Canning and Anwar 1968, Canning and Anwar 1969). The individual genotype in a strain derived from the blood of a wild rodent thus comprises the mitotic products of one of the meiotic products of a zygote. Such a

strain is thus presumed to comprise "individual genotypes" of a haploid generation.

The model, is constructed as follows.

In a closed population of malaria parasites a certain locus is polymorphic. Two alleles only, A and B, of respective frequencies p and q, occur at this locus. The number of "individual genotypes" in a single strain from the blood of a wild rodent depends, by definition, upon the number of meiotic products whose mitotic progeny are present in the strain. Among all strains derived by any standard procedure, there is an average number, "λ", of meiotic products which ultimately give rise to mitotic progeny in an individual strain. The value of "λ" is governed by the following factors:

a) the number of mosquito bites received by an individual rodent prior to collection of the isolate from which the strain is derived,

b) the number of meiotic products from which ripe infective sporozoites have developed at the time of biting,

c) the proportion of such meiotic products whose mitotic progeny are present in the blood sample from which the strain is derived.

The determination of these values is not necessary to the formulation of the model.

It is, however, essential to take into account the proportion of sporozoites derived from a single zygote, which ultimately give rise to blood parasitaemias. If a very large number of sporozoites derived from a single zygote give rise to blood parasitaemias we are, in effect, dealing with complete zygotes as the units of distribution of genotypes among isolates. In this case the units of distribution are diploid.

However, the infectivity of sporozoites derived from individual zygotes may be so low that each zygote is ultimately represented in the blood sample from which a strain is derived, by the progeny of a single sporozoite. In such a case the units of distribution of genotypes among strains are haploid.

Finally it is assumed that the distributions of events governing all variable parameters are entirely governed by chance and are therefore, described by the Poisson distribution.

In order to take into account all parameters postulated for the construction of the model, equations in four variables must be derived. To avoid such complexity however, two alternative models are proposed. Thus Model I assumes that large numbers of sporozoites derived from a single zygote give rise to blood parasitaemias; Model II assumes that individual zygotes are represented in the blood parasitaemia by the progeny of a single sporozoite. A full derivation of the equations proposed and the method of application of results to each model, is presented in the Appendix.

For Model I the following relationships are derived:

$$f(A) = \frac{e^{p^2\lambda/2} - 1}{e^{\lambda/2} - 1}$$

$$f(B) = \frac{e^{q^2\lambda/2} - 1}{e^{\lambda/2} - 1}$$

where "e" is the base of natural logarithms and f(A) and f(B) are the proportions of isolates examined in which are found only allele A or only allele B respectively. f(A and B), the proportion of isolates in which both alleles are found is thus simply given by

$$f(A \text{ and } B) = 1 - f(A) + f(B).$$

For Model II the relationships derived are as follows:

$$f(A) = \frac{e^{p\lambda} - 1}{e^{\lambda} - 1}$$

$$f(B) = \frac{e^{q\lambda} - 1}{e^{\lambda} - 1}$$

In fitting results to the general model it is assumed that the observation of two enzyme variants in a single strain represents the presence of distinct lines of haploid cells in such a strain, each line being characterized by one variant only. This assumption was verified by the results of the analysis of the polymorphic strain 6AD, which was resolved into "cell lines" each characterized by one variant only.

It was found that the data from the distribution of variants of both GPI and 6PGD was incompatible with Model I for all four "populations" studied. In polymorphic populations of parasites in which the least common allele at a locus occurs at a frequency of 10% or more, a high proportion of strains must show the simultaneous presence of both alleles in order to comply with Model I. However, while all variants occurred at high frequency, polymorphic strains represented only a small proportion of each sample or were absent altogether. All possible combinations of results for variation at a specific locus can be fitted to Model II (see Appendix). Since the data were not compatible with Model I it was concluded, therefore, that the assumptions invoked in the construction of Model II form the most satisfactory basis for the explanation of the observed distributions. It is implied in this conclusion that the infectivity of sporozoites under wild

conditions is very low and that individual zygotes or oocysts are generally represented in strains from wild rodents by the progeny of a single sporozoite.

The analysis on Model II of the distributions of variants of GPI and 6PGD among strains of P.b. yoelii, P.b. killicki, P.v. chabaudi and P.v. lentum is presented in Tables 9 and 10. The accuracy of the values of parameters derived from the results of the present work was unfortunately limited by the numbers of strains which it had been possible to examine from each "population" of parasites. These numbers were very low, varying from 7 to 14 (cf. 57 to 224 for the analysis of polymorphism in mouse populations by Selander and Yang 1969). It was, nevertheless, considered that a good estimation of the order of size of the three variables "p" "q" and "λ" was obtained.

In the three instances in which no polymorphism was observed in individual strains, i.e. GPI variants in isolates of P.b. killicki and 6PGD variants in strains of P.v. chabaudi and P.v. lentum, values of "λ" derived from the results are theoretically zero. In the three instances, however in which polymorphism in individual strains was observed, "λ" values of between 0.5 and just over 1.0 were derived. Statistical tests, however, showed that, for the sample sizes involved, "λ" values of zero are not significantly different from values of 1.0. Similarly it was shown that the observed distributions of polymorphic strains were unlikely to have derived from "λ" values greater than 2 or 3.

It was concluded, therefore, that the individual strains examined represent at the most two or three, but probably not more than one, "individual

Sub-species; total no. of strains examined	Distribution of variants among strains				Estimated frequency of variant alleles		"λ"-estimated mean no. of haploid genotypes per strain
P.b. yoelii 14	variant(s)	GPI-1	GPI-2	GPI-1+GPI-2	GPI-1	GPI-2	
					0.83	0.17	0.5
	no. of strains	11	2	1			
	proportion of strains	0.79	0.14	0.07			
P.b. killicki 7	variant(s)	GPI-1	GPI-2	GPI-1+GPI-2	GPI-1	GPI-2	
					0.71	0.29	0
	no. of strains	5	2	0			
	proportion of strains	0.71	0.29	0			
P.v. chabaudi 9	variant(s)	GPI-4	GPI-5	GPI-1+GPI-5	GPI-4	GPI-5	
					0.85	0.15	1.0
	no. of strains	7	1	1			
	proportion of strains	0.78	0.11	0.11			
P.v. lentum 8	variant(s) present in each strain	GPI-4	GPI-5	GPI-4+GPI-5	GPI-4	GPI-5	
					0.50	0.50	1.0
	no. of strains	3	3	2			
	proportion of strains	0.38	0.38	0.24			

TABLE 9 Analysis on Model II of GPI polymorphism in populations of P. berghei and P. vinckei.

haploid genotype". Such a deduction is of particular interest as it sets an unexpectedly low limit to the variability which can be attributed to genetic variation within a single strain (see Peters et al. 1970). It is pointed out, however, that the values of "λ" cannot necessarily be equated with the number of "individual parasite genotypes" present in individual wild rodents. Thus a single strain is derived from a single blood specimen; it cannot, however, be assumed that the "individual parasite genotypes" represented in such a specimen represent all "parasite genotypes" present in the rodent host. The relationship between "λ" and the number of "parasite genotypes" present in a wild rodent is, therefore, a matter for speculation.

To sum up, therefore, the model presented here was devised as a means of determining the frequencies of variant alleles in a population of malaria parasites on the basis of the distribution of variant phenotypes among strains collected from wild rodents. However, the model provides information in addition to values for the frequencies of variant alleles. Thus a value for the average number of "individual parasite genotypes" in single strains and other information concerning the distribution of "parasite genotypes" in a natural enzootic were also obtained.

The available data was not adequate to provide a verification of the validity of the model. However, since it was formulated largely upon the basis of known facts concerning the life cycle of the rodent malaria parasites, the model is considered to provide a credible basis for the study of malaria enzootics. It is predicted, therefore, that further examination on this basis, of enzyme variation among larger samples of strains of malaria

Sub-species; total no. of strains examined	Distribution of variants among strains				Estimated frequency of variant alleles			"λ"-estimated mean no. of haploid genotypes per strain
P.b. yoelii 14	variant	6PGD-4			6PGD-4 1.0			-
	no. of strains	14						
	proportion of strains	1.0						
P.b. killicki 7	variant	6PGD-4			6PGD-4 1.0			-
	no. of strains	7						
	proportion of strains	1.0						
P.v. chabaudi 8	variant	6PGD-2	6PGD-3		6PGD-2 0.50	6PGD-3 0.50		0
	no. of strains	4	4					
	proportion of strains	0.50	0.50					
P.v. lentum 8	variant	6PGD-2	6PGD-3	6PGD-5	6PGD-2 0.13	6PGD-3 0.50	6PGD-5 0.37	0
	no. of strains	1	4	3				
	proportion of strains	0.13	0.05	0.37				

TABLE 10 Analysis on Model II of 6PGD polymorphism in populations of P. berghei and P. vinckei.

parasites, would provide valuable information concerning the mechanism of distribution of parasite genotypes in natural enzootics.

3) Uptake of host cell components by the malaria parasite

The application of starch gel electrophoresis to the problem of identifying the biological and biochemical origin of enzyme activity in individual parasite preparations was demonstrated in the experiments described in section 5) of the results. The methods used to identify extraneous enzyme activity on gels incubated for GPI, and to distinguish between host and parasite enzymes in the studies of GPI, MDH and G6PD require little further comment. They demonstrate, however, the power of gel electrophoresis as a technique for identifying specific proteins.

The analysis of the origin of activity on starch gel electrophoretograms, of MDH and G6PD in parasite preparations, however, raises some points of interest. The results of this investigation had initially suggested that the parasite may incorporate the host forms of these enzymes into its own cytoplasm. This hypothesis had arisen from the identification of electrophoretic forms of MDH and G6PD specific to the host blood, in preparations of parasites released from the red blood cells by immune lysis. In the case of G6PD, this hypothesis was particularly attractive in view of the apparent relationship between deficiency of G6PD in human red blood corpuscles and resistance to falciparum malaria.(Allison and Clyde 1961). The idea was abandoned, however, when it was found that the activity of the host enzyme in parasite preparations was markedly reduced on applying added precautions during the purification procedure.

While the results of this analysis thus lead to a negative conclusion, it is considered worth recording as it serves as an illustration of the dangers of misinterpretation of data in terms of a host-parasite relationship. Moreover, similar proposals have been made from time to time by other workers. These are, therefore, examined in the light of the results presented here.

Literature relating to the uptake of material from the host cell by the malaria parasite is fairly extensive. In most instances in which good evidence exists for such uptake, small molecules such as cofactors and coenzymes for instance folic acid and acetyl CoA, and substrate molecules, e.g. haemoglobin, are involved (for references see Garnham 1966). The dependence upon substrate molecules and cofactors or vitamins derived from another organism is, however, a universal phenomenon among heterotrophic organisms, and as such does not reflect a unique aspect of the host-parasite relationship.

Suggestions of the incorporation and exploitation by the parasite of the specific functions of macromolecules of the host cell, however, invokes a rather different phenomenon. The utilization by the parasite of host enzymes such as MDH and G6PD belongs to this hypothetical class of phenomena. Situations of this kind have been postulated recently in several contexts. Cenedella et al. (1969) presented evidence indicating that the parasite may incorporate fatty acids synthesized by its host, directly into its own lipid complex. Tokuyasu et al. (1969) were unable to account for the synthesis of the 16S particle of the parasite ribosome in P. berghei. They suggested that this particle may be derived from the host cell. In no instance, however, has the utilization by the malaria parasite of macromolecules synthesized by the host cell been substantiated.

A similar situation to that originally postulated in the present work has been suggested in the relationship between Schistosoma mansoni and its mammal host. Thus Smith and Brown (1970) attributed activity of G6PD in S. mansoni to the presence of the mouse host enzyme in the parasite. This conclusion was based on the results of Conde-del-Pino et al. (1968) who found that the G6PD of the worm and the mouse host migrated identically on electrophoresis, and on their own observation that G6PD activity in axenically maintained S. mansoni decreased with time; they attributed this loss to the decline of host enzyme activity in the worms during culture.

Coles (1970) maintained that the interpretation put upon their results by Smith and Brown implied absorption of the host enzyme by the worms, as they had found that G6PD activity continued to fall after the erythrocyte debris had been lost from the gut of the parasite. It was thus implied that the major activity of G6PD in the worm was due to the presence of the host enzyme. However Coles also pointed out that the fact that the G6PD of the worm and the host migrate identically on electrophoresis does not mean that the proteins are identical. Moreover he quoted results from an unpublished study of another schistosome, S. mattheei, which showed that the G6PD of the worm differed from that of the red blood cells of its hamster host.

The interpretation of their results, by Smith and Brown, cannot, therefore, be considered to be substantiated. Coles concluded that the more likely explanation is that the fall in G6PD activity during axenic culture of S. mansoni represented the metabolic decline of the worms in an inadequate medium.

It is clear, therefore, that confusion between components of the host and parasite systems may frequently arise. It may be easy and tempting to interpret results in terms of the incorporation by the parasite of components of the host system. Artifical contamination of parasite preparations by residual host material must, however, be rigorously eliminated and the components of the host and parasite must be clearly distinguished.

4) Comparison of enzymes of the blood parasites and the oocyst

A comparison of the electrophoretic mobilities of GPI, 6PGD and MDH was made in the blood parasites and the oocyst. The mobility of the GPI of the oocyst was found to correspond exactly to that of the blood parasite in all strains examined. Since the same electrophoretic variation of GPI among different strains occurred in both the oocyst and blood parasite, it was concluded that the same structural gene locus controlled this enzyme at both stages of the parasite life cycle. The mobility of 6PGD was also indistinguishable in the oocyst and the blood parasites. However 6PGD showed no variation in electrophoretic mobility in either the oocyst or the blood parasites. It was not possible, therefore, to confirm that identical proteins catalyse the 6PGD reaction at both stages of the life cycle.

The two regions of MDH activity found in the blood parasites were absent in the oocyst. However an anodal band of MDH activity was present in the oocyst, migrating to a position distinct from that of the anodal band of MDH in the blood parasite. It was concluded that distinct genetic loci probably control the oocyst and blood parasite forms of MDH.

The transformation from the blood parasite to the sporogonic stages in P. berghei, is known to involve the emergence of mitochondria in the sporogonic stages (Vanderberg et al. 1967, Garnham et al. 1969, Howells and Fullard 1970). It is presumed that the transformation is also accompanied by a switch from mainly non-aerobic respiratory metabolism in the blood parasites, to aerobic respiratory metabolism in the sporogonic stages. MDH is found in the mitochondria where it functions as one of the enzymes of the citric acid cycle - the central pathway of aerobic respiratory metabolism. MDH, however, may also mediate the interconversions of malate in the cell cytoplasm, in metabolic circumstances not directly related to the activity of the citric acid cycle. Thus in the apparent absence of citric acid cycle activity in the blood parasite, it is probable that the MDH of the blood parasite performs a different metabolic role to that of the MDH of the oocyst. The mitochondrial and cytoplasmic forms of NAD-dependent MDH in mammalian sytems have been shown to be two distinct proteins (Thorne 1960). It is, therefore, of particular interest to record the apparent switch of gene loci controlling MDH, between the two stages.

In contrast to MDH, both GPI and 6PGD have unique metabolic roles in the Embden-Meyerhof pathway of glycolysis and in the pathway of "direct oxidation" of glucose, respectively. Both these pathways are common to all cell systems. It is not surprising, therefore, to find that the same genetic loci appear to control the formation of these enzymes in both the blood parasites and the oocyst.

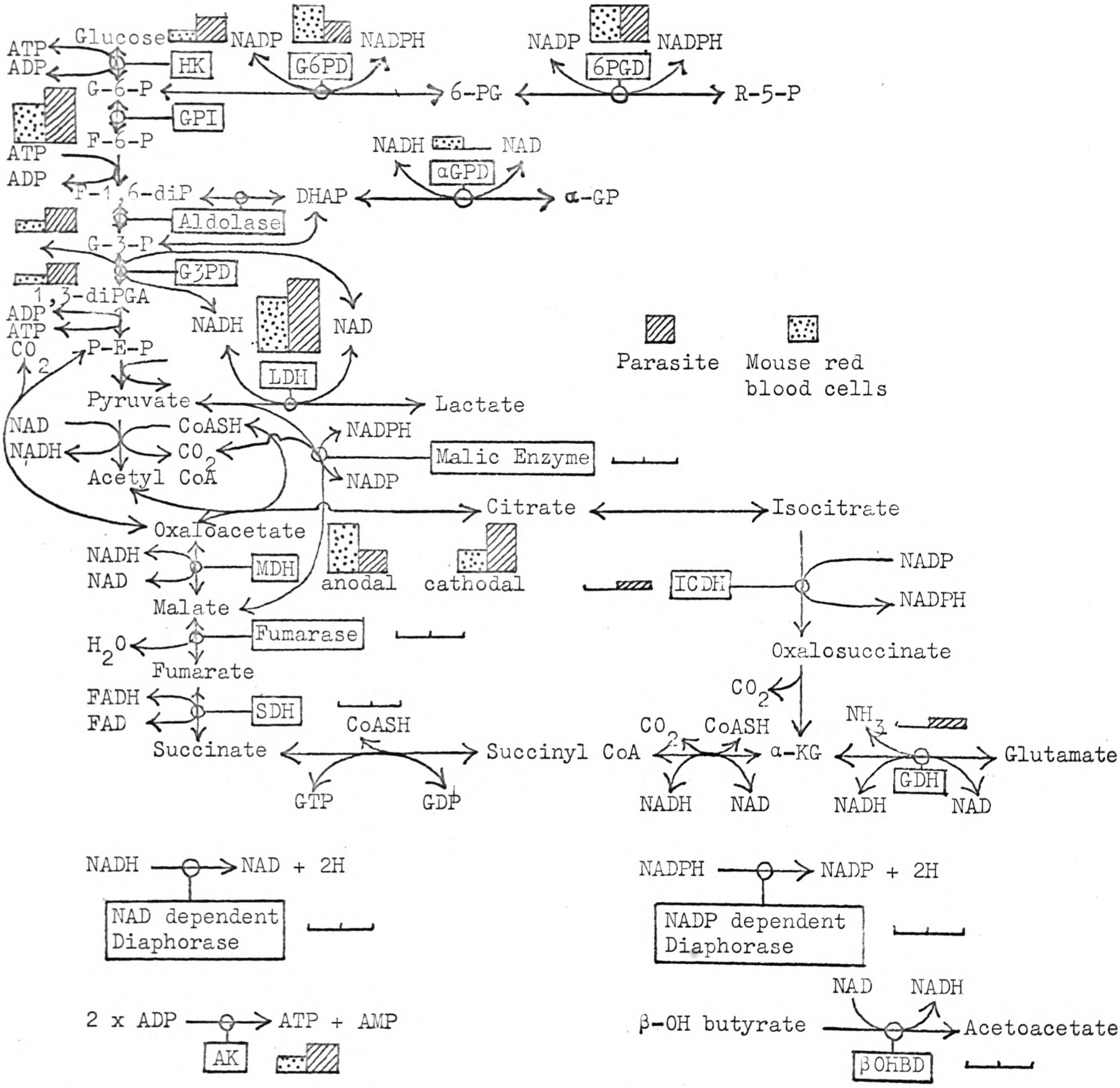


FIGURE 10 Metabolic scheme showing the relative activitie of enzymes of P. berghei blood parasites and mouse red blood cells.

5) Metabolic implications from the survey of the activities of enzymes of the blood parasites of P. berghei and the host red blood cells

Table 7 of the results presents a complete list of the enzymes whose activities on starch gel were assayed in preparations of mouse red blood cells and blood parasites of P. berghei. It was possible to demonstrate, within the limitations of the method of estimation, that a unique level of activity was consistently associated with each of the enzymes examined in both parasite and blood cell preparations. Since, in every case, it was possible to distinguish the parasite and the host enzyme, the results could be interpreted in terms of the relative activities of enzymes in the host and parasite cells. These results are represented in Figure 10 in association with a scheme of the metabolic pathways involved.

With the exception of the α-naphtyl acetate esterases all the enzymes examined are involved in, or closely associated with, the main pathways concerned in energy production. These are the Embden-Meyerhof pathway of catabolism of glucose, the pathway of direct oxidation of glucose-6-phosphate (G-6-P), or the phosphogluconate pathway, and the reactions associated with the citric acid cycle and the production of reducing equivalents supplying the process of oxidative phosphorylation.

The results reflected an apparent low degree or absence in both the parasite and blood cells, of activity of enzymes involved in pathways associated with aerobic respiration. Thus of the enzymes of the citric acid cycle and the NADH and NADPH dependent diaphorases, only NAD-dependent MDH showed significant activity on starch gel from either the parasite or the red blood cell. However, NAD-dependent MDH is involved in pathways other than those directly associated with the main respiratory activities of the cell. As

pointed out in the previous section, the observation of an apparent switch of loci controlling MDH, from the blood parasite to the oocyst, may be interpreted to indicate a difference in the metabolic role of MDH in the two stages of the life cycle. Thus while the MDH of the sporogonic stages is probably involved in the metabolism of aerobic respiration, the MDH forms found in the blood parasite may well have other functions.

Nagarajan (1968) found, on incubating P. berghei freed from the host red cells, with glucose and ^{14}C labelled bicarbonate, that 60% of the label became incorporated into malate, 6% into succinate and 6% into fumarate. The rest of the label was unaccounted for. Siu (1966) partially purified phosphoenol pyruvate carboxykinase from P. berghei blood parasites. This evidence suggests that oxaloacetate formed by the carboxylation of phosphoenolpyruvate may provide the substrate for the MDH reaction in the blood parasite. The role of this reaction in the metabolism of the parasite is uncertain. Both NADP-dependent MDH, or malic enzyme, and fumarase, the only two enzymes other than NAD-dependent MDH known to mediate the interconversions of malate, were apparently inactive in both blood cell and parasite. These observations suggest that malate may represent a "dead end" in the metabolism of the red blood cell and the malaria parasite, similar to that represented by lactate.

Other than MDH, the only enzymes associated with the citric acid cycle, which were examined and for which activity was detected, were ICDH and GDH. Both these enzymes were inactive in preparations of red blood cells and their activity in preparations of parasitized cells was low. The results indicate, therefore, that P. berghei blood parasites do not possess the full complement of enzymes necessary for the operation of the citric acid cycle.

In contrast to the general absence or low activity of enzymes of aerobic metabolism, moderate or high activity was demonstrated in both red blood cell and blood parasite among enzymes involved in pathways of anaerobic respiration - AK, HK and enzymes of the Embden-Myerhof pathway - and in G6PD and 6PGD from the phosphogluconate pathway. Among these enzymes the highest activity in both the host red cell and the malaria parasite, was found for GPI and LDH. These two enzymes respectively catalyse the formation of fructose-6-phosphate, the first intermediate of the glycolytic sequence proper, and the removal of pyruvate, the last intermediate of the glycolytic sequence, by its reduction to lactate. The conversion of pyruvate to lactate is closely coupled to the operation of glycolysis as the oxidizing equivalents, in the form of NAD, required for the function of this pathway are largely supplied by this reaction. Teleologically the high activity of the enzymes catalysing the formation and removal of the initial and terminal intermediates, can be interpreted as a mechanism designed to promote the maximum flow of intermediates through the glycolytic sequence. The high activities of GPI and LDH thus indicate a strong emphasis on anaerobic glycolysis as a source of metabolic energy in both the red cell and the malaria parasite.

Activity of AK and HK and of the two other enzymes involved in glycolysis - Aldolase and G3PD - was demonstrated in both red blood cells and parasites. In all cases, however, these enzymes appeared to be considerably less active than either LDH or GPI.

In general the observations discussed above demonstrate similarity between the parasite and the host cell in the activity of enzymes involved in pathways of energy production. However, among enzymes for which significant activity was detected, with the possible exception of MDH, the enzymes of

the parasite were all found to be more active than the corresponding enzyme in the blood cells. While the cathodal band of the MDH in the blood cells was more active than that of the parasite, the anodal band of the parasite MDH was more active than that of the red blood cells. It was not possible, therefore, to estimate the overall relative activities of MDH in the two cell systems.

Sherman (1961) working with P. lophurae and Phisphumvidhi and Langer (1969) working with P. berghei made similar observations to those reported in the present work, concerning the relative activity of LDH in the parasite and the red blood corpuscle. Sherman suggested that the higher activity of the parasite enzyme might endow the parasite with a "competitive advantage" over its host cell. By the same criterion this interpretation could be applied to the results reported in the present work. It is not, however, axiomatic that the parasite should exhibit a superiority over its host cell in its ability to assimilate resources or in its capacity to supply its energy requirements. On the contrary, it is to the advantage of the parasite to disrupt its host as little as possible. Nevertheless, in contrast to the metabolically rather inactive red cell, the malaria parasite as an actively growing cell, has a high requirement both for resources and for metabolic energy. The simplest interpretation of these results is, therefore, as a reflection of the difference in energy requirements of the two cell systems.

The activities in the red blood cell and the parasite, of the two enzymes from the pathway of "direct oxidation" of G-6-P, namely G6PD and 6PGD, reflect a different aspect of the metabolic priorities of the host and parasite cells. These enzymes function consecutively to mediate the

oxidation of G-6-P to ribulose-5-phosphate, two molecules of NADP being reduced to NADPH in the process. The primary role of the NADPH so formed is as a source of reducing power in non respiratory processes.

Both parasite and host cell appeared to exhibit similar moderately high levels of activity of 6PGD, the second enzyme of the sequence. The activity of G6PD, on the other hand, while, in the red cell, similar to that of 6PGD, was considerably less active in the erythrocyte freed parasite. A major difference in the metabolic priorities of the host and parasite cells, is, therefore, indicated with respect to the fate of G-6-P. Thus the relative activities of GPI and G6PD in the parasite are such as would be expected to provide for the metabolism of G-6-P largely through the glycolytic pathway but very little through the phosphogluconate pathway. In the red blood cells, on the other hand the activities of GPI and G6PD appear to be similar suggesting that comparable amounts of G-6-P are directed through each pathway.

The high priority given by the red cell to the dehydrogenation reactions of G6PD and 6PGD may be partially, if not largely, accounted for in terms of the necessity of maintaining haemoglobin in its reduced ferrous state in an environment of high oxygen tension. Both NADH, derived from the oxidation of G-3-P during glycolysis, and NADPH, formed in the oxidative pathway of G6PD and 6PGD, are oxidized by methaemoglobin reductase, the enzyme mediating the reduction of methaemoglobin to haemoglobin. Since as much as 50% of haemoglobin is normally oxidized to methaemoglobin in the course of 24 hours, the functioning of these reducing systems is obviously of vital importance to the red blood cell.

The same priority for maintaining the level of cellular reduction

does not presumably apply in the case of the malaria parasite. Nevertheless, as an actively growing cell, the malaria parasite must make considerable demands on the products of synthetic processes and hence ultimately on a supply of NADPH. The low activity of the parasite G6PD, however, suggests either that the consumption of NADPH by the parasite is low, or else that NADPH is furnished by a mechanism other than the phosphogluconate oxidative pathway.

The observation of low activity of parasite G6PD is of particular interest in relation to the association of deficiency of G6PD in the erythrocytes among human populations, with resistance to falciparum malaria, first reported by Allison and Clyde (1961). The apparent low priority given by the parasite to metabolism of G-6-P by the phosphogluconate pathway, with its associated regeneration of NADPH, may be reflected in a dependence by the parasite upon its host cell for the functioning of this system. In P. knowlesi infections in monkeys, Fletcher and Maegraith (1962) found that the levels of activity of both G6PD and 6PGD in parasitized monkey erythrocytes were elevated over those in the uninfected erythrocytes. This system differs from that found in P. berghei infections in mice, where the activities of erythrocyte G6PD and 6PGD are intrinsically high. Nevertheless both systems reflect an association of parasitaemia with high activity of these enzymes in the host cell, while most evidence indicates low activity of G6PD in the parasite. Such a relationship could, therefore, form the basis of the explanation of the observations of Allison and Clyde in that the parasite may depend for its synthetic requirements either directly or indirectly on the supply of NADPH mediated by an adequately functioning G6PD and 6PGD in the host cell.

The observations recorded during the present work, reguarding the relative activities of G6PD and 6PGD in red blood cells and parasites, conflict with the results of Langer et al. (1967). In preparations of P. berghei freed from the host cell these workers reported similar levels of activity for both enzymes to those found in uninfected erythrocytes. The results of Bryant et al. (1964) are, however, in accord with present observations. Working with P. berghei in pulse labelling experiments using U-^{14}C glucose, Bryant found little or no incorporation of label into 6-phosphogluconate, thus indicating an absence or very low activity of G6PD in the parasite. Bowman et al. (1961) found, on incubating both free parasites (P. berghei) and parasitized reticulocytes with 1-^{14}C labelled glucose, that less than 1% of label was released as CO_2, indicating a low activity of the phosphogluconate pathway.

In conclusion, therefore, the results of the present work are generally consistent with those reported by other workers. The present work, however, differs in approach from the majority of previous studies on the metabolism of malaria parasites. Most such studies have been concerned with determining the levels and fates of intermediates of metabolism and thence attempting to reconstruct the nature of the enzyme reaction sequences in operation. The present study takes the converse approach. From a survey of the relative activities of a number of enzymes an attempt was made to predict the metabolic priorities of the cell systems studied. The two approaches thus compliment each other in the reconstruction of metabolic events.

6) Genetic studies:

The original object of the study of enzyme variation among isolates of rodent plasmodia was to find markers suitable for use in genetic studies on the malaria parasite. The work of the project, of which the studies presented here form a part, was concerned with the development of techniques to enable genetic studies to be performed on malaria parasites using P.b. yoelii as the experimental organism. In the present study, however only one variant locus, that controlling GPI, was identified among strains of this subspecies. The prospects for performing such experiments, therefore, depended on the identification or induction of other variant characters among the strains of P.b. yoelii. The induction of electrophoretic variation of proteins at other loci by U.V. irradiation or by treatment with chemical mutagens was considered unlikely to be productive. The ability to select for a mutant organism or cell depends upon the possibility of devising conditions whereby the mutant is given a growth advantage over the wild type. That a mutant protein manifesting electrophoretic variation with respect to the wild type should endow an organism with a growth advantage over the wild type is highly unlikely. The search for genetic markers was therefore extended to other characters of the parasite.

A strain of pyrimethamine resistant P.b. yoelii was recently developed in this laboratory from strain 17X (Morgan, unpublished results). The level of resistance was shown to be clearly different from that of the wild type strain from which it was derived, and to be stable to transmission through mosquitoes. Using this pyrimethamine resistant line of 17X, characterized by GPI-1, and a wild type strain 3L, pyrimethamine sensitive and

characterized by GPI-2, an attempt to demonstrate genetic hybridization was made by feeding mosquitoes on the blood of an animal infected with both strains. On subsequent analysis of blood infections derived from sporozoites from these mosquitoes, reassortment of the pyrimethamine resistance characteristics and the GPI variants appeared to have taken place. This result suggested that hybridization had taken place between genotypes of the two strains. These results, however, have yet to be verified.

7) Conclusion:

While progress in the use of enzyme variants for genetic studies on malaria parasites was limited, the study of enzyme differences among parasites and between the parasite and its host cell, yielded results of interest from several points of view.

It was found that variation in the electrophoretic mobility of enzymes provided an unambiguous basis for the clarification of the taxonomic relationship between isolates of parasites from different regions of Africa. By the application of a set of criteria different degrees of relationship could be demonstrated. It was thus possible to categorize groups of isolates into a number of "taxonomic classes", on the basis of enzyme variation, while enzyme variation within "taxonomic classes" could be identified in the form of apparently allelic polymorphisms. By the application of a model, proposed on the basis of theoretical considerations, it was possible to present an analysis of the distribution of variants at polymorphic loci, among the populations of malaria parasites. The consequences of the model, moreover, provided information regarding

the mechanism of distribution of genotypes in terms of transmission, at different stages of the life cycle, of unique genetic lines among wild populations of malaria parasites. Unfortunately, due to the small numbers of isolates available from each area studied, few of the conclusions reached could be validated on a firm statistical basis.

The technique of starch gel electrophoresis was used to provide a method for distinguishing enzyme components of different biological and biochemical origins. This application was used in the resolution of ambiguous results arising from the confusion of enzyme activity of different origins in preparations of parasite material.

Comparison of the electrophoretic mobility of MDH, 6PGD and GPI in the blood stage parasite and the oocyst indicated that while the same genetic locus appeared to operate in both stages for GPI and 6PGD; a complete switch of loci controlling MDH appeared to take effect during the transformation from the blood stages to the mosquito stage parasite.

A comparative survey on starch gel, of a range of enzymes in both blood stage parasite and host erythrocyte, provided information concerning the similarities and differences in the metabolic priorities of the parasite and the host cell. The results suggested possible metabolic relationships in the red blood cell and the parasite.

APPENDIX

<u>Derivation of models describing the distribution of "individual genotypes" of malaria parasites among strains derived from blood samples from wild rodents</u>

(see Discussion, section 2))

Model I

Assume diploid units of distribution of genotypes among strains.

The distribution of A and B among all diploid units,

$$= (p + q)^2 = p^2 + 2pq + q^2 = 1$$

of all diploid units, the proportion containing only A = p^2

and " " " " " " " " B = q^2

" " " " " " " " A and B = $2pq$

λ = mean number of haploid genotypes represented in a strain.

$\therefore$ mean number of diploid units represented in a strain = $\frac{\lambda}{2}$

$\therefore$ mean number of diploid units per strain characterized by

$$A.A = p^2.\ \lambda/2$$

$$\text{by } B.B = q^2.\ \lambda/2$$

$$\text{and by } A.B = 2pq.\lambda/2 = pq.\lambda\ .$$

The Poisson distribution gives

$$e^{-x}\Sigma\left(1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \ldots\ \right) = 1$$

By substituting for x, either $p^2.\ \lambda/2$, or $q^2.\ \lambda/2$, or $pq.\lambda$, we have that the proportion of strains in which are represented no diploid units characterized by

$$A.A = e^{-p^2 . \lambda/2}$$

$$\text{by } B.B = e^{-q^2 . \lambda/2}$$

$$\text{and by } A.B = e^{-pq.\lambda}$$

Hence the proportion of strains in which A.A is represented,

$$= (1 - e^{p^2 . \lambda/2})$$

The proportion of strains in which only diploid units characterized A.A are represented,

= the proportion of strains in which A.A is represented x the proportion in which neither B.B nor A.B is represented,

$$= (1 - e^{-p^2 . \lambda/2}) \times e^{-p^2 . \lambda/2} \times e^{-pq.\lambda}$$

$$= (1 - e^{-p^2 . \lambda/2}) \times e^{-(q^2 + 2pq). \lambda/2}$$

$$= e^{-(q^2 + 2pq). \lambda/2} - e^{-(p^2 + 2pq + q^2). \lambda/2}$$

$$= e^{-(1 - p^2). \lambda/2} - e^{- \lambda/2}$$

= proportion of all possible strains, in which only diploid units characterized by A.A are represented. However from the Poisson equation it is predicted that a proportion of all possible strains equal to $e^{- \lambda/2}$, do not represent any diploid units at all. In other words such strains comprise unparasitized blood. Obviously such strains were not examined. Nevertheless their hypothetical existance must be taken into account. Thus the sum of all strains in which parasites occur represents a proportion of the hypothetical total of strains, which is less than unity by the amount of $e^{- \lambda/2}$, and is thus equal to $(1 - e^{- \lambda/2})$.

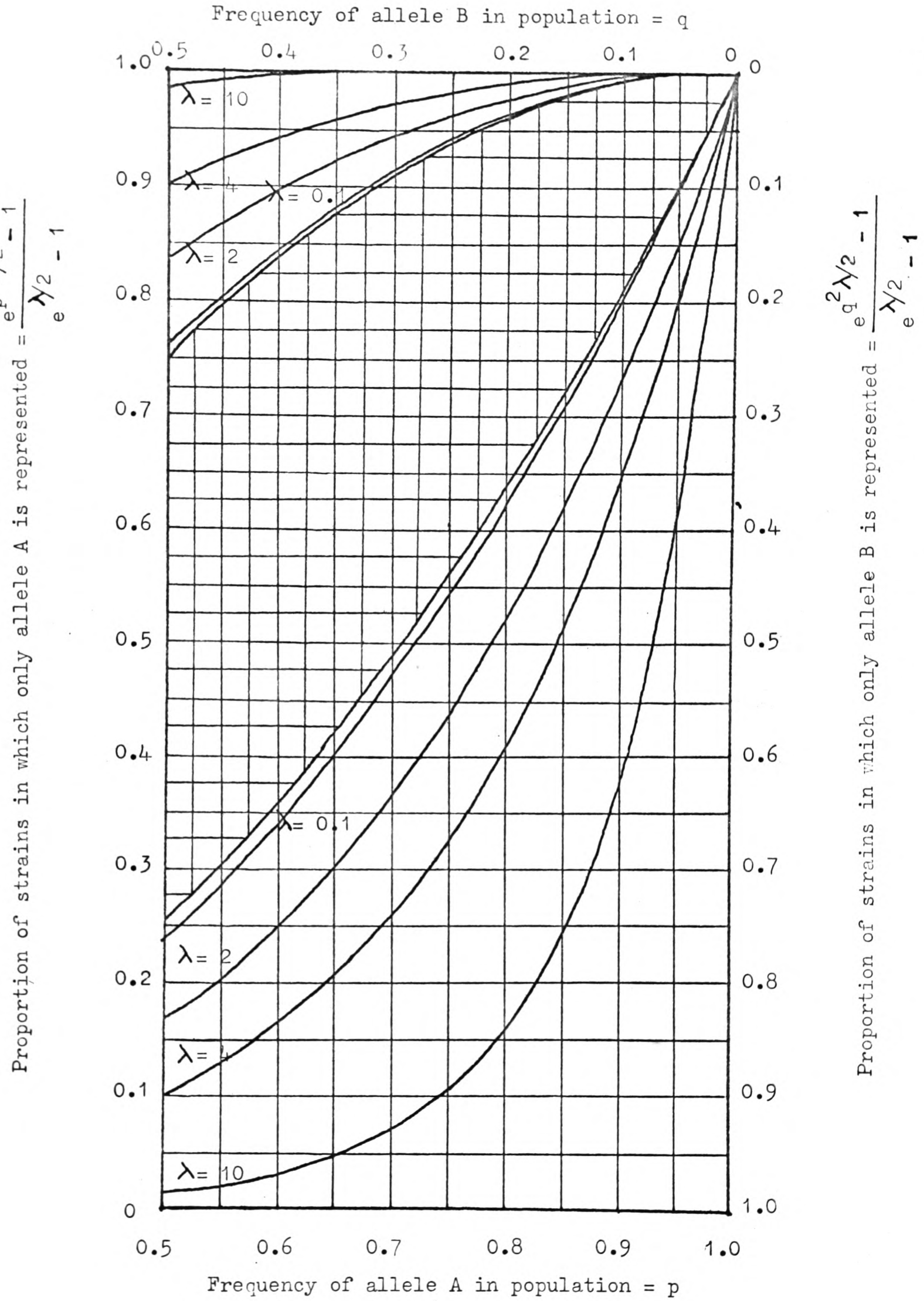


FIGURE 11 Analysis of distribution of variant alleles in natural populations of malaria parasites - Model I

Thus the proportion of strains representing only A.A diploid units among strains in which parasites occur

$$= \frac{(e^{-(1 - p^2).\lambda/2} - e^{-\lambda/2}}{(1 - e^{-\lambda/2})}$$

$$= \frac{e^{p^2\lambda/2} - 1}{e^{\lambda/2} - 1} = f(A)$$

Similarly $\frac{e^{q^2.\lambda/2} - 1}{e^{\lambda/2} - 1} = f(B)$

Model II

Assume haploid units of distribution of genotypes among strains. Then distribution of A and B among all haploid units

$= p + q = 1$

= mean number of haploid genotypes represented in a strain

mean number of haploid units per strain characterized by

$$A = p\lambda$$

and by $B = q\lambda$

The proportion of strains in which are represented no haploid units characterized by

$$A = e^{-p\lambda}$$

and by $B = e^{-q\lambda}$

Hence the proportion of strains in which A is represented

$$= (1 - e^{-p\lambda})$$

The proportion of strains in which only haploid units characterized by A, are represented

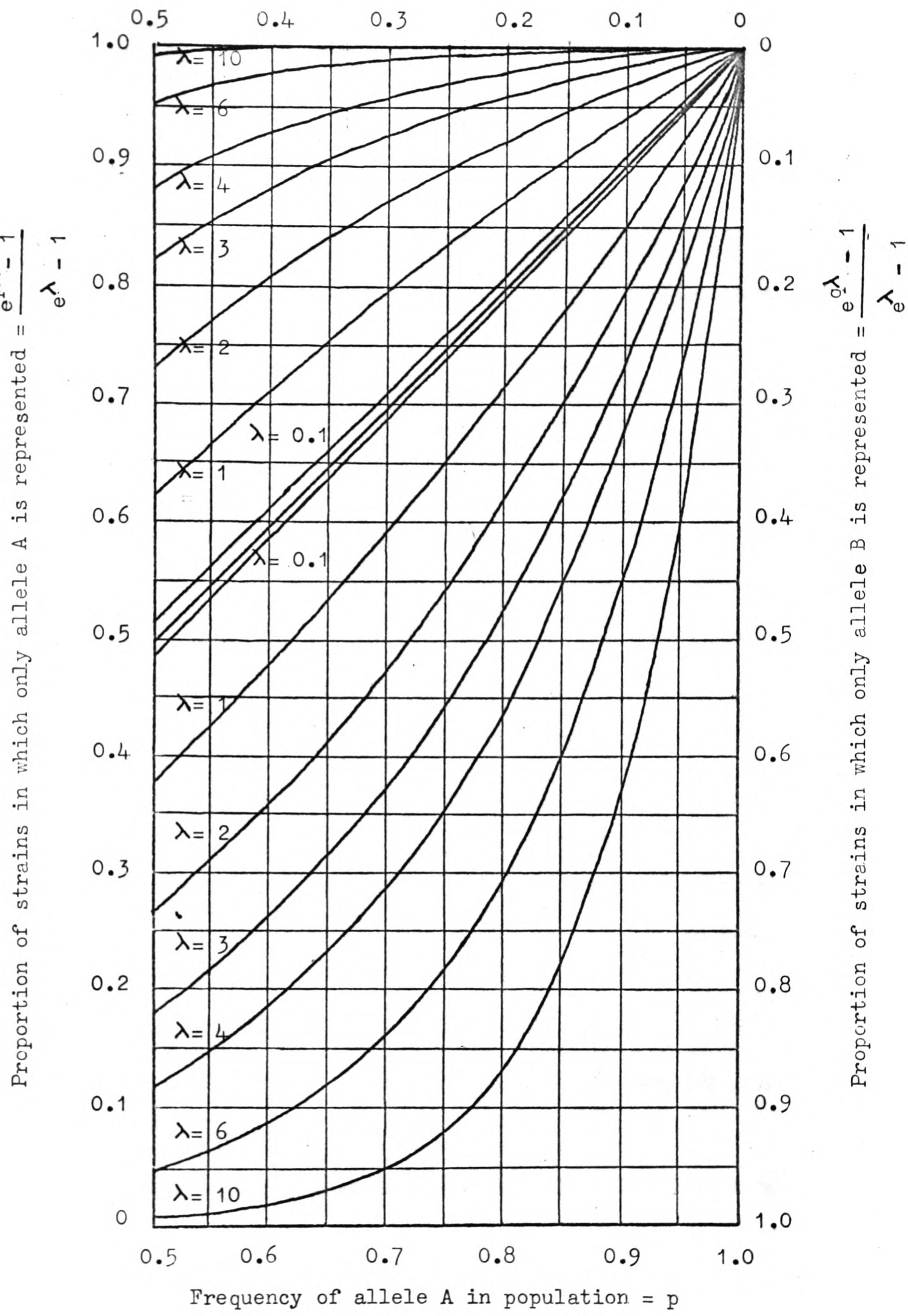


FIGURE 12 Analysis of distribution of variant alleles in natural populations of malaria parasites.- Model II

= the proportion of strains in which A is represented x the proportion in which B is not represented

$$= (1 - e^{-p\lambda}) \times e^{-q\lambda}$$

$$= e^{-q\lambda} - e^{-(p+q)\lambda}$$

$$= e^{-(1-p)\lambda} - e^{-\lambda}$$

By the same arguement that was applied for Model I, we have that the proportion of strains representing only A haploid units, among all strains in which parasites occur

$$= \frac{(e^{-(1-p)\lambda} - e^{-\lambda})}{(1 - e^{-\lambda})}$$

$$= \frac{e^{p\lambda} - 1}{e^{\lambda} - 1} = f(A)$$

Similary $\frac{e^{q\lambda} - 1}{e^{\lambda} - 1} = f(B)$

The two models are represented graphically in Figures 11 and 12. For every value of "λ" the relationship between f(A) and "p" and between f(B) and "q", is represented on each model by a unique pair of curves, the "λ curves". In order to derive values for "p" and "q", the observed values for f(A) and f(B) must be fitted to a pair of "λ curves" having the same value of "λ". It is seen that by fitting any two values of f(A) and f(B) to a pair of "λ curves" unique values of p, q and "λ" are obtained. It is evident that all possible combinations for f(A) and f(B) can be fitted to the "λ curves" of Model II. There is, however, on Model I, a series of combinations of f(A) and f(B), represented by the hatched area on the graph, which do not correspond to any pair of "λ curves". Nevertheless, for

values of "λ" greater than 4, it can be seen that the relationships between "λ" and the values of f(A) and f(B) correspond very closely on the two models, as do values of "p" and "q" for all combinations of f(A) and f(B) which can be fitted to both models.

If, therefore, all assumptions made in formulating the models are valid, the values of "p" and "q" obtained from either model approximate very closely to the true frequencies of alleles A and B. The estimation of values of "λ" less than 4, however, depends the model to which values of f(A) and f(B) are fitted.

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REFERENCES

ADAM, J. -P, LANDAU, I., & CHABAUD, A. -G. (1966). C. r. Acad. Sci., Paris, 263, 140.

ALLISON, A.C. & CLYDE, D.F. (1961). Br. med. J., i, 1346.

BAFORT, J. (1967). Annls Soc. belge Med. trop., 47, 271

BAFORT, J. (1968a). Nature, Lond., 217, 1264.

BAFORT, J. (1968b). Parassitologia, 10, 107.

BOWMAN, I.B.R., GRANT, P.T. & KERMACK, W.O. (1960). Expl Parasit., 9, 131.

BOWMAN, I.B.R., GRANT, P.T., KERMACK, W.O. & OGSTON, P. (1961). Biochem. J., 28, 472.

BRUCE-CHWATT, L.J. (1967). Trans. R. Soc. trop. Med. Hyg., 61, 412.

BRYANT, C., VOLLER, A. & SMITH, M.J.H. (1964). Am. J. trop. Med. Hyg., 13, 515.

CANNING, E.U. & ANWAR, M. (1968). J. Protozool., 15, 290.

CANNING, E.U. & ANWAR, M. (1969). Trans. R. Soc. trop. Med. Hyg., 63, 4.

CARTER, R. (1970). Trans. R. Soc. trop. Med. Hyg., 64, 401.

CENEDELLA, R.J., JARRELL, J.J. & SAXE, L.H. (1969). Milit. Med., 134, 1045.

COLES, G.C. (1970). Trans. R. Soc. trop. Med. Hyg., 64, 647.

CONDE-DEL-PINO, E., ANNEXY-MARTINEZ, A.M., PEREZ-VILAR, M. & CINTRON-RIVERA, A.A. (1968). Expl Parasit., 22, 288.

DIGGENS, S.M. (1970). Trans. R. Soc. trop. Med. Hyg., 64, 9.

FLETCHER, K.A. & MAEGRAITH, B.G. (1962). Nature, Lond., 196, 1316.

GARNHAM, P.C.C. (1966). Malaria Parasites and other Haemosporidia, Oxford: Blackwell.

GARNHAM, P.C.C., BIRD, R.G., BAKER, J.R., DESSER, S.S. & EL-NAHAL, H.M.S. (1969). Trans. R. Soc. trop. Med. Hyg., 63, 187.

GREENBERG, J. (1956). Expl Parasit., 5, 359.

GREENBERG, J. & TREMBLEY, H.L. (1954). J. Parasit., 40, 336, 667.

HARRIS, H. (1966). Proc. R. Soc. Lond. B, 164, 298.

HARRIS, H., HOPKINSON, D.A. & LUFFMAN, J. (1968). Ann. N.Y. Acad. Sci., 151, 232.

HOWELLS, R.E. & FULLARD, F.J. (1970). Trans. R. Soc. trop. Med. Hyg., 64, 6.

KILLICK-KENDRICK, R., SHUTE, G.T., & LAMBO, A.O. (1968). Bull. Wld Hlth Org., 38, 822.

KREBS, H.A. & EGGLESTON, L.V. (1940). Biochem. J., 34, 442.

LANDAU, I. (1965a). C.r. Acad. Sci., Paris, 260, 3758.

LANDAU, I. (1965b). Progress in Protozoology, p. 173. Second Int. Conf. on Protozoology, London; Excerpt Medica Foundation Amsterdam.

LANDAU, I. & CHABAUD, A.G. (1965). C. r. Acad. Sci., Paris, 260, 230.

LANDAU, I. & KILLICK-KENDRICK, R. (1966). Trans. R. Soc. trop. Med. Hyg., 60, 633.

LANDAU, I. MICHEL, J. -C. & ADAM. J. -P. (1968). Annls Parasit. hum. comp., 43, 545.

LAUDAU, I., MICHEL, J. -C., ADAM, J. -P. & BOULARD, Y. (1970). Ann. trop. Med. Parasit., 64, 315.

LANGER, B.W., Jr., PHISPHUMVIDHI, P. & FRIEDLANDER, V. (1967). Expl Parasit., 20,68.

LUMSDEN, W.H.R. & HARDY, G.J.C. (1965). Nature, Lond., 205, 1032.

LUMSDEN, W.H.R., ROBERTSON, D.H.H. & McNEILLAGE, G.J.C. (1966). Br. J. vener. Dis., 42, 145.

LEWONTIN, R.C. & HUBBY, J.L. (1966). Genetics, Princeton, 54, 494.

NAGARAJAN, K. (1968). Expl Parasit., 22, 33.

PETERS, W. (1969). Trans. R. Soc. trop. Med. Hyg., 63, 25.

PETERS, W., BAFORT, J. & RAMKARAN, A.E. (1970). Ann. trop. Med. Parasit., 64, 41.

PHISPHUMVIDHI, P. & LANGER, B.W., Jr. (1969). Expl Parasit., 24, 37.

RODHAIN, J. (1952). Annls Soc. belge Med. trop., 32, 275.

SELANDER, R.K. & YANG, S.Y. (1969). Genetics, Princeton, 63, 653.

SHERMAN, I.W. (1961). J. exp. Med., 114, 1049.

SHERMAN, I.W., ~~JUDITH, A.R.~~ RUBLE, J.A. & ~~IRWIN, F.T.~~ TING, I.P. (1969). Expl Parasit., 25, 181.

SIU, P.M.L. (1966). Fedn Proc., 25, 756.

SMITH, I. (1968). Chromatographic and Electrophoretic Techniques, Vol. II, zone electrophoresis, chapts. 8 & 9, London: Heinman Medical Books Ltd.

SMITH, T.M. & BROWN, J.N. (1970). Comp. Biochem. Physiol., 32, 573.

THORNE, C.J.R. (1960). Biochim. biophys. Acta., 42, 175.

TOKUYASU, K., ILAN, Judith & ILAN, J. (1969). ~~J. Parasit., 40, 475.~~ Milit. Med., 134, 1032.

TREMBLEY, H.L. & GREENBERG, J. (1954). J. Parasit., 40, 475.

VANDERBERG, J., ~~RODAIN~~ RHODIN, J. & YOELI, M. (1967). J. Protozool., 14, 82.

VINCKE, I. & LIPS, M. (1948). Annls Soc. belge Med. trop., 28, 97.

WORLD HEALTH ORGANISATION (1965). Tech. Rep. Ser., 296.

YOELI, M., UPMAINS, R.S. & MOST, H. (1969). Parasitology, 59, 429.

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ENZYME VARIATION IN *PLASMODIUM BERGHEI*

R. CARTER*
Protozoan Genetics Unit, Institute of Animal Genetics, University of Edinburgh

Since 1960 a number of isolations of *Plasmodium berghei* have been made from different regions of Africa. The purpose of the present work was to make a comparative study of the electrophoretic mobility on starch gels of some enzymes of a selection of strains derived from these isolations.

Materials and methods

Strains. The strains of *P. berghei* used in this study are listed in the Table.

Preparation of free parasites from blood stages. For the purpose of this work the most easily prepared material was that from the blood stages of the parasite in laboratory mice. To obtain preparations from each strain 5-7 outbred male mice, 20-35 days old, were used. The mice were inoculated intraperitoneally with 0·1 ml. of infected blood diluted 1 : 10 in citrate saline (0·9% NaCl, 1·5% Na citrate adjusted to pH 7·2). When parasitaemia had reached a level of at least 5%, the mice were etherized and bled from the brachial vessels. A little under 1 ml. of blood was collected from each mouse. The blood was transferred immediately into an equal volume of citrate saline and 4% calcium chloride was added (one tenth of the volume of citrate saline). Each preparation was then shaken manually with 3 mm. glass beads for 10 minutes, until the beads had clumped with precipitated fibrin. This procedure removed platelets from the blood. Each defibrinated sample was filtered through glass wool and centrifuged at 1,000 g. for 5 minutes, yielding a brown layer of parasitized cells on top of uninfected erythrocytes. The brown layer was resuspended in up to 10 ml. of phosphate saline, pH 7·4 (KREBS and EGGLESTON, 1940) and recentrifuged at 1,000 g. for 5 minutes. The final parasitized layer was resuspended in phosphate saline (10% v/v).

The parasites were freed from the erythrocytes by immune lysis by the method of BOWMAN, GRANT and KERMACK (1960), using specific anti-mouse erythrocyte serum with preserved guinea-pig serum as a source of complement. After lysis, parasites were freed from the red cell stroma by vigorous manual treatment with a teflon pestle. One drop of antiserum was added and the samples were allowed to stand for about 10 minutes until the stroma had flocculated. Each sample was then centrifuged at 300 g. for 30 seconds. The supernatants, containing most of the freed parasites, were retained

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We are indebted to the following for their kindness in providing us with this material, to Professor A. G. Chabaud and Madame I. Landau for strains 55X, 32X, 86X and 194ZZ, to Dr. J. Bafort for strains Anka and RLL, to Dr. D. Warhurst for strain NK65, to Mr. R. Killick-Kendrick for strain N67 and to the London School of Hygiene and Tropical Medicine for strains 17X and 33X.

and another drop of antiserum added to each. The sediments were suspended in about 5 ml. phosphate saline and again broken up with the teflon pestle, treated with antiserum and centrifuged as before. The combined supernatants, to which a further drop of antiserum was added, were allowed to stand for about 10 minutes and again centrifuged at 500 g. for 30 seconds. The final supernatant was centrifuged at 5,000 g. for 10 minutes, yielding a hard packed, dark brown pellet of parasites.

By this method parasite preparations entirely free from contamination with platelets and with very little contamination from red blood cells were obtained. No precautions were taken against contamination by white cells, but these were estimated to be less than 10% of total cell mass by examination of preparations under a phase contrast microscope.

TABLE. List of strains and their origins

Subspecies	Strain	Locality of collection
P. b. berghei	NK65	River Kisanga, Katanga
	Anka	River Kasapa, Katanga
	RLL	River Kisanga, Katanga
P. b. yoelii	17X	Bangui, Central African Republic
	33X	" " " "
	55X	" " " "
	32X	" " " "
	86X	" " " "
P. b. killicki	194ZZ	Brazzaville, Congo
"*P. berghei-like*" parasite	N67	Ilobi Forest, Nigeria

The "*P. berghei-like*" parasite is an unclassified strain of *P. berghei* isolated in 1967 (KILLICK-KENDRICK et al., 1968).

The final pellet was resuspended in about 5 volumes of phosphate saline and stored at 0° to 5°C. until required for use. For most enzymes examined samples could be stored in this way for up to 5 days without significant loss of activity, as judged by the intensity of band staining after separation by starch gel electrophoresis.

Samples of unparasitized mouse blood diluted 1 : 5 in citrate saline were used as blood enzyme controls.

Lysis of all samples prior to electrophoresis was achieved by adding 5% Triton X to a final concentration of 1% in resuspended parasite preparations. Each sample was then centrifuged at 5,000 g. for 5 minutes and the clear supernatant decanted.

Electrophoresis and staining of enzyme bands

Samples were separated by horizontal starch gel electrophoresis on the same day as lysis of the samples. Strips of filter paper were soaked in extract samples and inserted into slots in the gel along the line of origin (see Fig. 1). During electrophoresis the gels were maintained at 3° to 5°C. For a full description of the general technique see SMITH (1968).

The conditions of electrophoresis varied according to the enzyme under study. All buffer systems were continuous, the same buffer being used both in the electrophoresis tanks and in the gel. Malate dehydrogenase, adenylate kinase and glucose phosphate isomerase were run on 0·01M citrate phosphate gels at pH 7·0 in a field strength of 10 volts per cm. for 6 hr.; 6-phosphogluconate dehydrogenase was run on a 0·01M tris citrate gel at pH 6·0 in a field strength of 5 volts per cm. for 6 hr.

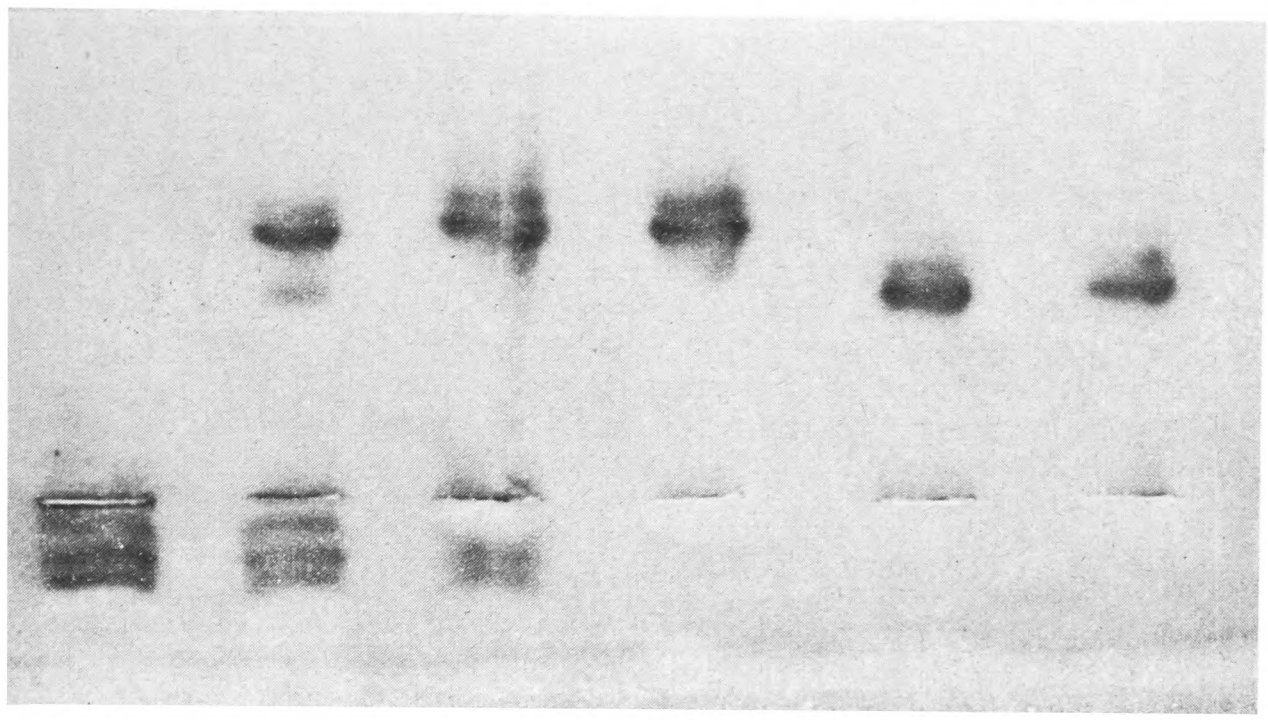

FIG. 1. Starch gel zymogram of glucose phosphate isomerase on a 0·01M citrate phosphate gel at pH7·0 showing a comparison between mouse blood and the GPI-1 and GPI-2 variants in four strains of *P. berghei*.

The positions of enzymic activity on the gels were made visible, after slicing the gels horizontally, by incubating with specific substrate solutions, the enzymic reaction being linked in each case to the reduction of the dye MTT—tetrazolium by reduced pyridine nucleotide via the catalyst phenosine methosulphate to give blue bands at the regions of activity.

The reaction conditions for each enzyme were as follows:

Adenylate kinase: 40 mg. D-glucose, 4 mg. $MgCl_2$, 20 mg. ADP, 7 mg. NADP, 20 units hexokinase, 6 units glucose-6-phosphate dehydrogenase, 1 mg. phenosine methosulphate, 6 mg. MTT—tetrazolium made up to 30 ml. in 0·1M tris HCl buffer at pH 7·4; gels incubated at 37°C. for about 4 hr.

6-*phosphogluconate dehydrogenase:* 200 mg. D-6-phosphogluconate, 7 mg. NADP, 1 mg. phenosine methosulphate, 6 mg. MTT—tetrazolium made up to 30 ml. in 0·1M tris HCl buffer at pH 7·0; gels incubated at 37°C. for about 2 hr.

Malate dehydrogenase: 5 ml. 1 M L-malate adjusted to pH 8·5 10 mg. NAD, 10 mg. KCN, 1 mg. phenosine methosulphate, 6 mg. MTT—tetrazolium made up to 30 ml. 0·1M tris HCl at pH 8·5; gels incubated at 37°C. for about 1 hr.

Glucose phosphate isomerase: 50 mg. D-fructose-6-phosphate, 6 mg. NADP, 30 mg. $MgCl_2$, 2 units glucose-6-phosphate dehydrogenase, 1 mg. phenosine methosulphate, 5 mg. MTT—tetrazolium made up to 30 ml. in 0·1M tris HCl at pH 8·0; gels incubated at 37°C. for about 2 hr.

Results

In a preliminary investigation extracts of *P. berghei* were tested for the activity of about 20 enzymes, after electrophoretic separation on starch gels. In order to demonstrate the presence of enzymic activity specific to the parasites it was necessary to show not only that enzymic activity existed in the parasite extract but also that this activity was not a product of residual contamination by blood enzyme. This was easy to demonstrate on starch gels for the four enzymes included in this study, as the mobilities of the host and parasite enzymes could be clearly seen to be different in most cases, using as controls enzymes of unparasitized blood. The single exception is the adenylate kinase band of the three strains of *P.b. berghei* which migrates at an almost identical rate to that of the blood enzyme under the conditions of electrophoresis used in this work.

Fig. 1 shows the results for glucose phosphate isomerase from 4 strains of *P. b. yoelii*, from blood and from a mixture of blood and the 4 parasite extracts. The distinction between the blood and parasite enzymes is clear, and all but one of the parasite extracts show no trace of the blood enzymes.

Of those enzymes which were shown to be present in the parasite extracts and distinct from the host enzyme the following were chosen for inclusion in this study:

Glucose phosphate isomerase,
Adenylate kinase,
6-phosphogluconate dehydrogenase,
NAD dependent malate dehydrogenase.

Fig. 2 summarizes the observations on the electrophoretic forms of these enzymes in the 10 strains of the *P. berghei* complex. These results fall into two categories:

(1) enzymic differences between subspecies

(2) enzymic differences within subspecies

Enzymic differences of the first category are shown by comparing the three strains of *P.b. berghei* with *P.b. yoelii*, *P.b. killicki* and the "*P. berghei-like*" parasite. It will be seen that *P.b. berghei* differs from all the other subspecies in regard to all 4 enzymes.

Variants of glucose phosphate isomerase constitute the second category. There are two migratory forms among the strains of *P.b. yoelii*, a fast moving form designated GPI-1 and a slow moving form designated GPI-2. The single strains of *P.b. killicki* and the "*P. berghei-like*" parasite contain the types GPI-1 and GPI-2, respectively. Fig. 1 shows these variants in 4 strains.

Discussion

The material examined in this work is representative of all known forms of *P. berghei*. The findings confirm the distinction between the subspecies *P.b. berghei* and the other subspecies, but do not show any differences between *P.b. yoelii* and *P.b. killicki*. On the basis of our results the "*P. berghei-like*" parasite from Nigeria belongs to the same enzymic class as *P.b. yoelii* and *P.b. killicki*.

The findings of the two forms of glucose phosphate isomerase among strains of *P.b. yoelli, P.b. killicki* and the "*P. berghei-like*" parasite is of interest in connection with the genetic relationship between these isolations. Whether or not any close genetic relationship exists between the 3 subspecies cannot be decided until further isolations of all 3 have been made and examined. Nevertheless, the finding of a variant form among 5 isolations of *P.b. yoelii*, collected in the same area and within a few days of each other, strongly indicates the existence of a polymorphic population in this subspecies. We hope to be able to study this problem further using new isolations of *P.b. yoelii* from a number of specimens of the Congolese tree rat, *Thamnomys rutilans* the natural host of this parasite, recently collected from the Central African Republic.

Finally we hope to be able to use the two forms of glucose phosphate isomerase as markers in genetic studies on *P. berghei*.

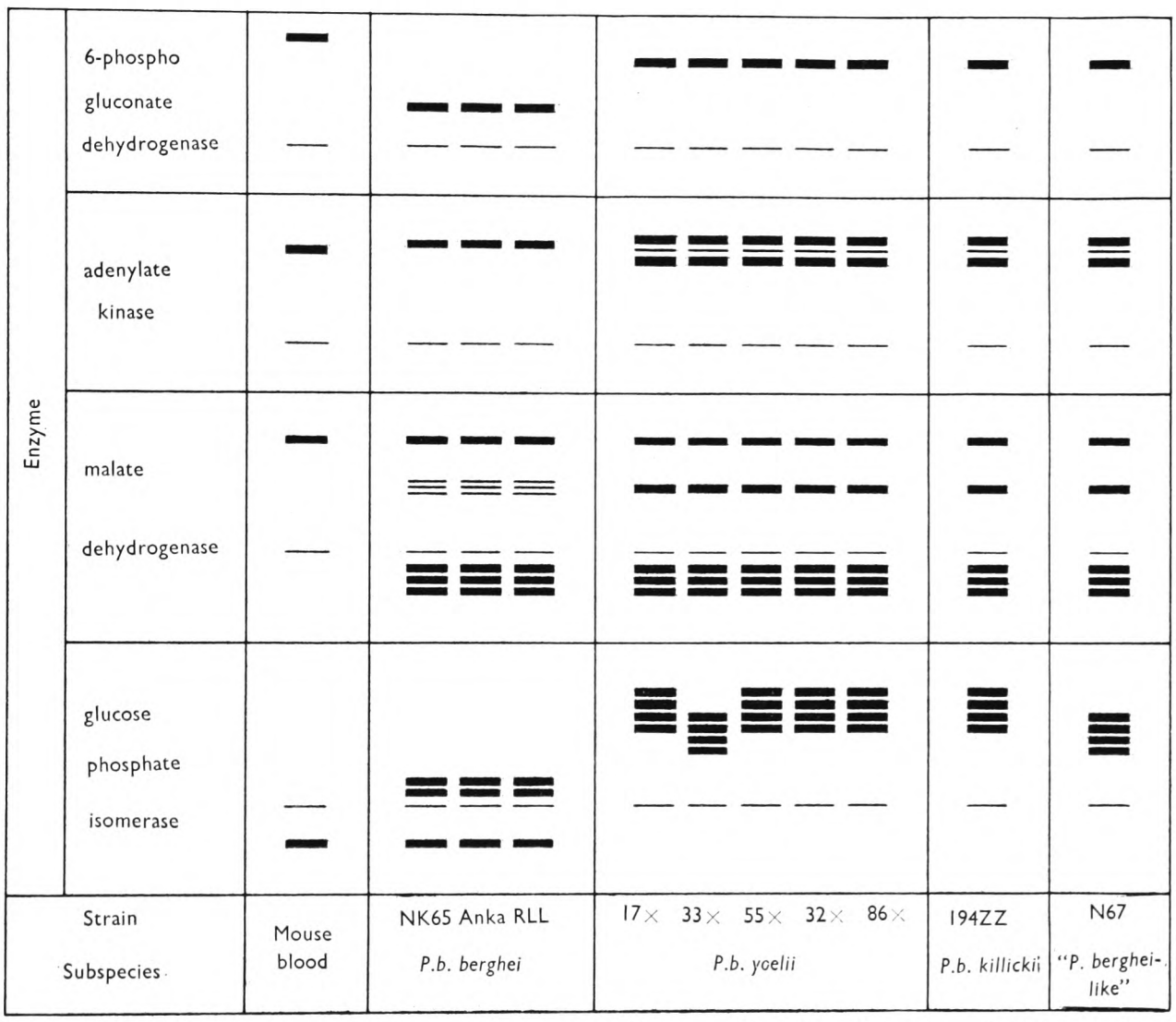

FIG. 2. Electrophoretic forms of enzymes of mouse blood and strains of *P. berghei*.

Summary

10 strains of *P. berghei* representing 4 subspecies—*P.b. berghei, P.b. yoelii, P.b. killicki* and a "*P. berghei-like*" parasite from Nigeria were used in this study. The electrophoretic band patterns of 4 enzymes—glucose phosphate isomerase, adenylate kinase, 6-phosphogluconate dehydrogenase and malate dehydrogenase were examined in all strains.

With the exception of glucose phosphate isomerase the enzymic patterns of all strains of *P.b. yoelii, P.b. killicki* and the "*P. berghei-like*" parasite were identical for each enzyme studied. The strains of *P.b. berghei* were distinct from the other 3 subspecies in regard to all 4 enzymes. 2 migratory forms of glucose phosphate isomerase were identified among the strains of *P.b. yoelii, P.b. killicki* and the "*P. berghei-like*" parasite.

REFERENCES

BOWMAN, I. B. R., GRANT, P. T. & KERMACK, W. O. (1960). *Expl. Parasit.*, **9**, 131.
KREBS, H. A. & EGGLESTON, L. V. (1940). *Biochem. J.*, **34**, 442.
KILLICK-KENDRICK, R., SHUTE, G. T. & LAMBO, A. O. (1968). *Bull. Wld Hlth Org.*, **38**, 882.
SMITH, I. (1968). *Chromatographic and Electrophoretic Techniques, Vol. II, Zone electrophoresis.* London: Heinemann Medical Books Ltd.

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