THE GENETICS OF DRUG RESISTANCE IN MALARIA PARASITES

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Submitted to the University of Edinburgh

as a thesis in fulfilment of the

requirement for a degree of

Doctor of Philosophy

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August 1977

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SUMMARY

This thesis describes work performed on the genetics of resistance to the drugs sulphadiazine and pyrimethamine in the rodent malaria parasite Plasmodium chabaudi.

A number of lines resistant to pyrimethamine were obtained by direct exposure to the drug in a single step. Characteristically these were some 20 times resistant to pyrimethamine and had an increased dependence on PABA. Subsequent genetic analyses showed that this class of mutant was genetically homogeneous and that the primary resistance to pyrimethamine and the concomitant PABA dependence were inseparable by recombination - both consistent with a single gene model of resistance.

A number of lines resistant to sulphadiazine were obtained in a single step either by direct exposure to the drug or following PABA restriction. Characteristically these lines were some 4 times resistant to sulphadiazine and required a reduced PABA growth supplement but exhibited a variable spectrum of cross-resistance to pyrimethamine uncorrelated with their mode of selection. A phenotypically indistinguishable example of this type of line was also obtained following exposure to pyrimethamine. Evidence from subsequent selection experiments was taken to show that at least two types of separate heritable change might result in the acquisition of sulphadiazine resistance. An example of one of these was later shown to segregate in a simple mendelian fashion when crossed with a wild line.

A number of lines were obtained by selection in two discrete steps: either following the PABA restriction of a pyrimethamine

resistant line; or by the direct exposure of a sulphadiazine resistant line to pyrimethamine. While these lines were some 40 times resistant to pyrimethamine they exhibited wild type responses to sulphadiazine and PABA.

One such line was crossed with a wild line and two types of non-parental clone were detected among the products of the cross. These resembled either the pyrimethamine resistant or sulphadiazine resistant lines obtained by selection in a single step. This result was consistent with a genetic recombination event having taken place between the genetic elements proposed to confer resistance to either of these two drugs. Evidence from a reconstruction experiment suggested that the linkage between these elements was either loose or non-existent. (An unsuccessful attempt was made to analyse this cross in the greater precision afforded by the characterisation of clones derived from single oocysts obtained by micro-dissection.) In another cross between two single step lines resistant to pyrimethamine and to sulphadiazine respectively both wild type and double mutant parasites were detected among the cross-products - thus confirming that recombination could link these mutants to produce multiple drug resistance.

During the development of a rat-adapted line of *P.chabaudi* (which was later shown to be unsuitable for use as a selective marker) it was discovered that gametocytogenesis was enhanced in unadapted parasites during subsistence in rats. This led to the substitution of rats for mice as rodent carriers of the bi-parental mixed-blood infection prior to cyclical passage in crosses.

Estimates of the rates of acquisition are presented for each

type of resistance in the Discussion together with a critical appreciation of the extent to which these represent true mutation rates.

INTRODUCTION

Malarial resistance to antifolates was first reported in the case of proguanil by Bishop and Birkett (1947) and by Williamson, Bertram and Lourie (1947) in P.gallinaceum. Clinical reports of proguanil resistance soon followed from Malaya (Field and Edeson, 1949) in P.falciparum.

Pyrimethamine which was synthesised on the basis of its structural resemblance to cycloguanil, the active metabolite of proguanil, was introduced soon afterwards and almost immediately McGregor and Smith (1952) reported a case of apparent resistance in P.falciparum. By the end of the decade clinical reports of pyrimethamine resistance had come from as far afield as Venezuela (in P.falciparum and in P.vivax) and Pakistan (in P.falciparum) as well as from the African continent (in P.falciparum and in P.malariae). For references see inter-alia: Jones (1954, 1958); Afridi and Rahim, (1962); Maberti (1960) and for a comprehensive review, Peters (1970).

Rollo (1951, 1952) had forseen this resistance problem and in a series of experiments carried out with P.gallinaceum and P.berghei showed that the frequency with which resistance to pyrimethamine arose seemed to be related to the numbers of parasites undergoing selection. Anne Bishop and her co-workers who pioneered much of the early work on antifolate and sulphonamide resistance had already shown that proguanil resistance in P.gallinaceum was stable after cyclical passage (Bishop and Birkett, 1947) and after repeated passage in the absence of drug pressure (Bishop and MacConnachie, 1950a): and in a study of metachloridine resistance (Bishop, 1958) she also found a

correlation between the size of the infective inoculum and the ease with which resistance developed. The suddenness and stability of resistance suggested a mutational origin, a finding confirmed when she extended her study to cover proguanil and pyrimethamine (Bishop, 1962), though no correlation was found between the size of the infective inoculum and the ease with which resistance developed to these drugs. However, as Bishop herself pointed out, her drug test was such that resistant parasites might not be detected until a subsequent passage.

In addition to confirming the earlier reports that pyrimethamine resistance was stable through multi passages in the absence of drug pressure, Jacobs (1964) made the key observation that his pyrimethamine resistant line had acquired an increased growth requirement for PABA (para aminobenzoic acid) a precursor in the biosynthesis of folate coenzymes - a pathway blocked by the action of sulphonamide drugs. Morgan (1974) found that the majority of pyrimethamine resistant lines which she obtained by single step selection possessed this increased PABA growth requirement. (It is interesting and relevant to note that the only exception to this rule was a line which though it had also been selected by direct exposure to pyrimethamine, appeared to be resistant to sulphadiazine.) This observation was consistent with those of Bishop and her co-workers (Bishop and MacConnachie, 1948, 1950a,b,c) who had also pioneered the experimental study of sulphonamide resistance again using the <u>P.gallinaceum</u> model. As well as showing this resistance to be stable through cyclical passage, they showed that sometimes it was accompanied by cross-resistance to

proguanil and indeed in one such case the primary resistance manifested itself after the cross-resistance had been detected. In addition Bishop and Birkett (1947) showed that continued treatment with proguanil appeared to have superimposed cross-resistance to sulphadiazine on a line already resistant to proguanil itself. (Bishop conducted her selection experiments using the multi-passage technique with repeated bouts of drug pressure).

Finally Ramakrishnan et al. (1956) showed that restricting the dietary PABA intake of infected mice could lead to the production of sulphonamide resistance in P.berghei: Jaswant Singh et al. (1954) had already shown that a sulphadiazine resistance line of this parasite (obtained after direct exposure to the drug) required a lower PABA supplement than the line it had been derived from.

Clearly then resistance to sulphonamides and to anti-folates was linked phenotypically by their effects on PABA dependence. Recent studies have enabled us to trace this link backwards to the biochemical level and it is now possible to identify the sites of action of these two classes of drug in pyrimidine synthesis. This pathway has been summarised in Fig. 12 and discussed in the accompanying text.

The close relationship between these two classes of drug resistance has prompted their combined study in the present work. Their known stability suggestive of a mutational origin and the relative ease with which they may be obtained are also encouraging from a genetic point of view. Indeed, Walliker et al. (1975) had shown that pyrimethamine resistance in P.chabaudi appeared to be controlled by a mendelian genetic element which

underwent free recombination with markers controlling enzyme polymorphisms.

It was the main aim of the present study to continue these investigations in both the cases of pyrimethamine and sulphadiazine resistance, paying special attention to the responses of mutants in each category to both these drugs and to their PABA dependences also. Thus it was felt necessary to obtain a wide variety of mutants using the selective techniques pioneered in these earlier studies just mentioned given obvious logistic constraints such as the numbers of mice available. Thus for example, some selections were conducted at various intensities of drug pressure at various PABA regimes and with immunosuppression.

At the outset however it was decided to limit bouts of selection to the single step method favoured by Diggens (1970) and by Morgan (1974) since this technique was thought to favour the selection of single gene mutants. Although possibly easier to measure, the large degrees of resistance induced following multi passage selections were suspected of being inherited by more than 1 genetic change. Such mutations are notoriously hard to analyse genetically even in micro-organisms more amenable to study than Plasmodium. In addition it was considered crucial that lines be cloned before and after selection for obvious reasons. Having done this, mutant lines were then crossed with sensitive lines in order to investigate the mode of inheritance. Having established the genetic nature of a mutant line it was then possible to superimpose further genetic changes by selection and so on.

However such a classical approach has its inherent

limitations and it was felt that a more detailed analysis was desirable than had been hitherto possible using the available techniques. Before these are introduced, it is worth considering the "state of the art."

The first reports of a genetic study of malaria parasites appeared in a series of papers authored by Trembley and Greenberg (Greenberg and Trembley, 1954a,b; Trembley and Greenberg, 1954; Greenberg, 1956). They crossed a mild line of P.gallinaceum with a virulent resistant one and after joint mosquito passage detected non parental parasites resistant to pyrimethamine and with a mild growth characteristic. However as they themselves were aware (Trembley et al, 1951), this growth characteristic was an unstable one. This and the fact that they failed to clone their parent lines prior to the cross renders their interpretation of their results open to question.

In another study Yoeli and his co-workers (Yoeli, Upmanis and Most, 1959) claimed to have shown evidence of an infective gene transfer mechanism which they christened "sympholia." They inoculated a mouse with a mixed infection of P.vinckei parasites which had been made resistant to pyrimethamine and a sensitive P.berghei line. Pyrimethamine resistant P.berghei parasites were later detected in 6 out of 12 hamsters subinoculated from the mixed mouse infection. Without an intervening mosquito passage there had been no opportunity for a sexual mechanism to generate these non parental parasites so Yoeli proposed that the transfer of genetic information had taken place during the simultaneous invasion of red blood cells by parasites from each of the parent lines, thus mimicing the transformational or transductional processes known to occur in certain bacteria.

(See Hayes 1969 for a review of these). However, no further reports of sympholia have come from Yoeli's laboratory and Walliker (1971, 1973, 1975) in his studies of genetic recombination on P.yoelii and P.chabaudi was unable to find evidence of this mechanism though he did not specifically look for it.

The most satisfactory study of genetic recombination in plasmodia has been that carried out by Walliker in this laboratory (Walliker et al, 1971, 1973, 1975, 1976) and by his co-workers Morgan (1974); Rosario (1976a,b) and Oxbrow (1972). These studies have all confirmed the existence of a conventional mendelian mechanism in rodent malaria parasites leading to the reciprocal exchange of genetic information following mixed mosquito passage. These techniques (described elsewhere pp 33-40) were given invaluable aid and stimulus by the work of Carter in developing electrophoretic techniques for the detection of enzyme polymorphisms in different species of rodent plasmodia. (Carter, 1970, 1973; Carter and Walliker, 1975). These enzyme polymorphisms represent the best evidence of genetic variation in malaria parasites to date as their detection is straight-forward and their interpreation unequivocal. By using them in genetic studies it has been possible to demonstrate recombination between variants of glucose phosphate isomerase and a marker conferring pyrimethamine resistance in P.yoelii (Walliker et al, 1971, 1973). In a later study recombination was shown to have taken place between enzyme variants themselves in Lactate dehydrogenase (LDH) and 6 phosphogluconate dehydrogenase (6PGD) in P.chabaudi (Walliker et al, 1975). This last must be considered a paradigm for genetic recombination in plasmodia.

In the same laboratory genetic studies aimed at elucidating the inheritance of drug resistance have been carried out by Morgan (1974) in the case of pyrimethamine resistance in P.yoelii and by Rosario (1976a,b) in the case of chloroquine resistance in P.chabaudi and in the same organism in the cases of pyrimethamine and sulphadiazine resistance with special reference to their combined inheritance by the present author. The work of Morgan may be considered of particular relevance to the present study as she pioneered many of the techniques and approaches described herein.

She investigated the behaviour of 2 types of marker conferring resistance to pyrimethamine after crossing each with a wild type line. In the first of these types of cross the marker had been accompanied after selection by a decreased dependence on PABA. These appeared to segregate independently as separate mendelian factors although Morgan herself admitted the number of cross product clones examined was too small to permit general conclusions to be drawn from this observation. Moreover, the character PABA dependence was not easy to distinguish owing to the rather small differences in PABA dependence observed between the parent lines she used. In another cross the pyrimethamine resistance marker had been accompanied after selection by an increased PABA dependence and although analysis confirmed the apparent mendelian inheritance of this type of pyrimethamine resistance, the cross product clones were not characterised for their dependence on PABA.

In the present study screening techniques exploiting the increased PABA dependence of some pyrimethamine resistance mutants have been widely exploited. This adjunct to the

characterisation of individual clones is widely used in the genetics of other micro-organisms (see Hayes, 1969 passim) and makes possible the detection of certain rarer genetic events which might be missed using conventional techniques. Clearly care has to be exercised in the use of such techniques lest mutants, selected de novo, be scored as recombinants but this danger can be avoided by the use of appropriate controls and by restricting numbers to submutational levels; and in any case these dangers do not apply to the interpretation of negative results.

At the other end of the scale it was considered desirable to perform an analysis of individual genetic events to be revealed by the characterisation of clones derived from the products of single hybrid oocysts harvested before rupture. Although single oocysts were known to be infective (Walliker, 1972) there was some doubt as to the infectivity of the individual sporozoites contained therein (Vanderberg, 1975). In the conventional genetic analysis, clones selected at random from among the mixed population of hybrid and non-hybrid oocyst derived cross-product blood infections were characterised. Thus the reciprocity of genetic exchange within oocysts could only inferred and more direct evidence was desirable particularly in the case of characters whose inheritance might be complex such as drug resistance. Proof of reciprocity demanded the analysing of the segregation of as many character pairs as possible and prompted the development of the rat adapted line of P.chabaudi (see below). Walliker et al. (1975) had expressed the hope that such a study would lead to the more precise location of the meiotic reduction division in Plasmodium particularly in the

light of a report based on cytological evidence that such a division took place within the oocyst itself (Sinden and Canning, 1978).

Partly in order to satisfy the demand for additional markers to be analysed by the single oocyst technique and partly in order to provide a selective marker for screening techniques but one unconnected with drug resistance and therefore probably free from interactions with drug resistance markers it was decided to develop a line of P.chabaudi adapted to growth in rats. This technique had been reported before (Coombs and Gutteridge, 1975) and it brought an unexpected but vital bonus.

During these selection experiments it was found that non-adapted lines of P.chabaudi produced greatly increased numbers of gametocytes when injected into rats and that the normal processes of exflagellation took place subsequently (MacLeod and Brown, 1976). This was important since P.chabaudi, although possessing several advantages over the other rodent model used in earlier crosses, P.yoelii was more difficult to passage though mosquitoes and perform crosses with and these difficulties had been attributed to the paucity of gametocytes in P.chabaudi infections. Consequently, the conventional crossing technique was modified by the substitution of rats for mice as rodent carriers of the mixed parental blood infection.

These techniques were exploited to investigate a number of questions regarding the inheritance of resistance to sulphadiazine and pyrimethamine together with the selection experiment data.

1. Were pyrimethamine resistance and the concomitant increase in PABA requirement pleiotropic characters of a single genetic

change or were these inherited separately?

2. Could the above class of mutants be ascribed to changes in the same gene locus in independently arisen examples?

3. Was resistance to sulphadiazine inherited in a simple mendelian fashion as had been desmonstrated in the case of pyrimethamine resistance?

4. Did the genetic elements controlling resistance to pyrimethamine and to sulphadiazine recombine freely and could an estimate be obtained of this recombination frequency?

While it has been considered that satisfactory answers were obtained to these questions, several lines of investigation were attempted unsuccessfully. Those that failed for reasons considered to be other than the merely technical have also been included: e.g. the results of the attempted characterisation of hybrid single oocysts in the section dealing with the relevant cross which had been already performed using more conventional techniques and the results of the cross using the rat adaptation marker.

Finally, two assumptions underlay the interpretation of cross results. Firstly, it was assumed that no gross distortion in the relative frequencies of parental and recombinant classes had taken place such as might have resulted due to mutant lines being at a significant disadvantage over their wild counterparts in mixed infections. Consideration of the results of Walliker et al. (1975) when they crossed a wild and a pyrimethamine resistant line of P. chabaudi showed no evidence of natural selection - but this evidence was not wholly unequivocal. In an experiment specifically designed to detect natural selection for

or against pyrimethamine resistant lines of P.chabaudi, Hall (unpublished results) found no evidence of gross distortion in input frequencies following mixed passage though his technique would not have detected minor ones. Secondly, it was assumed that parasites were haploid in respect of their genetic complement. Both Carter (1970, 1971, 1973) and Walliker et al. (1971, 1973, 1975) had been unable to find an instance where a cloned parasite exhibited more than one type of an enzyme variant either in the case of parasites obtained from wild infections or following a cross between parasites differing in respect of the enzyme variant they possessed. Thus the evidence for haploidy was strong though indirect.

MATERIALS AND METHODS

(1) Parasite Species:

a. Nomenclature and Provenance

Apart from one experiment using P.vinckei all the work in this study concerns *Plasmodium chabaudi*.

P.chabaudi was described by Landau (1965) who isolated this parasite from thicket rats (*Thamnomys rutilans*) of the Central African Republic. The development and infection patterns of these parasites were studied by Landau and Killick-Kendrick (1966); Wery (1968) and by Landau *et al.* (1970).

Bafort (1968) reclassified the parasite as a subspecies of *P.vinckei* but following electrophoretic studies Carter and Walliker (1975) renamed the parasite *P.chabaudi* on account of its range of enzyme polymorphisms.

The parasites used in the present study were obtained from infected blood samples obtained from thicket rats trapped in the Central African Republic in 1969.

b. Life cycle of P.chabaudi in Mice and Mosquitoes

P.chabaudi infections in this laboratory were typically synchronous over a 24 hour division cycle with schizogony occurring around midnight. Schizonts typically yielded between 4 and 8 merozoites.

Gametocytogenesis reached its greatest intensity in mice after the peak of the blood infection, that is about 10 days after inoculation. However it never occurred with anything like the intensity observed in other rodent plasmodia such as *P.yoelii* or *P.vinckei* in this host.

Cyclical passages were initiated by permitting mosquitoes (Anopheles stephensi) to gorge themselves on blood containing macro - and microgametocytes.

Sporozoites were detectable in such mosquitoes after about 2 weeks and these could then be used to initiate blood infections by a variety of techniques described elsewhere (p.16).

c. Definition of "isolate", "line", "clone" and "stabilate"

Isolate:- a sample collected from a wild rodent or mosquito on a single occasion and preserved either by repeated passage or cryogenically. Isolates are not necessarily genetically uniform and may contain parasites from different species.

Stabilate:- a sample of parasites preserved in a viable condition on a unique occasion (Lumsden and Hardy, 1965) such as following cryopreservation.

Line:- in the present work this refers to any number of parasites which had the same special treatments together since their last cloning. Thus recloning or selection procedures created new lines but routine maintainance did not.

Clone:- a group of parasites thought to be genetically uniform following the asexual multiplication of a single organism. However the random occurrence of spontaneous mutation was unavoidable and given this, these mutants were probably omnipresent.

(2) Host Species:

a. Rodent

Both rats and mice were used. For drug and PABA tests, male inbred C57 black mice (Centre for Laboratory Animals, The

Bush, Edinburgh) were used exclusively. For cloning and routine passage these and mixed strains obtained from the main mouse colony of this department were used. Male inbred white rats (*Rattus norvegicus*) were exclusively used in rat experiments (Obtained from "The Bush" also.)

The temperature of the mouse house was nominally 18°-22°C and was lit by daylight.

b. Mosquito

For cyclical transmissions *Anopheles stephensi* maintained in this laboratory were used. These were nominally maintained at 25°C at 90% humidity under artificial light conditions of alternating 12 hours light and darkness. Mosquitoes were fed on rabbits prior to experimental use and on a 10% solution of glucose supplemented with PABA (0.05%) thereafter.

Stray mosquitoes were discouraged from entering the mouse house by the use of double doors and as a last resort, by swotting.

(3) Routine Maintenance of Parasites in the Laboratory:

a. Preservation in Liquid Nitrogen

Stabilates of infected blood were stored in sealed capillary tubes kept in liquid nitrogen (-196°C) after the method of Lumsden *et al.* (1966). When required stabilates were thawed and after dilution in citrate saline (0.9% sodium chloride, 1.5% sodium citrate, adjusted to pH 7.2) injected into mice intraperitoneally.

b. Syringe Passage

Routine blood passage was effected by tail bleeding of an

infected rodent into a syringe containing citrate saline and by injection of about 0.1 ml of the diluted blood into an uninfected rodent.

c. Cyclical Passage

i) Rodent to Mosquito

Routine mosquito passage was not considered necessary since lines showed no diminution in fertility even after 50 or more syringe passages. Cyclical passages were carried out from time to time in order to measure fertility as well as forming a basis control procedure in crosses.

The standard procedure involved feeding mosquitoes (Anopheles stephensi) which had been starved for one or two days on mice inoculated some ten days previously i.e. when the amount of exflagellating gametocytes had reached a peak. From the following day the mosquitoes received 10% glucose made up in PABA (0.5 g/l). However, the success rate of this method in transmitting P.chabaudi through mosquitoes was found to compare rather unfavourably with rates of P.y.nigeriensis and P.y.yoelii transmission. A further complication arose while attempting to transmit virulent lines of P.chabaudi. These killed most if not all mice before the infections contained sufficient sexually mature parasites.

Fortunately, the discovery made while developing a rat adapted line of P.chabaudi that a substantial proportion of parasites injected into young splenectomised rats developed into gametocytes enabled these hosts to be used with more success in transmission. (Method described fully in this Section (pp 35-37).

ii) Mosquito to Rodent

About 8 days after the blood meal a sample of around 10 mosquitoes was dissected and oocysts were counted microscopically as described in this section (p. 18).

To begin with sporozoites were harvested after about 14 days from triturated salivary glands dissected into cold tissue culture medium. (Modified Kitamura's medium and VP12 medium (Pudney and Varma, 1971)). Where more than about 20 mosquitoes were being dissected, these were triturated whole after the removal of legs and wings. The triturate was centrifuged at 2000 RPM for 4 minutes at 4°C and the sediment discarded. Up to 0.5 ml of the sporozoite suspension was then inoculated intravenously into each of a group of mice.

After April 1974 in order to maximise the number of sporozoites contributing to the resulting mouse infection, the "natural" method of transmission was used in the majority of instances. Thus infected mosquitoes were allowed to feed on mice about 14 days after the blood meal. If need be the process was repeated the following night.

Mouse infections resulting from sporozoites could be detected some 4 to 8 days later.

(4) Counting Red Blood Cells and Parasites:

a. Red Blood Cells

These were counted using a Coulter counter. Routinely dilutions for counting were made with 2 micro-litres of tail blood injected from a micro capillary tube into 100 ml formalin saline solution. Counts were corrected for coincidence using the chart provided.

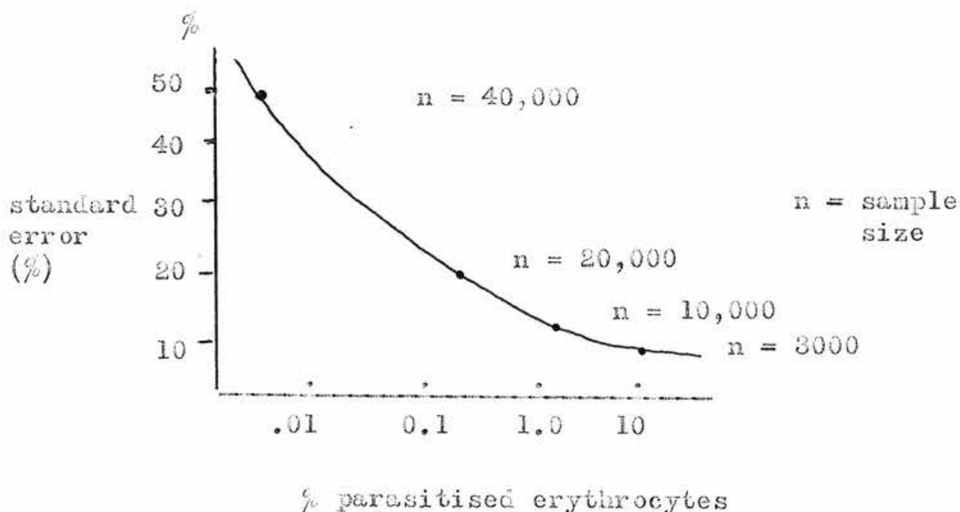
b. Malaria Parasites

The percentage of parasitised erythrocytes (hereafter percent parasitaemia) was estimated from counts performed on thin smears of mouse tail blood and stained with Giemsa's stain. (BDH Chemicals Ltd.) When accurate counts were required the sample size was related to the limits of error deemed acceptable. When measurement errors were required to be known these were estimated using Hayter's formula (cited in Morgan, 1974). The relationship between measurements of different parasitaemia levels and their errors for different sample sizes is illustrated graphically in Fig. 1.

When a mean of several measurements was required this was obtained geometrically (logarithmically) and individual measurement errors summed by the method of least squares. Errors have therefore been expressed as geometric ranges of ± 1 standard error.

FIG. 1

Standard Errors in parasitaemia counts (expressed as a percentage of the measurements to which they are attached) over a range of parasitaemia levels at their usual sample sizes:



c. Oocysts and Sporozoites

The number of oocysts per infected gut was determined by examination of dissected guts in coverslip preparations magnified 400 times using a phase contrast objective.

Accurate sporozoite counts were not carried out. Their abundance was merely noted after examination of coverslip preparations magnified 400-1000X and viewed under phase contrast illumination.

(5) Preparation of Inocula Containing Known Parasite Numbers

Except during routine serial passage, animals were inoculated with known numbers of parasites. These ranged from inocula containing fewer than one parasite in dilution cloning up to and occasionally exceeding 10^{10} parasites in some experiments using rats. The volume of all inocula prepared by dilution was normally 10^{-1} ml. When undiluted or centrifuged infected bloods were inoculated, the volumes of these inocula ranged up to 2 ml in some rat experiments.

a. Collection of Bloods

Prior to exsanguination parasitaemia counts and erythrocyte counts were performed on donor animals. When more than one donor was required these counts were performed on the mixed bloods *in vitro*. Blood was collected either from the heart or from the brachial vessels using minimal heparin (approx. 5 i.u. ml^{-1}). In some experiments requiring a large number of donors time saving was accomplished by a rapid accurate system of subinoculation: thus donors were bled into microcapillary tubes of known capacity and each of these were then drawn into a syringe containing about 0.3 ml of heparinised serum ringer and kept on ice until

inoculation.

b. Dilutions (Including Clonal Dilutions)

The vast majority of dilutions fell into two numerical categories: those containing between 10^6 and 10^8 parasites per ml, e.g. in the cases of drug or PABA tests and selection experiments; and those containing fewer than one parasite per ml - mostly cloning procedures.

When accuracy was required potential donors with parasitaemias lower than about 1% were rejected since these were regarded as being over prone to counting errors: those exceeding parasitaemias of 20% were also rejected since this figure represented the approximate limit of exponential growth in *P. chabaudi*. However, when cloning the upper limit was set at about 5% to minimise the chance of inducing infections derived from erythrocytes containing more than one parasite.

Dilutions were effected using calibrated pipettes for dilution factors not exceeding 5×10^3 per step or by using microcapillary tubes ranging in capacity from 1 to 20 microlitres for dilution steps exceeding that figure. Dilutions exceeding 5×10^5 required more than one step. (Even in the case of clonal dilutions most of which required two dilution steps, the limiting factor on accuracy was that of the parasitaemia counts themselves.)

No significant difference was found in infectivity of clonal inocula between the intravenous or the intraperitoneal routes so the latter was used in the present study for logistic reasons. When performing such inoculations poorly injected mice were rejected immediately lest their inclusion among mice scored as uninfected artificially improved the results of that cloning experiment. Blood smears were taken of the remaining mice after

8 days and for up to 20 days following inoculation although in practise on only one occasion did an animal become patent after 20 days which had been ajudged clear at 16 days.

Clonings were considered successful if fewer than one in 5 mice became infected: given a Poisson distribution of parasites among mice this figure implies that 90% of such infections had arisen from a single parasite. Since routine infection rates following cloning were less than about 10%, the chances of infections having arisen from more than one parasite were proportionately diminished. On only one occasion was a "clone" discovered to have arisen from such a multiple infection. (However, it is worth noting that while electrophoresis of enzyme variants during the analysis of cross-product clones made possible the detection of "doubles" which differed in these variants, it is unlikely they would have been spotted by drug testing alone since drug resistance may be considered dominant over sensitivity.)

c. Inocula Containing More Than 10^8 Parasites

In some experiments rats were inoculated with large amounts of blood typically from 0.5 to 2.0 ml. The intravenous route was preferred for these inoculations since that method seemed to give more predictable results and made it possible to immediately count parasites before such possible factors as cell division or host passive immunity might have affected their numbers.

On some occasions mouse bloods for these rat inoculations were packed by centrifugation (1000g for 4 minutes): in this way it was possible to inject up to 10^{10} parasites into each rat, resulting in instantaneous parasitaemias which typically ranged from 15 to 30%.

(6) The Diet of Experimental Animals:

Routinely all the animals used in this study were maintained on "Quayside" rat cake fed *ad libitum* (MacGregor's of Leith, Scotland).

In one series of experiments designed to compare the standard diet with a milk diet known to be deficient in PABA (Hawking, 1953), mice were allowed to feed *ad libitum* on a dry paste prepared from "Ostermilk" suspended in minimal tap water.

No special attempt was made in these studies to prevent coprophagy.

Drinking water was supplemented with para-aminobenzoic acid, hereafter PABA, (Sigma) at 0.5 gl^{-1} unless otherwise stated. Animals were maintained on a given PABA regime for at least seven days prior to inoculation.

(7) The Source Preparation and Administration of Drugs:

a. Pyrimethamine

Pyrimethamine ("Daraprim": 2, 4 diamino-5-p-chlorophenyl-6-ethyl-pyrimidine) in the form of powdered base was obtained from the Wellcome Research Laboratories, England, courtesy of Dr. R.A. Neal.

Drug doses were related to the mean body weight of a group of experimental mice. Doses were calculated in mg/kgm from weighings taken on the first day of drug administration.

A range of stock solutions of pyrimethamine in distilled water at 0.5, 5 and 25 mg/ml were prepared according to the method of Yoeli *et al.* (1969). Thus a known weight of pyrimethamine powder was suspended in distilled water to which two drops of Tween 80 per 10 ml had been added. The suspension was

sonicated in bursts for a total of 3 minutes using a Soniprobe and having been made up to the required volume in a measuring flask and transferred to a thick walled glass container for storage. Further dilutions from the stock solutions were made in sodium carboxymethyl cellulose (2%) and shaken on a vortex mixer immediately prior to administration. 0.1 ml aliquots of the final drug solutions were given via the intraperitoneal route. Routinely control mice received 0.1 ml sodium carboxymethyl cellulose solution 2%.

b. Sulphadiazine

Sulphadiazine (2-sulphonamido-pyrimidine) in the form of a dry powder was obtained from May & Baker Ltd., England.

Drug doses were calculated by the same method as that used for pyrimethamine.

A range of stock solutions of Sulphadiazine between .05 and 100 mg/ml were prepared by suspending the dry powder in warm 2% Sodium carboxymethyl cellulose. The stock suspension was stirred magnetically before use and further dilutions were made in 2% Sodium carboxymethyl cellulose and shaken on a vortex mixer immediately prior to administration. As with pyrimethamine 0.1 ml of the final drug solution was given intraperitoneally - the controls receiving 0.1 ml Na CM C only.

(8) Techniques for the Measurement of Variation:

a. Starch gel electrophoresis - method of Carter (1970, 1971)

i) Preparation of material:- Mice (strain immaterial) with high blood infections (i.e. less than 20% parasitised erythrocytes) were etherised and bled from the brachial artery into sufficient citrate saline to make up to 10 ml in a centrifuge tube. After

centrifugation at 1000g for 5 minutes, the supernatant was discarded and the packed blood cells mixed thoroughly with half their volume of 0.15% saponin in 0.85% Na Cl. Each sample was then incubated for 20 minutes at 37°C in a shaking water bath. Samples were then removed and after resuspension in 10 volumes of ice-cold 0.85% Na Cl centrifuged again - this time at 5000g for 10 minutes at 4°C. The supernatant was then discarded and only the upper greyish layer of parasite material retained.

Material for immediate electrophoresis was freeze thawed twice in three times its volume of distilled water (to disrupt the parasite membranes).

Usually however samples were preserved and different batches run together for reasons of economy. This meant re-suspending the parasite material in about 1 ml of distilled water in a bijou container. These were left overnight to freeze in a dry ice refrigerator. Frozen samples were then vacuum dried in an apparatus comprising a desiccator jar connected to a condenser flask immersed in a dry-ice/ethanol freezing mixture in a Dewar container. Vacuum was maintained by an electric pump. Freeze dried samples were preserved in an evacuated desiccator jar and kept at 20°C.

ii) Enzyme assay and electrophoresis:- *P. chabaudi* is polymorphic for iso-enzymes of 6PGD (6 phosphogluconate dehydrogenase) and LDH (Lactate dehydrogenase) Carter and Walliker (1975). The basic principles of electrophoresis were of course the same for both these enzymes but the methods in each case differed in the specifics of buffer system, incubation system and in the duration and intensity of electrophoresis. These details together with the gel formula are given in Carter (1971).

Prior to electrophoresis about 1 mg of sample material was dissolved in a droplet of distilled water delivered from a syringe through a narrow gauge needle. Cellulose acetate strips soaked in these samples were then inserted into the prepared gel with the assistance of a metal comb thus defining the origin line at a right angle to the axis of electrophoresis.

Each gel was then placed on a cooling plate between two electrode troughs each containing 500 ml of the appropriate electrode buffer and wicks laid over the edges of the gel so that they would just meet the edges of the cooling plates. A thin sheet of polythene was then laid over the gel and wicks and covered by a second cooling plate. The appropriate current and voltage were then applied across the gel from a stabilised power supply for between 8 and 14 hours - depending on the system being used.

After switching off the power supply the cellulose acetate strips were removed and the gel sliced horizontally. Slices could then be incubated with the appropriate enzyme assay solution to reveal the positions of both host and parasite enzyme activities.

iii) Band identification:- Isoenzyme type was normally identified by comparison with samples of known identity run on the same gel. Since in the vast majority of cases only two configurations were possible for each enzyme - those of the lines herein designated AS (LDH type 3, 6PGD type 2) or AJ (LDH type 2, 6PGD type 3) - identification resolved itself into questions of the type either, or. (For an explanation of isoenzyme nomenclature see Carter (1974).) Identification was further simplified in the case of LDH since the isoenzyme

characteristic of lines AS and AJ migrate in opposite directions from the origin.

b. Measurement of Drug Resistance

The 4 day test developed by Thurston (1950) was routinely used for drug testing. Groups of a 4 C57 mice, standardised for sex (male) and age and therefore of approximately similar weights were set up in different cages and inoculated with parasite aliquots (routinely 10^6 parasites per inoculum). Drug dosage was based on cage weight and the appropriate amount of drug administered via the intraperitoneal route 3 hours after inoculation, and on each of the following 3 days at about the same time of day. (In this study druggings were usually carried out between 4 and 7 pm). Control cages received injections of Sodium carboxymethyl cellulose only as did PABA test cages if they were included in the test.

Parasite counts were made on the fourth and subsequent days after inoculation. The geometric mean count for each drugged group was expressed as a percentage of the control group. These percentages were converted into logs. and plotted against log. dose. A straight line plot was drawn between the points spanning the line of 10% survival, and the drug dose corresponding to the point where the plot crossed this line (ED_{90}) read off. Measurement errors and within-group-variation errors were summed by the method of least squares and the resultant total standard error expressed as a range in the text.

(The dose required to reduce the infection to the size of the original inoculum was estimated by extrapolation after making the assumption that each mouse contains about 3×10^{10} red blood cells. This very roughly estimated dose (ED_{ps} p.s. =

plasmodiostatic) was only used for guidance in planning some selection experiments in which continuous drug pressure was to be applied for longer than just 4 to 6 days.)

Having obtained base line ED_{90} estimates for wild type lines. It was possible to express the increase in resistance achieved after selection in the mutant lines derived from them. Thus where in the text a mutant line is described as having a pyrimethamine resistance of 20X this is a shorthand way of saying its ED_{90} for pyrimethamine is some 20 times greater than that of the wild type.

When large numbers of lines were being tested simultaneously it was logistically impossible to carry out full drug tests. In these cases "mini" tests were carried out using only 2 control animals and 2 drugged and sometimes 2 animals receiving a low PABA supplement. While this had the advantage of reducing the number of mice required by at least half, it did mean that drug responses could only be expressed as a percentage growth (compared with the untreated controls) rather than as an ED_{90}. Data from such tests were interpretated with more caution but with a judicious choice of dose a single dose test could be used to detect qualitative differences in drug responses: (i.e. growth <u>vs</u> no growth). Moreover, having made due allowance for the greater variance of these mini-tests they agreed fairly well with the results of the full 4 day tests which were subsequently performed on a selection of lines.

Routine quantitative 4 day tests were carried out on all lines used in this study both as precaution against mixups and as a check on stability. (No instances of changes in resistance were attributed to instability in this work.)

Finally, it is worth repeating again that all estimates of drug response were obtained from animals fed on the standard rat cake. As a general rule all estimates of pyrimethamine resistance were obtained with the standard PABA supplement of 0.5 gl^{-1}; and whereas this supplement was used for estimations of sulphadiazine resistance obtained after January 1975, estimates before this date were obtained without any such supplements. However, there were several exceptions to this general rule and these have been indicated in the text.

c. Measurement of the Requirement for Dietary PABA Supplementation

Altered requirements for PABA growth supplementation were measured using a modification of the tests used to measure drug responses. Since growth to a peak at a given PABA level either did or did not occur - the alternative being no growth at all or very rarely a low-level fleeting infection soon after inoculation - it was decided to use a simple qualitative index of PABA dependence: growth vs no growth (symbolised in the text as + and - respectively).

Groups of 2 to 4 mice, standardised for age and sex and of similar weights were set up in different cages and maintained on experimental PABA levels for at least a week prior to inoculation with standard aliquots of 10^6 parasites.

Parasite counts were carried out regularly and usually until the control animals (receiving the normal PABA supplement of 0.5gl^{-1}) were past peak infections. At the chosen experimental PABA levels of 0.05 and 0.025 gl^{-1} and with no supplement at all (zero PABA in the text) growth conformed to the either or pattern. (Other PABA levels were abandoned due to the variable results

they gave.)

An added complication arose sometime between Christmas 1974 and January 1975 when all lines showed a significant increase in their requirements for PABA supplementation. The change occurred in all lines and the PABA "rank order" remained unchanged. Drug testing and electrophoresis confirmed no mixups had occurred and the manufacturers denied any change in the composition of their rat cake had taken place. Unfortunately no samples of the old rat cake were available for comparison or analysis. (see pp94-96 for a discussion of this phenomenon.)

PABA requirement data have therefore always been bracketed with the period to which the tests refer. Thus the expression that a line required zero PABA (post Jan. 1975) is a shorthand way of saying that the line in question required no PABA supplement to reach peak levels of infection when tested after the upward shift in requirements took place.

d. Determining the Ability to Grow in Rats

P.chabaudi although able to reach parasitaemias exceeding 80% in mice infected with single parasite inocula is quickly eliminated from intact rats. Growth in splenectomised rats tends to be erratic and this ability diminished as the rats grow to adulthood. Since it had been planned to use rat adaptation as a genetic marker a simple series of growth tests was devised to distinguish between wild and adapted parasites.

Animals used in these tests, male norway wistar rats, intact or splenectomised, newly weaned or adult, were inoculated intraperitoneally with 10^6 parasites. Blood smears were taken and daily parasitaemia counts estimated. Accurate counts were made of the blood smears representing the peak of each infection and

the group geometric means computed.

Because it almost always proved impossible to obtain more than 8 rats standardised for age and sex comparative tests of growth in rats used only 2 animals for each test.

(9) Selection Experiments:

a. Selection for Drug Resistance

All mice in selection experiments were inoculated intraperitoneally with 0.1 ml aliquots of blood parasites diluted in citrate saline. Selection pressures were commenced so that parasitaemias did at no time exceed 10% to minimise the risks of subsequent immune host reaction. In computing the number of parasites subjected to selection pressure, sample parasitaemia counts were continued until peak infection and this value used. Mouse blood volumes were roughly estimated on a mouse weight basis-at a rate of 2 mls blood per 10g body weight. Mouse blood was assumed to contain 8×10^9 red blood cells per ml.

Drug doses were calculated from mean cage weights and drugs administered intraperitoneally from 4 to 6 days in the case of short-term, high-dose therapy and for up to 20 days in the case of continuous drug therapy. Thin blood smears were made 12 days after the commencement of drugging and every 4 days subsequently. Animals without patent infection after 20 days were considered to be free of parasites and discarded. (Subinoculations from a small sample of these were occasionally carried out and the results of these seemed to bear out the last assumption.)

In the case of selection by PABA restriction tap water was merely substituted for the normal supplement of $0.5gl^{-1}$ in the drinking water. Otherwise selection was conducted as described

above.

If recrudescent parasites were observed following selection these were tested for the appropriate responses using an abbreviated version of the 4 day suppressive test (Thurston, 1950). A sample of these were then tested more rigorously after cloning using the full 4 day suppressive test and their quantitative drug responses worked out. Recrudescent parasites reckoned to be sensitive after the preliminary tests were discarded although the possibility that they concealed resistant parasites was not ruled out.

b. Selection for Adaptation to Growth in Rats

Newly weaned inbred male Norway rats were splenectomised as previously described, and inoculated intraperitoneally with 0.5 ml of blood harvested from mice at peak infection. After 10 days growth in rats bloods were harvested again and used to infect a second group of splenectomised rats and so on until they had undergone seven such passages. The whole process was repeated this time using intact rats and cloned in mice. Four mouse passages followed, the parasites being harvested after peak infection. The line was then passaged through mosquitoes (after several attempts) - an adult intact rat being used as rodent carrier prior to mosquito passage, and a male C57 mouse used as a recipient for the sporozoite infection which was accomplished by the natural feeding method. The line was then cloned in mice.

Growth tests in both rats and mice were carried out periodically, the test animals receiving inocula of 10^6 parasites via the intraperitoneal route. One test was carried out in which the mean inoculum size was only one parasite per

aliquot. After selection, responses to pyrimethamine and PABA requirements were also estimated by the methods previously described.

(10) Immunosuppression:

Immunosuppression was effected either by splenectomy (Schnitzer *et al*, 1946) or following the administration of the corticosteroid prednisone (Carrescia, 1961; Schmidt and Squires, 1951).

Corticosteroid immunosuppression was used as an adjunct to some selection experiments in which the importance of host immunity acting against recrudescent lines was being investigated with the hope of eventual whole or partial elimination. A few mice were splenectomised for the sake of this cause also.

Rats were splenectomised during the development of the rat adapted line of *P. chabaudi* and prior to use as rodent carriers of the biparental blood infections.

a. Splenectomy

Before being operated on each animal was anaesthetised in an ether jar and shaved on the left flank thus exposing the concentration of splenic blood vessels underneath the skin. Instruments were flamed beforehand but no other sterile precautions were taken and in no case was septicaemia observed afterwards. After incision the spleen was removed intact by cutting the blood vessels without cauterisation. Bleeding was hardly ever fatal and animals were normally active within 24 hours. The incisions were closed with metal suture clips.

b. Corticosteroid Immunosuppression

Prednisone (Sigma Chemicals) was chosen for immuno-

suppression for its cheapness and relative efficacy (Carrescia, 1961). 100 mg was dissolved in 50 ml warm ethanol which was then used to supplement 2.5l of drinking water with or without PABA. Since a 20g mouse was observed to consume about 4 ml of water per day - this corresponded to a daily dose of 8 $mg.kg^{-1}$ which was probably in excess of the dose required.

(It was observed that non s.p.f. mice very often became ill about 2 weeks after the commencement of immunosuppression and this was reckoned to be due to the weakening of immune controls on subpatent infections of unknown origin.)

(11) Estimating Acquisition Rates of Drug Resistance

During single session drug selection experiments which are commonly conducted over a few days, very high doses approaching the maximum tolerated by the host species, are used to prevent the recrudescence of those sensitive parasites which might escape drug treatment. This type of survival can be viewed as an inevitable consequence of biological variation and its extent is a consequence of the numbers of parasites being treated. (See Martin and Arnold, 1968 for a discussion of this point.)

Another undesirable implication of short-term, high-dose drug therapy is that many resistant parasites will perish since these doses commonly exceed their plasmodiostatic doses as well as those of the sensitive parasites. Moreover, at the height of such a selection pressure the number of resistant parasites will be at its lowest so they will be hypersusceptible to the vagaries of biological variability.

These twin dangers can be largely avoided by applying a prolonged constant dose between the plasmoidiostatic doses of

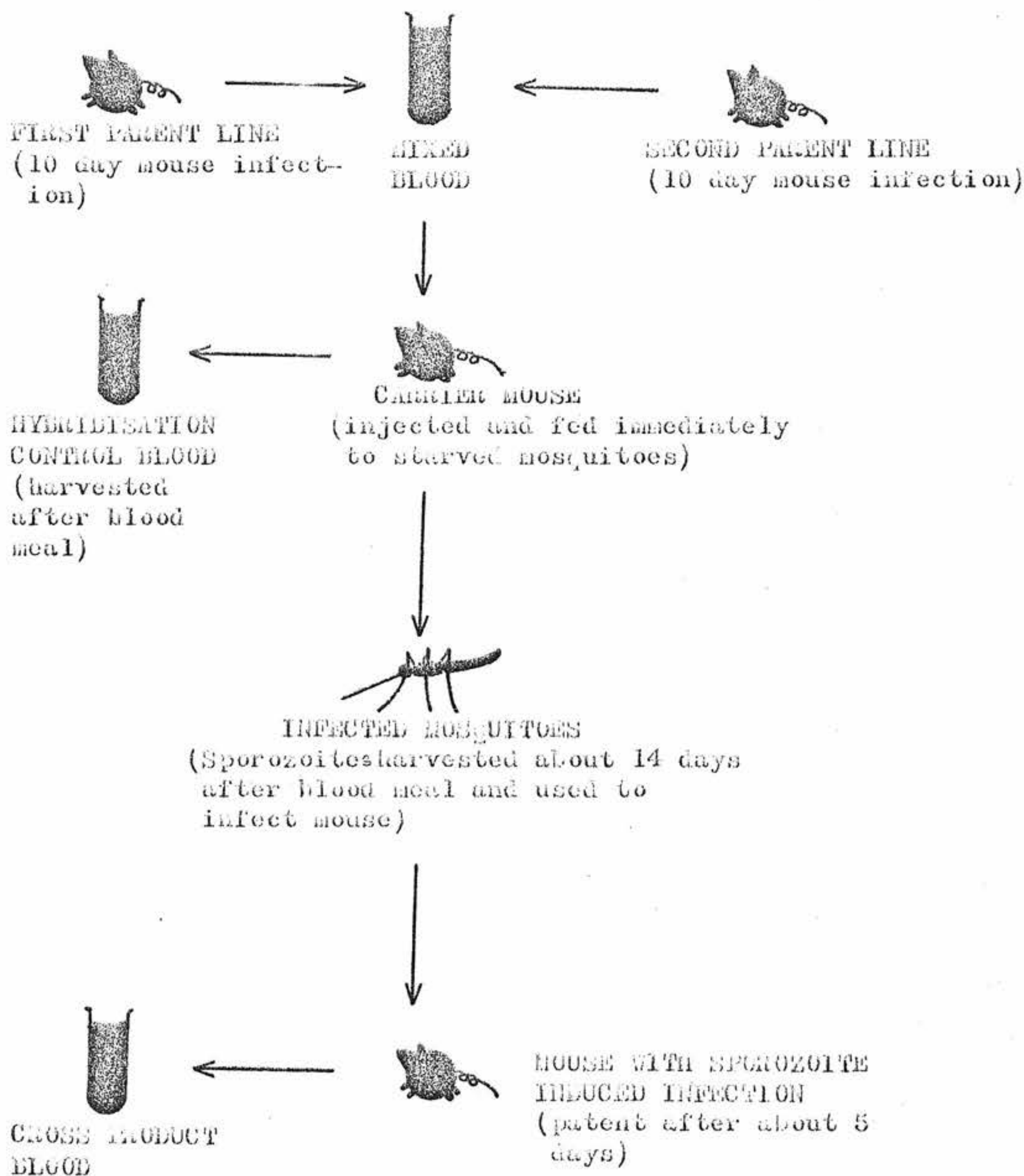
sensitive and resistant parasites. While such methods are common place in vitro they are well nigh impossible to achieve in vivo. The nearest approach to this ideal in vivo in the present study has been the use of total withdrawal of PABA as a selective agent against parasites with a growth requirement for this substance such as some pyrimethamine resistant lines. Thus the estimates of acquisition rates of sulphadiazine resistance superimposed in this way are considered the most reliable to date. Furthermore, for such a transformation the scoring system of growth vs no growth on a PABA free diet is simpler and easier to interpret than quantitive measurements of drug resistance. This had made possible the scoring of larger numbers of mutants than would have been possible otherwise. In the case of estimates of the acquisition rate of pyrimethamine resistance a constant drugging technique has been used. Doses approximating the midpoint of the plasmoidiostatic doses of sensitive and resistant parasites have been applied up until the emergence of recrudescent parasites or until 20 days if these fail to emerge. Since recrudescent parasites so treated tended to emerge at or around the sixteenth day of treatment compared with the twelvth day in the case of short-term, high-dose therapy, the parasite population sizes in either case cannot have differed that much nor the consequent risk of scoring multiple mutants. Furthermore the rather long biological half life of pyrimethamine (Brooks et al., 1968) would further tend to minimise any such discrepancy.

(12) Genetic Techniques:

a. Standard crossing procedure

The general method for conducting crosses between lines of rodent malaria parasites has been described elsewhere (Walliker *et al.*, 1971, 1975). This method has been summarised diagrammatically in Fig. 2.

FIG. 2



Standard Crossing Procedure for Rodent Plasmodia Using Mice as Rodent Carriers of the Mixed Parental Blood Mixture

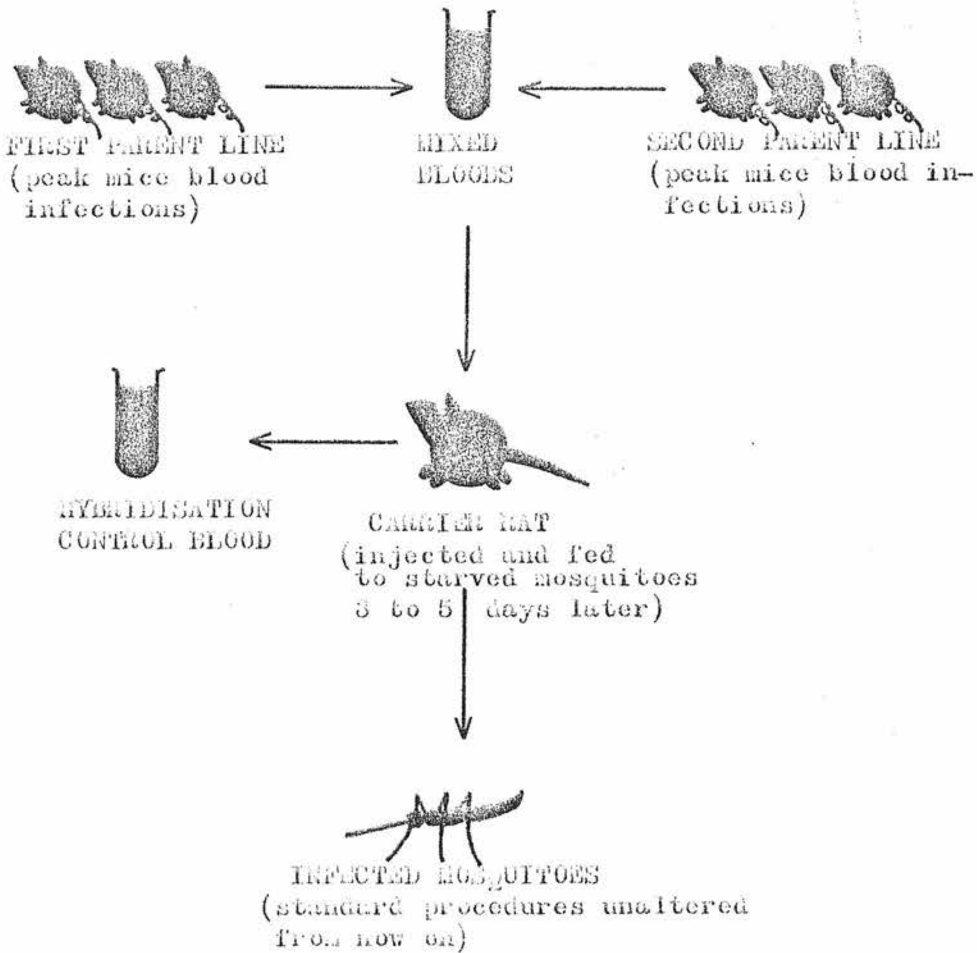
Routinely bloods from both the lines being crossed were harvested from mice at the time of peak fertility which was taken as being when exflagellating micro gametocytes were easily detectable in a coverslip blood preparation examined under phase contrast at 400X magnification. Typically this occurred at or around the tenth day after inoculation. The two bloods were then mixed and adjusted so that the final mixture contained roughly equal numbers of such exflagellating micro gametocytes. About 0.5 to 1.0 ml of this blood mixture was then injected intravenously into each carrier mouse which was then placed inside a cage of starved mosquitoes.

Samples of these were then removed after 8 days and on successive days and dissected. Oocyst counts were then performed and given a sufficiency of mature oocysts sporozoites were harvested at or around the fourteenth day after the blood meal.

b. Modified Crossing Procedure using Splenectomised Rats as Rodent Carriers of the Biparental Blood Mixture

Since *P.chabaudi* was found to produce a greater number of gametocytes in splenectomised rats than in mice (see pp65-68 and MacLeod and Brown, 1976), this host was substituted for mice in most of the later crosses reported in this study. (Some difficulty had been experienced in crossing *P.chabaudi* by the standard method and this had been attributed to its relatively low fertility when compared with *P.yoelii* which had been rather less troublesome in this respect.) The modification has been summarised in Fig. 8.

FIG. 3

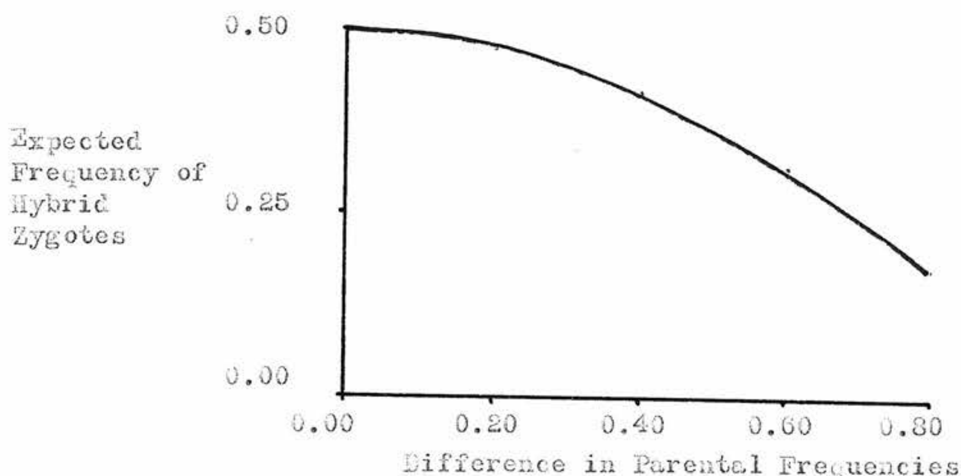


Modification to Standard Crossing Procedure by
The Substitution of Splenectomised Rats as
Rodent Carriers of the Biparental Blood Mixture

Following the use of the modified technique a large increase in oocyst numbers was observed though accurate counts were not performed and the variation between individual mosquitoes was large. It seemed reasonable therefore to attribute the greater success rate in crosses and transmissions to the enhancement of gametocytogensis in rats.

In the modified technique peak infected bloods were harvested from mice infected by the two parental lines and the pooled mixture adjusted to contain equal numbers of parasites from each of the parents. However in some crosses between AS and AJ lines, the mixture was adjusted to contain a slight preponderance of the latter since control studies suggested that AS parasites outgrew AJ parasites in rats (see Table 15). Whenever this was done it has been mentioned in the results section. Fortunately if parasite matings occurred at random the effects of unequal numbers of parents would have been minimised by the buffering effect of the consequent binomial production of hybrid zygotes. This effect has been illustrated graphically in Fig.4. Up to 2 ml of the pooled biparental blood mixture was injected

FIG. 4



Hybrid Zygote Production as a Function of the Difference in Parental Input Frequencies

into each splenectomised rat, by the intravenous or intra-peritoneal routes. If the blood had been packed by centrifugation and the supernatant discarded it was possible to inject as many as 10^{10} parasites in this way and typically instantaneous rat parasitaemias of 20-30% were produced. Since the numbers of gametocytes produced was related to the size of the infection (see Discussion) large rat parasitaemias were considered conducive to success in crossing P.chabaudi lines using the modified procedure.

Peak gametocytogenesis in rats usually took place between the third and sixth day after inoculation. Carrier rats were then anaesthetised (Nembutal) and left in a cage of starved mosquitoes. Subsequent procedures remained unaltered.

c. Crossing Procedure using the Membrane Feeding Apparatus

For some crosses neither rats nor mice were considered suitable hosts for carrying parental blood mixtures prior to mosquito passage. (It had been planned to use rat adaptation as a marker in crosses with unadapted lines and the use of rats might have conceivably had a selective effect on parasites of the unadapted parent line.) Instead bloods containing peak levels of gametocytes were harvested from each parent line, and after mixing transferred to a water jacketed flask with an inner diameter of about 3 cm. The temperature of the flask was maintained at 35°C by a circulating water supply from a thermostatically controlled water bath. Mosquitoes were allowed to feed on these bloods through a thin parchment membrane. In prolonged feeds the bloods were replaced every 20 minutes.

Subsequent procedures remained unaltered.

d. Techniques used in analysing crosses

Blood parasites were harvested from mice inoculated with cross product sporozoites and then either preserved in liquid nitrogen or analysed immediately. Parasites from the original biparental blood mixtures were used as controls for hybridisation.

Although the analysis of each cross differed in procedural detail the common substantive features of cross analysis have been summarised below and any departures from this convention indicated in the relevant parts of the results section.

(i) Testing for biparental transmission: In most crosses the parents possessed distinguisable variants of the enzymes LDH and 6PGD. The presence of both variant forms of either enzyme among the products of the cross was taken as proof of biparental transmission. However, electrophoresis was usually restricted to LDH since variants of this enzyme were easier to detect on starch gels by staining.

(ii) Testing for hybridisation: A variety of tests were used. In crosses where these enzyme variants were segregating and one of the other parental traits was amenable to selection conditions restrictive to the other parent were enforced e.g. by drug pressure or by PABA restriction. The presence then of both forms of LDH or 6PGD was taken as proof of recombination and therefore of hybridisation.

Some crosses were expected to generate a non-parental type of recombinant whose phenotype permitted it to grow in conditions restrictive to both parents. The presence of these following the enforcement of such restrictive conditions among the products of the cross but not among the biparental crontrol mixture was taken as proof of genetic recombination and there-

fore of hybridisation.

(iii) Clone analysis: The basic method of analysing crosses involved isolating lines derived from blood infections induced from dilution clones. Each cloned line was then tested for a number of characters such as drug response and isoenzyme type. The majority of time spent in analysing crosses was occupied doing this type of work.

(iv) Parent line controls: The stability of drug resistance phenotypes among the parental lines used in the crosses was tested routinely after mosquito passage. For logistic reasons these control studies were not carried out at the same time as the crosses themselves but rather when the opportunity arose. In no case was resistance found to have significantly altered due to mosquito transmission and consequently no data have been presented.

e. Technique for harvesting single oocysts:

Oocysts were removed from guts by micro dissection on a depression slide using tungsten needles sharpened in molten potassium chloride. Guts were dissected from mosquitoes 12 to 13 days after a mixed blood meal i.e. before oocyst rupture was widespread. Micro dissection and washing were carried out in modified Kitamura's medium and VP 12 medium (Pudney and Varma, 1971) by circulating ice-cold water. After washing each oocyst was ruptured using a sharp tungsten needle and the sporozoite suspension injected intravenously into a stock mouse. Control mice received the wash only. Both the appearance of each oocyst (mature or immature) and the ease with which the suspension were injected were noted. A third group of mice received larger

numbers of ruptured oocysts to determine sporozoite viability.

RESULTS

1

Selection for Drug Resistance Mutants

In this section the details of the selection experiments themselves have been preceded by base line drug response data of the wild type lines which were the ultimate ancestors of all the mutant lines. In classifying a line as resistant therefore, it was required to exhibit a drug response significantly different from that of the parent line undergoing selection. The characteristics of these so called resistant lines have been presented in the results section together with their frequency among all types of recrudescent parasites and the experimental details of the selection experiments themselves.

The rationale of classification has been explained in each case in the discussion section.

In some cases lines which had been made resistant by one treatment were subjected to a further bout of selection to create doubly resistant mutants. In one case a putative triple mutant was selected, but this was not tested genetically. To help in following these rather confusing lines of ancestry a genealogy of all these types of line together with the main examples of each type has been presented in the appendix in Table 29.

a. Wild Type Baseline Data

(All data refer to experiments performed using the standard rat cake diet and PABA supplement of $0.5gl^{-1}$) unless otherwise stated.

(i) PABA supplement: Whereas the CR line of *P.petteri* exhibited a shallow response curve to PABA restriction (R. Carter

unpublished results, confirmed by the present author) both the AJ and AS lines of P.chabaudi exhibited a sharp decline in growth at restrictive PABA levels. However before January 1975, both the wild lines of these parasites were able to grow in the absence of any PABA supplement but after this date the requirement for peak growth (symbolised by "+" in the text) had increased to $0.025 gl^{-1}$. At zero PABA no growth (symbolised by "-" in the text) was possible. (For all lines used in this study supplements stepped on the series zero, 0.025, $0.050 gl^{-1}$, enabled all or none growth whereas supplements between these steps gave variable growth responses and data from these have therefore been omitted. Consistent growth responses at these PABA steps were exhibited by all lines within the two time zones: before; or after Jan. 1975). Baseline wild type PABA responses for these two time zones have been summarised in Table 1, and a summary of growth responses for all lines has been presented in Table 28 in the discussion.

TABLE 1 - on accompanying page

TABLE 1

Wild type lines: all or none growth responses (+ or –) at normal and diagnostic levels of PABA supplementation before and after Jan. 1975.

Wild Line	Before/After Jan. 1975	Solid Diet	Prednisone ($40mg.l^{-1}$)*	PABA Supplement (gl^{-1}) 0.5	0.05	0.025	Zero
AS	Before	Rat Cake	Not Given	+	+	+	+
AJ	"	" "	" "	+	+	+	+
AS	"	" "	Given	+	+	+	+
AJ	"	" "	"	+	+	+	+
AS	After	" "	Not Given	+	+	+	–
AJ	"	" "	" "	+	+	+	–
AS	"	Milk Diet	" "	+	+	+	–
AJ	"	" "	" "	+	+	+	–

* administered for at least 7 days prior to inoculation.

Thus, while the minimum PABA growth supplement for wild type lines irrespective of diet was Zero before Jan. 1975 it rose to $0.025gl^{-1}$ afterwards. The reason for this change has been considered in the discussion.

The prednisone data have been included for convenience of comparison and were obtained as formal control data for the selection experiments conducted using this compound as an immunosuppressive agent. There was no evidence that prednisone affected PABA dependence.

(ii) Wild type responses to Sulphadiazine: ED_{90} estimates ± 1 standard error range have been presented in Table 2. Whereas the estimates obtained before Jan. 1975 refer to responses without a PABA supplement those after that date were obtained with the standard supplement as neither wild line was able to grow in the absence of PABA.

TABLE 2

Wild type lines: responses to Sulphadiazine (ED_{90})

Wild Line	Before/After Jan.1975	PABA Supplement (gl^{-1})	Response to Sulphadiazine ED_{90} ($mg.kg^{-1}$) ± 1SE range
AJ	Before	Zero	0.054(0.049-0.059)
AS	"	"	0.061(0.047-0.074)
AJ	After	0.5	80.3 (51.3 - 126)
AS	"	0.5	61.0 (52.5 - 70.8)

(iii) Wild type responses to Pyrimethamine: ED_{90} estimates ± 1SE range have been presented in Table 3. All the estimates were obtained with the standard rat cake diet whether before or after Jan. 1975.

TABLE 3 - on accompanying page

TABLE 3

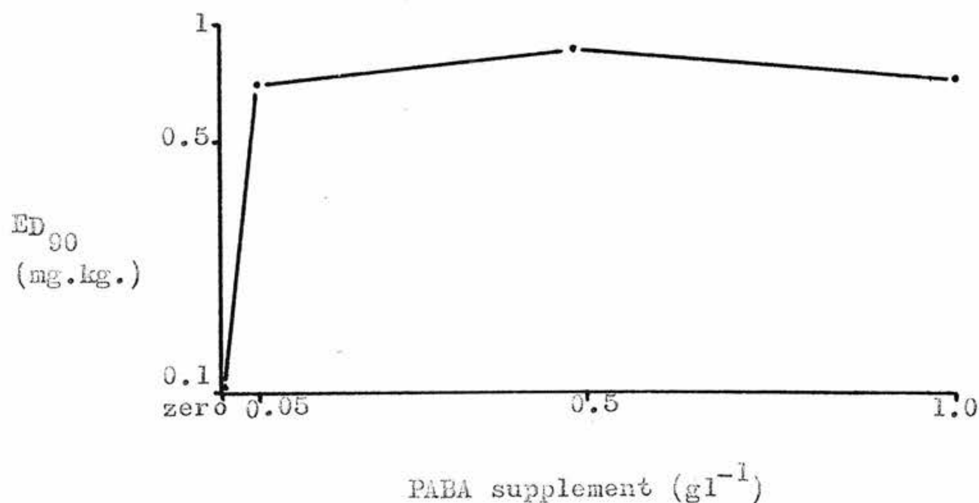
Wild Line	Before/After Jan. 1975	PABA Suppl. $g.l.^{-1}$	Prednisone 40 $mg.l^{-1}$	Response to Pyrimethamine $ED_{90}(mg.kg^{-1}) \pm$ ISE
AJ	Before	Zero	Not Given	0.116 (0.096 - 0.141)
AJ	"	0.05	" "	0.95 (0.48 - 1.89)
AJ	"	0.50	" "	0.87 (0.53 - 1.41)
AJ	"	1.00	" "	0.90 (0.59 - 1.47)
AJ	"	0.50	Given	1.03 (0.81 - 1.30)
AJ	After	0.50	Not Given	0.98 (0.87 - 1.10)
AS	"	0.50	" "	0.58 (0.38 - 0.89)

The variations between these estimates except in the case of the Zero PABA supplement were not significant arising from random errors and measurement errors.

The relationship between response of a wild type line to Pyrimethamine and the level of the PABA supplement has been expressed graphically in Fig. 5.

FIG. 5

AJ wild type: Pyrimethamine sensitivity at 4 levels of PABA supplementation (data obtained before Jan. 1975)



b. Wild Type Lines: Selection Experiments

(i) Pyrimethamine: Data concerning the frequency of recrudescent infections after pyrimethamine selection at various doses and dose rates and with or without PABA supplementation, are presented in Table 4. Data on the effects of immuno-suppression by splenectomy or by administration of prednisone in the drinking water (40 mg.l^{-1}) have also been included.

A summary of the characteristics of a selection of these recrudescences - based on initial drug test and PABA test data is presented in Table 5 along with comparable wild type and resistant line data. (The resistant line was selected Dr. D. Walliker of this laboratory) by exposing a growing infection of the AS wild type line of P.chabaudi to 4 consecutive daily doses of pyrimethamine (50 mg.kg^{-1}) and called AS PR DW.)

More comprehensive data (obtained after cloning) have been presented for a selection of these lines in Table 6. These included both some apparently "resistant" lines as well as those lines which could not be classified with reasonable certainty after the initial tests - i.e. so called anomalous lines. The initial classification was based on the test data in Table 5 revised if necessary after more comprehensive tests (see Table 6). In all but 2 dubious cases the comprehensive tests performed on a sample of lines (n = 12) agreed with the initial classification.

TABLE 4 - on accompanying page

TABLE 4

Wild type lines: pyrimethamine selection: Effects of varying dose. Dose rate. PABA supplement and immunosuppression on the frequency and characteristics of recrudescent infections. (All data pre Jan. 1975)

Expt. No.	line under-going selection	Pyrimethamine		PABA supple-ment (gl^{-1})	Immuno-suppression	Total No. treated parasites ($\times 10^{10}$)	Recrudescences				
		dose ($mg.kg^{-1}$)	times given				Total	Tested	Resist.	Sensi.	Anomal.
401	AS wild type	50	4	0.5	not given	10	0	0	0	0	0
402	AJ " "	50	4	0.5	" "	42	9	9	7	2	0
403	AJ " "	50	4	zero	" "	33	0	0	0	0	0
404	AJ " "	50	4	0.5	splenectomy	4.7	4	4	0	4	0
405	AJ " "	50	4	0.5	prednisone	13	7	6	1	5	0
406	AJ " "	50	5	0.5	not given	18	1	1	1	0	0
407	AJ " "	50	6	0.5	" "	17	0	0	0	0	0
408	AJ " "	25	6	0.5	" "	6.8	5	4	0	4	0
409	AJ " "	20	contin.	0.5	" "	8.1	0	0	0	0	0
410	AJ " "	10	"	0.5	" "	11	11	9	9	0	0
411	AJ " "	10	"	zero	" "	19	0	0	0	0	0
412	AJ " "	5	"	0.5	" "	4.1	6	6	5	0	(1)*
413	AJ " "	2.5	"	0.5	" "	3.4	16	9	1	7	1

* lost before comprehensively tested

TABLE 5

Wild type lines: pyrimethamine selection. Results of initial tests performed on lines derived from recrudescent infections. (Comparable data on wild type and pyrimethamine resistant lines included)

Ref. No.	Selection expt. obtained from ref. no. (see Table 4)	Response to *1 Pyrimethamine (% control)	Growth on 2 Zero PABA* (% control)	Initial 3 * classification	Fate
AJ wild type	-	(<0.24(2.5) (<0.32(5)mg.kg	+ 200	-	-
ASP^{r} (DW)	-	(163 (2.5) (180 (5)	-<0.40	-	-
4021	402	<0.3	+104	w.type	abandoned
4022	402	<0.5	- 38	" "	"
4023	402	25	-<0.4	resis.	"
4024	402	30	-<0.11	"	"
4025	402	138	-<3.7	"	"
4026	402	76	-<0.17	"	"
4027	402	131	-<0.08	"	retested
4028	402	56	-<0.16	"	"
4029	402	339	-<0.35	"	"
4041	404	<0.24	+ 22	w.type	abandoned
4042	404	<0.13	+ 76	"	"
4043	404	<0.54	+ 42	"	"
4044	404	<0.30	+<0.30	anom.	retested (sensitive)
4051	405	<0.30	+ 218	w.type	abandoned
4052	405	<0.31	+ 17	"	"
4053	405	<0.75	+ 49	"	"
4054	405	no data	+ 98	"	"
4055	405	132	+ 85	anom.	retested (sensitive)
4056	405	180	no data	resis.	"
4061	406	15	-<0.13	"	"
4081	408	<1.7	+ 169	w.type	abandoned
4082	408	<0.22	+ 165	"	"
4083	408	<1.5	no data	"	"
4084	408	<0.11	+ 25	"	"
4101	410	132	-<0.30	resis.	"
4102	410	54	-<0.17	"	"
4103	410	59	-<0.14	"	"
4104	410	92	-<0.14	"	"
4105	410	249	-<2.2	"	"
4106	410	22	-<0.32	"	"
4107	410	119	-<0.31	"	"
4108	410	122	-<0.19	"	retested

/Cont

TABLE 5 continued:

Ref. No.	Selection expt. obtained from ref. no. (see Table 4)	Response to *1 Pyrimethamine (% control)	Growth on 2 * Zero PABA (% control)	Initial 3 * classification	Fate
4109	410	100	-<0.34	resis.	retested
4121	412	41	-<0.12	"	abandoned
4122	412	133	-<0.32	"	"
4123	412	71	-<0.33	"	"
4124	412	61	-<0.41	"	"
4125	412	108	-<0.17	"	retested
4126	412	158	+ 106	anom.	lost
4131	413	<0.13	+ 41	w.type	abandoned
4132	413	<0.48	+ 129	"	"
4133	413	<1.37	+ 169	"	"
4134	413	<1.00	+ 48	"	"
4135	413	<0.69	+ 18	"	"
4136	413	<0.23	+ 135	"	"
4137	413	<0.10	+ 164	"	"
4138	413	284	-<0.61	resis.	retested
4139	413	22.6	+ 127	anom.	" (sulphadiazine resistant)

*1 4021-4034 received 4 doses of 5 mg.kg^{-1}
4101-4139 " 4 " of 2.5 mg.kg^{-1}

*2 + = reached peak; - = failed to grow

*3 The resistant class includes all those resembling ASpR DW

w. type = wild type
resis. = resistant
anom. = anomalous

TABLE 6

Wild type lines: pyrimethamine selection: Results of further tests performed on selected lines obtained from recrudescent infections. (Comparison data on a wild type line and on a resistant line (ASpR DW) included).

Ref. Number	Pyrimethamine ED_{90} (range ± ISE)	% Growth on Zero PABA (range ± ISE) (pre Jan. 1975)	Classification w.r.t. pyrimethamine
wild type	0.87 (0.53/1.41)	51.4 (19.4/136)	sensitive (1X)
$ASP^{R}DW$	19.5 (13.3/26.9)	<0.26	resistant (22X)
AJ 4027	18.2 (13.0/25.7)	<0.21	resistant (21X)
AJ 4028	7.8 (3.6/16.7)	<0.32	" (9.0X)
AJ 4029	21.8 (13.3/35.6)	<0.22	" (25X)
AJ 4044	1.10 (0.64/1.89)	57.5 (28.2/117)	sensitive (1.3X)
AJ 4055	0.32 (0.14/0.74)	128 (38.6/429)	" (0.37X)
AJ 4056	24.5 (15.0/41.8)	<0.95	resistant (28X)
AJ 4061	22.5 (13.2/33.4)	<0.40	" (26X)
AJ 4108	12.2 (8.60/17.4)	<0.25	" (14X)
AJ 4109	17.3 (13.0/23.1)	<0.31	" (20X)
AJ 4125	18.5 (12.9/26.9)	<0.24	" (21X)
AJ 4138	10.9 (6.4/19.1)	<0.14	" (13X)
AJ 4139*	2.14 (1.56/2.95)	122 (48/310)	anom. (2.5X) or sensitive

* for data concerning the response of this line to sulphadiazine - see Table 8.

It was concluded that the lines classed as resistant resembled $ASP^{R}DW$ both in the level of pyrimethamine resistance acquired and in the concomitant PABA dependence. The basis for the classification has been considered in more detail in the discussion along with estimates of the acquistion rates. The anomalous line AJ 4139 was later classified as resembling sulphadiazine resistant lines obtained by direct exposure to the drug or following PABA restriction and has been discussed along with these.

(ii) Wild Type Lines: Selection with sulphadiazine (pre Jan. 1975) and by restriction of dietary PABA (post Jan. 1975).

The direct drug pressure selection experiments were completed prior to the PABA shift in Jan. 1975 but drug testing of the recrudescent lines was not completed until after that date. Consequently all lines still available were retested under the new PABA regime and only these data are presented. Both the PABA restriction experiment and the testing of the recrudescent lines obtained after selection in this way were carried out under the new PABA regime after Jan. 1975. All experiments were performed using the AJ wild type parasite line.

Data concerning the frequency and characteristics of recrudescent lines obtained after direct drug pressure or after indirect selection by PABA restriction are presented in Table 7. Sulphadiazine and Pyrimethamine response data for some of these lines are presented in Table 8 together with data on one of the anomalous lines obtained after pyrimethamine selection by exposure to a constant dose of 2.5 $mg.kg^{-1}$ pyrimethamine. Comparison data on a wild type line and on a member of the predominant class of pyrimethamine resistant lines (AS P^R DW) have been included.

All the direct drug selection experiments were performed with no PABA supplement being given.

TABLE 7 - on accompanying page

TABLE 7

Wild type lines: selection experiments using sulphadiazine and by restriction of dietary PABA.

ref. number	date	Sulphadiazine		total number treated parasites ($x 10^{10}$)	Recrudescences *2			
		dose (mg.kg*1)	times given		total	tested	wild	sulpha. resist.
701	pre. Jan '75	20	x 4	4.3	0	0	0	0
702	" " "	10	x 4	4.4	0	0	0	0
703	" " "	5	x 4	10	3	3	1	2
704	" " "	2.5	x 4	7.3	5	5	1	4
705	" " "	1.25	x 4	2.4	7	6	5	1
801	post Jan '75	PABA restriction		6.6	9	9	0	9
802	" " "	"	" *1	5.9	12	10	0	10

*1 immunosuppression by administration of prednisone in drinking water ($40 mg.l^{-1}$)

*2 of the 26 lines classed as sulphadiazine resistant only 8 were directly tested. The resistance of the others has been inferred from their PABA independence as PABA independence (after Jan. 1975) was always found to be associated with sulphadiazine resistance.

TABLE 8

Wild type lines: selection with sulphadiazine and by PABA restriction: responses to sulphadiazine and pyrimethamine and growth on a PABA free diet (after Jan. 1975) together with relevant comparison data.

Line	how obtained	ED_{90} (± ISE range) $mg.kg^{-1}$) Sulphadiazine	Pyrimethamine	All or none *1 grow on zero PABA (after Jan.'75)	Classification
AJ wild type	-	80.3 (51.3 - 126)	1.11 (0.82 - 1.53)	-	
AS P^R DW	) exposure to	3.35 (2.76 -4.12)	19.1 (13.8 - 26.3)	-	pyrimethamine resist. *3
AJ 4139	) pyrimethamine	478 (274 - 619)	2.18 (1.81 - 3.59)	+	anomal. sulphad. resist.
AJ 7031	)	851 *2 (372 -1947)	2.13 (1.58 - 2.91)	+	sulphadiazine resist.
AJ 7032	) exposure to	428 (313 - 587)	2.19 (1.71 - 2.57)	+	" "
AJ 7041	) sulphadia-	327 (257 - 403)	0.89 (0.64 - 1.60)	+	" "
AJ 7042	) zine	199 (152 - 259)	1.40 (0.93 - 2.10)	+	" "
AJ 7054	) (expts. 703	250 (204 - 305)	2.11 (1.66 - 2.69)	+	" "
AJ 7056	) - 705)	43.5 (22.7 - 83.2)	0.53 (0.37 - 0.74)	-	sens. recrudescence
AJ 8015	) PABA re-	245 (182 - 330)	1.72 (1.20 - 2.48)	+	sulphadiazine resist.
AJ 8016	) striction	327 (219 - 488)	2.52 (1.30 - 4.89)	+	" "
AJ 8017	) (expt. 801)	158 (63.5 - 392)	1.20 (0.82 - 1.74)	+	" "

*1 + = reached peak: - = failed to grow
*2 ED_{90} sulphadiazine (pre Jan. 1975) = 1.30 $mg.kg^{-1}$ (0.64 - 2.62)
*3 See Table 6

Characteristically the lines classed as sulphadiazine resistant were all PABA independent after Jan. 1975 and showed a continuously variable cross-resistance to pyrimethamine also, thus resembling the anomalous line AJ 4139 obtained after exposure to pyrimethamine. There seemed to be no correlation between the mode of selection (direct or indirect) and the phenotypes produced. Although sensitive recrudescences were detected (following direct exposure to drug pressure only) no intermediate types were identified. These are further explained in the text.

c. Mutant Lines - Selection Experiments

The main aim of the selections performed on the wild lines was to obtain an estimate of the range of basic genetic variation available by inference from the range and frequency of the different resistance phenotypes produced. Having obtained these it was planned to demonstrate their combination by genetic means-as shown in Cross 5.

On the other hand selections were performed on mutant lines with a view to investigating the ease with which single gene mutants could be combined by selection and later dissected by genetic means-as shown in Cross 4.

It was hoped that the combination of these two approaches could be used to investigate to what extent simple mendelian genes could be invoked to explain the inheritance of multiple drug resistance.

Sections (i) and (ii) deal with the case where single gene mutants which had been made resistant either to pyrimethamine or to sulphadiazine by direct drug exposure were subjected to secondary bouts of selection designed to induce resistance to

the alternative drug also: thus both may be considered as alternative routes to the same genetic endpoint.

In section (iii) this double mutant was exposed to a third bout of selection (by PABA restriction) to create a presumed triple mutant - though this line was not tested genetically.

In addition the selection experiments involving PABA restriction of lines in which this substance was required as a growth supplement following the acquisition of pyrimethamine resistance were used to detect any back mutations to pyrimethamine sensitivity. (PABA independence due to the acquisition of sulphadiazine resistance would be distinguishable from that due to back mutation as in the former case no loss of pyrimethamine resistance would be expected.)

(i) <u>Pyrimethamine resistant PABA dependent lines - secondary selection by PABA restriction</u>: The minimum level of PABA supplement required for normal growth by these lines rose from 0.025 gl^{-1} to 0.050 gl^{-1} after Jan. 1975. However despite this increase all the PABA restriction experiments involved the total withdrawal of the PABA supplement. Since the suppressive effect of this restriction was not immediately apparent the PABA was removed at modal parasitaemias between 0.1 and 1.0%. Typically this meant that the mean maximum parasitaemias of lines undergoing selection were between 5 and 15%. These maxima were used in calculating the number of parasites which had undergone selection.

Data on the frequency and characteristics of recrudescent lines following selection have been presented in Table 9 along with data on the effects of the administration of prednisone as an immunosuppressive (40 mg l^{-1} in the drinking water.) The

TABLE 9

Pyrimethamine resistant PABA dependent lines: secondary selection by PABA restriction and the effects of immunosuppression (by prednisone) on the frequency and characteristics of recrudescent lines.

Ref. No.	line undergoing selection	Minimum PABA Growth Requirement (gl^{-1})	Experiment Before/After Jan. 1975	Special Treatment	No. treated parasites ($\times 10^{10}$)	Frequency and Phenotypes of Recrudescences				
						Total	PABA Tests		Pyr. Res. Tests	
							No. tested	result	No. tested	result
901	AS P^R DW	0.025	before	-	21	40	26	all recruds. PABA independent Now	11	all recruds. more resist. or the same as the line which underwent selection
903	AS P^R DW	0.025	before	Prednisone	2.7	5	5		4	
904	AJ 4027	0.050	after	-	3.1	0	-		-	

TABLE 10

Pyrimethamine resistant PABA dependent lines PABA restriction selection experiments. Growth of recrudescent lines in the absence of PABA and after pyrimethamine therapy (4 x 20 mg.kg^{-1}) - both expressed as a percentage of the untreated control. Comparison data included. All data obtained before Jan. 1975.

Line Ref. No.	How Selected: Summary of Treatment	All or None & % Growth On Zero PABA	% Growth After Pyrimethamine (4 x 20mg.kg^{-1})
AS P^R DW	4x50mg.kg Pyrimeth.	- <0.26	9.3
AJ 4027	4x50mg.kg Pyrimeth.	- <0.21	7.6
AJ wild type	not selected	+ 51.4	(eliminated at 5mg.kg x 4)
AS 90101 *1	PABA restriction *2	+ 45	49
AS 90102 *1	" "	+ 7.5	78
AS 90103 *1	" "	+ 250	280
AS 90104	" "	+ 24	143
AS 90105	" "	+ 5.4	27
AS 90106	" "	+ 22	76
AS 90107	" "	+ 3.6	183
AS 90108	" "	+ 450	173
AS 90109	" "	+ 613	20
AS 90110	" "	+ 100	36
AS 90111	" "	+ 96	28
AS 90112	" "	+ 45	Not tested
AS 90113	" "	+ 121	" "
AS 90114	" "	+ 32	" "
AS 90115	" "	+ 190	" "
AS 90116	" "	+ 508	" "
AS 90117	" "	+ 5.5	" "
AS 90118	" "	+ 1201	" "
AS 90119	" "	+ 67	" "
AS 90120	" "	+ 118	" "
AS 90121	" "	+ 340	" "
AS 90122	" "	+ 17	" "
AS 90123	" "	+ 45	" "
AS 90124	" "	+ 44	" "
AS 90125	" "	+ 116	" "
AS 90126 *1	" "	+ 131	2.6
AS 90301 *1	PABA rest. & immun.	+ 31	73
AS 90302	" " " "	+ 103	17
AS 90303	" " " "	+ 17	39
AS 90304	" " " "	+ 115	29
AS 90305	" " " "	+ 35	Not tested

*1 retest data presented in Table 11   *2 of AS P^R DW

immun. = immunosuppression

brief classification of these recrudescence included in Table 9 have been obtained from results of the preliminary tests summarised in Table 10 and from the more detailed tests, the results of which have been summarised in Table 11.

TABLE 11

Pyrimethamine resistant PABA dependent lines: secondary selection by PABA restriction. Response to pyrimethamine (ED_{90} ($mg.kg^{-1}$ $\pm$ ISE range) and growth in the absence of PABA (results of repeated tests) of a selection of recrudescent infections. Comparison data on the ancestral line included. (All data obtained before Jan. 1975.)

Ref. No.	All or None and % Growth on *1 Zero PABA	Response to Pyrimethamine ED_{90}($mg.kg^{-1}$) $\pm$ ISE range	Increase of Pyrimeth. *3 resistance
AS P^R DW	- (not retested prev. est. < 0.26)	19.5(13.8-26.9)	-
AS 90101	+ 113(41.9-303)	40.9(27.4-61.0)	2.1 X
AS 90102	+ 78.5(42.2-146)	33.8(26.6-43.0)	1.7 X
AS 90103	+ 82.9(47.1-146)	39.7(26.7-59.9)	2.0 X
AS 90126	+ 43.1(26.1-71.1)	16.8(11.9-23.7)	0.86 X
AS 90207 *2	+ 107 (data insufficient for SE determination	38.2(19.4-76.7)	2.0 X
AS 90213 *2	+ 69.4(48.6-99.4)	22.5(14.9-33.6)	1.2 X
AS 90307	+ 18.3(6.68-50.1)	53.5(34.3-85.5)	2.7 X

*1 + = reached peak — = failed to grow
*2 both these lines were selected by PABA restriction also, but details have not been included in the text.
*3 the mean value for increase of resistance = 1.7 X

While PABA restriction had restored the PABA independence of all these recrudescent lines in no case was the resistance to

pyrimethamine abolished either following the preliminary or the quantitative drug tests where these were performed. On the contrary, there was a mean increase of pyrimethamine resistance of 1.7 X.

(ii) Sulphadiazine resistant lines: continued selection with pyrimethamine: (see previous section) AJ 7082 which had been made resistant to Sulphadiazine by direct exposure to the drug (4 x 5 mg.kg) was also cross resistant to Pyrimethamine (ED_{90} = 2.19 $mg.kg^{-1}$) i.e. about two fold compared with wild type lines. A second bout of selection was imposed by direct exposure to pyrimethamine administered continuously until recrudescences appeared for up to 20 days following commencement of drug treatment. Mice received the normal PABA supplement of $0.5gl^{-1}$. Details of the frequency and characteristics of recrudescences have been summarised in Table 12 and their responses to pyrimethamine and PABA have been summarised in Table 13. (All experiments and tests were performed after Jan. 1975.)

See the text for a further discussion together with acquisition frequency estimates and an interpretation of the results. Line AS 90301 was later used in a cross with a wild type line (Cross 4) and in another selection experiment. (See results, Section 1c (iii)).

TABLE 12 - on accompanying page

TABLE 12

Sulphadiazine resistant lines: secondary selection with Pyrimethamine. Effects of varying dose on the frequency and characteristics of recrudescent lines. (After Jan. 1975)

line undergoing select.	expt. ref. no.	pyrimeth. dose (mg.kg^{-1})	total no. treated paras. (x10^{10})	Recrudescences					
						Pyrimeth. resistance		PABA Depend.	
				tl.	tst.	unch.	incr.	unch	inc
AJ7032	1201	40	10.0	0	0	0	0	0	0
AJ7032	1202	20	8.3	8	8	0	8	0	8
AJ7032	1203	10	4.9	3	3	0	3	0	3

Key: tl. = total tst. = tested unc. = unchanged
incr. = increased
paras. = parasites

TABLE 13

Sulphadiazine resistant lines: secondary selection with Pyrimethamine. Growth (% control) after pyrimethamine treatment (4 x 20 mg.kg^{-1}) and in the absence of a PABA supplement. (Data after Jan. 1975). Comparison data on AJ 7032 included.

line ref. no.	seln. expt. ref. no.	% Growth after pyrimethamine treatment	% Growth without a PABA supplement *1
AJ 7032	703	< 0.23	+ (37)
AJ 12021 *2	1202	111	- (< 0.37)
AJ 12022	1202	17.8	- (< 0.19)
AJ 12023	1202	30	- (< 0.75)
AJ 12024	1202	61	- (< 0.40)
AJ 12025	1202	236	- (< 0.28)
AJ 12026	1202	100	- (< 2.5)
AJ 12027	1202	56	- (< 0.95)
AJ 12028	1202	59	- (< 0.30)
AJ 12031	1203	4	- (< 0.13)
AJ 12032	1203	637	- (< 1.0)
AJ 12033	1203	267	- (< 1.2)

*1 + = reached peak
- = failed to grow

*2 (ED$_{90}$) pyrimethamine was later found to be 31.62 mg.kg^{-1} ± 1SE range 23.44 - 42.66 mg.kg^{-1})

It was concluded that the secondary selection by exposure to pyrimethamine had significantly increased the resistance to this drug in all the recrudescences tested. Although only one quantitative measurement of this increase was obtained, there was no reason to suppose this to be atypical. This line AJ 12021 resembled closely the double mutant lines obtained by PABA restriction of pyrimethamine resistant lines which required this growth supplement. In effect both were thought to constitute alternative routes to the same end. (This has been further discussed in the text together with estimates of the acquisition rates.)

(iii) AS 90301 double mutants: tertiary selection by PABA restriction: AS 90301 which had been isolated after two selection steps: firstly by direct exposure to pyrimethamine (4×50 mg.kg^{-1}) followed by PABA withdrawal was able to grow in the absence of PABA before Jan. 1975 but required a supplement of 0.025 gl^{-1} after that date. Consequently the line was allowed to undergo a second round of PABA restriction after that date. Mice with growing infections of AS 90301 parasites had their PABA supplement removed entirely and the maximum number of parasites was estimated to have reached 2.2×10^{10}. Subsequently 4 recrudescent lines were detected and preliminary tests indicated that all of these had become PABA independent. After cloning measurements were made of their responses to pyrimethamine and PABA growth requirements. These data have been summarised in Table 14.

TABLE 14 - on accompanying page

TABLE 14

AS 90301: characterisation of 4 recrudescent lines isolated after a second step of PABA restriction along with characteristics of the original line that underwent selection. (All data obtained after Jan. 1975.)

Ref. Number		Pyrimethamine	PABA
		ED_{90} (mg.kg^{-1}) ± ISE range	min. Growth suppl. to reach peak(g.l^{-1})
AS 90301(original line)		34.5 (25.7-46.3)	0.025
AS 90301141	)	42.4 (32.4-55.6)	Zero
AS 90301142	) recrud-	32.3 (24.0-43.5)	Zero
AS 90301143	) escent	no data	Zero
AS 90301144	) lines	28.3 (25.1-32.0)	Zero

This second round of PABA restriction had resulted in a return to PABA independence in all the recrudescences tested. There was no evidence of any significant change in pyrimethamine resistance though it was inferred that sulphadiazine resistance had been increased. The significance of these data have been discussed in the text.

(iv) Conclusions: Data from the PABA restriction experiments in (i) and (iii) showed no loss of pyrimethamine resistance following selection and therefore no evidence of back mutation.

The mutant phenotypes described in sections (i) and (ii) appeared indistinguishable on phenotypic grounds and were presumed to have resulted from the combination of two single gene mutants conferring resistance to pyrimethamine and sulphadiazine respectively.

The mutant described in section (iii) was presumed to be a triple consisting of one pyrimethamine mutant and two sulphadiazine resistance mutants. Its existence demonstrated that more than one type of mutant conferring sulphadiazine resistance was available - a finding possibly relevant to the rather wide range of pyrimethamine cross resistances observed following selection for sulphadiazine resistance e.g. in section (i). (This point has been considered in more detail in the discussion.)

2

Selection for Rat Adaptation in P.chabaudi

In order to obtain a selective marker unconnected with drug resistance intended for use in more detailed analyses of the genetics of drug resistance, a rat adapted line was obtained by selection.

Data on the mean peak percentage parasitaemias in male C57 mice and in newly weaned and 8 week old norway wistar male rats reached by the wild type AJ line before and after selection and after 4 mouse and 1 mosquito passage after selection are presented in Table 15. Data on the responses to pyrimethamine and PABA dependence before selection and after selection and mouse/ mosquito passage have been included (Post Jan. 1975). All inoculum sizes = 10^6.

TABLE 15 - on accompanying page

During the initial development of the rat adapted line, large numbers of male and female gametocytes were observed following inoculation with more than 10^8 parasites or so. With these large inocula non adapted parasite infections peaked at between 4 and 6 days in splenectomised rats and gametocytes could be observed from initial appearance at about 2 days through a peak at between 4 and 7 days and remained visible for up to between 14 and 28 days after inoculation.

Data on peak levels of infection and gametocyte production for three lines in the different hosts used in this study are presented in Table 16.

TABLE 15

Wild type and rat adapted lines: effects of rat adaptation

line/description	mean maximum % parasitaemia (± ISE range)	♂ norway wistar rats		Response to Pyrimethamine $mg.kg^{-1}$	minimum PABA supplement for growth to peak *1
	C57 ♂ mice	splenectomised newly weaned	intact at 8 weeks	(ED_{90} ± ISE range)*1	
AJ wild type	42.2 (48.1) (37.0)	1.30 (1.60) (1.06)	<.002 (-)*2	0.98 (1.10) (0.87)	.025%
AJ rat adapted	35.8 (40.8) (31.4)	23.1 (26.3) (20.3)	6.67 (7.60) (5.85)	not tested	not tested
AJ rat adapted (after 4 mouse & 1 mosquito passage)	37.5 (42.8) (32.9)	22.9 (26.1) (20.1)	7.06 (8.05) (6.19)	0.96 (1.12) (0.83)	.025%

*1 tested in mice
*2 failed to grow

TABLE 16

Wild type and rat adapted lines: gametocyte production in rats and mice

line	inoculum size	HOST: ♂ C57 mice: mean max. % parasitaemia	HOST: ♂ C57 mice: mean max. % gametocytes	HOST: ♂ Splenectomised Norway Wistar rats (newly weaned): mean max. % parasitaemia	HOST: ♂ Splenectomised Norway Wistar rats (newly weaned): mean max. % gametocytes	HOST: intact ♂ Norway Wistar rats (8 weeks old): mean max. % parasitaemia	HOST: intact ♂ Norway Wistar rats (8 weeks old): mean max. % gametocytes
AJ wild type	10^6	42.2 (48.1) (37.0)	0.34 (0.41) (0.27)	1.30 (1.60) (1.06)	0.49 (0.63) (0.38)	not	tested
AS wild type	10^6	65.6 (82.7) (52.1)	0.47 *	5.90 (7.97) (4.37)	1.30 (1.71) (0.98)	not	tested
AJ wild type	10^9	not	tested	17.02 (22.1) (13.1)	1.80 (2.14) (1.51)	not	tested
AJ rat adapted	10^6	35.8 (40.8) (31.4)	0.24 (0.41) (0.14)	23.1 (26.3) (20.3)	0.64 (0.81) (0.51)	6.67 (7.60) (5.85)	0.54 (0.63) (0.46)

* too few animals for standard error to be determined

The data in Table 15 show that not only had selection rendered the surviving parasites capable of growing to a higher parasitaemia in splenectomised rats than wild types, but also had given them the novel ability to grow in intact rats; and although data have not been presented, this extended to infections initiated with clonal inocula. Growth in mice seemed unaffected by rat adaption as were PABA dependence and pyrimethamine resistance. Rat adaptation appeared to be stable also.

On the other hand the data in Table 16 show that rat adaptation seemed to have significantly reduced the phenomenon of abundant gametocytogenesis observed in wild type infection of rats - suggesting that this phenomenon was symptomatic of growth in hostile or abnormal conditions.

All these phenomena have been discussed in the text. However, the technique of selection by repeated passage prohibited the calculation of the rate of acquisition of rat adaptation.

The rat adapted line was later crossed with a sulphadiazine resistant line with a view to elucidating the mode of inheritance of the former character (see Cross 6).

3

Cross Results

Apart from the presumptive triple mutant type all the main types of mutant and mutant combination described in the present work were used in Crosses.

Crosses 1 and 2 concern the inheritance of pyrimethamine resistance and Cross 3 deals with sulphadiazine resistance. The main type of question posed in these crossing experiments is to what extent the inheritance of these characters and their correlated effects can be attributed to mendelian single gene mutations - the simplest possible model.

Crosses 4 and 5 deal with the combined inheritance of resistance to these two drugs with special emphasis on the occurrence of recombination between them. Included also in Cross 4 is a brief description of an unsuccessful attempt to analyse this cross by the analysis of clones obtained from single oocyst infections.

Cross 6 was intended as a necessary preliminary investigation into the nature of rat adaptation prior to the use of this marker as a selective tool in the screening of crosses with drug resistance markers, and although this marker was shown to be unsuitable the data have been included and discussed and illustrate some of the problems attendant upon a genetic analysis of mutants with complex modes of inheritance.

In each cross, the results have included a brief rationale followed by a description of the parents and the cross products and end with a short conclusion. Each cross has been discussed in detail in the text. The discussion ends with general conclusions regarding the nature of the inheritance of drug

resistance drawn both from the selection experiments and from the crosses themselves.

Cross 1 - AS P^R (DW) (pyrimethamine resistance, PABA dependent obtained by direct exposure to the drug in a single step) x AJ wild type: further analysis to detect recombination between primary resistance to pyrimethamine and the concomitantly developed increased dependence on PABA and thereby test for pleiotropy.

The analysis of this cross by clone analysis has been reported elsewhere (Walliker et al., 1975). He kindly allowed me to perform the further analysis using blood samples preserved from the original cross.

(i) Description of parental phenotypes: The responses to pyrimethamine and minimum PABA requirements (pre Jan. 1975) are presented in Table 17.

TABLE 17

Cross 1: AS P^R DW x AJ wild type. Responses to pyrimethamine, minimum PABA supplements required for growth (Data pre Jan. 1975) and enzyme markers.

Parent lines	Response to Pyrimethamine (ED_{90} $\pm$ ISE range) $mg.kg^{-1}$	Min. PABA suppl. for growth to peak (gl^{-1})	enzyme variant	
			LDH	6PGD
AJ wild type	0.87 (0.53-1.41)	Zero	2	3
AS P^R DW	19.5 (13.3-26.9)	0.025	3	2

(ii) Experimental details: Walliker et al. (1975) showed free recombination between variants of the two enzyme markers LDH and 6PGD and the qualitative character pair pyrimethamine resistance/sensitivity. It remained uncertain however whether or

not pyrimethamine resistance and PABA dependence segregated together. To choose between these alternatives a test system was set up to find any parasites in which pyrimethamine resistance had lost its accompanying high PABA dependence.

The procedure was analogous to a 4 day drug test. Two groups of mice were inoculated with 10^6 aliquots derived from a cross product parasite stabilate preserved in the laboratory. One group received 5 $mg.kg^{-1}$ pyrimethamine intraperitoneally 3 hours later and on the 3 days following. The other group was treated as a control and received carboxymethyl cellulose only. On the fourth day after inoculation parasite counts were performed on both groups of mice and these agreed closely, doubtless due to the presence of resistant parasites in the drugged group. Aliquots of 10^5 parasites were then obtained from both groups of mice and each set used to inoculate a group of mice which had been maintained without a PABA supplement and a group of control mice receiving the standard supplement of 0.5 gl^{-1} making 4 groups in all. (The choice of 10^5 parasites was made as a compromise between running the risk of selecting a mutant or a parental survivor by weight of numbers and risking the exclusion of rare recombinant types.) Animals in all groups except those which had received both drug and PABA restriction became patent. These animals were still clear after 20 days. (Had parasites been observed, as well as being drug and PABA tested, the experiment would have been repeated using smaller aliquots than 10^6 and 10^5.)

This negative result was taken to demonstrate the absence of pyrimethamine resistant PABA independent parasites among the products of the cross, supporting the conclusion that the increased level of PABA dependence found in pyrimethamine

resistant lines resulted from a pleiotropic effect induced by the primary genetic change.

Crosses 2a: 2b: 2c - AS P^R DW x AJ 4027; AS P^R DW x AJ 4028; AJ 4027 x AJ 4028. All these were of the type: pyrimethamine resistant, PABA dependent. While both parents in each of these three crosses had more or less indistinguishable phenotypes, they had been obtained independently and had possibly arisen due to mutations at different loci. It was the purpose of these crosses detect any recombination between these possible loci, by the use of a screening technique.

(i) Origin of parents: All three lines used in these crosses were made resistant to pyrimethamine by single step selection and had become concomitantly dependent on PABA. AS P^R DW, as mentioned previously, had been selected by Dr. D. Walliker of this department using 4 doses of pyrimethamine (50 mg.kg^{-1}); AJ 4027 and AJ 4028 had been selected using 4 doses of pyrimethamine (50 mg.kg^{-1}). A summary of the parental phenotypes has been presented in Table 18.

TABLE 18

Crosses 2a: 2b: 2c: parental phenotypes. Responses to pyrimethamine, dependence on PABA (pre Jan. 1975) and enzyme markers.

Line	Response to Pyrimethamine (ED_{90} ± ISE range) mg.kg^{-1}	Min. PABA suppl. for growth (gl^{-1})	enzyme variant	
			LDH	6PGD
AS P^R DW	19.5 (13.3-26.9)	0.025	3	2
AJ 4027	18.2 (13.0-25.7)	0.025	2	3
AJ 4028	7.3 (3.6-16.7)	0.025	2	3

(ii) Experimental details: 3 pair wise crosses were performed, the first of these (AS P^R DW x AJ 4027) was performed twice using both the standard crossing procedure and the modified procedure using splenectomised rats as a rodent carrier. The other two crosses were performed using the modified procedure only. Sporozoites were harvested as previously described.

Cross product bloods were examined electrophoretically in crosses 2a and 2b and parental co-transmission confirmed in both cases following the modified crossing procedure, but not in cross 2a following the standard procedure and this blood was discarded. Parental electrophoretic identity in the case of cross 2c prevented this check being made.

Serial dilutions of 10^6, 10^4 and 10^2 parasites per 0.1 ml were prepared from each of the remaining bloods and aliquots used to inoculate groups of 2 or 4 mice (♂♂C57) which had received no PABA supplement for at least 7 days. Mice receiving the full PABA supplement of 0.5 gl^{-1} were inoculated with 10^2 parasite aliquots as viability controls. A further group of 2 zero PABA mice were inoculated with 10^2 parasite inocula obtained from the AJ wild type line as a check on PABA dependence. Mice were checked for patent infections after 5 days and subsequently; the results of these tests are presented in Table 19.

TABLE 19 - on accompanying page

TABLE 19

Crosses 2a: 2b: 2c: growth of cross product parasites in mice receiving no PABA supplement. (pre Jan. 1975)

Composition of inoculum	Size of inoculum	mice inoculated		No. mice with patent infections
		No.	PABA supplement	
2a cross-prods	10^2	2	0.5 gl^{-1}	2
2b cross-prods	"	2	"	2
2c cross-prods	"	2	"	2
2a cross-prods	"	2	zero	0
2b cross-prods	"	2	"	0
2c cross-prods	"	2	"	0
2a cross-prods	10^4	2	"	0
2b cross-prods	"	2	"	0
2c cross-prods	"	2	"	0
2a cross-prods	10^6	4	"	0
2b cross-prods	"	4	"	0
2c cross-prods	"	4	"	0
AJ wild type	10^2	2	"	0

The failure of cross product parasites to induce patent infections in mice receiving no PABA supplement was taken to indicate the absence of PABA independent parasites amongst them. Thus there was no evidence of recombination between these 3 independently selected mutants suggesting that these loci resided in the same or adjacent genes. This was taken as evidence in support of the view that the phenotypic homogeneity of this class of mutant resided in their genetic homogeneity.

Cross 3: AS wild type x AJ 7031 (Sulphadiazine resistant). The main purpose of this cross was to verify that the sulphadiazine

resistance marker segregated in a mendelian fashion as a single genetic factor.

(i) <u>Origin and characteristics of parent:</u> The AS wild type line closely resembled its AJ counterpart in its response to pyrimethamine (see Table 3), in its PABA dependence (see Table 1) and in its response to sulphadiazine (see Table 2); but differed from the AJ line in its electrophoretic variants of 6PGD and LDH. The AJ line used in this cross, 7031, had been made resistant to sulphadiazine after receiving 4 doses of the drug (5 mg.kg^{-1}). It had acquired a two fold cross resistance to pyrimethamine also and grew to peak without PABA supplementation unlike its wild type mate in the cross. (All data post Jan. 1975). The phenotypes of the parents have been summarised in Table 20.

TABLE 20 - on accompanying page

(ii) <u>Experimental details:</u> The cross was performed using the modified crossing procedure with newly weaned splenectomised rats as rodent carriers. Each rat was inoculated intravenously with about 0.5 ml of peak infected blood composed of 2 parts AJ 7031 parasites to 1 part AS parasites. This ratio being chosen to partially compensate for the apparently greater fertility of AS parasites in this host (see Table 16). After 5 days exflagellation was observed and starved mosquitoes allowed to feed on the rats.

Sporozoites were harvested 14 days later as previously described and used to inoculate 3 C57 mice intravenously. All

TABLE 20

Cross 3: AS wild type x AJ 7031 (Sulphadiazine resistant) Parental phenotypes: responses to pyrimethamine and sulphadiazine and PABA dependence (post Jan. 1975).

Line	Response to Pyrimethamine ($ED_{90} \pm$ ISE range) $mg.kg^{-1}$	Response to Sulphadiazine ($ED_{90} \pm$ ISE range) mg.kg 0.5 gl^{-1} PABA post Jan.'75)	Minimum PABA growth suppl. required (gl^{-1})	Enzyme Variant	
				LDH	6PGD
AS wild type	0.58 (0.38 - 0.89)	61.0 (52.5 - 70.8)	0.025	3	2
AJ 7031	2.13 (1.58 - 2.91)	851 (372 - 1947)	Zero	2	3

of these mice became patently infected within 5 days indicating the original sporozoite infection to have been a heavy one.

Parental co-transmission was confirmed electrophoretically and parasites from 2 of the three mice cloned by dilution (0.33 parasites per inoculum). Of 103 mice so inoculated 14 became patently infected and 12 of these clones were tested for their responses to pyrimethamine and sulphadiazine and for their ability to grow in the absence of any PABA supplement along with samples from the parents making 14 in all. Control animals from each test were later sacrificed and the bloods harvested for electrophoresis. 11 C57 male mice were used in each test there being: 3 controls; 3 dosed with sulphadiazine (160 $mg.kg^{-1}$ x 4) and 3 dosed with pyrimethamine (1.25 $mg.kg^{-1}$ x 4). All of these received the normal PABA supplement of 0.5 gl^{-1}. In addition there were two animals which had received no PABA supplement for at least 7 days beforehand. Otherwise this 4 day test was conducted as previously described. Growth expressed as a percentage of the controls was calculated for both drug doses and PABA restriction. The results have been presented in Table 21.

Two clones (CX 304 and CX 309) whose responses to sulphadiazine rendered them difficult to classify were retested using the same dose (160 $mg.kg^{-1}$ x 4) of this drug. This time 4 mice were used in each treatment group.

After retesting the growth after drug treatment (expressed as a percentage of the control) was 7.02 (4.03 - 12.21) for clone CX 304 and 94.5 (59.4 - 150) for CX 309.

Using these retest data the mean percent growth after drug treatment with sulphadiazine (4 x 160 $mg.kg^{-1}$) was 124% for

TABLE 21

Cross 3: AS wild type x AJ 7031 (sulphadiazine resistant). Responses to Sulphadiazine and Pyrimethamine and growth in the absence of PABA supplement.

Clone/line	Sulphadiazine (4 x 160mg.kg^{-1}) (% growth ± ISE range)	Pyrimethamine (4 x 1.25mg.kg) (% growth ± ISE range)	PABA no supplement (% growth ± ISE range)	Enzyme Variant LDH	Enzyme Variant 6PGD
AS wild type	0.81 (0.40 - 1.62)	1.23 (0.58 - 2.62)	- < .186	3	2
AJ 7031	150 (47.9 - 469)	40.1 (10.7 - 151)	+ 389 (1244) (121)	2	2
CX 301	1.09 (0.43 - 2.77)	0.25 (0.13 - 0.50)	- < 0.11	3	2
CX 302	3.93 (1.41 -10.93)	14.95 (4.31 -51.87)	- < 0.84	3	2
CX 303	0.59 (0.23 - 1.51)	0.72 (0.28 - 1.86)	- < 0.25	3	2
CX 304	(8.90 (4.02 -19.7)) (7.02 (4.03 -12.21))*	0.53 (0.20 - 1.40)	- < 0.19	3	2
CX 305	0.68 (0.22 - 2.14)	2.38 (0.70 - 8.00)	- < 0.27	3	2
CX 306	1.19 (0.60 - 2.35)	0.68 (0.16 - 2.95)	- < 0.79	2	dubious
CX 307	4.34 (2.75 - 6.86)	1.08 (0.60 - 1.95)	- < 0.62	2	2
CX 308	no data	1.97 (0.76 - 5.10)	- < 0.23	2	2
CX 309	(20.6 (8.09 -52.6)) (94.5 (59.4 - 150))*	8.96 (4.04 -19.9)	+ 55.4 (18.6 - 165)	2	2
CX 310	91.2 (29.9 - 278)	44.9 (16.5 - 122)	+ 124 (65.6 - 234)	2	3
CX 311	137 (71.7 - 262)	107 (72.8 - 157)	+ 140 (110 - 178)	3	3
CX 312	203 (71.0 - 579)	145 (51.8 - 406)	+ 272 (95.8 - 722)	3	3

* = retested (+) = reached peak (-) = failed to grow

those clones which grew in the absence of PABA and 1.80% for the non-growers. The figures for pyrimethamine (4 x 1.25 mg. kg) were 50.0% for growers and 1.22% for non growers.

This enabled the 12 clones to be dichotomised: clones CX 301 to 308 all exhibited wild type responses to sulphadiazine and to pyrimethamine and in respect of their PABA growth requirements; on the other hand, clones CX 309 to 312 resembled the resistant parent both in respect of their responses to sulphadiazine and in their PABA growth requirements. While on average the latter clones appeared more resistant to pyrimethamine there was some overlap in respect of the measurements of this character and this has been considered in the discussion.

Both sulphadiazine resistance and PABA independence appeared to segregate together. Although this was probably true of cross resistance to pyrimethamine also the evidence was less clear cut. This suggested a pleiotropic origin for the correlated characters.

There appeared to be free recombination with both the enzyme markers 6PGD and LDH.

Cross 4: AJ wild type x AS 90301 (pyrimethamine resistant, PABA dependence wild type): detection of non parental phenotypes among cross products as evidence for recombination between markers conferring resistance to pyrimethamine and to sulphadiazine.

(i) Origin and characteristics of parents: Line AJ wild type has been described previously (see Tables 1-3). Line AS 90301 was obtained by selection in two steps: AS wild type was made 20 times resistant to pyrimethamine by direct exposure to

the drug (4 x 50 $mg.kg^{-1}$) in a single step (Walliker <u>et al.</u>, 1975); and this line which was PABA dependent was selected by PABA restriction (Tables 9-11) — this constituting the second step of selection. After the second step of selection which had increased the level of PABA resistance to between 35 and 61 (range of estimates, mean = 46) times that of the wild type AJ line used in the cross, the line was no longer PABA dependent before Jan. 1975 but required 0.025 gl^{-1} for normal growth after that date. Thus its PABA dependence (and sulphadiazine sensitivity) was indistinguishable from that of the wild type line in the cross. These phenotypes have been summarised in Table 22.

<u>TABLE 22</u>

Cross 4: AJ wild type x AS 90301: Parental phenotypes: responses to pyrimethamine and to sulphadiazine and PABA growth requirements before and after Jan. 1975.

Parent Line	Response to: ($ED_{90} \pm 1SE$) $mg.kg^{-1}$				PABA growth requirement (gl^{-1})
	Before/ After Jan.'75	Pyrimethamine	Sulphadiazine		
		($0.5gl^{-1}$) PABA	($0.5gl^{-1}$) PABA	Zero PABA	
AJ wild type	before	[1]0.87(1.41) (0.53)	no data	[4]0.054(.059) (.049)	Zero
	after	[1]0.98(1.10) (0.87)	[3]30.3(12.6) (51.3)	no data	0.025
AS90301	before	[2]53.5(85.5) (84.3)	no data	[4]0.060(.081) (.045)	Zero
	after	[2]34.5(46.3) (25.7)	[3]67.2(87.1) (51.9)	no data	0.025

1, not sig. diff. 2, not sig. diff.
3, " " " 4, " " "

(ii) Experimental details: The cross and preliminary analysis were carried out before Jan. 1975. However the detailed characterisation of clones was performed after that date and this has been indicated elsewhere as appropriate.

The cross was performed using the modified crossing procedure with newly weaned splenectomised rats as rodent carriers of the biparental blood mixture which was adjusted to contain equal numbers of parasites from each parent line.

The mixed parental blood meal was permitted to take place on the fifth day after these rat inoculations and sporozoite infections were induced by natural transmission. Cross product bloods were split into samples for cryopreservation and immediate analysis. The original biparental blood mixture was also preserved and used as a hybridisation control.

Enzyme electrophoresis confirmed parental co-transmission.

Dilutions containing 10^7 parasites per ml were prepared from both cross product and control samples. Aliquots (0.1 ml) from each sample were then used to inoculate two groups of mice (C57 male) receiving no PABA supplement: the first group received 1.25 $mg.kg^{-1}$ sulphadiazine and on the following 3 days; and the other group was inoculated with Na CMC only as a growth control.

Undrugged control mice inoculated with both blood samples became patent after 3 days. Drugged mice inoculated with cross product blood parasites became patent between 2 and 3 days later whereas those which had been inoculated with the control mixture remained clear of infection even after 16 days, suggesting that all the parasites had been eliminated. These data revealed the presence of a non parental class of parasites amongst the products of the cross: inferentially these were probably

TABLE 23

Cross 4: AJ wild type x AS 90301. Characteristics of 30 cross product clones and preliminary classification. (Data pre Jan. 1975)

	Enzyme Variant		Response to (%UNT. control)		% Growth Zero PABA (% Full PABA	Initial Classification
	LDH	6PGD	① Pyrimeth. 4x2.5mg.kg-1	② Sulphad. 4x1.25mg.kg		
401	3	2	72.8	- < 0.20	+ 46.4	)
402	3	2	36.3	- < 0.30	+ 74.4	)Sulphadiazine
403	3	2	74.6	- < 0.18	+ 101	)sens. PABA
404	3	2	41.9	- < 0.45	+ 933	)Independent
405	2	2	171	- < 0.42	+ 30.7	)Pyrimethamine
406	3	-	122	- < 0.86	+ 30.2	)Res.
407	3	2	58.4	- < 0.17	+ 104	)(Parental
408	3	2	54.5	- < 0.22	+ 37.0	) Type 1)
409	2	2	56.6	- < 0.22	+ 52.1	)
410	2	2	211	- < 0.24	+ 46.3	)
411	3	3	116	- 0.31	+ 38.6	)
412	3	3	216	- 0.26	+ 99.8	)
413	3	3	34.7	- 0.10	+ 15.2	)
④ (414	Ambi.	2	45.8	- < 0.42	+ 45.4	)
415	3	2	213	③ -	- < 0.66	)Sulph. Sens.
416	3	2	10.0	③ -	- < 0.11	)PABA
417	3	2	12.5	③ -	- < 0.15	)Dependent
418	3	2	301	③ -	- < 1.05	)Pyrimeth.
419	3	3	16.9	③ -	- < 0.26	)Res. (Non
420	3	3	2230	③ -	- < 3.1	)Parental Type
421	2	3	119	③ -	- < 0.27	) 2)
422	2	3	< 0.66	- < 0.48	+ 48	)Sulphad. Sens.
423	2	3	< 1.61	- < 0.41	+ 141	)PABA Independ.
424	2	3	1.73	- < 0.27	+ 317	)Pyrimeth.
425	2	3	< 1.89	- < 1.89	+ 134	)Sens. (Parental
426	2	3	< 0.16	- < 0.16	+ 14.2	)Type 2)
427	2	3	< 0.22	- < 0.22	+ 137	)
428	2	3	< 1.22	- < 0.83	+ 3.0	)
429	2	3	46.9	+ 6.74	+ 154	)Sulphad. Res.
430	2	3	2.9	+ 31.7	+ 464	)PABA Independ.

Variable
Resp. to
Pyrimeth.
(Non Parental
Type 1)

/Cont

TABLE 23

CONTINUATION

	Enzyme Variant		Response to (%UNT Control)		% Growth Zero PABA (% Full PABA	Initial Classification
	LDH	6PGD	① Pyrimeth. 4x2.5mg.kg^{-1}	② Sulphad. 4x1.25mg.kg		
AS90301	3	2	124	- 0.48	+ 68	First Parent
AJ Wld Type	2	3	0.39	- 0.91	+ 90	Sec. Parent

① normal PABA supplement
② normal PABA supplement
\+ reached peak
\- failed to grow

③ quantitative drug response presented in Table 24 clones 415-421 failed to grow on zero paba which was used as a control for these estimates therefore no estimates of % growth was possible

④ because of double enzyme type this line was recloned and retested (LDH 2 6PGD 3)

sulphadiazine resistant and had arisen as a result of genetic recombination.

30 dilution clones were then established over a period of time from the cross products and each of these tested for its responses to pyrimethamine (4 x 2.5 $mg.kg^{-1}$, normal PABA); to sulphadiazine (4 x 1.25 $mg.kg^{-1}$, zero PABA); and for their ability to grow in the absence of a PABA supplement. Untreated control mice were later sacrificed and their bloods freeze dried and enzyme tested (LDH and 6PGD). The results of these tests have been summarised in Table 23.

Following consideration of the data a 4 way classification was constructed comprising 2 parental types and 2 non parental types. In order to further test the validity of this classification, 7 clones were retested after Jan. 1975 and their quantitative responses to sulphadiazine, to pyrimethamine and their PABA growth responses estimated: 1 clone representing each parental category, 2 clones representing each non parental category and CX 414 the presumptive double clone. These data together with relevant comparison data have been summarised in Table 24.

These data (in Table 24) were taken to confirm the generation of two non parental classes of parasite phenotype among the products of the cross. These seemed to most closely resemble the two classes of mutant previously obtained by single step selection using pyrimethamine and sulphadiazine (or PABA withdrawal) - respectively.

Exploiting the rise in PABA dependencies after Jan. 1975 a series of reconstruction experiments was undertaken whose purpose was to measure the relative frequency of recombinant parasites among the products of the cross and thereby estimate

TABLE 24

Cross 4: AJ wild type x AS 90301 (Pyrimethamine resistant). Responses to Pyrimethamine and Sulphadiazine (0.5 gl PABA supplement) and growth in the absence of PABA. (Data obtained after Jan. 1975). Comparison data included.

Line/ Description	Response to (ED_{90} ± ISE range mg.kg^{-1}		Growth in the absence of any PABA supplement
	Pyrimethamine	Sulphadiazine	
AJ wild type	0.98 (0.87-1.10)	80.3 (57.3-126)	-
AS 90301	34.5 (25.7-46.3)	37.2 (51.9-87.1)	-
AJ 7032	2.13 (1.58-2.91)	851 (372 -1947)	+
AS P^R DW	19.1 (13.8-26.3)	3.35 (2.76-4.12)	-
CX 414	0.63 (0.47-0.86)	63.8 (52.5-77.62)	-
CX 401	45.4 (33.1-62.4)	57.5 (48.9-67.6)	-
CX 420	20.4 (13.5-30.9)	3.64 (2.82-4.70)	-
CX 421	17.2 (12.6-23.4)	3.50 (2.75-4.44)	-
CX 422	0.40 (0.28-0.57)	67.6 (52.5-87.1)	-
CX 429	1.41 (0.97-2.04)	204 (174 - 240)	+
CX 430	2.75 (1.91-3.96)	197 (104 - 372)	+

the frequency of recombination between the genes proposed to confer primary resistance to pyrimethamine and sulphadiazine.

To achieve this aim a series of dilutions was prepared from the products of the cross and aliquots from these used to inoculate 2 series of mice (C57 ♂♂) receiving the full PABA supplement (0.5 gl^{-1}), and no supplement. Control inocula were prepared from parasites obtained from the biparental mixture. The results of these tests are presented in Table 25.

TABLE 25

Cross 4: AJ wild type x AS 90301: Infectivity of 1 and 10 parasite inocula in the absence of PABA supplement. (Data post Jan. 1975)

Line	mean no. parasites per inoculum	PABA supple.	No. mice inocul.	No. sub-seq. patently infected	Poisson Expect-ation
Cross 4 prods.	1	0.5gl^{-1}	20	1	2.5
"	10	"	"	13	15
"	10^6	"	2	2	
"	1	Zero	20	9	12.5
"	10	"	"	18	20
"	10^6	"	2	2	
biparental mixt.	"	0.5gl^{-1}	"	2	
"	"	Zero	"	"	

Comparison of the number of patently infected animals in the test groups with those expected on the basis of the Poisson distribution (assuming 12.5% of cross product parasites to be PABA independent), showed no significant departures from that expectation. This suggested that there was no detectable linkage

between the genes proposed to control resistance to sulphadiazine and to pyrimethamine.

(iii) Analysis by examination of single oocysts obtained by microdissection: The parent lines used in Cross 4 proved to be fertile despite multi blood passage (AS 90301 produced abundant gametocytes and vigorous exflagellation even after some 100 serial passages). This and the fact Cross 4 had been analysed in the greatest detail of all the crosses described in this study, led to its choice for analysis by examination of single oocysts.

Bloods from both parents were harvested from splenectomised rats on the fifth day after inoculation and the mixture adjusted to contain equal number of exflagellating gametocytes from each parent.

The mixed bloods were fed to cages of starved mosquitoes using the membrane feeding apparatus already described. Mosquitoes were dissected on or before the sixteenth day after feeding and guts with ruptured oocysts discarded.

The remaining guts were then torn with sharpened tungsten needles to yield fragments containing 1 or 2 oocysts: Manipulation was continued until isolated oocysts remained. Each of these was removed in a pasteur pipette or on the end of a tungsten needle to the first of the three depressions on the water cooled slide and washed. After a second wash each oocyst was ruptured in the third of these depressions and the resultant sporozoite suspension injected into a stock mouse by the intravenous route. Both the apparent maturity of the oocyst and the ease with which the suspension was injected were noted. Control mice were inoculated with the bulk of the second oocyst wash.

Out of 30 single oocysts injections, 13 were considered to be 'good' injections of mature oocysts.

Only 1 mouse inoculated with a single oocyst subsequently became patently infected and that after 12 days. None of the control mice became patently infected.

Enzyme electrophoresis showed this infection to possess the variants peculiar to the sensitive parent only: (LDH 2 6PGD 3). However subsequent tests showed this infection to contain parasites able to grow in the absence of a PABA supplement, and so in all probability derived from a hybrid oocyst. Nevertheless, in view of the time taken for the infection to become patent and more importantly, on the grounds of enzyme monomorphism, the parasites were not further analysed.

The probable reason for the failure of this cross has been briefly stated in the text of the discussion.

<u>Cross 5</u>: AJ 7032 (Sulphadiazine resistant) x AS P^R DW (Pyrimethamine resistant). Cross conducted and analysed after Jan. 1975. This cross, the reverse of cross 4, was performed to show that multiple drug resistance may be generated by recombination as well as being broken up by it.

(i) <u>Origin of Parents</u>: Both parent lines have been described previously (AJ 7032, Table 3: AS P^R DW Walliker <u>et al</u>., 1975); a summary of their phenotypes has been included in Table 27.

(ii) <u>Experimental details</u>: The cross was performed after Jan. 1975 by the modified crossing procedure using splenectomised rats as rodent carriers of the mixed parental blood infection and starved mosquitoes were permitted to feed on these rats on the sixth day of infection: i.e. when the gametocytogenesis had reached a maximum. These mosquitoes were starved a second time 15 days after the blood meal and allowed to feed on stock mice

two days later. Patently infected mice were sacrificed and exsanguinated and the bloods used to prepare samples for electrophoresis. The presence of both parental enzyme types confirmed the transmission of both parents. 14 clones were obtained from the products of the cross by dilution (0.25 parasites/inoculum). Each of these was tested for its ability to grow at two PABA levels (0.025 gl^{-1} and zero PABA) and at two doses of pyrimethamine (4 x 1.25 $mg.kg^{-1}$ and 4 x 40 $mg.kg^{-1}$). Bloods harvested from control mice were subsequently used to provide samples for electrophoresis. The results of these tests have been summarised in Table 26. (Anomalous clones CX 507 and 513 were retested and these data used for classification). Two clones CX 512 and CX 514 appeared to be non parental after the preliminary tests and these clones were tested again and their quantitative responses to pyrimethamine and sulphadiazine estimated. These data together with relevant comparison data have been summarised in Table 27. (The pyrimethamine response of clone CX 512 was re-estimated after a second series of tests and this figure is also included).

TABLE 26 - on accompanying page

TABLE 26

Cross 5: AJ 7032 x AS P^R DW: Growth (expressed as a percent of the control) of cross-product clones at two restrictive PABA tests and at 2 pyrimethamine dose levels. (Data after Jan. 1975) Comparison parental and mutant line data included)

line/ clone	% Growth				Clone Classification	
	PABA supplement		Pyrimethamine (mg.kg x 4)			
	.025mg.kg^{-1}	Zero	1.25	40		
clone CX501	+ 32.7	+ 14.7	28.0	< 0.26	pr.	(res.AJ7032)
" CX502	+ 102	+ 16.3	11.6	< 0.11	"	"
" CX503	+ 37.9	+ 3.9	1.51	< .066	"	"
" CX504	+ 286	+ 177	11.2	< 0.45	"	"
" CX505	+ 44.2	+ 2.30	13.6	< 0.20	"	"
" CX506	+ 528	+ 12.6	55.7	< 0.86	"	"
" CX507*	+ 254	+ 177	107	< 0.46	"	"
" CX508	- < 0.14	- < 0.14	43.5	0.91	"	(res.ASP^{r}DW)
" CX509	- < 0.11	- < 0.11	205	0.117	"	"
" CX510	- < 0.12	- < 0.12	283	0.44	"	"
" CX511	- < 0.056	- < 0.056	47.8	0.076	"	"
" CX512	+ 38.6	- < 0.22	65.0	54.3	npr.	(res.AS90301)
" CX513*	+ 94.9	- < 0.27	116	26.9	"	"
" CX514	+ 183	- < 0.22	1.10	< 0.22	"	(res.AJ w.typ)
AJ 7032	+ 78.8	+ 62.3	5.59	< 0.16	–	–
AS P^R DW	- < 0.13	- < 0.18	45.2	1.78	–	–
AS 90301	+ 5.02	- < 0.20	102	17.9	–	–
AJ w.typ.	+ 185	- < 0.27	9.53	< 0.27	–	–

* after retesting
± growth/no growth
res. = resembled

pr. = parental
npr = non parental
w. typ. = wild type

TABLE 27

Cross 5: AJ 7032 x AS P^R DW: Clones CX 512 and 514 responses to pyrimethamine and sulphadiazine. Comparison data obtained from both parents and AS 90301 and AJ wild type included. (Data after Jan. 1975) (All mice received the full PABA supplement of 0.5 gl^{-1})

Line	Pyrimethamine (ED_{90} ± ISE range) $mg.kg^{-1}$		Sulphadiazine (ED_{90} ± ISE range) $mg.kg^{-1}$	
clone CX 512	22.0	(17.4-27.7)	31.6	(15.1-66.1)
CX 514	1.04	(0.81-1.32)	55.3	(38.1-80.4)
CX 512 retest.	38.5	(30.5-48.4)	-	-
AJ 7032	2.13	(1.58-2.91)	851	(372 -1947)
AS PR DW	19.1	(13.8-26.3)	3.35	(2.76-4.12)
AS 90301	34.5	(25.7-46.3)	67.2	(51.9-87.1)
AJ wild type	0.98	(0.87-1.10)	80.3	(51.3-126)

± growth/no growth

These data were taken to demonstrate that genetic recombination had generated both double mutant and wild type resembling parasites and thus mimiced the reverse of the process thought to have occurred in the case of Cross 4.

This cross has been further discussed in the text.

Cross 6: AJ 7032 (Sulphadiazine resistant) x AJ rat adapted.

(i) Origin and characteristics of Parents: Both lines have been described previously: AJ 7032 (see Table 8 p 54) AJ rat adapted (see p 66 Table 15).

The aim of the cross was to establish the suitability of the rat adapted marker for use selectively in crosses with drug resistance mutants; whether the inheritance of this character was mendelian and if so whether polygenetic or not. An ideal selective marker would have been inherited by a single gene.

(ii) Experimental details: This cross was performed after Jan. 1975 by the modified technique using splenectomised rats as rodent carriers of the biparental mixed bloods. It was also repeated using the membrane feeding apparatus with a blood mixture containing parasites obtained from mice on or around the tenth day of infection in the case of both parents. In both cases the blood mixtures were adjusted to contain equal numbers of gametocytes from each parent.

In both crosses sporozoites were harvested by allowing infected mosquitoes to feed on stock mice. Parasites from these mice were used to subinoculate both adult intact rats and mice receiving no PABA supplement. Both groups of animal became patently infected and this was taken to confirm biparental transmission in the case of the cross performed using splenectomised rats as rodent carriers, but in the case of the cross performed using the membrane feeding apparatus no rat adapted parasites were detected.

In the successful cross a screening technique was employed to detect recombination between the genetic elements controlling

sulphadiazine resistance and adaptation to growth in rats. 8 newly weaned intact rats receiving no PABA supplement were each inoculated with 10^6 parasites obtained from the cross product bloods. Control animals, C57 mice receiving no PABA supplement and newly weaned intact rats receiving the normal PABA supplement, received the same inoculum. Only the control animals became infected.

Lest the rats provided a different PABA environment from mice although preliminary evidence seemed to militate against this the screen was repeated in a modified form. Thus parasites harvested from the control mice in the first screen were used to inoculate intact rats receiving a full PABA supplement. Different groups of these rats were inoculated with a range of aliquots containing 10^2, 10^4 and 10^6 parasites. None of these became patently infected.

This negative result was open to ambiguous interpretations and these have been considered critically in the discussion Section V, cross 6.

DISCUSSION

1. Baseline Data:

Reference to Table 1 which summarises the effects of different PABA levels on the growth of wild type lines under different conditions both before and after Jan. 1975, allows two main conclusions to be drawn. Neither the supplementation of the drinking water with prednisone (40 $mg.l^{-1}$) nor the substitution of a milk diet for the natural solid rat cake had any discernible effects on the PABA requirements of the wild type parasites. While the first of these two tests was merely a formal control for some of the selection experiments in which prednisone was used as an immunosuppressive agent, the latter suggests that at least after Jan. 1975 there were very few PABA related compounds contained in the rat cake available for parasite utilisation since it was known that milk diets are deficient in these (Hawking, 1953). This is specially relevant in view of the major evidence summarised in Table 1 which suggests that both wild type lines used in the present study (AS and AJ) needed a PABA supplement of 0.025 gl^{-1} to reach peak parasitaemias after Jan. 1975 whereas prior to that date no such supplement was necessary. That this finding was just as applicable to the other lines used in this study for which data are available can be seen from Table 28.

TABLE 28 - on accompanying page

TABLE 28

Levels of minimum PABA supplements required for growth to peak levels by wild type and resistant lines of P.chabaudi before and after Jan. 1975. (All mice fed on rat cake).

line	minimum PABA supplement for growth to peak (gl^{-1})	
	Before Jan. 1975	After Jan. 1975
AS wild type	zero	0.025
AJ wild type	"	"
AS P^{R} (DW)	0.025	0.050
AJ 4027	"	"
AJ 4028	"	"
AJ 7031	zero	zero
AS 90301	"	0.025

The sulphadiazine resistant line (AJ 7031) was evidently unaffected by the change whatever its cause - but this was only to be expected in view of the association between PABA independence and sulphadiazine resistance: the line maybe viewed as being so resistant as to be immune from minor fluctations in the availability of PABA related compounds. Had curves of PABA dependence against date of estimate been plotted for each of these lines these would have all been flat with a step-up during the period around Jan. 1975 - excepting the case of AJ 7031 whose plot would have remained flat the whole time.

Since this upward quantum jump in PABA requirements affected all lines to about the same extent - leaving aside AJ 7031 as a special case - a common explanation was sought. Enzyme electro-

phoresis ruled out the possibility of a mistake having occurred and fresh and old supplies of PABA gave identical results. Multi passaged lines and lines which had recently been mosquito passaged exhibited identical PABA requirements so parasite ageing was ruled out also and anyway this was not a common explanation.

The most plausible explanation was that the manufacturers had knowingly or unwittingly allowed the composition of the rat cake to have been changed - although direct enquiry was unable to elicit confirmation of this. Comparisons of the new rat cake with milk diets were unable to detect any differences in the ability to sustain parasite growth at a range of PABA levels (Table 1). This suggested that the quantity of PABA related compounds in the new rat cake was very low since milk diets are known to be deficient in these (Hawking, 1953). Unfortunately none of the old rat cake had remained for comparison, nor had milk diet studies been carried out prior to Jan. 1975. Thus no direct evidence of the hypothesis was available.

All estimates of sulphadiazine resistance obtained before and after Jan. 1975 used mice receiving no PABA supplement. Since after this date only sulphadiazine resistant lines were able to grow under these conditions direct comparisons of the responses of these lines only were possible. Thus, while the ED_{90} for sulphadiazine was estimated to be 1.25 $mg.kg^{-1}$ before Jan. 1975, the estimate obtained after that date had dropped to 0.095 $mg.kg^{-1}$ in the case of AJ 7031. The quantitative nature of the antagonism with sulphadiazine has been investigated by Thurston (1954) and given this, the difference between these two estimates would seem indirectly to have suggested that the amount of PABA related compounds available to the parasites had

diminished between the dates of the estimates.

Although the baseline responses of wild lines to sulphadiazine (Table 2) agreed closely with those of Peters (1967) the similarity was probably fortuitous in view of PABA antagonism and the difficulty in standarising the level of PABA supplementation between laboratories. The dramatic increase in baseline estimates of sulphadiazine resistance after Jan. 1975 merely reflected the increased level of PABA supplementation occasioned by the inability of most lines to grow in the absence of this compound.

Reference to the baseline pyrimethamine response data summarised in Table 3 shows that here too, resistance seemed to be related to the size of the PABA supplement but the relationship would seem to have been less straightforwardly competitive than that reported by Thurston for sulphadiazine. It has been shown graphically in the case of an AJ wild type line in Fig. 5. Thus while raising the PABA supplement from zero to $0.05gl^{-1}$ effectively increased the resistance of this line some sixfold further increases in the level of the PABA supplement up to $1.0gl^{-1}$ had no effect or sensitivity. (This type of saturation curve was also found in the case of AS 90301, the doubly resistant mutant line).

It is possible that the discrepancy in base line sensitivity estimates in P.chabaudi between those of the present author and those of Peters (1967) (his line was some 15 times more sensitive) may to some extent be accounted for by the routine use of rather high levels of PABA supplement in this laboratory. This may not be such a bad thing since it follows from the foregoing discussion that more reproducible estimates of drug

resistance will be obtained in the region of PABA levels encompassed by the flat part of the pyrimethamine response/PABA level curve since these will be less labile to minor fluctuations in PABA supply such as might be expected between batches of rat cake and further aggravated by the tendency of animals to vary their intake of food and water. (Another reason for maintaining animals on high level PABA regimes is to avoid unwanted selections for sulphadiazine resistance.) On the other hand it is clearly preferable to obtain estimates of sulphadiazine sensitivity without any PABA supplement since total restriction probably offers the best means of maintaining a constant supply of this substance that is, if the parasites will grow under these conditions.

The close correspondence between estimates of pyrimethamine sensitivity obtained before and after Jan. 1975 (Table 3) is unremarkable. From consideration of the pyrimethamine sensitivity/PABA level curve it follows that the removal of 0.025 gl^{-1} equivalent-PABA (the probable size of the PABA deficit in the new rat cake) would not shift the amount of residual PABA off the flat part of the curve and would therefore be unlikely to affect pyrimethamine sensitivity.

Finally, the data in Table 3 demonstrate that the AS and AJ wild type lines were indistinguisable on the basis of their responses to sulphadiazine and to pyrimethamine: i.e. there was no evidence of wild resistance to these drugs or that either had acquired resistance inadvertently such as by PABA restriction. This was a subtly important result since it implied genetic uniformity - a vital factor if these lines were to be used in crosses together or to be compared in selection experiments.

2. Selection Experiments:

Table 4 summarises the results of the pyrimethamine selection experiments all of which were carried out before the PABA shift in Jan. 1975.

Two types of experiment were performed as judged by the intensity and prolongation of drug pressure: experiments 401 to 407 inolved the use of short-term, high-dose therapy whereas in those numbered 409 to 415 a lower dose was used and therapy maintained till the emergence of recrudescent lines or 20 days whichever was sooner. (Control studies showed that no recrudescent lines emerged after this time suggesting the complete elimination of parasites.) Experiment 408, intermediate in character between these two classes, was designed to facilitate comparisons between them. The results of the preliminary tests performed on recrudescent lines following selection have been presented in Table 5. More rigorous quantitive drug responses and PABA dependences have been summarised for some of these lines in Table 6. While the latter data were used in drawing up the basis of a classification the preliminary data in Table 5 were used in classifying these lines where no further tests were performed. Thus the numbers of lines scored were enlarged for statistical reasons when calculating frequency estimates. (This procedure has been followed in all the selection experiments in this study.)

Recrudescent lines following pyrimethamine selection were divided into two main classes on the basis of these responses: resistant lines which resembled AS P^R DW; and sensitive lines which were indistinguishable from wild types. The line AJ 4139 was considered to be anomalous i.e. fitting into neither of these

categories. Since further tests revealed this line to be sulphadiazine resistant (see Table 8) it has been discussed along with the other sulphadiazine resistant lines later on in this section. The sensitive lines were considered to consist of wild parasites which had survived selection for any reason and were not further investigated.

All the so-called pyrimethamine resistant lines were obtained following direct exposure to the drug. They were characteristically about 19 times resistant to pyrimethamine: the mean ED_{90} (n = 9) was 16.41 ($\pm$ ISE range 14.61 to 18.43 mg. kg^{-1}). They were about 0.04 times resistant to sulphadiazine (0.5gl^{-1} PABA supplement, after Jan. 1975) though too few estimates were made to attach a range to this figure. All of these lines required a dietary PABA supplement to reach peak growth: the supplements being 0.025gl^{-1} before Jan. 1975 and 0.05gl^{-1} after this date. In this last respect they may be said to have resembled the lines described by Jacobs (1964) in P.berghei; and in P.yoelii by Morgan (1974) which she termed 17 x PRI. Thus the resistant lines formed a fairly homogeneous group and this phenotypic similarity was taken to be suggestive of genotypic similarity. Although it is tempting to invoke the absence of lines intermediate in character between these resistant lines and the sensitives as strong evidence that a point-mutation was responsible for conferring pyrimethamine resistance both the imprecision of drug tests and the possibility that the modes of selection imposed a "procrustean bed" on the types surviving drug pressure, cannot be ruled out. However such genetic evidence as was available (see crosses 1 and 2) was also in favour of the single gene hypothesis and for the purpose of

calculating acquisition rates this hypothesis was taken as given.

As drug dosage increased while the frequency of recrudescence decreased, the proportion of resistant lines among them increased (see Table 4). This was expected and leads to the notion of an optimum selection pressure for estimating acquisition rates. In the case of the short-term, high-dose experiments this ideal was most closely approached in those experiments where 4 doses of 50 $mg.kg^{-1}$ were administered; and in the case of the continuous dose experiments by those in which 5 or 10 $mg.kg^{-1}$ were administered. By confining frequency estimates to these regimes this was found to be 1.66×10^{-11} in the case of the short-term, high-dose experiments - agreeing very closely with the figureof 1.59×10^{-11} obtained by Morgan (1974). However the estimate obtained from the continuous dose experiments was some six times more frequent at 1.06×10^{-10}.

The reason for this discrepancy probably lay in the use of such high dose regimes themselves under which even resistant parasites were probably not immune from damage and that sustained just at the time when numerically they were most susceptible to chance elimination (see Martin and Arnold 1968). In contrast the continuous dose regimes can be plausibly pictured as imposing a drug pressure which, while sufficient to gradually eliminate sensitive parasites, would have imposed only the mildest of restraints on the multiplication of resistant parasites.

Moving on to the experiments in which pyrimethamine was administered to animals receiving no PABA supplement (Table 4, expts. 403 and 411) it may be seen that no parasites of any sort survived such treatment. These results were not unexpected

in view of the pyrimethamine hypersensitivity induced by PABA restriction (see Table 3 and Fig. 5) and the inability of pyrimethamine resistant lines to grow in the absence of PABA.

In expts. 403 and 404 it had been hoped to accelerate the development of resistance to pyrimethamine by immunosuppression either using the steroid prednisone administered in the drinking water or by splenectomy. (Care had to be exercised in the choice of mice for these experiments since it was found that other than SPF mice tended to become sick after exposure to prednisone presumably on account of the consequent liberation of subpatent non pathogenic infections by organisms of unknown origin.) In fact a result opposite to that hoped for was obtained and only one resistant line was thus obtained corresponding to a rate of 5.7×10^{-12}. On the other hand many more sensitives seemed to survive drug treatment than had been the case without immuno-suppression (5.1×10^{-11} (data from splenectomy and prednisone treatment pooled) as opposed to 0.47×10^{-11} (no immuno-suppression)). This was taken to support the view that drug treatment may act in concert with host immunity in eliminating parasites during selection. Since the response of parasites to pyrimethamine seemed unaffected by the presence of prednisone (Table 3) the most likely explanation for its effect of re-versing the ratio of sensitives to resistants when used in selection experiments is that this co-operation between the drug and the hosts immuno system is synergistic. Thus, while normally we may picture the hosts immune system picking out the more damaged sensitive parasites, but unable to affect the less damaged resistant ones after heavy drug treatment, no such discriminatory mechanism would be operable under

immunosuppression thus allowing time for these sensitive parasites to convalesce. This is really equivalent to stating than in the present case immunosuppression defeated its own purpose.

Alternatively, though perhaps less plausibly, one might explain the apparent deficiency of resistant parasites by stating that their appearance had been merely masked by a majority of sensitive parasites. There are two main objections to this proposal however. Firstly, such evidence as was available from drug tests suggested that such a numerical superiority would have had to have been overwhelming (in the order of about 10 to 1) to disguise the presence of resistant parasites. Secondly, such an explanation would have had to have taken into account the assumed coincident recrudescence of sensitive parasites and the resistant parasites supposedly masked by them.

Immunosuppression has been used to promote the acquisition of drug resistance in Trypanosomes. Following the pioneer efforts of Jansco and Jansco (1934), Schnitzer <u>et al.</u> (1946) obtained a higher rate of P-rosaniline resistance in <u>T.equiperdum</u> after splenectomising the rodent hosts. More recently, Hawking and his co-workers (Hawking, 1966, Hawking and Gamage, 1967) obtained resistance to chloroquine in <u>P.berghei</u> following immunosuppression effected by injections of ethyl palmitate; however their data were insufficient to say whether this rate was any higher than would have been the case without immunosuppression.

The details of the sulphadiazine selection experiments have been summarised in Table 7 and the characteristics of the resistant lines so obtained, have been summarised in Table 8. For ease of comparison only the response data obtained after Jan. 1975 have been included. Lines 7051 to 7056 were obtained by

direct exposure to the drug before Jan. 1975 and lines 8015 to 8017 by PABA withdrawal after that date when it had become an absolute requirement for the growth of wild type lines. It was impossible to identify the mode of selection used to obtain resistant lines either on the basis of their responses to sulphadiazine or to pyrimethamine as both of these characters appeared to vary continuously. To what extent this was merely due to the imprecisions of the tests used, remains unknown.

Thus pooling all data the sulphadiazine resistant lines (n = 8) were 3.80 times resistant to sulphadiazine: ED_{90} = 305.1 (± 1SE range, 254.2 to 366.1 $mg.kg^{-1}$). None required PABA for growth either before or after Jan. 1975. Line AJ 4139, the so called anomalous line obtained after exposure to pyrimethamine (2.5 $mg.kg^{-1}$ continuously) had a similar response to sulphadiazine: ED_{90} = 478 (± 1SE range 274 to 619 $mg.kg^{-1}$) and needed no PABA supplement either. Its ED_{90} for pyrimethamine of 2.48 (± 1SE range 1.81 to 3.59 $mg.kg^{-1}$) while significantly higher than that of sensitive lines was indistinguishable from those of the sulphadiazine resistant lines whose mean ED_{90} was 1.68 (± 1SE range 1.48 to 1.91 $mg.kg^{-1}$). Though as a group this was just significantly higher than that of the wild types, individuals were in some instances not so distinguishable. Morgan (1974) also reported an instance where a sulphadiazine resistant line had been selected indirectly in this way. There seemed to be no greater cross resistance to pyrimethamine exhibited by lines obtained by direct exposure to the drug than those obtained by PABA restriction which contrasts with the findings of Nowell (1970, 1972) in the case of P. berghei. Nor was there any evidence that cross-resistance to pyrimethamine had manifested

itself prior to the expression of the primary resistance itself (Bishop and McConnachie, 1950c) though the experimental conditions were not really conducive to recognising such a phenomenon.

Although it was impossible to further classify the sulphadiazine resistant lines on the basis of their responses to pyrimethamine, it was far less certain than in the case of the pyrimethamine resistant lines that they formed a homogeneous category; and although the acquisition frequency estimates have ignored such a possible heterogeneity this caveat should be borne in mind. (In fact, evidence presented in the next section on selection experiments using mutant lines would appear to support the notion of at least two classes of sulphadiazine resistant mutant.)

Among the sulphadiazine selection experiments described in Table 7, the optima for obtaining acquisition frequency estimates were judged to be found in the case of the short term, high dose experiments in those in which 4 doses of 2.5 or 5 $mg.kg^{-1}$ were administered. The pooled acquisition estimate from these was 3.5×10^{-11}. The PABA withdrawal experiments yielded a rate of 1.34×10^{10} which rose without statistical significance to 2.03×10^{10} when prednisone was administered in the drinking water as an immunosuppressive.

In short therefore, all these varieties of selective treatment had succeeded in producing only two classes of resistant parasite: pyrimethamine resistant with an increased dependence on PABA; and sulphadiazine resistant with a reduced dependence on this compound. In addition it was found that continuous selection pressure maintained until the emergence of

recrudescent lines was the most efficient means of producing resistant lines of parasite. The significance of this in calculating true mutation rates will be considered in the general discussion.

3. Selection Experiments Using Mutant Lines:

In Tables 9 - 12 are summarised the results of selection experiments involving the withdrawal of PABA from PABA dependent pyrimethamine resistant lines. Expts. 901 and 902 were performed before Jan. 1975 and expt. 904 was performed after that date. As has already been noted, the PABA requirement of pyrimethamine resistant lines doubled from 0.025 $mg.kg^{-1}$ to 0.05 $mg.kg^{-1}$ after Jan. 1975 and the selection pressure imposed by removal of PABA may be regarded as having been correspondingly intensified. Thus the failure to obtain recrudescent lines after that date was not unexpected. In the experiments performed prior to the increase in PABA requirements, all recrudescent lines tested proved to have regained independence from PABA without loss of pyrimethamine resistance (n = 44). It had been hoped to isolate wild type revertant parasites in this way and their absence from among those recrudescent lines characterised suggested that the reversion rate for pyrimethamine resistance was very low.

On the contrary PABA independence had been accompanied by a further increase of pyrimethamine resistance amounting to some 1.7 times that of the line subjected to continued selection (with a range from 0.86 to 2.74). The mean ED_{90} for the seven lines tested was 32.98 $mg.kg^{-1}$ ($\pm$ 1SE range 28.43 - 38.26 $mg.kg^{-1}$). This continuum of cross resistance closely paralleled that observed among the recrudescent lines isolated after PABA withdrawal and

sulphadiazine selection of wild type lines. It seemed likely therefore that the result of PABA withdrawal had been to superimpose a second genetic change which on its own would have conferred sulphadiazine resistance. However the pre-existing PABA independence had so to speak cancelled out this sulphadiazine resistance. In fact, when the responses to sulphadiazine of one of these lines (AS 90301) was compared with those of a wild type line, very little difference was to be observed (see Table 22) and this supposition was later confirmed genetically (see crosses 4 and 5).

The rate of acquisition of this resistance as obtained from expt. 901 was 1.9×10^{-10} agreeing closely with the rate observed with the wild genetic background. The rate after immuno-suppression was not significantly altered at 1.85×10^{-10}, again resembling the result obtained from the wild type selection experiments. However these results do not necessarily prove that there were no immune factors operative which might have reduced the observed rate of resistance acquisition to below that of true mutation rate. Furthermore, though all the recrudescent lines were homogeneous with regard to their responses to sulphadiazine or PABA, this does not necessarily imply genetic homogeneity. The extent of the range of cross resistances to pyrimethamine was some threefold and random errors might have disguised the presence of two or more classes of mutant.

In Tables 12 and 13 are summarised the results of selection experiments carried out after Jan. 1975 in which AJ 7032 which had already been made resistant to sulphadiazine by direct exposure to the drug (4×5 mg.kg^{-1} with no PABA supplement - before Jan. 1975) was subjected to continuous pyrimethamine

therapy for up to 20 days at doses of 10, 20 or 40 $mg.kg^{-1}$. This line had acquired a twofold cross resistance to pyrimethamine (ED_{90} = 2.19 $mg.kg^{-1}$) simultaneously with being made sulphadiazine resistant and was of course PABA independent both before and after Jan. 1975.

Recrudescent infections were only detected in the animals which received 10 or 20 $mg.kg^{-1}$ of pyrimethamine. These arose at rates of 1.0×10^{-10} at both levels of drug pressure i.e. not significantly different from the rates of recrudescence when wild parasites were subjected to continuous drug pressure with pyrimethamine at the correspondingly lower doses of 10 and 5 mg.kg. Thus as was the case with the acquisition of sulphadiazine resistance following PABA restriction the presence of the allele conferring resistance to the other drug did not appear to have any effect on the rate at which resistance was acquired.

Classification of recrudescences was based on the qualitative information obtained from abbreviated 4 day tests using pyrimethamine (4 x 20 $mg.kg^{-1}$) a dose sufficient to suppress infections of AJ 7032 (the line which originally underwent selection) and from PABA tests. All the recrudescent lines produced patent infections at that dose of pyrimethamine - but none were able to grow in the absence of PABA whereas the ancestral line could.

As a cross check one line (AJ 12021) was retested and its quantitative response to pyrimethamine estimated: (ED_{90} = 31.62 $mg.kg^{-1}$ $\pm$ ISE range 23.44 - 42.66 $mg.kg^{-1}$). Thus both in the respects of pyrimethamine resistance and of PABA dependence it closely resembled the series of lines obtained from AS P^{R} DW after PABA restriction. It was concluded that

both types of selection had led to the superimposition of pyrimethamine and sulphadiazine resistance. These had interacted in predictable though interesting ways: firstly, it had appeared that the rather high levels of pyrimethamine resistance exhibited by these double mutant lines were the result of the geometric summation of primary pyrimethamine resistance and the cross resistance to pyrimethamine concomitant with primary sulphadiazine resistance. Conversely the increased PABA dependence following primary resistance to pyrimethamine appeared to have been cancelled out by the acquisition of sulphadiazine resistance so that these double mutant lines were indistinguishable from wild types on this criterion alone.

Although, as has already been mentioned, it had been hoped to use cross resistance to pyrimethamine as a diagnostic character in the classification of sulphadiazine resistant lines, the rather wide spread of these made this impossible with the tests available. (The standard errors attached to these estimates routinely encompassed a twofold range of resistance alone.) If the significant differences between the extreme limits of resistance were real rather than due to error then this meant that sulphadiazine resistance mutants were heterogeneous and might be superimposed by repeated selection. Certainly, the levels of sulphadiazine resistance observed in the present study ranging from 2 to twelve-fold had been greatly exceeded by Jaswant Singh et al. (1954), suggesting that continued selection might be successful. If continued selection increased the level of sulphadiazine resistance this would show that these mutants were indeed heterogeneous unlike the case observed with pyrimethamine resistant lines.

This selection experiment was facilitated by the increase in PABA dependencies after Jan. 1975 since double mutant lines such as AS 90301 were unable to grow without a PABA supplement after that date and could therefore be selected using PABA restriction. This turned out to be a case of killing two birds with one stone: the attempt described previously to isolate wild type revertants (i.e. back mutants) from single step pyrimethamine resistance mutants by PABA restriction had been foiled by the intervention of superimposed sulphadiazine resistance. Since the double mutant already had one of these sulphadiazine resistance mutants, only the availability of more of these could allow the attempt to fail again.

The result of the experiment confirmed the heterogeneity hypothesis and has been summarised in Table 14, all 4 recrudescent lines although now PABA independent and therefore presumably sulphadiazine resistant, had experienced no perceptible alteration in the level of their responses to pyrimethamine: mean ED_{90} = 33.8 $mg.kg^{-1}$ (the estimates ranging from 28.3 to 42.4 $mg.kg^{-1}$) compared to 34.5 $mg.kg^{-1}$ for the ancestral line.

The rate at which this resistance was acquired was 1.8 x 10^{-10} - not significantly different from any of the other estimates obtained after PABA restriction - though the numbers were low.

These data were taken to show that at least two different types of mutant conferring sulphadiazine resistance existed but failed to demonstrate the occurrence of "back-mutation."

4. Rat Adaptation:

Following Coombs and Gutteridge (1975) rat adaptation was effected by repeated passage in splenectomised and intact, newly weaned rats.

Table 15 summarises the growth characteristics of wild and rat adapted lines in rats and mice as well as their principal drug responses before and after selection.

As was expected neither pyrimethamine resistance nor PABA dependence was significantly altered by rat adaptation and these tests were performed as formal controls since it had been planned to use the rat adapted line in crosses.

(Although in a thorough study of rat adaptation it would have been desirable, no samples were retained from infections observed during the course of the selective passages. Has this been done a graph of rat growth could have been plotted against number of rat passages. However since the main aim of the research was the study of drug resistance the obtention of a rat adapted line was regarded as peripheral.)

Neither of the wild P.chabaudi lines used in the present study were able to grow in intact rats whether newly weaned or adult. Growth of these lines in newly weaned splenectomised rats was erratic but it appeared that the AJ line had the edge over AJ in this respect. (AS also reached higher peak parasitaemias and was more virulent than AJ in mice as the data in Table 15 show.) It was felt that in view of its slightly poorer growth in rats AJ was a better baseline from which to commence selection and all the rat adaptation experiments used this line.

The principal consequence of selection was to permit the

growth of this AJ line in intact rats and to show this ability was stable after mouse and mosquito passage. Although parasitaemias rarely exceeded 10% in adult (8 weeks) rats, figures of 20 to 30% were common among those newly weaned. These were therefore somewhat lower than the figure of 35% which Coombs <u>et al.</u> (1975) mentioned in connection with their line. They also noted the loss of the ability to produce gametocytes after adaptation but did not say how many passages their line had undergone so it was impossible to say whether this was a consequence of adaptation or multi-passage. In the present study no such total lossof gametocytogenesis was observed and although the adapted line was no less fertile in rats than a wild line in mice would have been many fewer gametocytes were produced than in a wild line in intact or splenectomised rats. Thus, if looked at it this way, during adaptation a gradual decline in gametocyte producing potential had taken place.

Thus while a wild line growing in splenectomised rats (or subsisting in young intact rats after a large inoculum) would routinely produce an infection comprising about 30% gametocytes the adapted line rarely exceeded 10% in any host.

(As has been noted this enhancement of gametocytogenesis observed among non-adapted lines was exploited to facilitate crossing.)

Although this host specificity might have resembled the case of Duffy antigens in preventing the invasion of erythrocytes by <u>P. Knowlesi</u> (Miller <u>et al.</u>, 1975) this fails to account for the enhancement of gametocytogenesis in wild lines. PABA deficiency had been ruled out as a cause of the latter phenomenon since the administration of excess quantities of PABA failed to

affect the process. Nor had the administration of sulphadiazine been shown to increase gametocytogensis unlike the case reported by Bishop (1954a). Whatever the mechanism it appeared to operate as a positive stimulus since selection favouring the survival of gametocytes could not alone account for their abundance given the numbers of trophozoites and schizonts observed during the cycles of these infections.

Thus the most plausible explanation was that host immunity had been responsible both for the enhanced gametocytogenesis in wild lines and for the mechanism of host specificity in rats. Certainly the effects of splenectomy in encouraging the growth of wild parasites in rats was consistent with this hypothesis, but unfortunately attempts to investigate the effects of host immunity and erythrocyte barrier mechanisms failed due to technical reasons but the recent development of a successful _semi-in-vitro_ technique for the continuous culture of plasmodia (Trager _et al._, 1976; Haynes _et al._, 1976) offers some encouragement in this direction.

Whether or not an immune trigger is necessary for gametocytogenesis remains doubtful: although the process occurs late in _P.chabaudi_ in mice - after the time of peak fertility - the opposite is true in _P.yoelii_.

If the host specificity could be shown to reside in host immunity rat adapted lines could provide an interesting example of antigenic variation in plasmodia. The correlation of the degree of adaptation with such hypothetical variants would be specially amenable to genetic analysis augmented by the use of the continuous culture techniques referred to above.

5. <u>The Crosses:</u>

<u>Cross 1</u> - Walliker <u>et al.</u> (1975) demonstrated free recombination between markers conferring resistance to pyrimethamine and variants of the enzymes LDH and 6PGD. Dr. Walliker was kind enough to permit further analysis of the products of this cross with a view to finding out whether any segregation had taken place between the pyrimethamine resistance marker he used and the PABA dependence which had accompanied its original selection by direct exposure to the drug (4 x 50 $mg.kg^{-1}$). A screening technique was used rather than the normal method of scoring clones since the latter method was unsuitable for detecting rare events.

Although the precise number of oocysts contributing sporozoites to the initiation of the cross product blood infection remained unknown, in view of the abundance of oocysts observed on the mosquito guts of that cross (Dr. D. Walliker pers. comm.) these probably numbered several hundreds. Moreover the frequency of recombinant clones reported in that cross (Walliker <u>et al.</u>, 1975) was not significantly different from that expected to have arisen from an oocyst population of which 50% had been hybrid - the maximum possible frequency obtaining if the original blood mixture had included equal numbers of gametocytes from each product. (See Fig. 4 again for a graphical explanation of this point, if necessary.)

Double selection, by exposure to pyrimethamine to remove the sensitive parent and by withdrawal of PABA to remove the resistant parent, failed to reveal the presence of any parasites belonging to the hypothetical non-parental class in which resistance to pyrimethamine was accompanied by a normal wild type

PABA dependence. (As the cross was performed before Jan. 1975 this dependence was zero) Thus recombination had failed to split pyrimethamine resistance from its concomitant PABA dependence and it seemed much more likely that pleiotropy had been responsible for this association of characters. This conclusion was also consistent with the predominance of this class of pyrimethamine resistant mutant in this study (see Table 5) and in that of Morgan (1974) both following single step selection. It was unlikely that these two characters should have been so consistently found together if separately inherited unless the mode of selection had been conducive to this. There was no obvious reason why this should be so and on the contrary, the single step method was judged to favour the selection of point mutations. In short, this pleiotropic phenotype was considered the expression of a genetic "elementary particle" of pyrimethamine resistance though not necessarily the only one.

If this were so then the 9 pyrimethamine resistant lines whose detailed characterisation had revealed an increased level of PABA dependence (see Table 6) had arisen as a result of mutation in the same gene. In order to test this proposition, the series of crosses presented under the heading "cross 2" were carried out (see results). The three possible types of pairwise cross were performed between the three independently isolated lines, AJ 4027, AJ 4028 and AS P^R DW described in Table 18. (The last of these lines had been used in the cross reported by Dr. D. Walliker (Walliker <u>et al.</u>, 1975) and had been selected by him. All had been made resistant to pyrimethamine by direct exposure to the drug in a single step and were unable to grow in the absence of PABA when the experiment was performed before Jan.

1975.

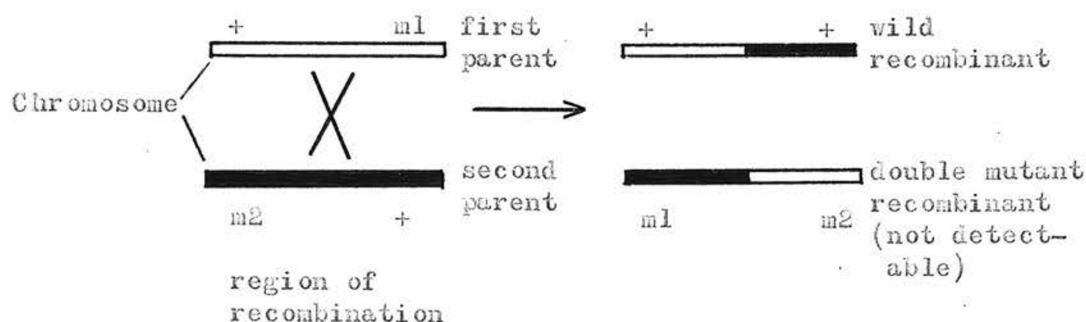
The analysis of these crosses again made use of a screening technique to detect rare recombinants. This consisted of passaging the three pairs of crossproduct bloods through mice receiving no PABA supplements. Even the largest inoculum size used (10^6) failed to induce patent parasitaemias in PABA free mice. (Larger inocula were not used for two reasons: firstly, these might have permitted spontaneous mutants to be scored as putative recombinants; also it was considered rather pointless in view of the probable sizes of the 3 hybrid oocyst populations which had contributed to the cross product blood parasites. These were unlikely to have each exceeded 10^4; and 10^3 was reckoned to be the most likely figure for the number of hybrid oocysts as judged from examination of mosquito guts and from consideration of the time delay between sporozoite inoculation and the appearance of patent blood infections.)

The failure of these crosses to produce PABA independent parasites was taken to suggest that the mutations in each of these parental resistant lines were very closely linked indeed. The upper limit of recombination frequencies in any of the pairwise crosses was set at 0.3%. This, together with the phenotypic similarity between these lines suggested in each case the mutation conferring resistance had probably occurred within the same gene.

Had PABA independent parasites been detected the most probable explanation for their appearance would have been as a consequence of a genetic recombination event generating wild type parasites. This process has been illustrated in Fig. 7.

FIG. 7

Model of recombination to generate wild types from cross between 2 pyrimethamine resistant lines.



m1 = mutation in first parent
m2 = mutation in second parent
\+ = wild allele

Reciprocal genetic exchange would also in this case have been expected to generate a completely new genotype with both the mutant alleles from the parents. (The phenotype of this recombinant would have been a matter for conjecture and it remained a possibility that the combination of both mutant alleles would have been lethal and therefore undetectable.)

It was not possible to pursue the analysis of the inheritance of sulphadiazine resistance as far as had been achieved in the case of pyrimethamine resistance. The main reason for this lay in the non availability of screening techniques and prompted the development of other screening techniques utilising the selective marker conferring rat adaptation.

Cross 3 (see results) was analagous to the cross reported by Walliker et al., (1975) with the substitution of sulphadiazine resistance for resistance to pyrimethamine. This sulphadiazine resistant line was crossed with a wild type line.

As the cross was carried out and analysed after Jan. 1975, only the sulphadiazine resistant parent could grow in the absence of a PABA supplement. Consequently, growth in the absence of PABA was used as the primary diagnostic character in the dichotomous classification of the cross-product clones. The details of these PABA tests have been summarised in Table 21. In addition abbreviated 4 day tests were used to estimate the growth percentage of each clone after sulphadiazine treatment (4 x 160 $mg.kg^{-1}$) and pyrimethamine treatment (4 x 1.25 $mg.kg^{-1}$) - both drugs being administered to mice receiving the normal PABA supplement of 0.5 gl^{-1}.

The dichotomous classification based on the all or none PABA test data was substantially confirmed in the case of the responses to sulphadiazine which with a mean growth percentage of 124.4% ($\pm$ ISE 103 - 150) for growers were significantly higher than the mean percentage for non-growers of 1.80% ($\pm$ ISE 1.24 - 2.62) with no overlap. On the other hand there was a slight overlap when growth after pyrimethamine therapy was measured - but the zero paba growers mean percentage of 50.0 ($\pm$ ISE 26.8 - 93.4) was still significantly higher than that of the non-growers at 1.22%. The overlap in pyrimethamine resistance however was expected in the light of the rather small difference in resistance between the parents - the resistant parent being only about twice as resistant as the sensitive parent at the ED_{90} level. In fact it was surprising that the overlap had not been greater.

As Walliker <u>et al.</u> (1975) had reported in the case of pyrimethamine resistance there appeared to be free recombination between the marker conferring resistance to sulphadiazine and

the two enzyme markers (LDH and 6PGD).

Although there was no evidence that the marker conferring sulphadiazine resistance had segregated polygenically, caution has to be observed in interpreting this result. Due to the crossing system which permitted incestuous matings to take place between micro and macrogametes within each parental line, at least and probably about 50% of all clones would not have been given the opportunity to undergo recombination and would therefore have exhibited their parental phenotypes anyway. The scoring bias thus incurred would have had the tendency to pull the mean growth percentages towards their respective parental means and possibly disguised any evidence of polygenic segregation. The number of clones scored was too few to have permitted an adequate statistical correction of this bias. Nevertheless, given this caveat the evidence from the clone characterisations was not inconsistent with a single gene model. The evidence was as good as could have been hoped for in the circumstances. Although it was not the main purpose of the experiment to test the hypothesis, the data showed no evidence of independent segregation of the primary resistance to sulphadiazine and the pyrimethamine cross resistance. Again the caveat mentioned above should be noted in this connection.

In Cross 4 (Tables 22-25) a wild type AJ line was crossed with the presumptive double mutant AS 90301 which had received two bouts of selection: firstly by direct exposure to pyrimethamine (4 x 50 $mg.kg^{-1}$; Walliker _et al._, 1975) and then by PABA restriction (both before Jan. 1975).

30 clones were briefly characterised before Jan. 1975 and a further more detailed characterisation were performed on seven

of these lines, thought to represent the 4 resistance phenotypic classes encountered in the cross, after that date.

In the preliminary tests as well as in the enzyme typing (LDH and 6PGD) three characters were measured for each of these 30 clones: growth in the absence of a PABA supplement; growth after sulphadiazine treatment (4 x 1.25 $mg.kg^{-1}$, without a PABA supplement); and growth after pyrimethamine treatment (4 x 2.5 $mg.kg^{-1}$, with the normal PABA supplement of 0.5 gl^{-1}). Growth in these tests was measured in two ways: both as a percentage of the untreated control and qualitatively - whether or not patent parasitaemias were observed in the group experimental mice receiving each treatment. Due to the rather large variations in the quantitative measurements of pyrimethamine resistance the classification into 4 types was in the main based on the responses to sulphadiazine and on the measurements of PABA dependence.

The 4 phenotypes observed were as follows (see Table 23):

1. Growth unaffected by pyrimethamine; growth abolised by sulphadiazine and growth unaffected by withdrawl of PABA. (Clones CX401 - 414: parental type 1).

2. Growth unaffected by pyrimethamine; growth abolished by sulphadiazine and growth abolished by withdrawal of PABA. (Clones CX415 - 421: non-parental type 2).

3. Growth abolished or nearly abolished by pyrimethamine; growth abolished by sulphadiazine and growth unaffected by withdrawal of PABA. (Clones CX422 - 428: parental type 2).

4. Growth variably affected by pyrimethamine; growth only slightly affected by sulphadiazine and growth unaffected

by the withdrawal of PABA. (Clones 429 - 430, non-parental type 1).

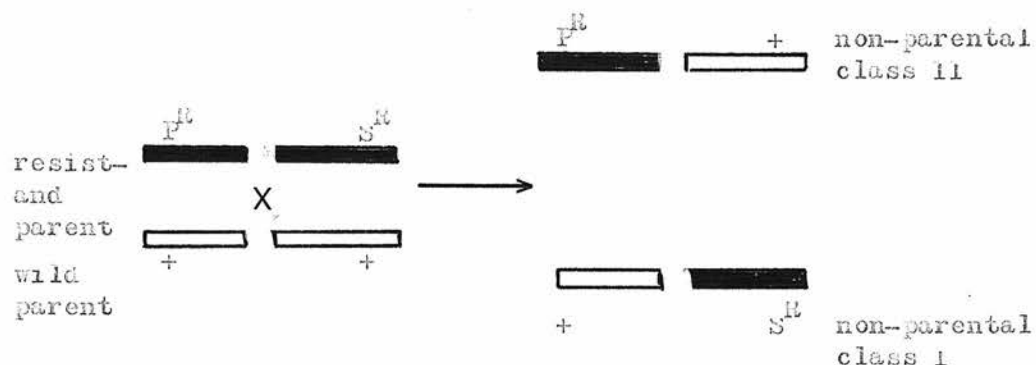
Following the PABA shift in Jan. 1975 seven clones were tested in more detail and their responses to sulphadiazine and to pyrimethamine have been summarised in Table 24 together with their updated PABA growth responses. Comparison of these responses with those of the parents and the two single step resistant lines AJ 7031 (sulphadiazine resistant) and AS P^R DW (pyrimethamine resistant) permitted the following similarities to be noticed: between AJ wild type and clones CX414 and CX422; between AS 90301 (the double mutant resistant parent) and clones CX401; between AJ 7031 and clones CX429 and CX430 (non parental class 1); and between AS PR DW and clones CX420 and CX421 (non parental class 2).

(Clone CX414 had originally been classed along with clone CX401 but following the discovery of a double LDH band it was recloned and reverted to a wild phenotype. This illustrated one of the hazards of cloning by dilution and underlined the value of enzyme typing in genetic studies of clone phenotypes. Presumably, the original "clone" had comprised two types of parasite: a pyrimethamine resistant type as well as the wild type later identified. Since drug resistance was "dominant" to sensitivity the wild parasites were not detected in the original series of tests.)

The occurrence of non parental parasites with phenotypes resembling those of single step mutants resistant to either pyrimethamine or sulphadiazine clearly suggested that genetic recombination had generated these types. The proposed mechanism has been summarised in Fig. 8.

FIG. 8

Recombination between a double mutant line and a wild type line:



P^R = pyrimethamine resistant allele

$+$ = wild type allele

S^R = sulphadiazine resistant allele

The reconstruction experiments summarised in Table 25 represented an attempt to estimate the frequency of class 1 recombinants (sulphadiazine resistant) among the cross-product blood parasites. Although this could have been done by scoring large numbers of clones this would have been laborious in the extreme considering the expected frequency of the type of clone and the numbers needed to render the estimate statistically significant. However following the PABA shift after Jan. 1975, only this type of parasite could grow in the absence of a PABA supplement thus allowing use to be made of the screening technique.

On the face of it the results show that the infection of 1 and 10 parasite inocula of cross product parasites in mice receiving no PABA supplement was consistent with the frequency

of PABA independent parasites being equal to 12.5%. This figure is the theoretical expectation given no linkage between the two genes proposed to control resistance to pyrimethamine and to sulphadiazine. However, given the conditions of the cross it was possible for lines to grow at unequal rates so the conclusion that there was no linkage between these two genes must be accompanied by that caveat.

(In order to resolve such ambiguities and also to study more precisely the genetic events following fertilisation, it was planned to extend the analysis of cross 4 by the characterisation of the products of single hybrid oocysts. As has been stated elsewhere (p. 87) this cross failed and it was thought most likely that the reason for this failure was the poor infectivity of pre-rupture oocysts. Following Vanderberg (1975) it was reasoned that sporozoites harvested from these early oocysts had not matured sufficiently to have developed full infectivity in mice.)

Recent work in this laboratory has confirmed that pre-rupture sporozoites in P.chabaudi are of low infectivity in mice and further attempts are being made to improve the technique. (Padua, pers. comm.)

Had the work been successful, it would have been possible to look directly at single genetic events instead of by the method of inference from scoring randomly selected clones from among a mixed population of hybrid and parental oocysts. Furthermore, as Walliker et al., (1975) have pointed out such a study might help in the more precise location of the meiotic reduction division particularly in the light of the report that such a division might occur during oocyst development (Sinden and

Canning, 1973).

Cross 5 (pp 88-91) may be considered the reverse of cross 4 with the two non parental types generated among the products of the latter used as parents in the former cross. Although it would have been possible to use as parents these actual recombinants themselves, it was decided to use the single mutants they most resembled, AJ 7032 and AS PR DW, in preference since these had been studied in greater detail and their genetic constitution better understood. The cross was conducted after Jan. 1975.

Characterisation of the 14 cross product clones (Table 26) revealed only three with non parental phenotypes. (One of these CX513, was classified as non parental after retesting.) Again all or none growth at restrictive PABA levels proved a more informative diagnostic aid in identifying non parental clones than did growth after pyrimethamine treatment. (In fact, the lower pyrimethamine dose (4 x 1.25 $mg.kg^{-1}$) was probably too low to completely distinguish sensitivities from non sensitivities and had the cross been repeated a slightly higher dose, say 4 x 2.5 $mg.kg^{-1}$, would have been used in preference.) However PABA growth although most useful in distinguishing the non parental clones did allow these to be classified into the two types expected and in this case the pyrimethamine response data were used.

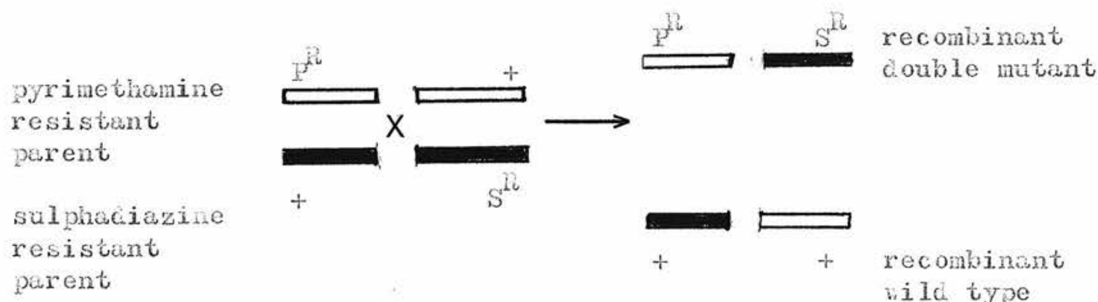
Two of these three presumptive non parental clones were again tested and this time their quantitative responses to pyrimethamine and sulphadiazine estimated. These data have been summarised in Table 27 along with comparison data for the parents and the wild type and double mutant lines with which

last two lines it was proposed to identify the non parental clones. Clone CX514 was classified as resembling the wild phenotype (ED_{90} pyrimethamine = 1.04 $mg.kg^{-1}$ ($\pm$ ISE range 0.81 - 1.32 $mg.kg^{-1}$)) and clone CX512 appeared to resemble the double mutant AS 90301 (ED_{90} pyrimethamine = 38.5 $mg.kg^{-1}$ ($\pm$ ISE range 30.5 - 48.4 $mg.kg^{-1}$, after retesting)). These diagnoses were supported by their respective responses to sulphadiazine - both being within the range expected of wild type and double mutant lines: (CX514 ED_{90} sulphadiazine = 55.3 $mg.kg^{-1}$ ($\pm$ ISE range = 38.1 - 80.4 $mg.kg^{-1}$) and CX512 ED_{90} sulphadiazine = 31.6 $mg.kg^{-1}$ ($\pm$ ISE range = 15.1 - 66.1 $mg.kg^{-1}$) - both estimates obtained with the normal PABA supplement of 0.5 gl^{-1}.))

Accepting this hypothesis that genetic recombination had generated a wild type and a double mutant from two single mutant parents we can visualise the cross thus: (Fig. 9)

FIG. 9

Cross between two singly resistant lines generating a double mutant and a wild type:



P^R = pyrimethamine resistant allele

+ = wild type allele

S^R = sulphadiazine resistant allele

The evidence from both crosses 4 and 5 demonstrate that genetic recombination can readily take place in P.chabaudi between these markers conferring resistance to sulphadiazine and pyrimethamine respectively and there is no reason to suppose that a similar process may not take place among human malarias. It is perhaps interesting to note in this connection that when 2 single step mutants resistant to pyrimethamine and sulphadiazine respectively appear in combination whether as a result of genetic recombination or following double selection, their pyrimethamine resistance appears to sum geometrically. Thus, for example (when measured at the ED_{90} level) while AJ 7032 was estimated to be twofold cross resistant to pyrimethamine and AS P^R DW was some 18 times resistant to this drug the figure for the double mutant was estimated to be 37-fold - almost their exact multiple. However the errors in these compound indices of resistance are of course considerable. Nevertheless if such a geometric combination of resistance were shown to be a general rule this would have serious implications in the field of human malaria chemotherapy.

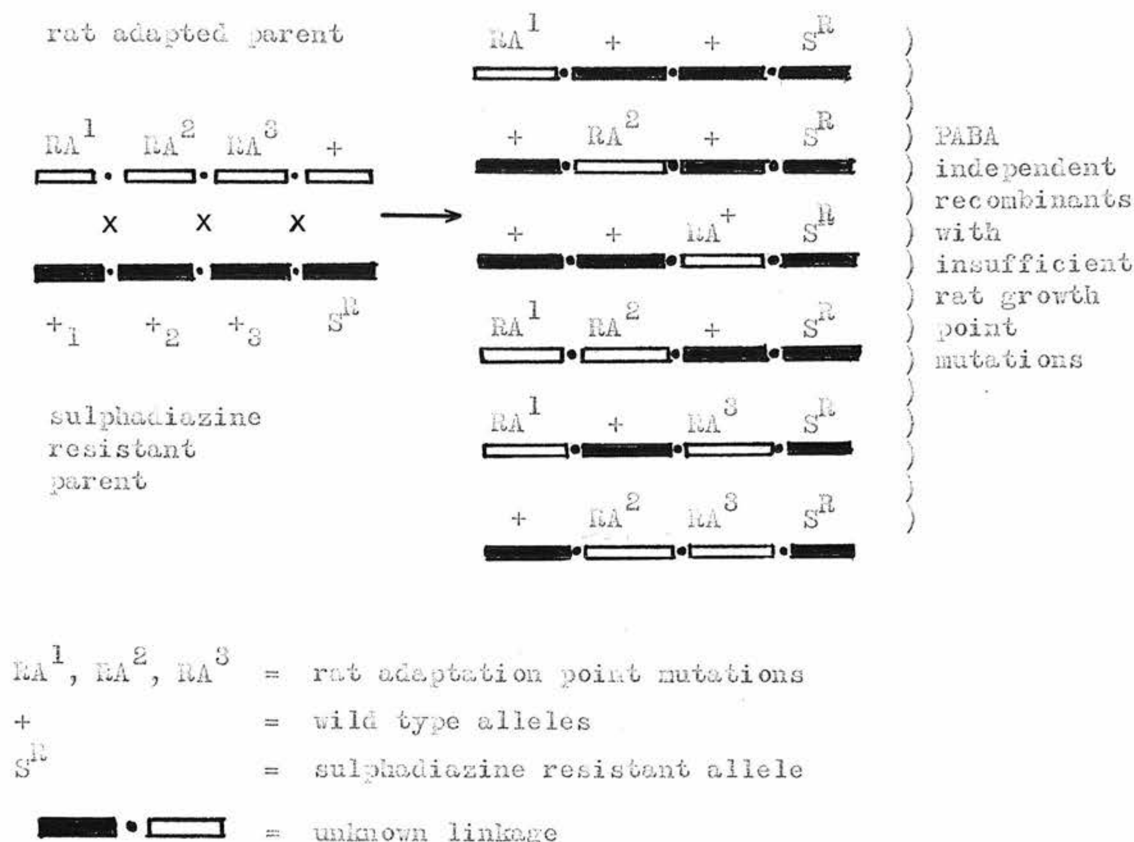
Although cross 6 (pp. 92-93) failed it has been reported here since it was felt that the failure was not due to merely technical causes. This cross was between two AJ lines: AJ 7032 which was sulphadiazine resistant; and AJ rat adapted which could grow from clonal inocula in adult intact rats unlike the other P.chabaudi lines described in this study.

Since the cross was conducted after Jan. 1975 the presence of PABA independent parasites among the products of the cross was taken to show the transmission of the resistant parent. The presence of parasites able to grow in intact rats was taken to

demonstrate the transmission of the rat adapted parent.

The use of the double screening techniques was originally intended merely to confirm the presence of recombinants and a positive result i.e. parasites still present after growth in rats receiving no PABA supplement would have required more rigorous confirmation from clone characterisation. However the negative result that was actually obtained did not necessarily mean that recombination had not occurred. It was possible the rat adaptation (which had been shown to be stable through mosquito passage) if due to genetic mutation might have been polygenic in origin: the selection procedure used, repeated passage through splenectomised and intact rats, was conducive to the accumulation of point mutations. Given this, recombination might have "diluted" these point mutations so that none of the cross products which had the sulphadiazine resistance mutation and were therefore able to grow in the absence of a PABA supplement had sufficient of the rat growth point mutations to survive the double selection. This model has been summarised in Fig. 10. E.g. given a three point model for rat adaptation only one in sixteen hybrid oocysts would have been expected to generate recombinants capable of surviving double selection and as the number of these point mutations increases so does the rarity of detectable recombinants. In view of the rather low fertility of the rat adapted line it was not unlikely that such was the paucity of hybrid oocysts as to further reduce this figure. Although a <u>semi-in vitro</u> test of rat adaptation was attempted this was unsuccessful. With this test it had been hoped to detect intermediate levels of rat adaptation. Clearly recent improvements in the continuous culture of Plasmodia (Trager

FIG. 10



et al., 1976) offer hope in this field.

In addition to this explanation two other plausible theories were considered. It was possible that the process of rat adaptation had altered the mating preference of the gametes produced by this line. Clearly a preference for incestuous matings would correspondingly reduce the frequency of hybrid oocysts.

Alternatively close linkage between any of the series of alleles conferring rat adaptation and the allele conferring sulphadiazine resistance would have reduced the frequency of rat adapted PABA independent recombinants.

In conclusion the marker conferring rat adaptation was

deemed unsuitable for use either as a selective marker or as an additional marker since its mode of inheritance remained unelucidated and was possibly polygenic. In fact the phenomenon of rat adaptation probably deserved a separate study. In such a study the line undergoing adaptation should ideally have been cloned between selective rat passages and tested for changes in rat growth ability along with controls. Lines exhibiting such changes could then have been analysed genetically. Such a procedure would tend to minimise the chances of selecting polygenic mutants.

Clearly then the attempt to analyse rat adaptation genetically illustrated the difficulty of handling polygenic mutants in ignorance of the details of their selective histories.

GENERAL DISCUSSION

Genetic mutations are widely believed to involve alterations in the DNA of living organisms. By the processes of transcription and translation these mutations find their way into the amino acid sequences of the proteins or polypeptides encoded. Many types of mutation have been discovered from studies on micro-organisms: substitution of purine or pyrimidine bases for one another; inversion of two or more bases and deletions of one or more base. These base changes can vary in effect from the total abolition of the function of that gene following the manufacture of a grossly defective protein or polypeptide chain to small alterations appearing to have little or no effect on the efficiency of the encoded enzyme such as these cases of enzyme polymorphism in plasmodia revealed by the work of Carter (1970, 1971, 1973). (For reviews of these mechanisms see Woese (1967) and Stahl (1969)).

Although technical constraints have made it impossible to demonstrate these processes in plasmodia with anything approaching the precision possible in other micro-organisms, notably certain bacteria and viruses (reviewed by Hayes, 1969), some interesting approaches at the molecular level have been made. In view of the widely accepted "mapping" of genetic mutations onto enzymes the report (Ferone, 1969; Ferone <u>et al.</u>, 1970) of differences between dihydrofolate reductases in <u>P.berghei</u> sensitive and resistant to pyrimethamine are strong supportive evidence that at least in this case resistance had occurred as a result of mutations in the DNA coding for this enzyme. Ferone's group found differences both between the kinetics and between the pyrimethamine inhibition

co-efficients of these enzymes. They had already shown (Ferone et al., 1968) that pyrimethamine inhibited this enzyme 1000 times more in P.berghei than in the host mice.

The second type of evidence supporting the mutational hypothesis concerns the stability of resistance to such drugs as pyrimethamine and sulphadiazine. The few cases of unstable resistance have been attributed to PABA deficiency (Peters, 1970) to which we may add the reluctance of certain authors to use cloned lines. In the present study, and in that by Morgan (1974), no examples of loss of resistance following serial or cyclical passages were observed. The suddenness and frequency of resistance to antifolates and sulphonamides are both consistent with the mutational hypothesis and in this respect the experience of the present author was no different from those of other authors (Young, 1957; Burgess and Young, 1959; Bishop, 1962; Diggens, 1970).

All the evidence of genetic studies (Walliker et al., 1971, 1973, 1975; Morgan, 1974) has shown pyrimethamine-resistance to segregate as a single mendelian factor. This was confirmed in the present study for both pyrimethamine and for sulphadiazine resistance. Furthermore all of these examples of drug resistance were obtained following single step selection which probably favours the selection of single gene mutations.

Following on from this question it is reasonable to enquire how closely did the estimated rates of acquisition of resistance come to true mutation rates. We can probably ignore estimates obtained from multi-passage experiments due to the possibility of different mutations accumulating in successive selective passages. We can also dismiss estimates obtained from

single step selection experiments using short-term, high-dose therapy since the rather high doses used in these probably kill some resistant parasites as well as sensitives. Single step drug selection experiments using continuous dose therapy while an improvement in the latter respect could not have been expected to guarantee very constant selection pressures during the course of selection: variations would have resulted from the mode of drug administration and from the complication of the host's speed of plasma clearance of the drug in question. (The importance of a constant selection pressure is in maintaining conditions which ensure the survival of all resistant parasites while at the same time favouring the elimination of sensitives.) In the present study, these conditions were most completely fulfilled in the PABA restriction experiments, particularly those carried out after Jan. 1975 when the rat cake was assayed to contain very little in the way of PABA related compounds. It is reasonable to suppose that the host homeostatic mechanisms would have maintained the parasites in a fairly constant PABA environment.

This leaves a complication common to all types of selection particularly in the case of relapse techniques - the effect of host immunity and its interaction with chemotherapy which if significant would tend to lead to the underestimation of true mutation rates. As to the significance of this immunity whether passive or acquired we can only attempt a guess: the evidence outlined in Table 9 that immunosuppression had no detectable effect on the number of recrudescences observed after continuous selection would seem to argue against the importance of immunity although the evidence pointed the other way in the case of

relapse selection (see pp102-103). However, we have no independent measurement of the efficacy of this immunosuppression other than the fact that normally mild lines of P.chabaudi were extremely virulent in mice receiving prednisone in their drinking water. Thus, although there was no evidence of factors operating which would have led to the serious underestimation of mutation rates in the PABA restriction experiments certainity about this was impossible.

The application of continuous culture techniques (Trager et al., 1976; Haynes et al., 1976) in selection experiments should go a long way towards meeting many of the criticisms: it should be possible to maintain constant selective conditions semi-in vitro and the problem of acquired immunity should be avoidable also.

A major assumption implicit in the equation of these estimated rates of acquisition with true mutation rates is that the resistant lines selected by any particular method arose as a result of mutation(s) in the same gene(s). The experiment in which pairwise crosses between 8 independently acquired lines resistant to pyrimethamine (cross 2) failed to detect recombination between any of these lines suggests very tight linkage. This added to the strong phenotypic resemblance noted between all the pyrimethamine resistant lines obtained in the present study (they were all accompanied by increased PABA dependence) suggests mutation within the same gene.

In the case of sulphadiazine resistance the position was much less clear. There seemed to be a spectrum of resistance from that accompanied by no cross resistance to pyrimethamine to that accompanied by a twofold or greater cross resistance. As

has been noted the imprecision of the drug response estimates may have accounted for the failure to discern discrete levels of cross resistance. However, the ease with which sulphadiazine resistance could be increased by superimposition without any detectable increase in the already enhanced cross resistance suggests that at least two distinct classes of resistance existed and though no evidence was obtained regarding segregation between these, there is no reason why this should not be tested.

In conclusion therefore although any estimate of the true mutation rate is vitiated by the factors mentioned in the foregoing discussion it is believed the acquisition rates obtained in the course of the present work constitute the closest approach yet to such an estimate in Plasmodia.

In view of the restriction of the present study to single step selection experiments at a narrow range of drug doses under defined levels of PABA supplementation it is not very surprising that so few phenotypes were observed and although there is no reason to suppose that a complete range of phenotypes was detected (and therefore a complete range of the genes implicated in resistance to antifolates or sulphonamides) it is worth pointing out that from these three proposed genes, no less than seven phenotypes can be generated following their combination in all the possible permutations. 6 of these permutations were obtained in the present study and the phenotypes of these lines together with those expected on the basis of the geometric summation of the resistances of each of the single mutations proposed to have combined to form these double and triple mutants have been summarised in Table 28.

TABLE 28

Gene combinations: observed and expected levels of resistance.

	Degree of Resistance to			
	Observed		Expected	
	PYR.	SULPHA.	PYR.	SULPHA.
- Pyrimethamine resistant (P^R)	18x	1/20x	-	-
- Sulphadiazine " with cross resistance to pyrimethamine (S^{R1})	2x	4x	-	-
- Sulphadiazine resistant with no cross resistance to pyrimethamine (S^{R2})	1x	4x	-	-
- P^R S^{R1}	37x	1x	36x	1/5x
- P^R S^{R2}	18x	1x	18x	1/5x
- S^{R1} S^{R2}	not obtained		2x	16x
- P^R S^{R1} S^{R2}	36	($\sim$ 4x)*	36x	0.8x

* = as estimated from PABA dependence

The point of all this is that given the fact of such combinations arising via the superimposition of such single mutations particularly following long term selection pressure, a wide variety of types can be generated. This added to the failure of most workers to use cloned lines and to control the levels of PABA supplements (Peters, 1970) may help to account for some of the wide range of resistance phenotypes reported in the literature.

The ease, demonstrated in the present study, with which recombination occurred between markers conferring resistance to pyrimethamine and to sulphadiazine has obvious significance in understanding the ways in which resistance to these drugs is spread. In this context the data of Rosario (1976a,b) merit consideration: he showed that recombination occurred readily between markers conferring resistance to pyrimethamine and to chloroquine in P.chabaudi. However although this chloroquine resistance was stable through unselected serial and cyclical passage it was considered a low level resistance and after further selection has been increased some 15-fold (Padua; pers. comm.). Thus whether or not the earlier example of chloroquine resistance was a suitable model for chloroquine resistance in P.falciparum remains to be seen. It may transpire that clinical examples of chloroquine resistance are inherited by polygenic mechanisms.

The inheritance of drug resistance in bacteria has been convincingly associated with cytoplasmic factors or episomes (Watanabe and Fukasawa, 1961; and Watanabe, 1963); and while erythromycin resistance in yeast (Thomas and Wilkie, 1968; Cohen et al., 1970) and in Paramecium aurelia (Beale, 1969; Beale et al., 1972) have been shown to be inherited mitochondrially no convincing examples of extrachromosomal inheritance have been demonstrated in Plasmodia. The description by Yoeli et al. (1969) of sympholia has already been criticised on the grounds that the technique used to detect drug resistance transfer between P.vinckei and P.berghei was equally capable of inducing resistance by selection (Diggens, reported in Peters, 1970). Although Ferone et al., (1970) claimed the presence of a dihydro-

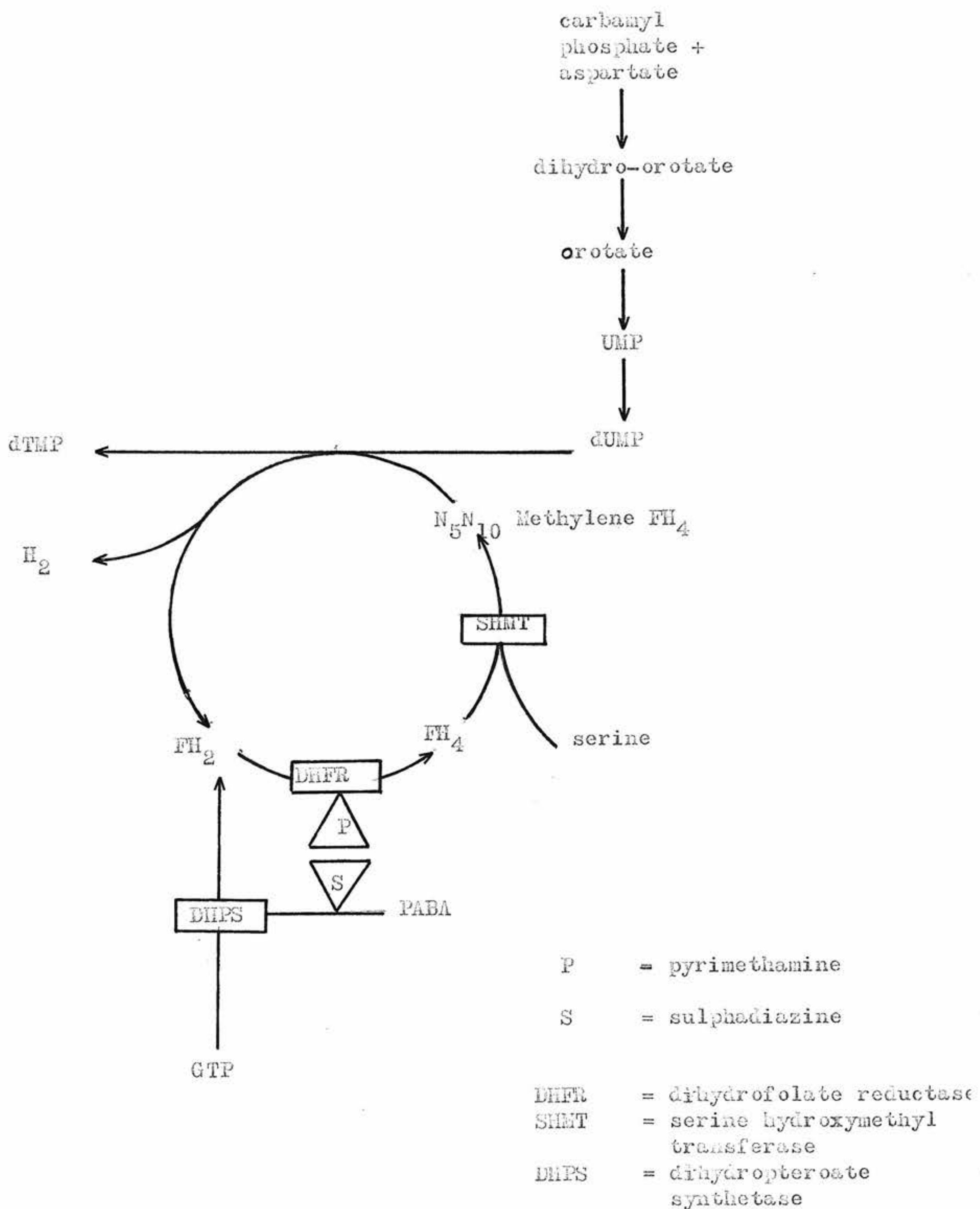
folate reductase in the recipient P.berghei which was intermediate in character between that of the donor resistant P.vinckei and that of a sensitive P.berghei was evidence in favour of syngholia - it was surely even better evidence that de novo selection had taken place.

Gene regulatory mechanisms in which the genes coding for enzymes involved in successive steps in a biochemical pathway are located together on the chromosome (co-ordinate induction) are well known. The most famous example of such mechanisms is the lac operon in E.Coli (Jacob and Monod, 1961). Although both antifolates and sulphonamides have been implicated in blocking pyrimidine synthesis the absence of linkage between markers conferring resistance to these drugs demonstrated in the present study, argues against such a control mechanism in plasmodia. However the relevance of this pathway to the study of the genetics of and mechanism of resistance to these drugs is obvious and the pathway has been summarised in Fig. 12 (modified after Jaffe and Gutteridge, 1974).

It is believed that in plasmodia de novo synthesis of pyrimidines (from carbamylphosphate and aspartate via orotate to UMP) predominates over any salvage mechanism since pyrimidines are only reluctantly incorporated into parasite DNA (Gutteridge and Trigg, 1970). UMP seems to be freely interconvertible into other pyrimidines and it is this conversion to dTMP which seems to be affected by the action of sulphonamides and antifolates. dUMP seems to be converted to dTMP by the action of an enzyme first shown in P.lophurae by Walsh and Sherman (1968) and in P.chabaudi by Walter et al. (1970) and by Walter and Konigk (1971). This synthesis involves the transfer of a carbon group

FIG. 12

Pyrimidine synthesis and the sites of action of Pyrimethamine sulphadiazine (adapted after Jaffe and Gutteridge, 1974)



and electrons from N_5N_{10} - methylene tetrahydrofolate to the 5 position of dUMP with the concomitant oxidation of tetrahydrofolate to dihydrofolate. The reduction back to tetrahydrofolate requires the enzyme dihydrofolate reductase which seems to be the prime target for the action of pyrimethamine (Ferone et al., 1969). The transfer of a 1C group back onto tetrahydrofolate from serine seems to be accomplished by serine hydroxymethyl transferase (Platzer, 1972). Dihydrofolate the essential co-factor required to maintain this cycle seems to be synthetised de novo from GTP and the condensation of pteridines such as PABA. A key enzyme in this pathway, dihydropteroate synthetase has been isolated (Konigk and Walter, 1973). Mammals are able to utilise preformed folates (unlike plasmodia) and the lack of this pathway is the reason for the clinical effectiveness of sulphonamides in bacterial and malarial infections.

From consideration of this pathway it is possible to construct ad hoc an explanation of the cross resistance patterns observed in this study. If we accept that pyrimethamine resistant lines contained altered dihydrofolate reductases then the correlated hypersensitivity to sulphadiazine and increased PABA dependence might have arisen as a result of such alterations: the reduced affinity for pyrimethamine had required some loss of the ensyme's efficiency in the conversion of FH_2. If the FH_2 pool size had been increased following PABA supplementation the effects of this sacrifice might have been minimised. On the other hand PABA restriction would have exposed the enzyme deficiency in maintaining an adequate supply of FH_4.

Some mutations affecting the synthesis of FH_2 from the

condensation of pteridines such as PABA are pictured in this model as causing sulphadiazine resistance by increasing the size of the FH_2 pool (for example following the breakdown of feedback inhibition resulting in an oversynthesis of enzyme or by another mechanism). As well as causing sulphadiazine resistance such a process would result in a small increase in pyrimethamine resistance following the increase of the FH_2 pool. Mutants which had caused sulphadiazine resistance without a concomitant increase in the throughput of FH_2 would not have been expected to have been accompanied by a cross resistance to pyrimethamine.

Proof of such speculations would depend upon accurately measuring pool sizes of FH_2 and FH_4 in sensitive parasites and in those resistant to either pyrimethamine or sulphadiazine at different levels of PABA supplementation and at different concentrations of the two drugs. Although ideally such experiments would require a true in vitro technique of parasite cultivation using defined media - the recent availability of a semi-in vitro technique (Trager et al., 1975; Haynes et al., 1976) offers some encouragement.

However perhaps the main stimulus to a genetic study of malaria parasites offered by these techniques is the opportunity they offer to study the genetics of drug resistance in human plasmodia particularly in the case of *P.falciparum* under experimental conditions; and indeed such a study is under progress in this laboratory. After the initial technical difficulties are overcome (among these one might mention such as that of transmission from semi-in vitro blood culture to mosquitoes or that of measuring drug resistance) the first

task must be to repeat the pioneer work which has been undertaken using the rodent model. The main difference in approach will be that as well as experimentally induced resistance crossing experiments will have available lines whose resistance has been acquired in the wild and not under controlled conditions. Furthermore such "wild" resistant lines will also have unknown genetic histories. Thus the problem will have additional degrees of complexity.

This is not to say that experiments using the rodent model are obsolete. The very fact that one can be sure rodent lines have not been tampered with genetically disallows that notion. More positively the ready availability of a feasible technique for the genetic study of rodent malaria parasites has generated a momentum of its own and several interesting lines of investigation are open and it will be while yet before *semi-in vitro* specialists catch up. Thus for example Rosario (1976a,b) demonstrated a case of mendelian inheritance of chloroquine resistance in *P.chabaudi*. However this was at a low level (3 $mg.kg^{-1}$) - about a twentieth that tolerated by the host. This suggested two possibilities: either it was a basic genetic building block of full chloroquine resistance; or it was an adventitious example. Only continued research can provide the answer.

Another example is provided in the present work by the unsuccessful attempt to obtain infections from single oocysts for cloning and characterisation. As well as confirming the reciprocal mechanism of inheritance of markers it had been hoped this study would help in more precisely locating the stage of the meiotic reduction division. Sinden and Canning (1973)

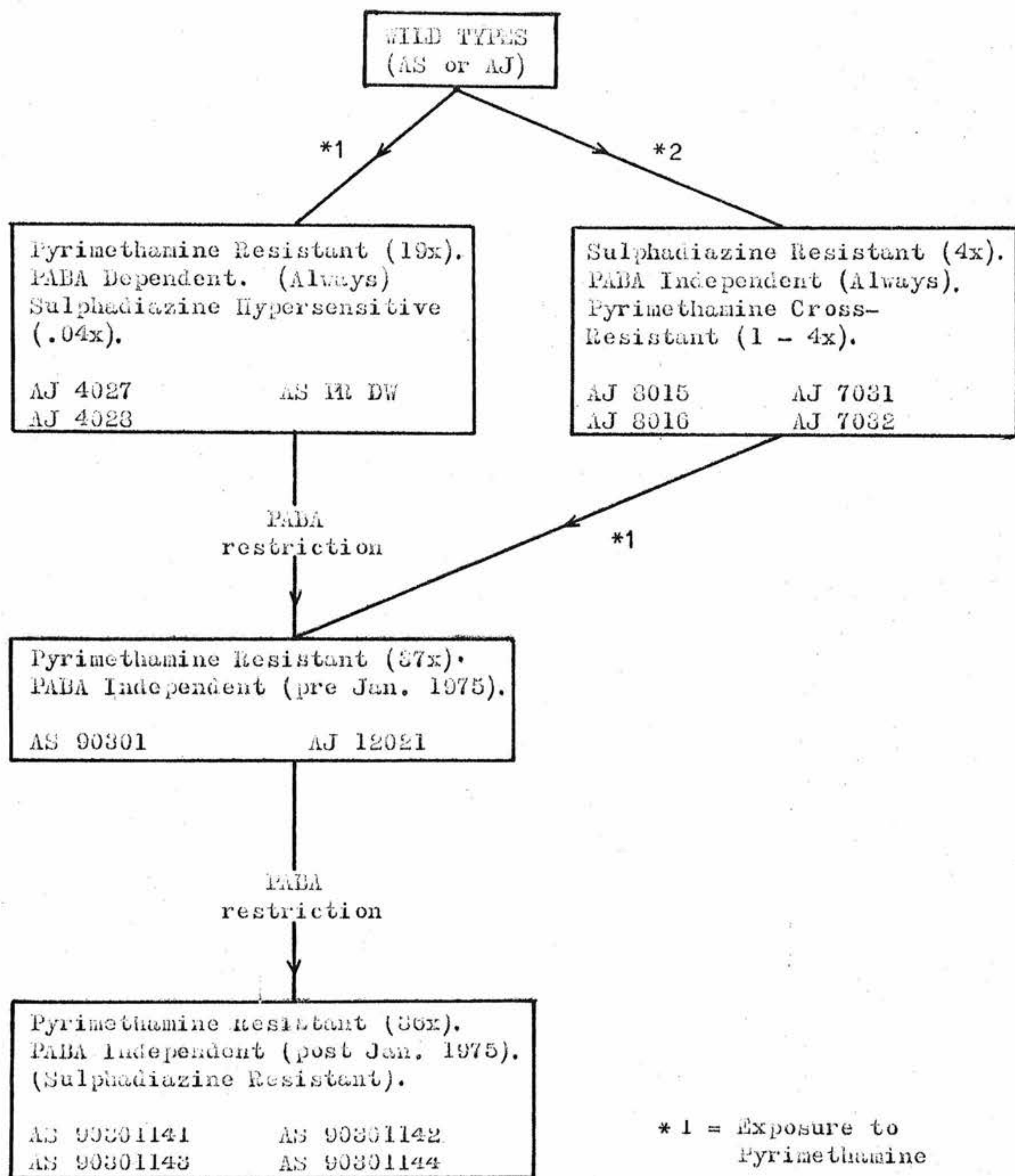
reported cytological observations consistent with a reduction division in oocysts of P.yoelii. Since the failure was possibly technical in origin further investigations are merited.

It seems likely that particularly in the wake of chloroquine resistant P.falciparum much effort will be employed in developing suitable vaccines - thus allowing chloroquine to be kept in reserve for acute cases once the vaccines have been shown to be effective. Given this it will be important to extend the genetic study of malaria to include the response of parasites to immune mechanisms. It may be that as with Trypanosomes the process of antigenic variation takes place (Grey, 1969) and the elucidation of its mode of inheritance would be the most interesting and useful achievement of protozoan genetics to date.

APPENDIX

TABLE 29

Genealogy of mutant lines with examples and modes of selection used: each box contains a different genotype.



ACKNOWLEDGEMENTS

I would like to thank: Professor G. H. Beale for providing laboratory facilities and for useful advice and encouragement; Dr. D. Walliker for explaining to me some of the mysteries of malaria and mosquitoes, for critically evaluating the manuscript and for allowing me to use his lines of rodent plasmodia; Mr. A. Sanderson, who performed the electrophoresis runs, for technical advice and help; Dr. Sonia Morgan for advice and inspiration and Mrs Edith Joyce who looked after my animals and for her patience despite some bizarre requests. These apart I wish to certify that all the experiments were planned and executed by myself as were the drawings and statistical tests and estimates of central tendency. This work was performed during the tenure of a Medical Research Council Scholarship.

27th August, 1977 Signature: R Walker

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