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MECHANISM OF ANTIMALARIAL ACTION

OF

TETRACYCLINE

BY

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(B.Sc. in Med. Tech.)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

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OF

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P. PRAPUNWATTANA

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ABSTRACT

Tetracycline showed a time-dependent antiparasitic activity on Plasmodium falciparum growth in vitro. It did not directly inhibit merozoite reinvasion processes and had no effect on normal erythrocytes. Cultivation of the infected erythrocytes in the presence of a therapeutic level of tetracycline resulted in a marked decrease in dihydroorotate dehydrogenase activity, but without or with a little effect on glutamate dehydrogenase activity. Dihydroorotate dehydrogenase was found mainly in the particulate fraction of parasite extract and presumed to be mitochondrial, whereas glutamate dehydrogenase, shown to be cytoplasmic in origin, was found mainly in the supernatant of parasite extract. The decrease in dihydroorotate dehydrogenase activity was not due to the direct effect of tetracycline but was shown to be due to the defect in the respiratory chain linked to it. Tetracycline could further enhance the $^{45}Ca^{2+}$ uptake by Plasmodium falciparum-infected erythrocytes after 16 h of drug-treatment, but only when the serum was omitted from the medium. This phenomenon may be due to the effect of tetracycline on the parasite, possibly on parasite mitochondria combined with the effect of ATP depletion of the infected cells. Tetracycline uptake was also greater in the infected erythrocytes, but no stoichiometry between [^{3}H]-tetracycline uptake and $^{45}Ca^{2+}$ uptake by tetracycline-treated infected cells was observed, indicating that tetracycline-enhanced $^{45}Ca^{2+}$ uptake was not

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due to the uptake of tetracycline-Ca^{2+} chelating complexes. By using two dimensional polyacrylamide gel electrophoretic technique, a protein of 95K, pI 4.5 was shown by its sensitivity to tetracycline and resistance to cycloheximide to be of possible mitochondrial origin. It is concluded that the action of tetracycline on the malaria parasite may be directed to the mitochondria, probably through its effect on mitochondrial protein synthesis.

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INTRODUCTION

The tetracyclines

History (see Sande and Mandell, 1980)

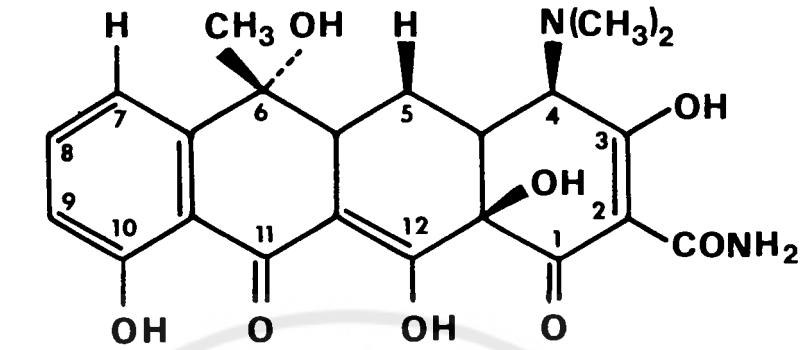
The tetracycline antibiotics were developed as a result of large-scale screening of soil specimens from around the world for antibiotic producing microorganisms. The first of these compounds, chlortetracycline, was introduced in 1948, followed by the discoveries of other derivatives, e.g. oxytetracycline, tetracycline, demethyl-chlortetracycline, methacycline, doxycycline and minocycline. The tetracyclines are all very closely congeneric derivatives of polycyclic naphthacene carboxamide. The structural formulas in Figure 1 are presented as the examples of the basic polycyclic unit of all these compounds.

Soon after their initial development, the tetracyclines were found to be highly effective against a wide range of microorganisms including many gram-positive and gram-negative bacteria, various species of rickettsia and mycoplasma, certain protozoa and large viruses (Laskin, 1967), and hence become known as "broad spectrum" antibiotics.

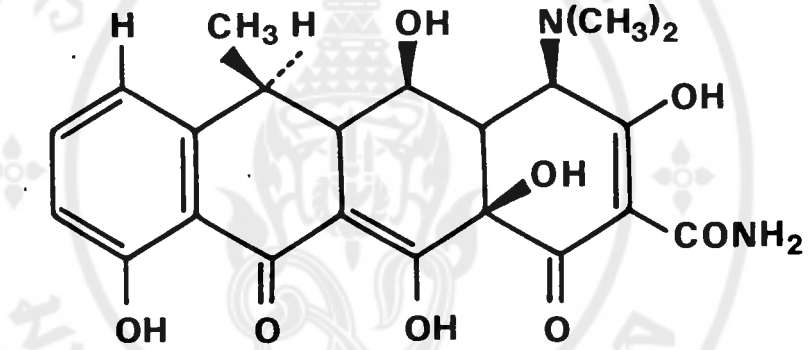
Action on prokaryotic system

At the blood concentrations achieved in antibacterial therapy, the tetracyclines are bacteriostatic and at much higher concentrations, they are bactericidal (Sande and Mandell, 1980). Various biochemical sites of action have been proposed for these drugs,

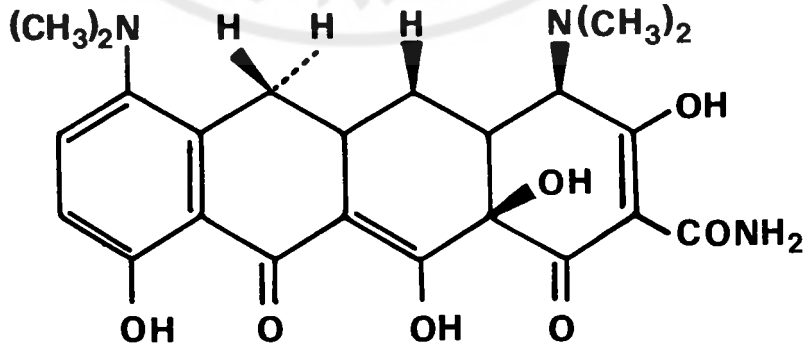
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Tetracycline



Doxycycline



Minocycline

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Fig 1 Structural formulas of the tetracyclines

based on reports of their inhibition of several bacterial enzyme systems, oxidative phosphorylation, glucose oxidation and membrane transport (for a review, see Laskin, 1967). However, the concentration required (or reported) to be inhibitory was in many instances much greater than those required for growth inhibition. In addition, many of these effects appeared to be secondary to other reactions and/or the ability of tetracyclines to chelate metal ions. It is doubtful, therefore, that any of these biochemical reactions can be considered to be the major site of tetracycline inhibition.

One of the earliest reports on the mode of action of the tetracyclines demonstrated that protein synthesis was particularly sensitive to inhibition by these drugs (Gale and Paine, 1950). It is now clear that the inhibition of protein synthesis is the major site of action of the tetracyclines which is responsible for the growth inhibition of prokaryotes by these drugs.

There are many reactions involved in the protein synthesizing system which may be outlined as follows:

1. Amino acid activation and attachment to t-RNA

2. Formation of the ribosome-messenger-aminoacyl-t-RNA complex. This has often been considered as consisting of two steps:

   a) the attachment of messenger RNA to the ribosomes, and

   b) the binding of aminoacyl t-RNA to the ribosome-messenger RNA complex (the "binding reaction")

3. Transfer of amino acid from t-RNA to the growing peptide chain (formation of peptide bond)

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Reactions 2 and 3 have been referred to as the "transfer reaction", i.e., the transfer of amino acid from aminoacyl t-RNA to protein, and all three are taken together can be considered as the "overall amino acid incorporation reaction".

Mechanism of action of the tetracyclines on protein synthesis is now well known (for reviews, see Laskin, 1967; Gale et al, 1981). The tetracyclines can bind to DNA, proteins, synthetic polynucleotides, transfer RNA and to ribosome, but only that drug bound to ribosome is inhibitory. This binding is largely reversible, and the bulk of the bound tetracycline is associated with the 30 S ribosome subunit. This binding of drugs to ribosome blocks the binding of aminoacyl t-RNA to the messenger RNA-ribosome complex and hence results in inhibition of protein synthesis. Once the aminoacyl t-RNA is bound to the 30 S particle, it cannot be dissociated by tetracyclines.

There are two sites for t-RNA binding on the m-RNA-ribosome complex: one is the acceptor site (A-site), the other is the peptidyl site (P-site). The latter is the site for the binding of N-formyl methionyl-transfer RNA (f-Met-t-RNA_F) which is the natural initiator of protein synthesis and for the binding of growing peptide chain attached t-RNA, whereas the former is for other aminoacyl-t-RNA binding. It is now known that drugs inhibit binding of aminoacyl-t-RNA to the A-site but not to the P-site.

This group of drugs has no effect on the following steps of protein synthesizing system:

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1) The steps involving activation of amino acid and transfer to t-RNA

2) Association of 30 S and 50 S ribosomal subunits to be 70 S ribosome

3) Binding of m-RNA to ribosome

4) Hydrolysis of GTP to GDP that is required for functional binding of aminoacyl-t-RNA to the A-site.

From the above mentioned information, the overall phenomenon is that tetracyclines inhibit protein synthesis in prokaryotes at the step called "transfer reaction".

Action on eukaryotic system

In the review of tetracyclines by Laskin (1967), it was clearly shown that, besides their action on prokaryotic systems, tetracyclines have some effects on eukaryotic systems as well. They can inhibit various biochemical reactions of mammalian cells, e.g. respiration, oxidative phosphorylation. Again, these effects require very high concentration of drug and appear to be secondary to other reactions and/or metal ion chelating properties of the tetracyclines.

It is also well known that tetracyclines can inhibit both cytoplasmic and mitochondrial protein synthesis of eukaryotic system. Inhibition of cytoplasmic protein synthesis require very high concentrations (>50 μg/ml) which normally will not be reached in patient treated with tetracyclines. (Laskin, 1967; De Jonge, 1973; Gijzel and Kroon, 1978). Again, like in prokaryotic system, the sensitive step is the 'transfer reaction' (Laskin, 1967). Impairment of mitochondrial protein synthesis by the tetracyclines can occur at concentration (6-10 μg/ml) that is nearly the same as necessary in serum of man for

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sufficient antimicrobial action during therapy, indicating the possibility that mitochondria, which also contain 70 S ribosome (Locker and Rabinowitz, 1979), is the target site of tetracyclines (De Vries and Kroon, 1970; De Jonge and Hulsmann, 1973; Van Den Bogert and Kroon, 1981; Van Den Bogert et al, 1983). It had been shown that, after prolonged administration at this level, tetracyclines inhibited the formation of mitochondrially synthesized proteins (or enzymes), e.g. cytochrome aa_3 (cytochrome oxidase), cytochrome b, cytochrome c_1 (De Vries and Kroon, 1970, De Jonge and Hulsmann, 1973, Van Den Bogert and Kroon, 1981). This may be accompanied by blocking of formation of entire mitochondria (De Vries and Kroon, 1970). The inhibition of mitochondrial protein synthesis may lead to arrest of cell proliferation (Van Den Bogert and Kroon, 1982).

Chelation of metal ions

The ability of tetracyclines to bind metal ions has long been of interest. They form strong coordination complexes with calcium (Ca^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}), nickel (Ni^{2+}) at the ratio of 2:1 metal ions to ligand complex and with magnesium (Mg^{2+}) at a 1:1 ratio (Doluisio and Martin, 1963; Benet and Gayon, 1966; Newman and Frank, 1976).

Many studies have been made but there is as yet no general agreement on the mode of binding. Proposed chelating groups are; a) the C_{10}-C_{11} and/or C_{12}-C_1, ketophenol group (Benet and Gayon, 1966; Mitscher, 1969; Newman and Frank, 1976), b) the C_{11}-C_{12} β-diketone group (Colaizzi et al,1965; Mitscher et al,1968; Newman and

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Frank,1976), c) the C_4-dimethylamine-C_3 or C_{12a} hydroxyl group (Doluisio and Martin, 1963; Mitscher et al, 1968), d) the C_1-C_3 tricarbonylmethane group (Colaizzi et al, 1965; Baker and Brown, 1966), e) the O_1-O_{12}-O_{11} and O_1-O_{12a}-O_{12} group (Jogun and Stezowski, 1976) and f) multidentate combination of C_{11}-C_{12} β-diketone group and C_1-C_3 tricarbonylmethane acheived through folding the molecule along the C_{4a}-C_{12a} axis (Caswell and Hutchinson, 1971).

This ability of tetracyclines to chelate ions is known to be essential for most of their effects on various biochemical reactions of both eukaryotic and prokaryotic system. Their effects can be reversed or stimulated by a suitable species of metal ion (Laskin, 1967). Loss of metal ion binding ability is often accompanied by the reduction or absence of the biological activity (Doluisio and Martin, 1963; Colaizzi et al, 1965; Brown and Ireland, 1978). The metal ion chelating properties, therefore, are sometimes considered as integral to the mode of action of tetracyclines.

Malaria

The disease

Of all parasitic diseases of man in the tropical areas of the world, malaria is still the most serious and prevalent cause of morbidity and mortality. It is a major health problem for both individuals and communities, and also impedes economic development resulting in continuing poverty in many countries (WHO, 1979).

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This disease is caused by obligate intracellular parasites of the genus Plasmodium, which are transmitted to victims by infected female mosquitoes. This genus includes protozoan parasites causing malaria in man (P. falciparum, P. vivax, P. ovale and P. malariae ,simians (P. knowlesi and P. cynomolgi), rodents (P. berghei, P. chabaudi, P. yoelii and P. vinckei) and birds (P. lophurae, P. gallinaceum).

The life cycle of the malaria parasite (see Bray and Garnham, 1982) .

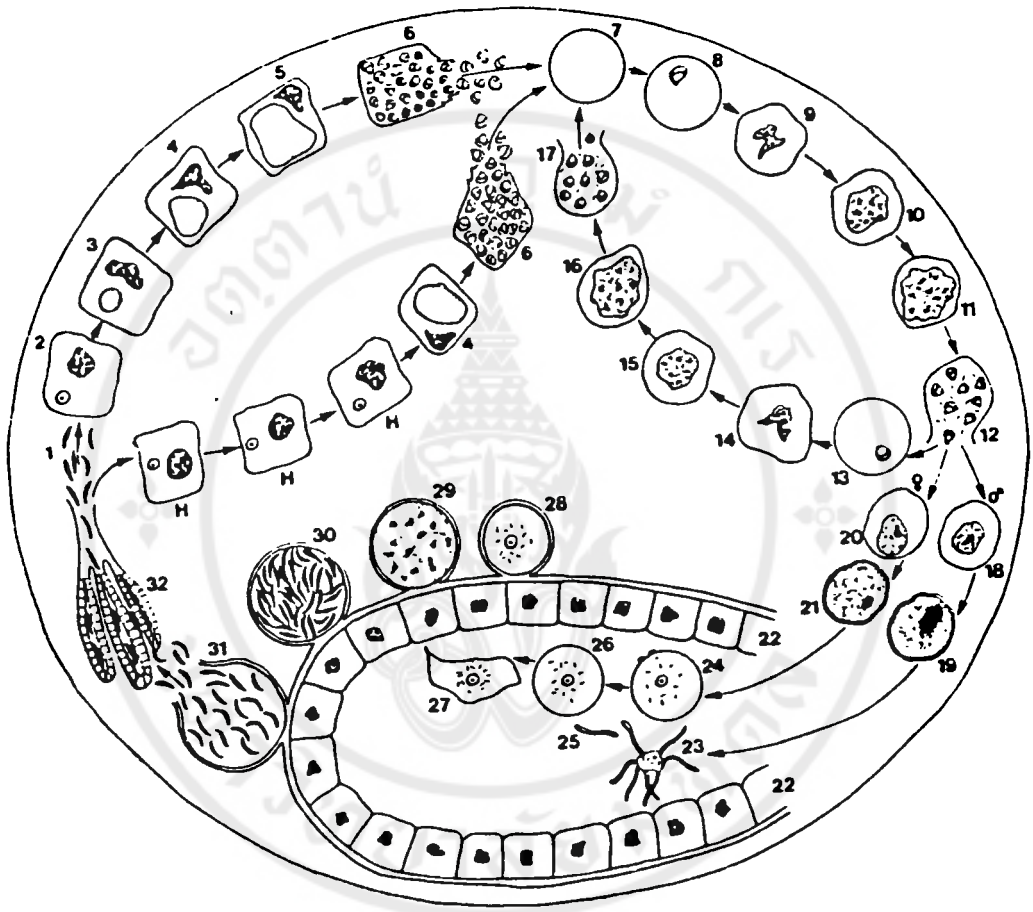
All species require two hosts, a vertebrate and an invertebrate, with 3 steps of differentiation : 2 steps of asexual division, called schizogony, in the vertebrate, and a sexual division, called sporogony, in the invertebrate.

Commonly, infection of the vertebrate host begins with the bite of an infected female anopheline mosquito (Fig 2). Sporozoites from salivary glands are inoculated and invade liver cells, where they differentiate into schizonts or, depending on whether the species of malaria parasite causes true relapse or not, into hypnozoites (latent form of tissue stage of schizonts). This step is called exoerythrocytic schizogony. The schizonts rupture, each releasing merozoites into blood stream. These merozoites invade erythrocytes where they form characteristic 'rings', which grow to become mature trophozoites and undergo another asexual multiplication to produce many new merozoites (erythrocytic schizogony). The erythrocytes erupt, releasing the merozoites which soon invade other

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Fig 2 The life cycle of the malaria parasite

(from Bray and Garnham, 1982)



1: sporozoites injected into skin by the mosquito; 2: 2-day-old exoerythrocytic form in a hepatocyte; 3, 4, 5: growing exoerythrocytic schizonts; H: hypnozoites in hepatocytes; 6. mature exoerythrocytic schizonts bursting, releasing merozoites into the blood; 7: erythrocyte; 8, 9: growing trophozoites; 10, 11: growing schizonts; 12: mature schizont releasing merozoites; 13, 14, 15, 16, 17: erythrocytic cycle repeated; 18, 19: growth of the microgametocyte; 20, 21: growth of the macrogametocyte; 22: mosquito has taken gametocytes up into its mid-gut; 23: exflagellation of the microgametocyte; 24: macrogametocyte escapes from erythrocyte to become a macrogamete; 25: microgamete; 26: macrogamete about to be fertilized; 27: zygote or ookinete; 28, 29, 30: oöcyst growth on the mid-gut surface; 31: oocyst bursting, releasing sporozoites; 32: sporozoites in the mosquito salivary glands

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erythrocytes and begin a new cycle. The cycle of growth of parasites tends to be synchronous, producing periodic fever-chill cycles which are the hallmark of malarial infections. Some parasites differentiate in erythrocytes into sexual forms called male and female gametocytes, which are infective to anopheline mosquitoes. The sexual cycle, called sporogony, is completed when a susceptible anopheline mosquito takes blood meal and ingests these gametocytes. These gametocytes develop into gametes, and fertilization takes place to form zygotes in the mosquito midgut. The zygote matures into a motile ookinete, which will penetrate through the gut epithelial cells to form an oocyst on the midgut surface. The oocyst bursts after maturation, releasing thousands of spindle-shaped sporozoites which in time enter the salivary glands from which they are able to infect a next host that the mosquito bites, by flowing with the saliva into the wound.

Malaria situation

According to WHO statistics (WHO, 1986 b) the overall malaria situation has not improved in the last 15 years. Although some countries, notably India and China, have been reporting downward trends, the malaria situation continues to deteriorate in rural areas undergoing intensive economic development, particularly in Asia and the Americas. In the remaining areas, the situation is fluctuating. At present, about 2.2 billion peoples, which is 48% of the total world population, still live in malarious areas, exposed to the risk of infection. It is estimated that the total actual incidence of malaria is in the order of 98 million cases annually.

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Approximately 80% is caused by P. falciparum, the causative agent of the most virulent and rapidly lethal form of human malaria.

In Thailand,the improvement in the situation which occured in 1983 did not continue in 1984. The number of cases reported which had decreased from 420,000 in 1982 to 244,000 in 1983, increased again to 301,000 in 1984. The most dangerous form, P. falciparum, caused approximately 71% of the infection (WHO, 1986 b). Like many of the malarious parts of the world, multidrug resistant P. falciparum is prevalent throughout the country.

The reasons for the spread of malarial infection to more and more victims all over the world can be explained partly by increasing worldwide economic crisis (with more expensive drugs and insecticides), partly by changes of agricultural technology producing irrigated land and increasing mosquito breeding (Chapin and Wasserstrom, 1981) and partly by growing resistance of the mosquitoes themselves to the currently used insecticides (WHO, 1980). Unfortunately, most of malarious areas are faced with further difficulties caused by the appearance of drug resistance in P. falciparum strains (WHO, 1986 a).

Emergence of drug resistance

At present, drug resistance is already a severe problem and the mechanisms of development and spreading are still poorly understood. The earliest description of drug resistance dates back to 1910 when resistance of P. falciparum to quinine was noted from Brazil (Nocht and Werner, 1910),

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followed by reports of more cases of resistance to the same and other conventional drugs. Even mefloquine, the most promising new anti-malarial drug which has been introduced recently, has also been shown to be less effective in some cases of P. falciparum infection (Boudreau et al, 1982; Lapierre et al, 1982; Bygbjerg et al, 1983; Harinasuta et al, 1983). The first recognition of resistance to conventional drugs of P. falciparum are summarized in Table 1. Presently there are about 40 countries reporting chloroquine resistance and 11 countries reporting resistance to sulfadoxine-pyrimethamine combination (Fansidar) (WHO, 1986 b). In addition, cross resistance and/or multi-resistance has often been found. However, there is some good sign in drug resistance situation since recent results from both in vivo and in vitro studies show that under the absence of chloroquine pressure for a suitable period, chloroquine-resistant P. falciparum became more sensitive to chloroquine (Sinha et al, 1984, 1985; Jacquier et al, 1985), and it is hoped that this drug can be used as an effective drug again in the future.

Malaria prevention

Up to now, an effective way of preventing malaria at the public health level is still mostly out of reach. The risk of infection has merely been lessened by mechanical methods (mosquito net, exposure avoidance) and chemical methods (repellants, insecticide sprays) used for individual protection against mosquito bites. These means are not enough to prevent or remove the risk of infections, and vector control by using insecticides now unfortunately faces a

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Table 1 Dates of introduction of antimalarials and the first recognition of resistance to them (Modified from Peters,1970)

Compounds	Year first used in therapy	Place and year in which resistance was first strongly suspected or confirmed
Quinine	c 1630	Brazil, 1910[a] (Nocht and Werner, 1910) Thailand, 1974[(b)] (Jaroonvesama et al, 1974)
Mepacrine	1935	New Guinea, 1946 (Fairley, 1946)
Chloroquine	1945	Venezuala, 1960[a] (Maberti, 1960) Colombia, 1961[b] (Moore and Lanier, 1961; Young and Moore, 1961) Thailand, 1962 (Harinasuta, et al 1962)
Proguanil	1948	Liverpool, 1949 (Adam and Seaton,1949) Malaya, 1949 (Field and Edeson, 1949) India, 1949 (Chaudhuri and Chaudhuri, 1949)
Primaquine	1951	Colombia, 1963 (Powell et al, 1963)
Pyrimethamine	1952	Gambia, 1952 (Mc Gregor and Smith, 1952)
Cycloguanil	1963	S. Rhodesia, 1964 (Lunn et al, 1964)
Sulphadoxine	1964	Kampuchea, 1968 (Verdrager et al, 1968)
Dapsone	1965	Kampuchea, 1968 (Verdrager et al, 1968)
Fansidar®	1965	Thailand-Kampuchea border, 1981 (Hurwitz et al, 1981)
Mefloquine	-	Thailand, 1982 (Boudreau et al,1982)

a) Only suspected b) First confirmation

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big problem since multiple resistance to insecticides has been reported from several countries (WHO, 1980). Another way to prevent malaria is the search for a safe, effective and economically feasible vaccine, but it is not yet possible to anticipate its place in malaria control strategies (Canavale and Mouchet, 1986). Chemotherapy is still the major weapon in a fight against malaria, although by reasons above-mentioned, drug resistance is a major problem.

Since various stages in the life cycle of malaria parasites show different susceptibility to antimalarial drugs, the latter may be classified into tissue schizontocides, blood schizontocides, gametocytocides and sporontocides (Fig. 3). The blood schizontocides are of particular interest as they are used for clinical treatment and suppressive prophylaxis of malaria. Treatment of P. falciparum infections from some areas of the world may require two blood schizontocides, the first to rapidly reduce parasitemia, e.g. quinine, chloroquine, amodiaquine and the second, a more slowly acting drug, e.g. tetracyclines, to acheive radical cure (Hoffman,1986).

The problems of drug resistance make it necessary to search continuously for new and/or more effective drugs. However, the development of new antimalarial drugs is a slow process. Moreover, minimising the risk of emergence of resistance to new alternative drugs is a crucial strategy. It is necessary, therefore to reconsider the therapeutic position of compounds once rated as having secondary importance as antimalarials, especially antibiotics, while waiting for the development of new effective drugs or gradual return to sensitivity to conventional drugs of the resistant parasites.

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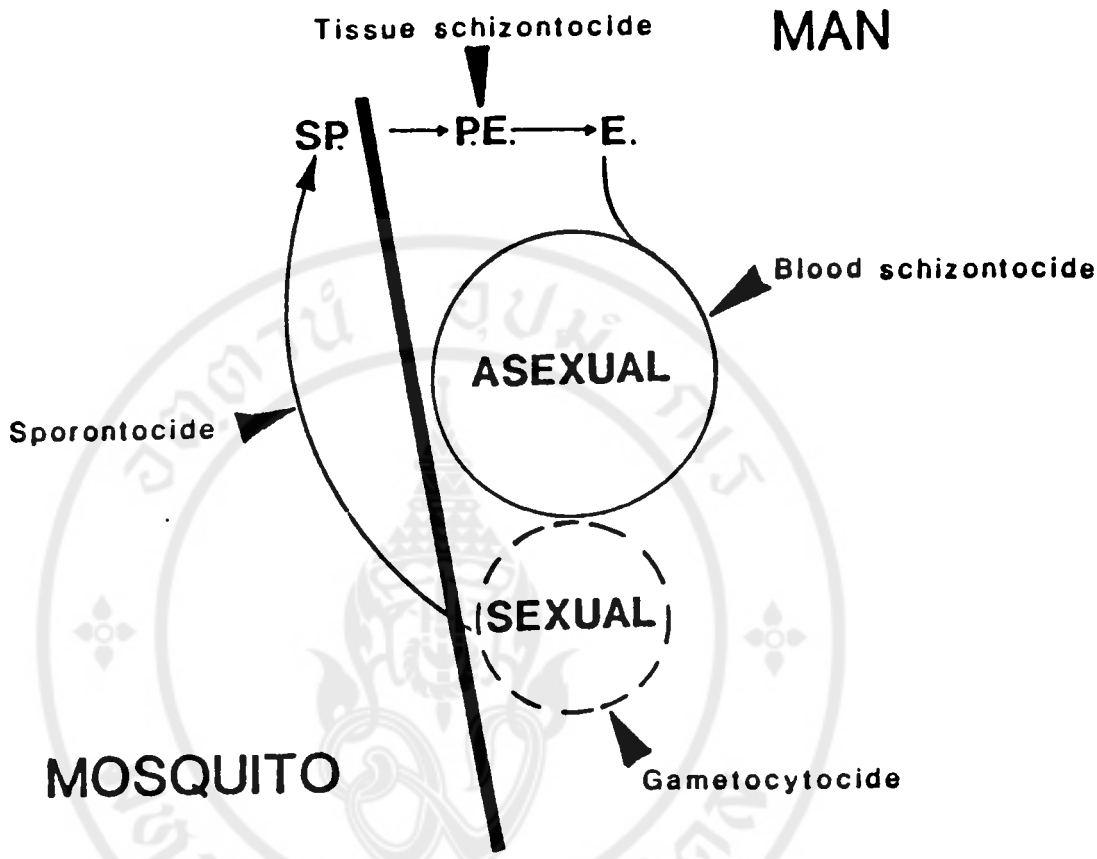


Fig 3 Action of antimalarial drugs (from Bunnag and Harinasuta 1986)

SP.- sporozoites PE.-preerythrocytic stage

E.-erythrocytes

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Tetracyclines and malaria

Tetracyclines as antimalarial drugs

Of the antibiotics which have shown antimalarial activity, tetracyclines are the predominant ones. They have been known as antimalarials since 1949 (Rieckmann, 1984). The results from many laboratories now cleary indicate that tetracyclines are highly effective in terminating both natural and experimentally induced infections of P. falciparum, including conventional drug resistant strains. The spectrum of activity of tetracyclines in human malaria, P. falciparum, is shown as follows;

1) Sporozoites : unknown

2) Primary exoerythrocytic stages : Tetracyclines are active against the primary exoerythrocytic forms of P. falciparum even when administration is started as late as 4 days (Rieckmann et al, 1972; Willerson et al, 1972)

3) Asexual erythrocytic stages : Tetracyclines exert a blood schizontocidal action against P. falciparum, including 4-aminoquinoline and/or dihydrofolate reductase inhibitor-resistant strains, either by using alone (Ruiz-Sánchez et al, 1951 a,b; Clyde et al, 1971; Rieckmann et al, 1971; Willerson et al, 1972) or in combination with other drug, e.g. quinine (Colwell et al, 1972 a,b; Willerson et al, 1972; Colwell and Hickman, 1973; Reacher et al, 1981; Pinichapongse et al, 1982; Bunnag et al, 1983; Schwarz et al, 1983), or amodiaquine (Rieckmann et al, 1972; Noeypatimanond et al, 1983).

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3) Gametocytes : Tetracyclines appear to have no gametocytocidal and sporontocidal activity against P. falciparum (Clyde et al, 1971; Rieckmann et al, 1971; Colwell et al 1972 a,b; Willerson et al, 1972).

All the early studies showed that tetracyclines act as blood schizontocides but, like other antibiotics, their abilities to clear fever and parasitemia was generally slower than other conventional antimalarials (Cooper et al, 1949; Imboden et al, 1950; Ruiz-Sanchez et al, 1951 a,b, 1952, 1956). Antibiotics were, therefore, previously not considered to be of practical value in the treatment of malarial infection (WHO, 1961).

With the widespread occurence of chloroquine-resistant strains of P. falciparum, a reconsideration of the value of some of the older antimalarials, including tetracyclines, was carried out in the early 1970s. It was emphasized that, because of their slow-acting properties, they should be administered in conjunction with a rapidly acting schizontocide, e.g. quinine or amodiaquine. It was shown that both combination therapies were highly effective in treatment (90-100% cure rate) of multidrug resistant P. falciparum-infections (Colwell, et al, 1972 a,b; Rieckmann et al, 1972; Willerson et al, 1972; Colwell and Hickman, 1973; Reacher et al, 1981; Pinichapongse et al, 1982; Bunnag et al, 1983; Noeypatimanond et al, 1983).

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Mechanism of antimalarial action

Although the antimalarial activity of tetracyclines have been known since 1949 (Coatney et al, 1949; Cooper et al, 1949), very little studies have been carried out on their mechanism of action. A possible mechanism has been suggested as occuring through action on parasite mitochondria (Geary and Jensen, 1983; Blum et al, 1984; Divo et al, 1985a)

P. falciparum possesses only a single mitochondrion, which can undergo a complex pattern of growth, development and replication during erythrocytic schizogony, and may or may not posses cristae (Langreth et al,1978; Divo et al, 1985b; Slomianny and Prensier, 1986). However, mitochondrial function in asexual erythrocytic stages of Plasmodium species is still poorly understood.

The parasite mitochondria may exert some function in energy metabolism, since it has been demonstrated that a variety of mitochondrial toxins could inhibit rhodamine 123 accumulation (Divo et al, 1985b) and could also kill the parasites (Divo et al, 1985a; Ginsburg et al,1986). However, it is generally accepted that mammalian malaria parasites derive most, if not all, of their ATP requirements via glycolysis (Sherman, 1984). The absence of α-ketoglutarate dehydrogenase activity in P. falciparum (Blum and Ginsburg, 1984) further indicates the lack of a fully functioning citric acid cycle in this species. If the maintenance of the mitochondrial membrane potential is not used for ATP generation, then the parasite may be expending energy for other purposes,

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probably of metabolic significance. The fact that the parasites are microaerophilic (Scheibel et al,1979) indicates that oxidation-reduction processes requiring oxygen do occur. Furthermore, cytochrome oxidase activity have been found in all species investigated (Scheibel and Miller, 1969 a,b; Scheibel and Pflaum, 1970). Therefore, it has been suggested that the exclusive role of this organelle of Plasmodium is to serve as an electron disposal mechanism in de novo pyrimidine biosynthesis (Gutteridge et al, 1979; Gero et al, 1981a,1984).

The mitochondria of parasite may be involved in Ca^{2+} regulation, a function commonly ascribed to this organelle (Carafoli, 1982). Calcium is known to be accumulated in Plasmodium (Bookchin et al, 1981; Leida et al, 1981; Tanabe et al, 1982; Krungkrai and Yuthavong, 1983).

It is well known that mitochondria possess their own genetic and biosynthetic systems including protein synthesis (Tzagoloff and Myers, 1986). Many species of Plasmodium were shown to possess mitochondrial DNA (Kilejan, 1975; Dore et al, 1983; Williamson et al, 1985). Additionally, by using electron microscope, P. falciparum mitochondria were shown to possess fine filaments which may be DNAs (Langreth, 1978). Plasmodial mitochondria, therefore, may have their own genetic and biosynthetic systems, especially protein synthesis, which may be indispensable for parasite living.

In order to find out whether tetracyclines exert their antimalarial activity through action on the mitochondria, three related hypotheses on the mechanism of action can be raised as follows:

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a) Inhibition of parasite mitochondrial protein synthesis

Since P. falciparum does possess mitochondria (Langreth et al, 1978), which may have their own protein synthesis, and tetracyclines can inhibit mitochondrial protein synthesis (see 'Action on eukaryotic system', page 5), it is possible that tetracyclines act as anti-malarial by inhibiting mitochondrial protein synthesis. Geary and Jensen (1983) demonstrated that potency of action of antimalarial activity of 70 S ribosome specific protein synthesis inhibitors including tetracycline was greater at 96 h of exposure than at 48 h. This result was consistent with the slow action of drug in clinical treatment. The explanation of the slow action was that the action of drug on mitochondria was limited by the permeability factor. Alternatively, Blum et al (1984) explained this result in such the way that it required a duration of time before the consequence loss of newly synthesized mitochondrial protein become significant for inhibition of growth and maturation of parasites. In addition, it was clearly demonstrated that drugs with antimalarial activity are only those which act as protein synthesis inhibitors for 70 S ribosomes, not 80 S ribosomes (Divo et al, 1985a). Since Plasmodium cytoplasmic ribosomes are of the 80 S type (Sherman, 1984), they are not likely to be the targets.

b) Alteration of the Ca^{2+} metabolism

Mammalian malaria parasites are known to have far higher Ca^{2+} uptake and content than that found within the uninfected host erythrocytes (Bookchin et al, 1981; Leida et al,1981; Tanabe et al,

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1982; Krungkrai and Yuthavong, 1983). Furthermore, Ca^{2+} is shown to be indispensable for normal growth and merozoite reinvasion of P. falciparum (Wasserman et al, 1982). Cultivation of P. falciparum infected erythrocytes in the condition that alter Ca^{2+} metabolism or transport such as in the media containing Ca^{2+} chelator; EGTA (Wasserman et al, 1982), erythrocyte transmembrane Ca^{2+} perturbant agents; vanadate, procaine and A 23187 (Mc Alister and Mishra, 1983) or Vitamin D (Vial et al, 1982), resulted in inhibition of growth, maturation and/or reinvasion of parasites. Since tetracyclines can chelate Ca^{2+} (see "Chelation of metal ions", page 6) and the mitochondrion is an organelle responsible for Ca^{2+} regulation (Carafoli, 1982), they may exert the antimalarial activity by altering Ca^{2+} metabolism, probably at mitochondrial level.

c) Inhibition of pyrimidine biosynthesis

One of the possible roles of mitochondria in malaria parasites is to serve for de novo pyrimidine biosynthesis by providing electron disposal mechanism. There are some results showing that agents which interfere with the electron transport chain, e.g. hydroxynaphthoquinone derivatives, can block pyrimidine biosynthetic pathway and result in highly effective antimalarial activity (Hudson, 1984; Hudson et al, 1985; Hammond et al, 1985). Additionally, pyrazofurin, a direct inhibitor of pyrimidine biosynthetic pathway, is effective against P. falciparum in vitro (Scott et al, 1986). It is possible, therefore, that if tetracyclines act at the mitochondrial level, the antimalarial mechanism is through action on pyrimidine biosynthesis. Furthermore, cytochrome oxidase (cytochrome aa_3), one

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of the enzymes in electron transport chain, is a mitochondrially synthesized protein (Tzagoloff and Myers, 1986) and its formation can be inhibited by tetracyclines (De Vries and Kroon, 1970; De Jonge and Hulsmann, 1973; Van Den Bogert and Kroon, 1981; Van Den Bogert et al,1983).

Since knowledge on the mechanism of antimalarial action of tetracyclines, presently still poorly understood, is of potential benefit in the use of the drugs and development of similar ones, the aim of the thesis is to gain insight into this mechanism through

1) studying their effect on mitochondrial protein synthesis.
2) studying their action on Ca^{2+} metabolism
3) exploring their effect on pyrimidine biosynthesis.

In order to achieve these aims, cycloheximide-resistant, tetracycline-sensitive protein synthesis in P. falciparum was explored by 2-dimensional polyacrylamide gel electrophoresis. The effect of tetracycline on Ca^{2+} uptake was studied in order to assess the functional disturbance on the mitochondria. Finally, the effect on a pyrimidine pathway enzyme, dihydroorotate dehydrogenase, which is located on mitochondria, was studied in comparison with another enzyme, glutamate dehydrogenase, of cytoplasmic origin. The results indicate the strong possibility that tetracycline indeed acts through the primary effect on parasite mitochondria.

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MATERIALS AND METHODS

Materials

A. Chemicals

The chemicals, of analytical grade, are listed in alphabetical order as follows,

Chemicals	MW	Companies
Radiochemicals		
Calcium-45 (^{45}Ca), Sp.act.10-40 mCi/mgCa	-	Amersham
[Carboxy-^{14}C]-L-5,6-dihydroorotic acid, Sp.act. 7855 dpm/nmol	-	*School of Biochemistry, University of New South Wales, Australia
L-[4,5-^{3}H]-Isoleucine, Sp.act.95 Ci/mmol	-	Amersham
L-[^{35}S]-Methionine, Sp.act.1270 Ci/mmol	-	Amersham
[7-$^{3}H(N)$]-Tetracycline, Sp.act.0.50 Ci/mmol		New England Nuclear
Non-radiochemicals		
Acrylamide	71.1	Sigma Chemical Co.
Acti-dione(Cycloheximide)	281.34	Schwarz/Mann
Agarose	-	Sigma Chemical Co.
Ammonia solution (NH_4OH)	35.05	Ajex Chemicals
Ammonium chloride (NH_4Cl)	53.49	J.T. Baker Chemical Co.
Ammonium persulfate ($(NH_4)_2S_2O_4$)	228.7	May & Baker Ltd.

*kindly provided by Prof. William J.O' Sullivan

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Chemicals	MW	Companies
Ampholine, pH 3.5-10	-	LKB
Amplify^{TM} RPM 100	-	Amersham
1,4-bis[2-(5-Phenyloxazolyl)] - benzene (POPOP)	364.4	Sigma Chemical Co.
Bovine serum albumin	68 K	Calbiochem-Behring Corp.
Bromophenol blue	670	Matheson-Coleman & Bell
Cellulose powder (CF-11)	-	Whatman Ltd.
Chloramphenicol	323.10	Sigma Chemical Co.
Coomassie Brilliant Blue R-250	-	Sigma Chemical Co.
Cupric sulfate ($CuSO_4 \cdot 5H_2O$)	249.69	J.T. Baker Chemical Co.
Cytochrome c(horse heart type II)	12384	Sigma Chemical Co.
Dibutyl phthalate	278.35	Eastman Kodax Co.
Diethyl ether	74.12	E Merck
Dihydroorotic acid (DHO)	158.1	Sigma Chemical Co.
2,5-Diphenyloxazole (PPO)	221.3	Sigma Chemical Co.
Di-sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)	177.99	Riedel-De Haën
Di-sodium tartrate ($C_4H_4Na_2O_6 \cdot 2H_2O$)	230.08	E Merck
Ethylenediamine tetraacetic acid (disodium salt) (EDTA)	336.2	Sigma Chemical Co.
Ethylene glycol-bis-(β-amino ethyl ether)-N,N'-tetraacetic acid (EGTA)	380.4	Sigma Chemical Co.
Formic acid, 99% (HCOOH)	46.026	Farmitalia Carlo Erba

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Chemicals	MW	Companies
Giemsa stain	-	Chroma
Gentamycin sulfate (Garamycin) injection	-	Schering Co.
Glycerol, anhydrous	-	J.T. Baker Chemical Co.
Glycine (H_2NCH_2COOH)	75.1	Sigma Chemical Co.
Hydrogen peroxide (H_2O_2)	-	Chemekit
N-2-Hydroxyl ethylpiperazine-N-2-ethanesulfonic acid (HEPES)	238.3	Sigma Chemical Co.
IEF pI calibration Kit	-	Pharmacia Chemicals
Isoleucine	131.2	Calbiochem
Menadione	172.17	Neutritional Biochemical
β-Mercaptoethanol	78.13	E Merck
Methanol (absolute)	-	J.T. Baker Chemical Co.
N,N´-Methylene-bis-acrylamide	154.17	Eastman Kodak Co.
β-Nicotinamide adenine dinucleotide phosphate, reduced form (βNADPH)	833.4	Sigma Chemical Co.
Nonidet-P-40	-	Imperial Shell Co.
Orotic acid, monosodium salt	178.1	Sigma Chemical Co.
Orthophosphoric acid (H_3PO_4)	97.995	Mallinckrodt
2-Oxoglutaric acid	146.1	ICN Pharmaceuticals
Perchloric acid, 70% ($HClO_4$)	100.47	Farmitalia Carlo Erba
Percoll	-	Pharmacia Chemicals
Phenol reagent (2N)	-	Fisher Scientific Co.

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Chemicals	MW	Companies
Potassium chloride (KCl)	74.85	Ajex Chemicals
Potassium cyanide (KCN)	65.12	E Merck
Potassium ferricyanide ($K_3Fe(CN)_6$)	329.25	BDH Chemical Ltd.
Potassium hydroxide (KOH)	56.11	BDH Chemical Ltd.
Powdered RPMI-1640, Formula No430-1800	-	GIBCO
Saponin	-	Sigma Chemical Co.
Sodium bicarbonate ($NaHCO_3$)	84.31	J.T. Baker Chemical Co.
Sodium carbonate (Na_2CO_3)	105.99	J.T. Baker Chemical Co.
Sodium chloride (NaCl)	54.443	Farmitalia Carlo Erba
Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)	156.01	Riedel-De Haën
Sodium dodecyl sulfate (SDS)	-	Sigma Chemical Co.
Sodium hydroxide (NaOH)	40.00	E Merck
Sodium penicillin G	-	Dumex
Sodium sulfite, anhydrous (Na_2SO_4)	142.04	E Merck
D-Sorbitol	182.17	Fisher Scientific Co.
Streptomycin sulfate	-	E Merck
Tetracycline-HCl	480.91	Sigma Chemical Co.
N,N,N'N'-tetramethyl ethylene diamine (TEMED)	116.2	Sigma Chemical Co.
Toluene	-	J.T. Baker Chemical Co.
Trichloroacetic acid (CCl_3COOH)	163.39	Farmitalia Carlo Erba

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Chemicals	MW	Companies
Tris-(hydroxymethyl) amino methane (Tris-base)	121.1	Sigma Chemical Co.
Triton-x-100	-	Sigma Chemical Co.
Urea, ultra pure grade (H_2NCONH_2)	60.06	Schwarz/Mann.

B. Other laboratory materials

Plastic tissue culture dishes (60x15 mm) were products of Nunc. 24-multiwells plates were products of Linbro. Sterile membrane filters, 0.45 μm pore sized, were obtained from Whatman Ltd. and Millipore Co. Ltd. Blood bags and blood donor sets were products of Terumo. DEAE ion exchange chromatographic papers (DE 81) and GF/A filter papers were purchased from Whatman Ltd. X-ray films were obtained from Fuji Photo Film Co. Ltd.

Normal type O bloods for preparation of uninfected erythrocytes and serum were from donors at Chulalongkorn Hospital, Rajvithi Hospital and Ramathibodi Hospital, Bangkok.

Plasmodium falciparum (K_1 strain) was obtained locally from an infected patient in Kanchanaburi Province, Westhern Thailand and was kindly provided by Professor Sodsri Thaithong, Division of Parasitology, Department of Biology, Faculty of Science, Chulalongkorn University.

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Methods

1. The in vitro cultivation of Plasmodium falciparum

The technique used was as described by Trager and Jensen (1976 a,b).

1.1 Preparation of RPMI-1640 medium

10.4 g of RPMI-1640 medium (see Appendix I for the formula) and 5.94 g of HEPES were dissolved in 960 ml of distilled water to give a final concentration of 25 mM HEPES with a pH of 6.75. 1 ml (40 mg) of Garamycin was added into the medium. The medium was sterilized by filtration through a 0.45 μM pore size membrane filter and then dispensed aseptically as 100 ml aliquots into sterile bottles. They were stored at $4^{o}C$ as stock medium and used within one month.

A 5% (w/v) solution of $NaHCO_3$ was prepared and sterilized by filtration through a 0.45 μM pore size membrane filter. It was then dispensed as 10 ml aliquots into sterile screw-capped tubes and kept at $4^{o}C$ with a shelf life of one month.

For washing of normal erythrocytes, stock medium (100 ml) was supplemented with 4.2 ml of 5% $NaHCO_3$ to yield a final concentration of 0.2% (w/v) $NaHCO_3$. After mixing, the color of the medium turned from yellow to orange indicating a shift in pH from 6.75 to 7.4. This medium, called incomplete medium, was always freshly prepared.

For cultivation, stock medium was made to pH 7.4 as described above. The medium was supplemented with 11.5 ml of thoroughly mixed

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type 0 human serum to give a final concentration of 10% human serum. This solution, named complete medium, was kept at $4^{o}C$ and used within one week.

1.2 Preparation of serum

Human serum was obtained from clotted blood. Type O blood (300 ml) was drawn without anticoagulant from each donor and allowed to clot at room temperature for a few hours. It was then subjected to complete coagulation at $37^{o}C$ for a further one hour prior to storage at $4^{o}C$, 16-18 h, for clot retraction process. The serum was separated from the clot by centrifugation at 700 x g at $4^{o}C$ for 20 min and then dispensed aseptically as 11.5 ml aliquots into sterile screw-capped tubes. They were stored frozen at $-20^{o}C$ and used within 3 months.

1.3 Preparation of normal erythrocytes

Erythrocytes used for culture were prepared from blood drawn from each donor into a sterile blood bag containing citrate phosphate dextrose (CPD) as anticoagulant. It was further aseptically transferred as 50 ml aliquots into sterile bottles, stored at $4^{o}C$ and used within 4 weeks after collection.

Before use, 10 ml of the packed erythrocytes from the bottom of the storage bottles were washed twice with incomplete medium and centrifuged at 700 x g at $4^{o}C$ for 10 min. As much as possible of supernate and buffy coat was removed to ensure that the cells would not be contaminated with platelets and leukocytes. The washed packed

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cells were resuspended to 50% (v/v) cell suspension with an equal volume of complete medium.

1.4 Cultivation of Plasmodium falciparum

Erythrocytes infected with P. falciparum were either obtained from culture material or from frozen storage.

If the culture was obtained from an already estrablished culture, it was centrifuged at 400xg at 4°C for 7 min and the supernate was discarded. The packed cells were resuspended to 50% (v/v) cell suspension with complete medium and the level of parasitemia was determined (see section 1.9). An appropiate dilution with freshly washed erythrocytes (obtained from section 1.3) was made to reduce parasitemia to a desired level, usually 0.3-0.5%. The complete medium was added to give a final cell suspension of 3%. 10 ml of this suspension were dispensed into 60x15 mm petridishes, which were then placed in a candle jar (desiccator containing a white candle). After lighting the candle, the lid of the jar was closed well (sealing with silicone grease) but the stopcock was left open. The stopcock was closed as soon as the candle went out. An atmosphere of relatively low O_2 and high CO_2 content (17% O_2, 3%CO_2, 80%N_2) was now produced. (Scheibel et al,1978) The candle jar was then placed in an incubator at 37°C.

If the source of culture was obtained from frozen storage, the detail would be further mentioned in section 1.8.2.

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1.5 Changing culture medium

Usually, the culture medium had to be changed every 24 h. As much as possible of the old medium was removed from the culture without removing the settled cells. The same volume of fresh complete medium was added to each culture dish and the cells were resuspended by gentle swirling. The dishes were then placed in the candle jar and incubated at 37°C.

For culture with high parasitemia (≥10%), it was essential to change the medium more frequently, e.g. every 12 h.

1.6 Synchronization of culture

Cultures of P. falciparum could be synchronized when most of parasites were in ring stage according to the method of Lambros and Vanderberg (1979). The cultures were centrifuged at 400xg at 4°C for 7 min and all supernate was removed. The packed erythrocytes were thoroughly mixed with 5 times of its volume of sterile aqueous 5% (w/v) D-sorbitol and allowed to stand at room temperature for 5 min, followed by centrifugation as above. During this treatment late stages of parasites (late trophozoite and schizont) were selectively destroyed, leaving only erythrocytes carrying ring stage. The remaining cells were now ready for use as described in section 1.4.

For some experiments, a stringent synchronization was required. Parasitized cells were synchronized by two successive treatments, 30 h apart, with D-sorbitol as described above. A homogeneous population of rings with a range of ages of 0 to 4 h was left after the second treatment.

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1.7 Concentration of schizont-infected erythrocytes

1.7.1 Preparation of 60% Percoll

Percoll was adjusted to osmolarity of 280-320 mOsm/kg H_2O by the addition of 1 part of 10 x concentrated physiological salt solution (8.5% NaCl) to 9 parts of Percoll. This is called 100% Percoll with a density of 1.130 g/ml. 60% Percoll (density 1.085 g/ml) was prepared from this stock Percoll by adding normal saline (0.85% NaCl) as diluent. Dropwise addition of normal saline with continuous, gentle stirring was required to prevent aggregation of silica particles. The 60% Percoll in normal saline was adjusted to pH 7.4 with a few drops of 1 M HCl. The 60% Percoll could not be sterilized by autoclaving since this will cause gelation, but it was sterilized by filtration through 0.45 µm pore size membrane filter. It was kept at $4^{o}C$.

1.7.2 Procedure for concentrating schizont-infected erythrocytes

Infected cells could be concentrated by a Percoll technique, modified from that described by Tosta et al (1980). Synchronous cultures were obtained from asynchronous ones by treating with 5% D-sorbitol (see section 1.6). These cells were cultured to a high parasitemia (∿10%), mostly at late stage (schizont). They were harvested and adjusted to 10% cell suspension with complete medium, gently overlayed onto an equal volume of 60% (v/v) Percoll and centrifuged at 700xg, at $4^{o}C$ for 20 min. Schizont-infected cells, with similar density to that of 60% Percoll, remained at the interphase

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as a brown layer, whereas other cells sedimented. The brown layer was carefully removed, centrifuged at 700xg at 4°C for 10 min and washed three times with incomplete medium. The parasitemia of this preparation was as high as 80-95% (Dejkriengkraikul et al, 1982).

1.8 Cryopreservation of malaria parasites

1.8.1 Freezing

It is best to store malaria parasites when the parasitemia exceed 5% and the parasites are mostly in ring stage.

1.8.1.1 Freezing solution

The solution was comprised of 28% glycerol, 3% sorbitol and 0.65% NaCl, prepared by adding 70 ml of 99% glycerol to 180 ml of 4.2% sorbitol in 0.9% NaCl solution. This solution was sterilized by filtration through a 0.45 μM pore size-membrane filter before use.

1.8.1.2 Freezing procedure

The culture material at ring stage was centrifuged at 400xg, at 4°C for 7 min and supernatant was discarded. The cells were resuspended in an equal volume of freezing solution. The cell suspension was gently mixed and 0.5 ml aliquots were placed into sterile small screw-capped vials (Cooke Laboratory). The vials were immediately placed into a liquid nitrogen tank.

1.8.2 Recultivation of malaria parasites

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The vial was removed from liquid nitrogen tank. The cap of the vial was slightly loosened and the vial was set upright

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in water at room temperature for a few minutes. The thawed suspension was immediately transferred to a centrifuge tube and centrifuged at 400xg, at $4^{o}C$ for 7 min. The supernatant was discarded. The cells were resuspended in an equal volume of hypertonic saline solution (3.5% NaCl) and gently mixed before centrifugation as above. Supernatant was again discarded. The packed cells was washed twice with an equal volume of incomplete medium. The cells were finally resuspended in an equal volume of the complete medium to give 50% cell suspension. Washed uninfected erythrocytes were added to obtain the desired parasitemia. The remaining steps were as previously described in section 1.4.

1.9 Parasitemia determination

The level of parasitemia was determined from Giemsa-stained thin blood films. Thin blood films were made from the cultures, fixed in acetone-free absolute methanol for 1 min and air-dried. The slides were stained with 3% Giemsa's stain (see Appendix II for formulation) in 0.067 phosphate buffer, pH 7.2 for 30 min followed by washing with tap water and air-dried. Using a microscope with an oil immersion lens (100 x magnification), 10,000 total erythrocytes were examined and the percentage of parasite infected cells was then calculated. An erythrocyte infected by more than one parasite (multiple infection) seen at the frequency of 10-20% was counted as one infected cell.

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2. Erythrocyte counting

The number of erythrocytes in cell suspension samples were counted on the Improved Neubauer Ruling hematocytometer using Gower's solution; made of 12.5 g anhydrous Na_2SO_4 and 33.3 ml of glacial acetic acid in 200 ml of distilled water. The error of number of erythrocytes obtained from this method was within 10%. The number of erythrocytes was expressed as cells/mm^3.

3. Preparation of parasite extract

3.1 Preparation of isolated parasites

Parasites were released from erythrocytes by saponin lysis and concentrated by centrifugation, using the modified method of Sanderson et al (1981). Parasitized cells from continuous culture (see section 1.4) or from some experiments were harvested and centrifuged at 400xg, at 4°C for 7 min. The supernatant was discarded and the packed cells were resuspended to 50% v/v with incomplete medium. An equal volume of 0.15% saponin in incomplete medium (see section 1.1) was added to the cell suspension. The thoroughly mixed suspension was incubated at 37°C for 20 min. The lysate was then resuspended in 5 times of its own volume of ice cold incomplete medium and, after thorough mixing, centrifuged at 2,000xg for 20 min. The supernate and the erythrocyte ghost layer, visible above the pellet of packed parasites, were carefully removed. The isolated parasites were washed 3 times in ice cold 0.15 M NaCl, 5 mM phosphate buffer, pH 7.4 (5 mM PBS, pH 7.4) and centrifuged as above.

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3.2 Preparation of parasite homogenate

The isolated parasites (obtained from above) were washed once with a suitable buffer for each experiment, resuspended in the same buffer, and lysed by sonication using sonicator cell disruptor model W-225 R. Sonication was performed at $4^{o}C$ for 5 sec, 50% duty cycle with output 40 watt. The sonicated suspension was then centrifuged at 10,000 x g for 10 min. The crude parasite lysate, 10,000 x g supernatant and 10,000 x g pellet were used immediately for each enzyme assay.

4. Procedure for determining the antimalarial activity of tetracycline

The technique used was as described by Trager and Polonsky (1981). A stock of tetracycline was prepared in distilled water and diluted further with complete medium at appropiate concentrations. A serial 3-fold dilution was normally used. 0.5 ml of the culture with 0.3-0.5% parasitemia, 3% cell suspension in complete medium without drug, was incubated in 24 multiwells-plate at $37^{o}C$ in a candle jar for 1 h. After that, the medium was changed by adding the serially diluted drug solution, and the culture was incubated further. The medium was changed and thin blood films were made from each well at every 24 h during 96 h of incubation. Percent parasitemia was determined. Calculation of percent inhibition at 48 and 96 h by various concentration of drug was made by comparing with drug-free controls. 50% effective concentration (EC_{50}) can be determined by plotting percent inhibition against log concentration of drug.

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5. Procedure for studying of the effect of tetracycline on merozoite invasion

High parasitemia, synchronized cultures (see section 1.6) were maintained until late schizont stages in the presence and absence of 10^{-5}M tetracycline for 24 h. These 2 groups of schizonts were concentrated by Percoll gradient centrifugation method (see section 1.7.2). Each group of concentrated schizonts was devided into 2 parts and each part was added with normal or 24 h - 10^{-5}M tetracycline-pretreated erythrocytes until the parasitemia was reduced to 0.3-0.5%. Each part was cultivated as 3% cell suspension, in complete medium with or without 10^{-5} M tetracycline, in 24 multi-wells plates under the candle jar condition at 37°C. At 15 h of incubation thin blood smears were prepared and the percent parasitemia (%PE) was determined (see section 1.9). The multiplication index was calculated as follows:

$$\text{Multiplication index} = \frac{\text{\% PE after 15 h of incubation}}{\text{\% PE at time -0}}$$

6. Analytical technique for determining protein synthesis

6.1 Metabolic labeling of parasite proteins

P. falciparum-infected erythrocytes from continuous culture (obtained from section 1.4) were harvested at the desired parasitemia and centrifuged at 400xg, 4°C for 10 min. Packed erythrocytes were washed twice in complete medium. Packed erythrocytes were incubated at 37°C in the RPMI 1640 medium depleted of the amino acid used for labeling plus the radiolabeled amino acid at desired specific activity.

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6.2 Method for determining the incorporation of radiolabeled amino acid into protein

Desired volumes of well mixed protein labeling suspensions (see section 6.1) were spotted onto GF/A filter papers. After the filter papers were allowed to dry, they were washed 3 times with 10% TCA (5 ml), twice with 10% hot TCA ($80^{o}C$, 5 ml), bleached overnight with bleaching solution (5 ml of 10% TCA : 35% H_2O_2 : 99% HCOOH = 2:1:1), washed twice with 70% ethanol (5 ml) and once with diethyl ether (5 ml). The GF/A filter papers were dried in an incubator ($50-60^{o}C$). The dried filter papers were counted for radioactivity by using Beckman liquid scintillation counter, model LS-1801 and using a toluene cocktail (PPO 20 g, POPOP 1.2 g added purified toluene to 4 litres) as the scintillation fluid. In some experiments, in order to reduce non-incorporated radiolabeled amino acid contamination, the same but non-radiolabeled amino acid was added into the 10% TCA solution at the concentration of 10^{-3}M.

7. Analytical two-dimensional gel electrophoresis

The technique used was as described by O'Farrell (1975). Proteins were separated according to isoelectric points by isoelectric focusing (IEF) in the first dimension, and according to molecular weights by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension.

7.1 Procedure for the first dimension

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7.1.1 Stock solutions and buffers for isoelectric focusing (IEF)

- Lysis buffer : 9.5 M urea, 2% (w/v) NP-40, 2% (w/v) Ampholines, pH 3.5-10 and 5% (v/v) β-mercaptoethanol.

- Sample overlay buffer : 8 M urea, 5% (w/v) NP-40, 1% (w/v) Ampholines, pH 3.5-10 and 5% (v/v) β-mercaptoethanol.

- SDS-sample buffer : 0.0625 M Tris-HCl pH 6.8, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 10% (w/v) glycerol

- 30% Acrylamide solution : 28.38% (w/v) acrylamide and 1.62% bisacrylamide.

- Nonidet P-40 solution : 10% (w/v) NP-40 in distilled water

- Lysis buffer and overlay buffer were stored as frozen aliquots. SDS sample buffer, 30% acrylamide solution, NP-40 solution were kept at $4^{o}C$.

7.1.2 Preparation of the isoelectric focusing (IEF) gel

To prepare 1 ml of gel mixture, 0.55 g of urea, 200 µl of 10% NP-40, 133 µl of 30% acrylamide solution, 60µl of Ampholine, pH 3.5-10 and 170 µl of distilled water were added together. The gel mixture was swirled until urea was completely dissolved. The solution was degassed, 7 µl of 10% TEMED and 10 µl of 10% ammonium persulfate were then added. The solution was mixed well and immediately loaded into gel tubes.

7.1.3 Procedure for casting gel tubes

Isoelectric focusing gels were made in glass tubings (150 x 20 mm inside diameter) sealed at the bottom with three layers of parafilm. The gel solution (obtained from section 7.1.2) was loaded

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into tubes using a hypodermic needle fitted with a long polyethylene tubing. To avoid trapping air bubbles, the tip of the tubing was inserted to the bottom of the tube and withdrawn slowly as the acrylamide mixture run in. The tubes were filled up to a specified mark (≈10 cm high) so that all gels were of the same length. The gels were overlayed with deionized distilled water and allowed to polymerize for 1 h. The polymerized gel surface was washed with distilled water and reoverlayed with 25 µl of lysis buffer. A small amount of water was layed over the lysis buffer to prevent urea from crystalizing at the top. The upper surface of the gels were allowed to equilibrate with lysis buffer for a further 1 h.

7.1.4 Running condition for isoelectric focusing (IEF)

The parafilm was removed and the gels were placed in a gel electrophoresis chamber, the lower reservoir being filled with 10 mM H_3PO_4. The solution above the gels was removed and gels were re-overlayed with 20 µl of fresh lysis buffer and enough 20 mM NaOH to fill the tubes. The upper reservoir was then filled carefully with 20 mM NaOH so as not to disturb the layers of solutions over the gel. The positive electrode was connected to the lower reservoir and the negative one to the upper reservoir. Gels were prerun as follows:

a) 200 Volts for 15 min

b) 300 Volts for 30 min

c) 400 Volts for 30 min

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After the prerun, the upper reservoir was emptied, lysis buffer and NaOH were removed from the surface of the gels, and the samples were loaded. After loading, 10 µl sample overlay buffer were carefully added, the tubes were filled with 20 mM NaOH, the upper reservoir was replenished. The gels were run for 14 h at 400 V and then at 800 V for a further 1 h.

7.1.5 Equilibration of IEF gels

At the end of the run, all gel tubes were removed from chamber and placed on ice. The gels were carefully removed from the gel tubes by rimming with a cold water filled syringe fitted with a long hypodermic needle. The extruded gels were then equilibrated in SDS sample buffer at room temperature for 30 min before loading onto slab gels of the second dimension.

7.2 Procedure for the second dimension

The second dimension was the discontinuous SDS slab gel system, modified from that of Laemmli (1970).

7.2.1 Stock solutions and buffers for SDS-PAGE

Solution A : 29.2% Acrylamide and 0.8% N,N'-methylene bisacrylamide in distilled water

Solution B : 1.5 M Tris-HCl buffer pH 8.8 contained 0.4% (w/v) SDS

Solution C : 0.5 M Tris-HCl buffer pH 6.8 contained 0.4% (w/v) SDS

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7.2.2 Preparation of the SDS separating gel

All slab gels were cast as uniform gel of 10% acrylamide concentration. The stock solutions were mixed in the following way to make 1 slab gel : 6 ml of solution A, 6 ml of solution B and 6 ml of distilled water. 50 µl of 10% ammonium persulfate and 10 µl of TEMED were added and then mixed well before pouring into the assembled glass plates (18x18x0.1 cm^3). The slab was 1.0 mm thick with 10 cm of 10% separating gel. After all solution was poured, the mixture was gently overlayed with about 3 ml of distilled water and allowed to stand for polymerization. The water and the residual unpolymerized gel mixture were removed before pouring of the stacking gel solution.

7.2.3 Preparation of the SDS stacking gel

The stacking gel of 4.65% acrylamide contained 1.5 ml of solution A, 2.5 ml of solution C, 6 ml of distilled water, 20 µl TEMED and 50 µl of 10% ammonium persulfate. The mixture was poured onto the top of the running gel after removing the covered water and the residual unpolymerized gel mixture. A teflon strip was inserted into the slab before the polymerization of the stacking mixture to make two slots (near the edge of the gel 0.5x1x0.1 cm^3) for loading samples or standard molecular weight proteins into the gel. The stacking gel was allowed to polymerize for 15 to 30 min.

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7.2.4 Loading isoelectric focusing (IEF) gel onto the slab gel

Before the first dimension gel was applied on top of the second dimension, the teflon strip was removed from the gel and all unpolymerized gel solution was removed from the surface. The IEF gel was removed from the SDS sample buffer and carefully laid on top of the stacking gel taking care to avoid any stretching or bending. The melted 1% agarose in the SDS sample buffer (see section 7.1.1) was used to keep the IEF gel in place and left to set for 3 to 5 min before the slab gel was clamped to the electrophoresis chamber.

7.2.5 Running of the slab gel

Running buffer (Tris-glycine electrode buffer, pH 8.3; 1 litre containing 3.0 g Tris-base, 14.4 g glycine and 1.0 g SDS) was added to upper and lower reserviors after the bottom teflon strip of the assembled set was removed. Air bubbles were removed from the bottom of the gel and then 2 drops of 0.1% bromphenol blue were added to the upper reservoir. The gel was run at 25 mA constant current until the dye front reached the bottom of the gel. At the end of the run the plate was removed from the apparatus and the gel was processed as desired. Standard molecular weight markers consisted of bovine serum albumin (68 K), ovalbumin (43 K), concanavalin A (27 K) and myoglobin (17 K).

7.2.6 Staining and destaining

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The proteins in the slab gel were stained for at least 2 h with 0.2% (w/v) Coomassie brilliant blue R-250 in 50% (v/v)

methanol and 10% (v/v) acetic acid at room temperature. The gels were then destained in 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h, and further destained in several changes of 5% (v/v) methanol and 7% (v/v) acetic acid.

8. Fluorography of radiolabeled proteins

Proteins in the gel to be processed for fluorography were fixed by staining and destaining or using a fixing solution of 7% acetic acid. Then, the gel was soaked in Amplify™ with gentle agitation for 30 min at room temperature. The gel was removed from the solution and dried between two sheets of cellophane paper under vacuum at 80-85°C by using a Bio-rad Model 224 Slab Gel Dryer with a pressure pump. The dried gel was exposed to X-ray film (Fuji RX 100) in a X-ray cassette with intensifying screen. The cassette was kept for appropriate time at -70°C. The image on the exposed film was developed using Kodak X-ray developer and Fixer (Eastman Kodak company) according to the standard procedure described by Kodak company.

9. Study of cycloheximide-resistant, tetracycline-sensitive fraction of protein synthesis

9.1 Inhibition of [^{3}H] -isoleucine incorporation by cycloheximide and tetracycline

Parasitized erythrocytes were harvested, washed twice with, isoleucine-free (IF) medium, as described in section 6.1, added with normal uninfected erythrocytes to reduce parasitemia to 3-4% and

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diluted to 3% cell suspensions with complete, IF-medium (IF medium plus 10% human serum group O), containing either cycloheximide or tetracycline at concentrations ranging from 10^{-7} to 10^{-3}M. Triplicates of 200 µl of each cell suspension were placed into 96 multiwells plate and incubated for 7 h at 37°C under candle jar atmosphere (see section 1.4). Each well contained 2.5 µCi of $[^3H]$-isoleucine (95 Ci/mmol) per well. For controls, medium without was added instead of drug solutions and non-parasitized cells were used in another set of controls.

After 7-h incubation, cell suspensions were mixed well and 180-µl-aliquots were spotted onto GF/A filter papers and processed further as described in section 6.2. The mean non-parasitized control cpm value was subtracted from the other cpm values, and at each drug concentration the $[^3H]$-isoleucine incorporation calculated as a percentage of the parasitized control value.

9.2 Inhibition of $[^3H]$-isoleucine incorporation by tetracycline in the presence of $3.3x10^{-3}$M cycloheximide

Incubation of $[^3H]$-isoleucine incorporation by tetracycline in the presence of $3.3x10^{-3}$M cycloheximide was assayed as described in section 9.1 with the following modifications. Two sets of parasitized control wells were employed, one set with no added drugs, and one set containing $3.3x10^{-3}$M cycloheximide. $3.3x10^{-3}$M cycloheximide was also present in non-parasitized control wells and in those containing 10^{-7} to 10^{-3}M tetracycline. In order to detect lower levels of $[^3H]$-isoleucine incorporation, the parasitemia was increased to 15-20%, and the amount of $[^3H]$-isoleucine was increased to 10 µCi per well.

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At the step of protein precipitation on GF/A by 10% TCA (see section 6.2), this solution was added with 10^{-4}M non-radiolabeled isoleucine.

10. Study of time course of incorporation of [^{35}S]-methionine into protein

High parasitemia of P. falciparum - infected erythrocytes were prepared for metabolic labeling as described in section 6.1. Parasites were metabolically labeled with [^{35}S]-methionine (1270 Ci/mmol) by incubating 5% cell suspension of infected erythrocytes at 37°C in methionine-free RPMI-1640 medium containing [^{35}S]-methionine at the specific activity of 20 µCi/ml mixture. At indicated intervals, 25 µl of the well mixed suspensions were spotted onto GF/A filter papers and processed further as described in section 6.2.

To study the effect on overall protein synthesis, cycloheximide or tetracycline at desired concentration was added into the mixture and the experiment performed as described above.

11. Analysis of cycloheximide-resistant, tetracycline-sensitive protein by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Parasites were metabolically labeled with [^{35}S]-methionine as described in section 10 but at the specific activity of 250 µCi/ml, with 10^{-4}M cycloheximide and without or with various concentrations of tetracycline. Controls were treated similarly but without drug additions. The incubation mixtures were incubated for a suitable

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period (≈7 h) (see result of the time course study of [^{35}S]-methionine, Fig. 8), At the end of the labeling, 5 µl well mixed suspensions were spotted on GF/A filter papers and processed as described in section 6.2. Then, each mixture was added with equal volume of lysis buffer (see section 7.1.1), freeze-thawed for 3-5 times and centrifuged at 10,000 xg for 10 min, the supernates were used for running 2D-PAGE.

To characterize protein by 2D-PAGE, a 50-µl aliquot of supernate of each sample was required for one run, the processes were as described in section 7. The protein patterns were obtained by fluorography as described in section 8.

12. $^{45}Ca^{2+}$ uptake studies

12.1 Preparation of *P. falciparum*-infected erythrocytes

P. falciparum - infected erythrocytes from continuous culture (obtained from section 1.4) at high parasitemia were harvested, centrifuged at 400xg, 4°C for 10 min, and the supernate was discarded. Packed erythrocytes were resuspended to 50% cell suspension in ice cold 5 mM PBS, pH 7.4 and the cell suspension was passed through a tightly packed volume of CF-11 cellulose powder to remove the remaining leukocytes. The eluted erythrocytes were washed 3 times with ice cold 5 mM PBS, pH 7.4.

12.2 Procedure for $^{45}Ca^{2+}$ uptake study

The PBS washed erythrocytes were further washed 3 times with about 5 volumes of ice cold washing medium consisting of 75 mM NaCl, 75 mM KCl, 10 mM Tris-HCl, pH 7.4 and 0.1 mM EGTA, and 3 times

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more with the same washing medium but without EGTA. The washed cells were packed at 400xg, 4° C for 10 min.

Washed packed erythrocytes were added into culture dishes containing RPMI medium plus $^{45}CaCl_2$ solution to give the final 10% cell suspension with 20 µCi of $^{45}Ca^{2+}$ per ml mixture. Culture dishes were placed in a candle jar and processed as described in section 1.4. Normal age-matched erythrocytes, treated in the same way as infected cells, were used as control.

12.3 Procedure of assaying ^{45}Ca radioactivity

^{45}Ca in all samples were determined according to the method described by Ferreira and Lew (1976) with a slight modification.

To determine ^{45}Ca radioactivity, triplicates of 0.2 ml samples of the cell suspension were placed in 1.5 ml microcapped centrifuge tubes containing 0.4 ml n-dibutyl phthalate and 0.9 ml of the initial washing medium but with 5 mM EGTA, all at 0-1°C. Each sample was centrifuged immediately for 15 s at 10,000xg in a TOMY microcentrifuge, model 15-A. The cells formed a compact pellet at the bottom of the oil layer and the aqueous supernate remained on top. The whole procedure from sampling to cell separation took about 1 min. The sample tubes were frozen in a dry ice-acetone bath, and each centrifuge tube tip was then cut at the oil layer near the packed cell interface to eliminate the contaminant of the supernate including almost of the oil. The residual oil on the packed cells surface was absorbed with blotting paper. The cells were lysed in 150 µl of distilled water. 50-µl hemolysate of each sample was withdrawn for determining the hemoglobin concentration. Proteins in the hemolysate

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were precipitated with 40 µl of 25% perchloric acid and pelleted by centrifugation at 10,000xg for 10 min. 100-µl alquot of the supernate of each sample was then taken into the scintillation vial containing 5 ml of 50% triton x-100-toluene cocktail, made up of 50% (v/v) toluene 50% (v/v) triton x-100, 0.5% (w/v) PPO and 0.03% (w/v) POPOP, and measured the radioactivity by using Beckman liquid scintillation counter, model LS-1801. Standard ^{45}Ca was also run using the same procedure. For determination $^{45}Ca^{2+}$ uptake, radioactivity in each sample was corrected to cpm per 20 µl packed erythrocytes by calculating as follows:

$$\text{Radioactivity in each sample (CPM/20 µl packed cells)} = \frac{\text{CPM in each sample} \times \text{OD 540}^{*} \text{ of 20µl packed cell}}{\text{OD 540* of each sample}}$$

(*see section 12.4)

and then converted into nmol $^{45}Ca^{2+}$ by using specific activity of ^{45}Ca (CPM/nmol ^{45}Ca), from a standard curve for ^{45}Ca. The results for all experiments were expressed as nmol $^{45}Ca^{2+}/10^{9}$ cells.

12.4 Determination of hemoglobin

Concentration of hemoglobin was determined by using Cyanmethemoglobin method (Drabkin, D.L., 1949).

50- 1 aliquot of hemolysate of each sample was added into 5 ml Drabkin's solution, made up of 1.0 g $NaHCO_3$, 0.2 g $K_3Fe(CN)_6$ and 0.06 g KCN in 1 litre of distilled water, and then solution was mixed well. After standing at room temperature for 10 min, the amount of cyanmethemoblobin formed was measured in a spectrophotometer, Shimadzu, model UV-120-01 at 540 nm, using Drabkin's solution as blank.

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13. Study of $^{45}Ca^{2+}$ uptake by P. falciparum (K_1)-infected erythrocytes carrying different stages of development

Parasites were stringently synchronized (see section 1.6) and cultivated for a further one cycle to get a high parasitemia of the early ring stages. Using this time as a starting point of the study (Time-0) and every 4 h to a time of 52 h, a part of cultures was harvested and processed as described in section 12. Each point of the study was from 4 h uptake experiment.

14. Study of the effect of tetracycline on $^{45}Ca^{2+}$ uptake by P. falciparum (K_1)-infected erythrocytes

All steps were carried out as described in section 12. Tetracycline at 3 µg/ml ($0.68x10^{-5}$M) was added in the incubation mixture and the samples were examined for the uptake of $^{45}Ca^{2+}$ at every 4 h of 20 h incubation.

15. Study of the effect of tetracycline pretreatment on initial $^{45}Ca^{2+}$ uptake rate of P. falciparum (K_1)-infected erythrocytes

Parasites were maintained in the presence of 3 µg/ml ($0.68x10^{-5}$M). At time intervals, parasites were harvested and processed for $^{45}Ca^{2+}$ uptake study in the presence of the same concentration of tetracycline as described in section 12. Each point of the study was from 4 h uptake experiment.

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16. Study of [^{3}H]-tetracycline uptake by infected erythrocytes

The method use was as described for studying $^{45}Ca^{2+}$ uptake (see section 12), except that 10^{-3} M tetracycline in 5 mM PBS, pH 7.4 was used instead of washing medium plus 5 mM EGTA at the step of cell separation (see section 12.3).

[^{3}H]-tetracycline, instead of $^{45}Ca^{2+}$, was added to the incubation mixture to a concentration of 3 µg/ml of tetracycline free base and specific activity of 1 µCi/ml incubation mixture. The time intervals for sampling were of 4 h duration along the incubation period of 20 h. The radioactivity was determined in the same way as $^{45}Ca^{2+}$ uptake study and the results were expressed as nmol [^{3}H]-tetracycline/10^{9} cells.

17. Dihydroorotate dehydrogenase assay

Dihydroorotate dehydrogenase (DHO-DHase, L-5,6-dihydro-orotate: oxygen oxidoreductase, E.C. 1.3.3.1) activity was dtermined by measuring the conversion of [carboxy-^{14}C]-L-dihydroorotic acid (DHO) into [carboxy-^{14}C]-orotic acid. The assay conditions, modified from the method of Gero et al (1984), were as follows : 10 mM HEPES-KOH, pH 8.0; 60 µM [carboxy-^{14}C]-L-DHO; the parasite extract in a final volume of 0.1 ml. The mixture was incubated at 30°C for 30 min in a waterbath shaker. The reaction was stopped by addition of 2 µl of 70% $HClO_4$ and the mixture was centrifuged for 10 min at 10,000xg. Dihydroorotic acid and orotic acid were separated by DEAE-cellulose paper chromatography (Hermann et al, 1973). 20-µl aliquots of the supernate were spotted onto the origin of a 23x57-cm sheet of DEAE-

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ion exchange cellulose paper. Four samples were spotted 5 cm apart along the origin 10 cm from one end of each sheet. A fold was made 4 cm from the other end of the sheet and the resulting flap was stapled to form a hem for the supporting rods. The DEAE papers were suspended in a chromatographic cabinet and equilibrated for 12 h over 0.6 N formic acid. Ascending chromatography was performed at room temperature until the solvent front ascended approximately 37 cm above the origin. The area containing orotic acid was that from R_f = 0.26 to 0.33 (mean R_f = 0.29); the area containing dihydroorotic acid was that from R_f = 0.42 to 0.52 (mean R_f = 0.47) (see Fig. 31).

Rectangular areas of each radioactive compound were cut from the chromatogram and placed into a glass scintillation counting vials. 10 ml of toluene cocktail (see section 6.2) were added into the vials and radioactivity was measured by a Beckman liquid scintillation counter, Model LS-1801.

The activity was calculated as follows:

$$\text{Total activity} = \frac{\text{Radioactivity of orotic acid x DHO concentration}}{\text{Radioactivity of DHO}}$$

$$\text{Specific activity} = \frac{\text{Total activity x 60}}{\text{incubation time x protein concentration of parasite extract}}$$

Specific activity was expressed as nmol orotic acid formed.$mg^{-1}.h^{-1}$

18. Study of the location of dihydroorotate dehydrogenase in P. falciparum

Parasite extracts were freshly prepared as described in section 3. Both 10,000xg supernatant and 10,000xg pellet were used for enzyme assay (see section 17). The results were presented as

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percentage of recovered activity which were calculated as described below,

$$\% \text{ of recovered activity} = \frac{\text{activity recovered in each fraction}}{\text{sum of activity in both fractions}} \times 100$$

19. Study of the direct effect of tetracycline on dihydroorotate dehydrogenase activity from P. falciparum

10,000xg pellet was used as enzyme located fraction (see table 3) for the study. Enzyme activity was assayed in the presence of various concentrations of tetracycline. The results were presented as relative activity as compared to the activity of control in the absence of drug.

20. Study of activity of dihydroorotate dehydrogenase of tetracycline-treated P. falciparum

P. falciparum-infected erythrocytes were maintained in the absence and presence of 10^{-5} M tetracycline. At different time intervals, parasitized erythrocytes were harvested and processed as described in section 3. Enzyme activity in each sample was assayed from 10,000xg pellet as described above. For some experiments, 0.3 mM menadione and/or 0.1 mM cytochrome c was(were) added into the assay mixture.

21. Glutamate dehydrogenase assay

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Glutamate dehydrogenase (Glu-DHase, L-Glutamate : NADP oxidoreductase deaminating, E.C. 1.4.1.4) activity was measured spectrophotometrically in a Shimadzu 240 spectrophotometer according

to a method modified from Schmidt (1974). The reaction mixture consisted of 0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, 40 mM NH_4Cl, 0.1 mM NADPH, 10 mM 2-oxoglutarate and approx 0.1 mg parasite extract, in a final volume of 1 ml. The reaction was initiated by the addition of 2-oxoglutarate at 340 nm and 25°C. The specific activity of Glu-DHase in the parasite extracts was calculated using extinction coefficient difference of 6200 M^{-1} cm^{-1}.

22. Study of the location of glutamate dehydrogenase in P. falciparum

Parasite extracts were prepared as described in section 3. Fresh parasite extracts, both 10,000xg supernatant and 10,000xg pellet, were used for enzyme assay (see section 21).

23. Study of activity of glutamate dehydrogenase in parasite extracts of tetracycline-treated P. falciparum

P. falciparum - infected erythrocytes were maintained in the absence and presence of 10^{-5} M tetracycline. At time intervals, parasitized cells were harvested and processed as described in section 3. 10,000xg supernatant of each sample was used as enzyme located fraction (see table 6) for activity assay (see section 21).

24. Protein determination

Protein concentrations were determined according to the method of Lowry et al (1951).

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The diluted sample (0.1 ml), containing no more than 100 µg of protein (1 mg/ml) was mixed with 3 ml of freshly prepared alkaline

copper reagent (1 part of 1% $CuSO_4.5H_2O$, 1 part of 2% sodium tartrate, and 100 parts of 2% Na_2CO_3 in 0.1 NaOH). The mixture was left at room temperature for 10 min before adding 0.3 ml of freshly prepared of 1 N Folin phenol reagent. The mixture was vigorously mixed and left for an additional 30 min. The absorbance at 660 nm was measured against blank which was treated as the sample except that 0.1 ml of distilled water was used instead. Crystalline bovine serum albumin (0.25-1.00 mg/ml) was used as standard.

When the sample was originally in 1% Triton x-100, SDS was added to alkaline copper solution to a concentration of 0.5% (w/v) to prevent the precipitate formation (Dulley and Grieve, 1975). Blank and standard protein also contained 1% Triton x-100.

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RESULTS

1. Effects of tetracycline on P. falciparum (K_1) growth in vitro

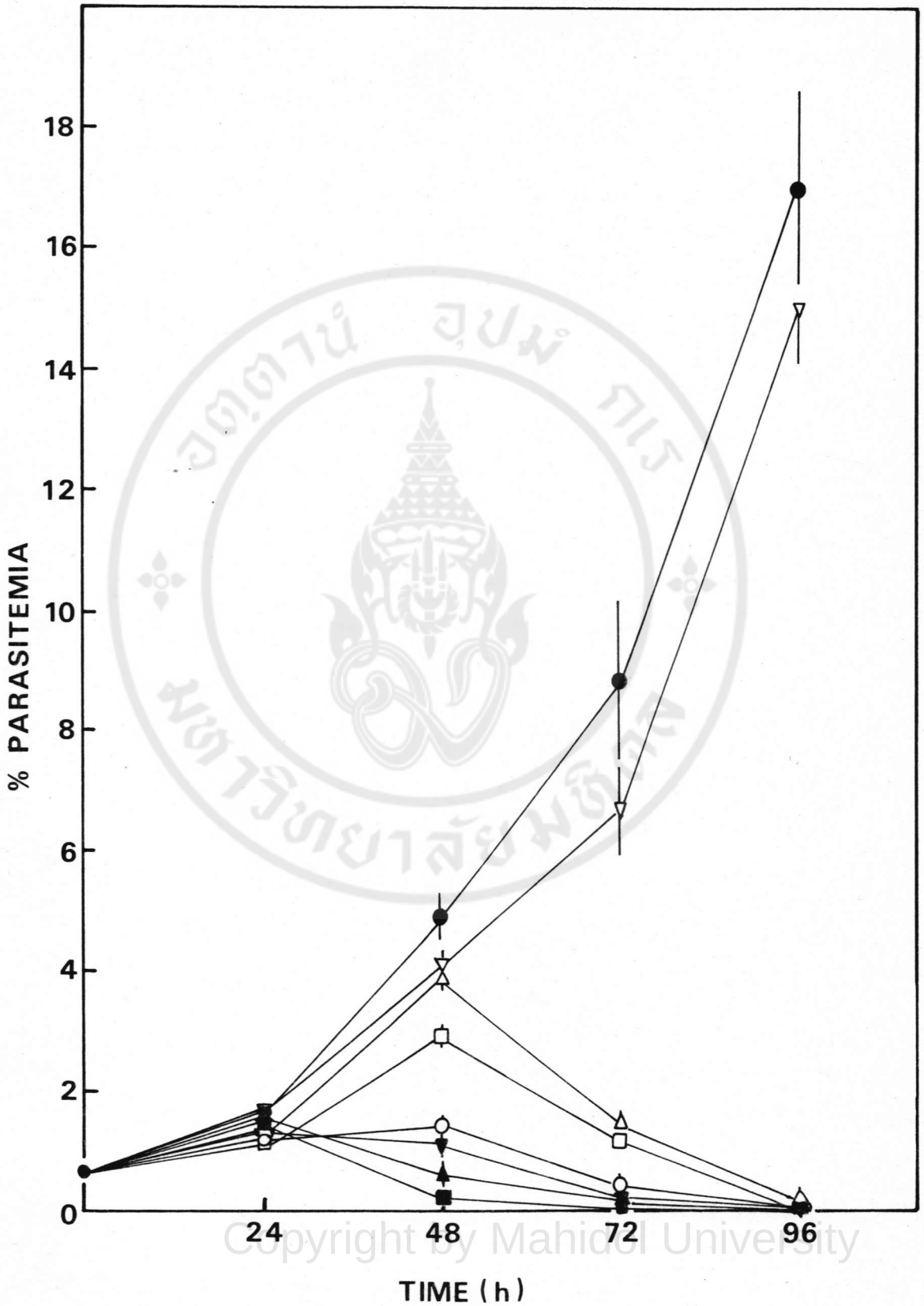
The parasite growth in the presence and absence of drugs was monitored every 24 h for 4 days. Fig. 4 shows the patterns of P. falciparum growth at various concentrations of tetracycline. After the cultures, started at 0.64% parasitemia, were maintained in the presence of tetracycline for 96 h, the lowest concentration of drug that showed significant antiparasitic activity was 4.11×10^{-7}M This concentration of drug inhibited the growth of P. falciparum nearly completely (0.17% parasitemia against 17.1% parasitemia for control without drug).

The antiparasitic activity of tetracycline was found to increase with time of exposure. EC_{50} for 48 h and 96 h were 1.7×10^{-6}M (0.76 µg/ml) and 2.1×10^{-7} M (0.09 µg/ml), respectively (Fig. 5). At 24 h of exposure, tetracycline, even at concentration as high as 1×10^{-4}M, showed very little or no effect on both parasitemia level (Fig. 4,6) and morphology of all stages (Fig. 7A,B, 8A,B, 9A,B). 24 h-treated schizonts contained the same amount of merozoites or nuclei per parasite as compared to control (Fig. 9A,B). At 48 h of exposure, at the concentration of the drug down to 1.23×10^{-6}M, parasitemia at all stages were markedly reduced and abnormal morphology were observed for the stages of trophozoite and schizont but not for ring forms (Fig. 7C, 8C, 9C). When the cultures were

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Fig. 4 Effect of various concentrations of tetracycline on P. falciparum (K_1) growth, under standard culture condition, with starting parasitemia of 0.64% (mixed stages of 0.30% ring form, 0.30% trophozoite and 0.04% schizont). Drug-free control, ●—● ; tetracycline $1x10^{-4}$ M, ■—■ ; $3.33x10^{-5}$ M, ▲—▲ ; $1.11x10^{-5}$ M, ▼—▼ ; $3.70x10^{-6}$ M, O—O ; $1.23x10^{-6}$ M, □—□ ; $4.11x10^{-7}$ M, Δ—Δ ; $1.37x10^{-7}$ M, ∇—∇ . Each point was the mean of triplicate values.

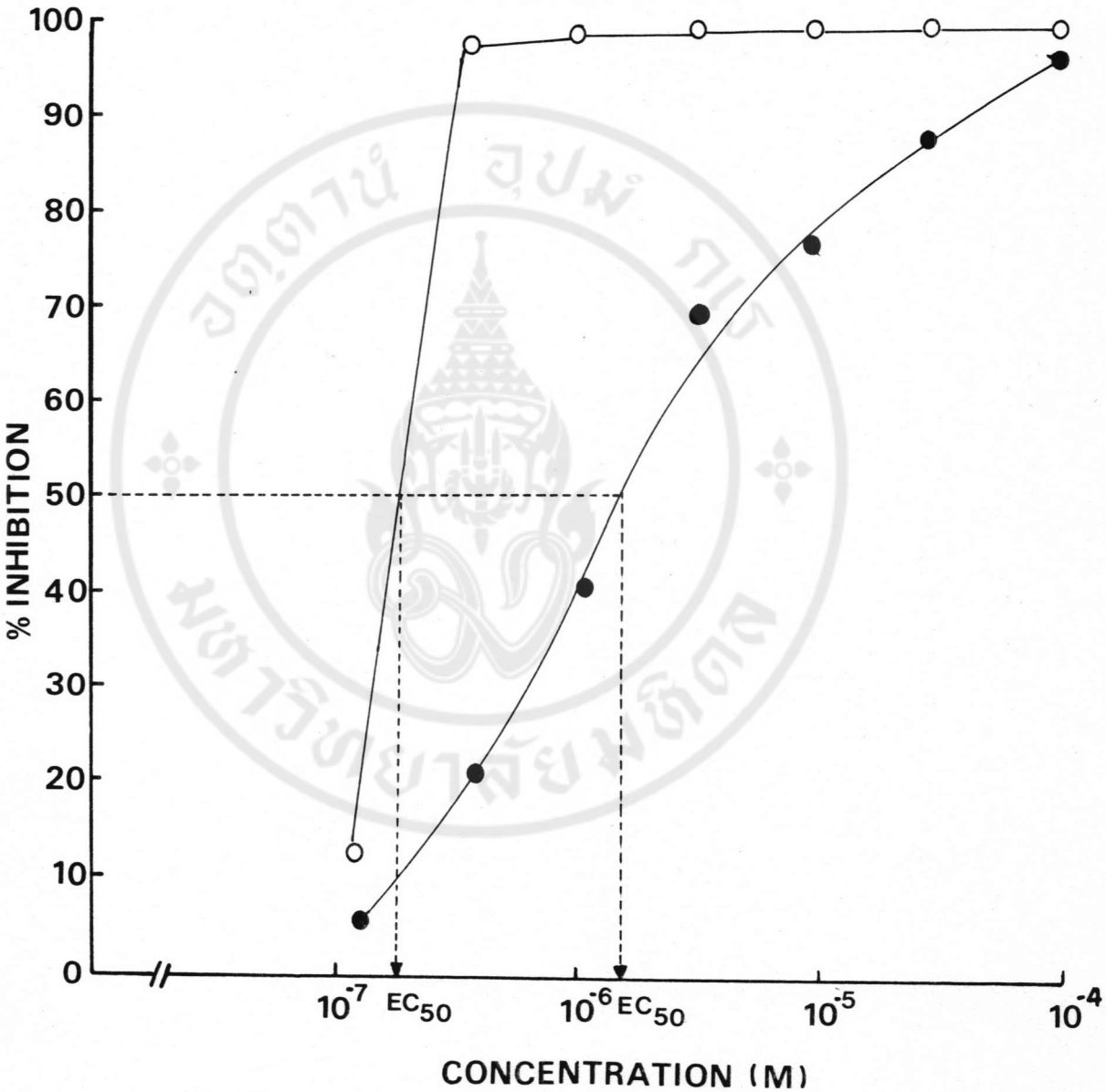
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Fig. 5 Log concentration-response curve of the inhibition of P. falciparum (K_1) growth by tetracycline. Cultures were exposed for 48 h (●—●), 96 h (○—○), and the control parasitemia were 4.91% and 17.01%, respectively. EC_{50} for 48 h and 96 h exposure were found to be 1.7×10^{-6} M (0.79 µg/ml) and 2.1×10^{-7} M (0.09 µg/ml) respectively.

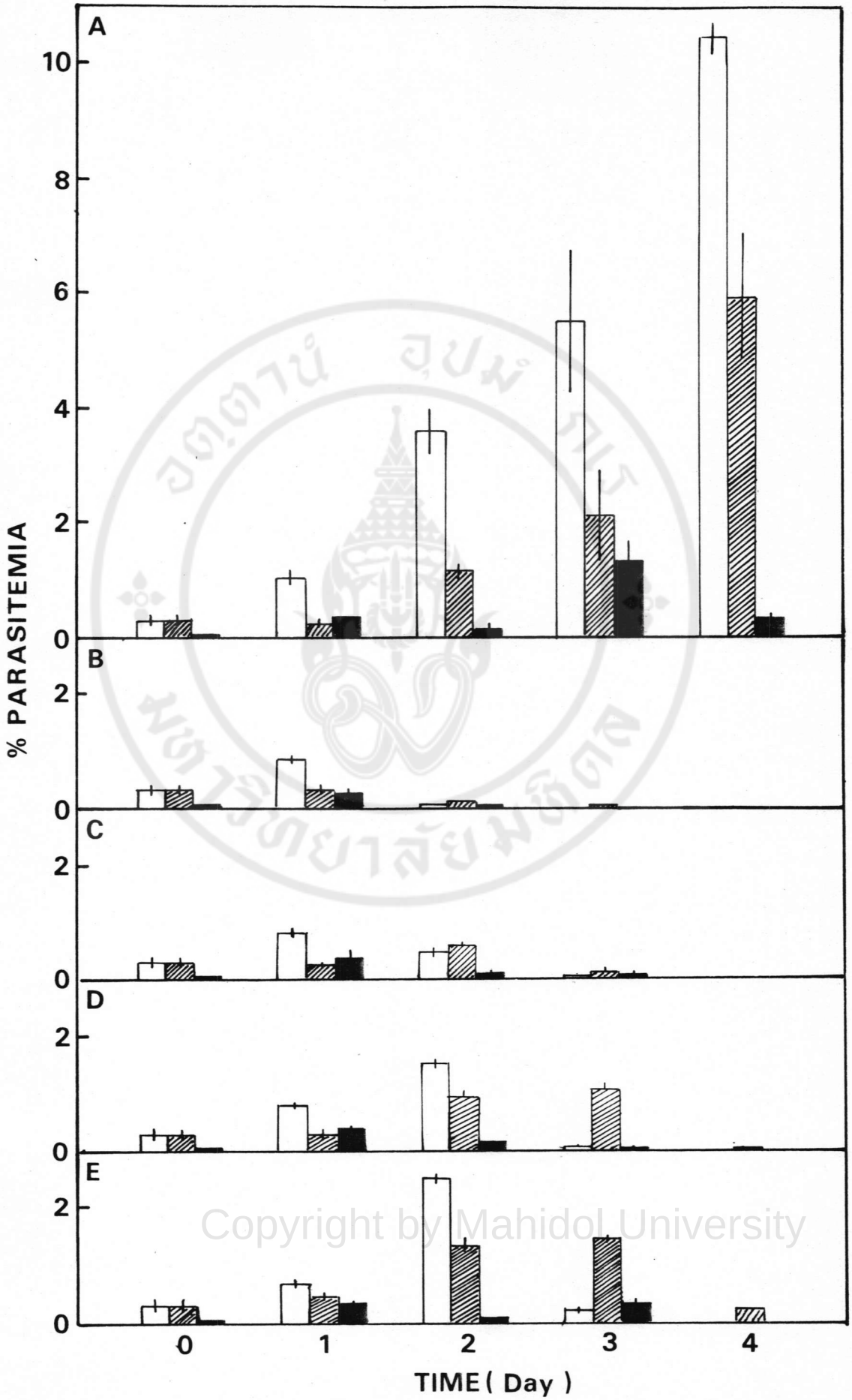
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Fig. 6 Effect of various concentrations of tetracycline on stage distribution of P. falciparum (K_1), under standard culture condition. Parasites were cultured in the absence (A) and presence of tetracycline at $1x10^{-4}$ M (B), $1.11x10^{-5}$ M (C), $1.23x10^{-6}$ M (D), $4.11x10^{-7}$ M (E). (ring form, ☐ ; trophozoite, ▨ ; schizont, ■)

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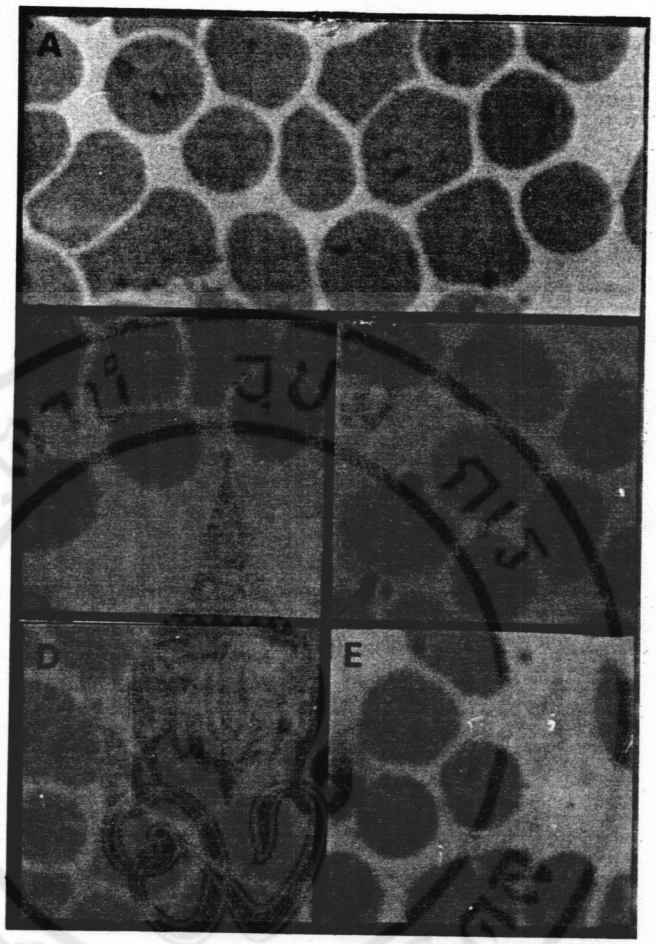


Fig. 7 Ring-stage of P. falciparum (K_1) at various times of tetracycline-treatment : drug-free control (A); $1x10^{-5}$ M tetracycline-treated for 24 h (B), 48 h (C), 72 h (D) and 96 h (E).

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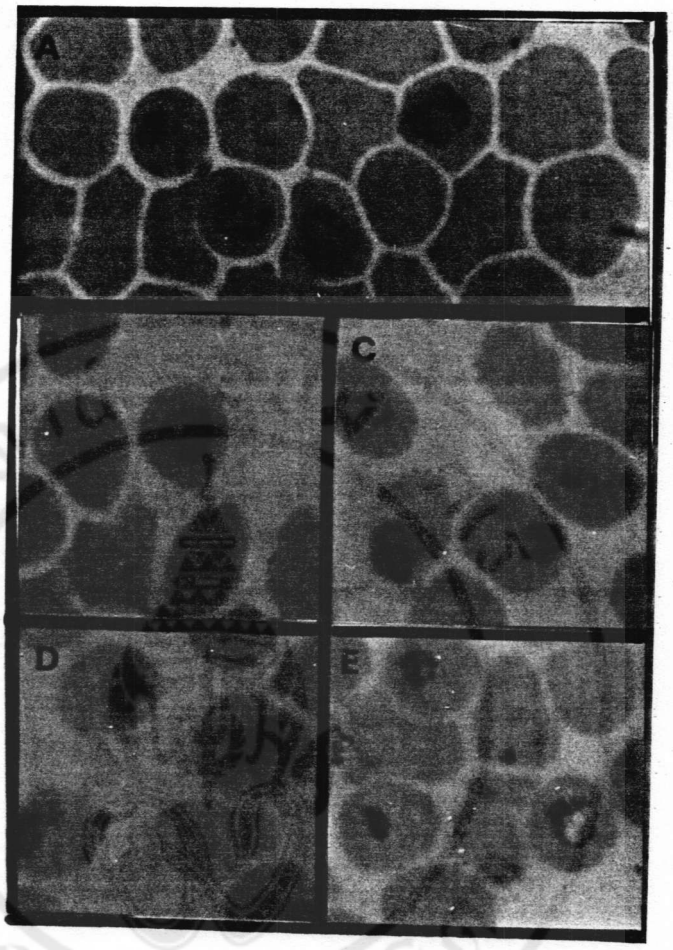


Fig. 8 Trophozoite of P. falciparum (K_1) at various times of tetracycline-treatment : drug-free control (A); $1x10^{-5}$ M tetracycline-treated for 24 h (B), 48 h (C), 72 (D) and 96 h (E).

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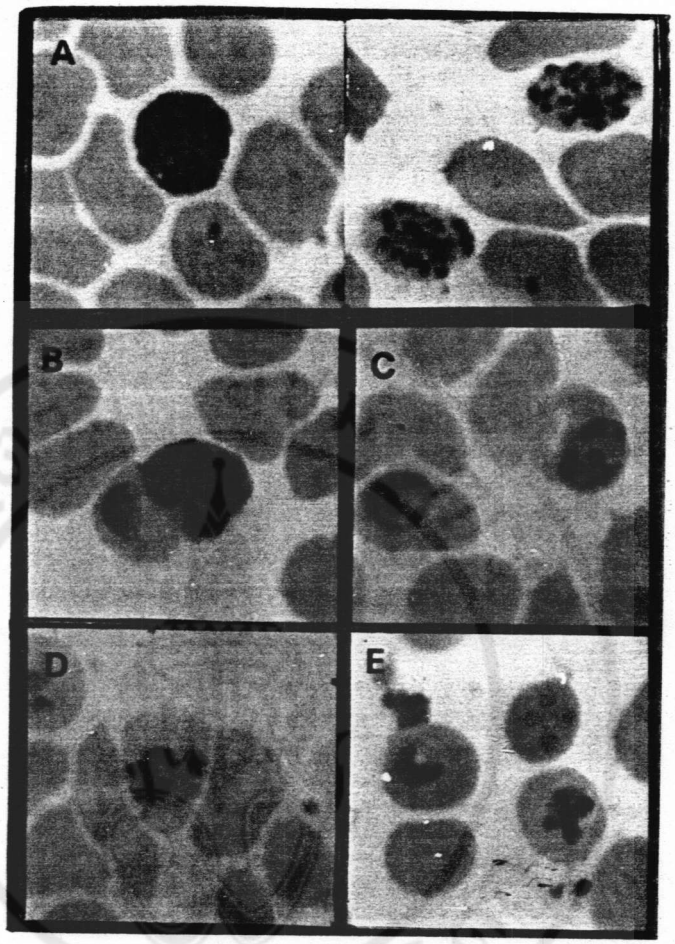


Fig. 9 Schizont of P. falciparum (K_1) at various times of tetracycline-treatment : drug-free control (A); $1x10^{-5}$M tetracycline-treated for 24 h (B), 48 h (C), 72 h (D) and 96 h (E).

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extended to 72 and 96 h of exposure, marked reduction in parasitemia level at all stages and abnormal trophozoites and schizonts were observed at the drug concentration down to 4.11×10^{-7} M, again without apparent effect on ring stages (Fig 7,8,9). Abnormal schizont contained reduced numbers of merozoites or nuclei (Fig. 9).

2. Effect of tetracycline on merozoite reinvasion of P.falciparum (K_1)

As shown in Fig. 6 and 9B, at 24 h of exposure, tetracycline had no effect on schizonts regarding parasitemia level and morphology. When the exposure was prolonged to 48 h, the percent parasitemia at ring stage of the culture in the presence of tetracycline at the concentration down to 1.23×10^{-6} M was markedly reduced (0.01-1.5% rings against 3.62% rings for control), this indicated the reduction in merozoite reinvasion. In order to see whether this result was the effect of tetracycline on normal erythrocytes or infected erythrocytes or both. Both normal and synchronized P. falciparum-infected erythrocytes at late ring stage were maintained in the absence and presence of 1×10^{-5} M tetracycline for 24 h. After 24 h, infected erythrocytes, which were at schizont stage, were concentrated by Percoll technique, subcultured with untreated and tetracycline-pretreated normal erythrocytes and maintained in the presence and absence of drug for another 15 h. Multiplication index of each condition is shown in Table 2. Tetracycline had no direct effect on merozoite reinvasion as shown by the result that multiplication index for the experiment performed in the presence of drug was unchanged (Table 2, row 2) as compared to control (Table 2, row 1). Tetracycline-pretreatment did not exert any effect on the ability of normal erythrocytes

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Table 2 Effect of tetracycline on merozoite reinvasion of P. falciparum

		Multiplication index*
1	UI + UN − TC	2.47 ± 0.05
2	UI + UN + TC	2.81 ± 0.17
3	UI + TN − TC	2.41 ± 0.30
4	UI + TN + TC	2.77 ± 0.17
5	TI + UN − TC	0.89 ± 0.31
6	TI + UN + TC	0.54 ± 0.05
7	TI + TN − TC	0.50 ± 0.08
8	TI + TN + TC	0.60 ± 0.08

*For calculation, see text

UI — untreated infected erythrocytes

UN — untreated normal erythrocytes

TI — 24 h-tetracycline pretreated infected erythrocytes

TN — 24 h tetracycline pretreated normal erythrocytes

TC — 10^{-5} M tetracycline

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to support parasite reinvasion since multiplication index for pretreated erythrocytes was unchanged (Table 2, row 3,4) as compared to control (Table 2, row 1). However, pretreated P. falciparum reinvaded both untreated and pretreated normal erythrocytes for less effectively, either in the presence and absence of drug (Table 2, row 5,6,7,8) when compared to control (Table 2, row 1). These results suggest that the reduction in percent parasitemia at ring form observed in 48 h of drug exposure (Fig. 6) was the result of the effect on the parasites.

3. Effect of tetracycline and cycloheximide on [^{3}H]-isoleucine incorporation by P. falciparum (K_1)

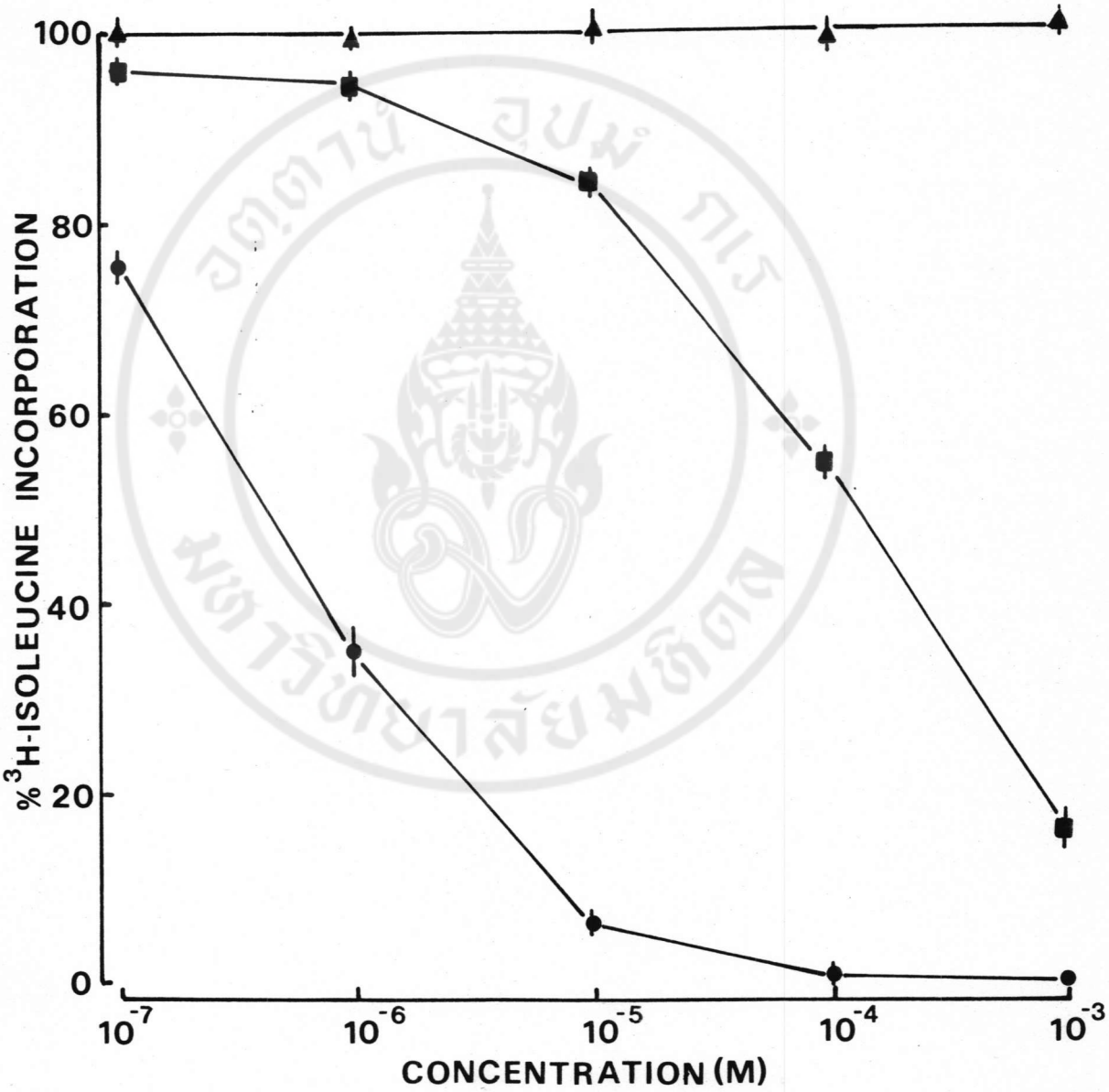
Results on the effect of tetracycline and cycloheximide are shown in Fig. 10. IC_{50} (50% inhibitory concentration value) of 4.2×10^{-7}M and 1.2×10^{-4}M were obtained, respectively. [^{3}H]-isoleucine incorporation was thus 286 times more sensitive to cycloheximide than to tetracycline.

At 10^{-3} M cycloheximide, isoleucine incorporation was reduced to 0.22% of control in the absence of drug (1146 cpm against 520850 cpm for control). In order to determine whether the cycloheximide-resistant fraction was sensitive to tetracycline, the incorporation in the presence of 3.33×10^{-4} M cycloheximide plus various concentrations of tetracycline was determined. The percentage of [^{3}H]-isoleucine incorporation in the presence of 3.33×10^{-4}M cycloheximide was about 0.48 (8886 cpm against 1870664 cpm for control). Fig. 1 shows that this fraction was not sensitive to tetracycline.

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Fig. 10 The effect of tetracycline and cycloheximide on $[^{3}H]$-isoleucine incorporation by P. falciparum (K_1). Starting parasitemia was 3.53% (0.51% ring form, 2.01% trophozoite, 1.02% schizont) for study of the effect of cycloheximide (●—●) or tetracycline alone (■—■), and 16.68% (2.51% ring form, 10.05% trophozoite, 5.10% schizont) for study of the effect of various concentrations of tetracycline in the presence of 3.33×10^{-4} M cycloheximide (▲—▲), for which 100% incorporation represents 0.48% of the total incorporation in drug-free medium. Each point was the mean of triplicate values.

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4. Analysis of P. falciparum (K_1) proteins with cycloheximide-resistant, tetracycline-sensitive synthesis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

In order to detect P. falciparum proteins whose synthesis were cycloheximide-resistant and tetracycline-sensitive, the parasite was metabolically labeled with [^{35}S]-methionine in the absence and presence of tetracycline, cycloheximide and tetracycline plus cycloheximide, and further analysed by 2D-PAGE and fluorography.

To determine the suitable time for metabolic labeling, the time course of [^{35}S]-methionine incorporation by P. falciparum was studied in the absence and presence of tetracycline or cycloheximide (Fig. 11). Normal erythrocytes showed no incorporation of [^{35}S]-methionine, indicating that the incorporation observed in the P. falciparum-infected erythrocytes was solely by the parasites themselves. The incorporation by P. falciparum in the absence, as well as in the presence of 10^{-6} M tetracycline, increased with time and was highest at 7 h of incubation. Incorporation in the presence of $5x10^{-4}$ M tetracycline and $1x10^{-7}$ M cycloheximide was markedly reduced when compared to control in the absence of drug, but constantly increased throughout the study. There was very little [^{35}S]-methionine incorporation in the presence of $1x10^{-4}$ M cycloheximide. Thus, the suitable time for metabolic labeling was 7 h.

Coomassie-blue staining after 2D-PAGE analysis was not sensitive enough to detect the cycloheximide-resistant, tetracycline-sensitive protein of P. falciparum, since the same pattern was obtained in all culture conditions and a very small number of spots was detected,

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either in the presence or absence of drugs (Fig. 14).

Fluorograms of 2D-PAGE analysis are shown in Fig. 15, 17-24 and their diagrams in Fig. 16,25,26. Cycloheximide was far more effective in reducing the intensities of protein spots than tetracycline. Most of the protein spots can be divided into various categories according to drug sensitivity : (a) sensitive to cycloheximide but resistant to tetracycline inhibition, (b) sensitive to both drugs used singly or in combination or (c) resistant to both drugs used singly or in combination. Most of the protein spots fell into the first category. The proteins in the third category could still be detected in the presence of high concentrations of the drugs, although the intensities of them were markedly reduced.

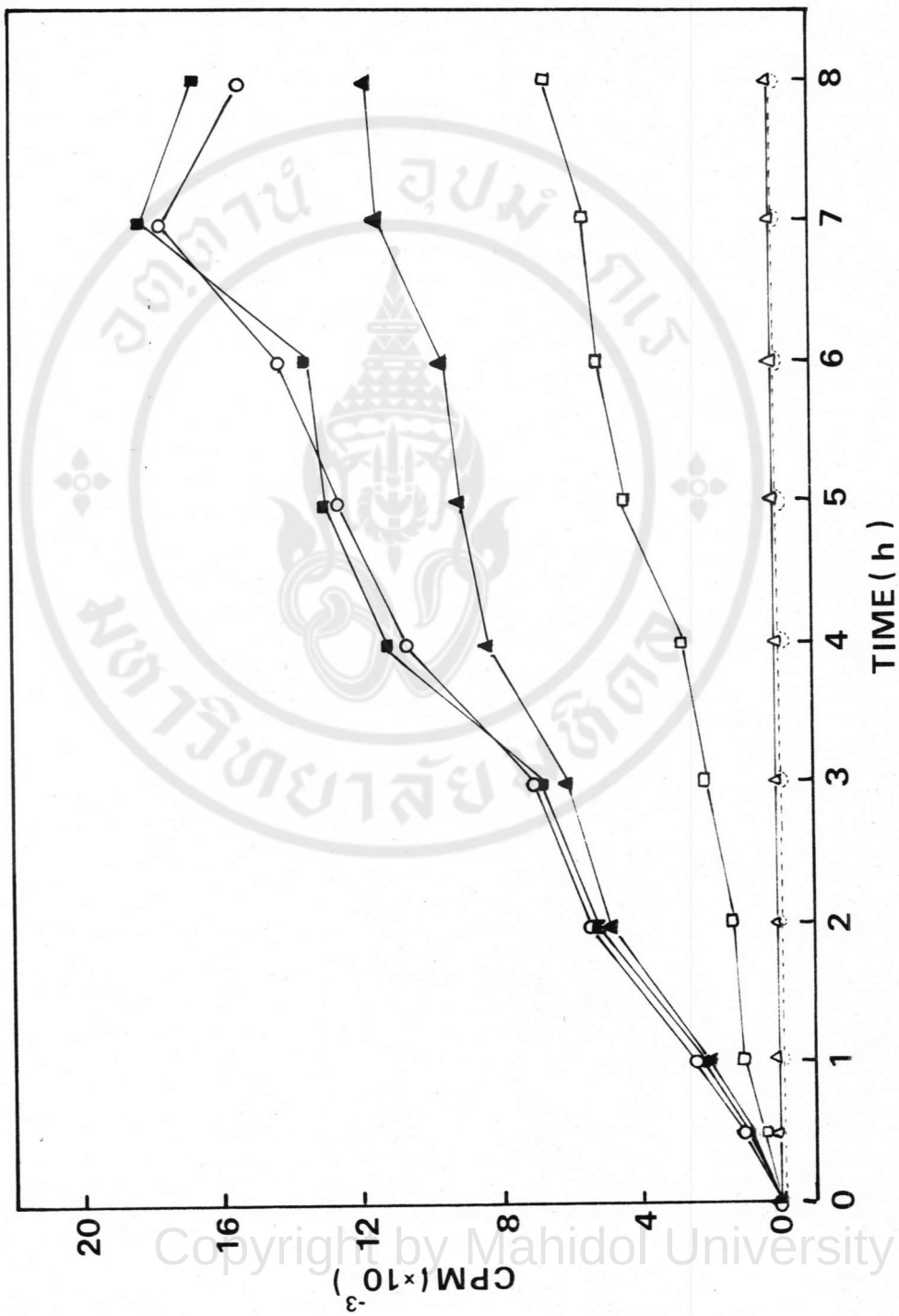
Only two protein spots are distinguished by their unique tetracycline sensitivity. The protein of 95 K, pI 4.5 (arrow 1) could be detected when P. falciparum was metabolically labeled in the presence of cycloheximide alone even at the concentration as high as $1x10^{-4}$ M (Fig. 18,19), but it disappeared when this parasite was metabolically labeled in the presence of tetracycline alone, at the concentration down to $1x10^{-5}$ M (Fig. 20,21) and $1x10^{-4}$ M cycloheximide plus $1x10^{-5}$ M or $1x10^{-6}$ M tetracycline (Fig. 23,24). This indicated that this protein spot was cycloheximide-resistant, tetracycline-sensitive.

Another protein of 85 K, pI 6 (arrow 2) was not inhibited by tetracycline alone (20-22), partially inhibited by cycloheximide alone (17-19) but could not be detected when this parasite was maintained in the presence of both drugs (Fig. 23,24).

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Fig. 11 Effect of tetracycline and cycloheximide on time course of $[^{35}S]$-methionine incorporation by P. falciparum (K_1). The experiment was performed in the absence (drug-free control, ○—○) and in the presence of tetracycline at $1x10^{-6}$ M (■—■), $5x10^{-4}$ M (□—□) or cycloheximide $1x10^{-7}$ M (▲—▲), $1x10^{-4}$ M (△—△), with normal erythrocytes as controls (◌·····◌). The starting parasitemia was 15.20% (3.10% ring form, 7.54% trophozoite and 4.56% schizont).

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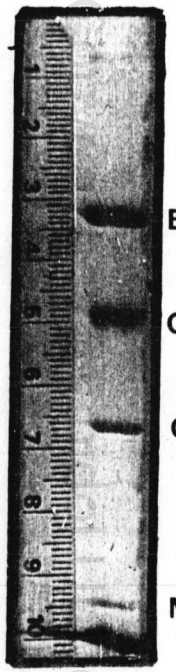
Fig. 12 A) SDS-polyacrylamide gel pattern of standard molecular weight markers

B) Standard curve for molecular weight determination on SDS-polyacrylamide gel electrophoresis.

Standard molecular weight marker proteins, composed of bovine serum albumin (BSA, 68 K), ovalbumin (43 K), concanavalin A (27 K) and myoglobin (17 K), were loaded into the precast 10% SDS-polyacrylamide gel. The electrophoresis was carried out according to the method described in 'Materials and Methods'. The values of relative mobility (the ratio of the distance of the proteins from the top of the gel against the the distance of the tracking dye) were plotted against log molecular weight of the proteins.

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A



Bovine serum albumin, 68 K

Ovalbumin, 43 K

Concanavalin A, 27 K

Myoglobin, 17 K

B

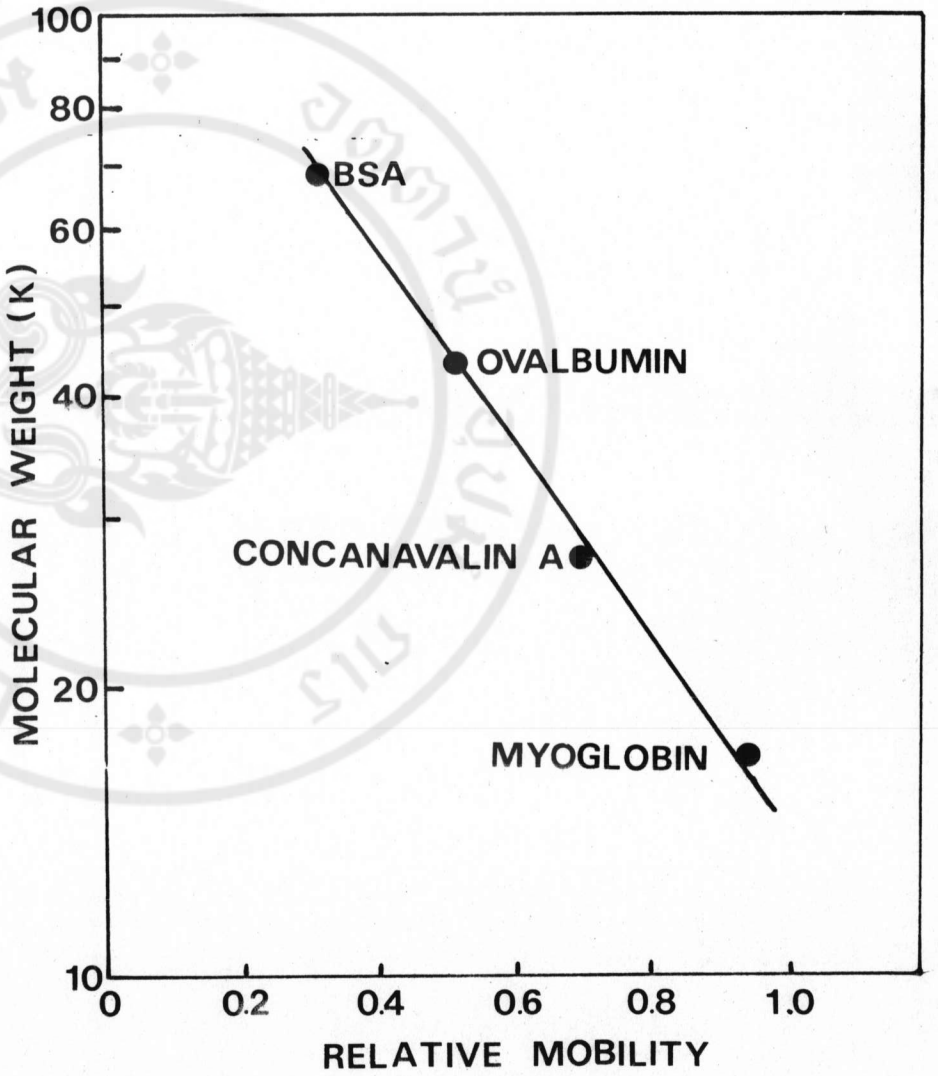
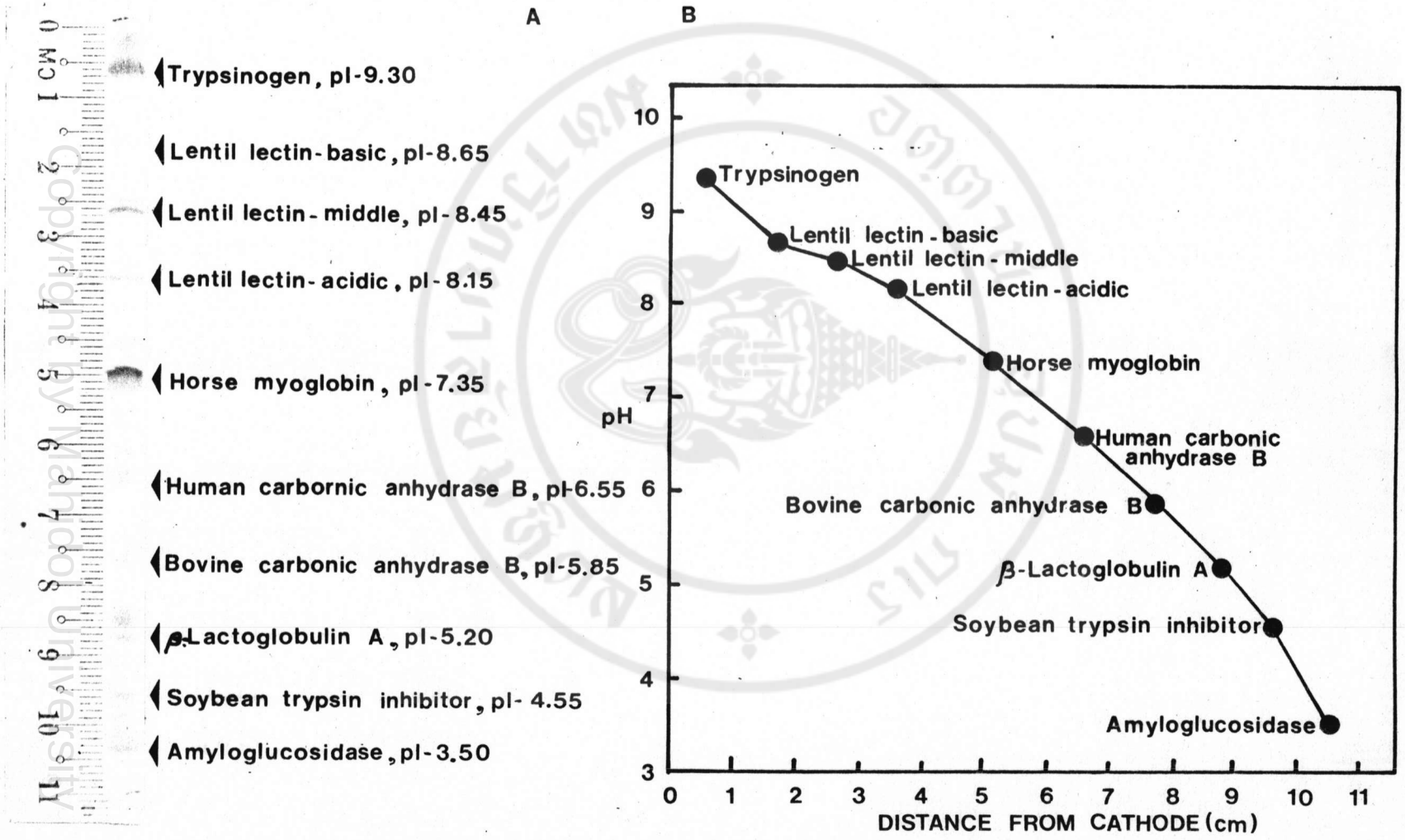


Fig. 13 A) Isoelectric focusing gel pattern of standard pI markers

B) pH gradient of isoelectric focusing gel as determined by standard pI markers.

Standard pI markers, composed of trypsinogen (pI=9.30), lentil lectin-basic (pI=8.65), lentil lectin-middle (pI=8.45), lentil lectin-acidic (pI=8.15), horse myoglobin (pI=7.35), human carbonic anhydrase B (pI=6.55), bovine carbonic anhydrase B (pI=5.85), β-lactoglobulin A (pI=5.20), soybean trypsin inhibitor (pI=4.55) and amyloglucosidase (pI=3.50), were separated by isoelectric focusing gel electrophoresis according to the procedure described in Methods. pI values were plotted against the distance migrated from the cathode.

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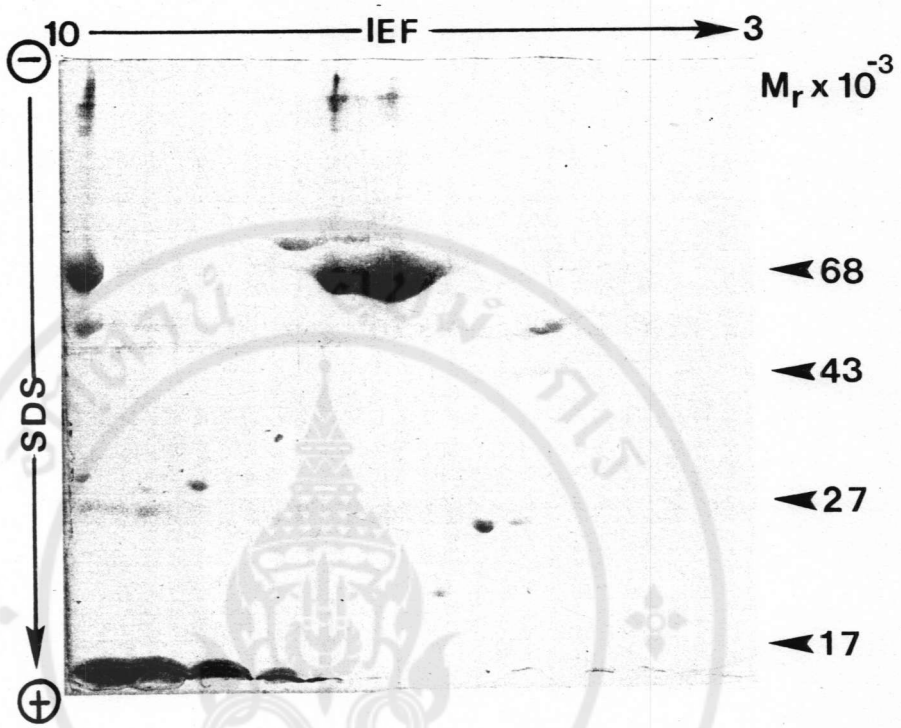


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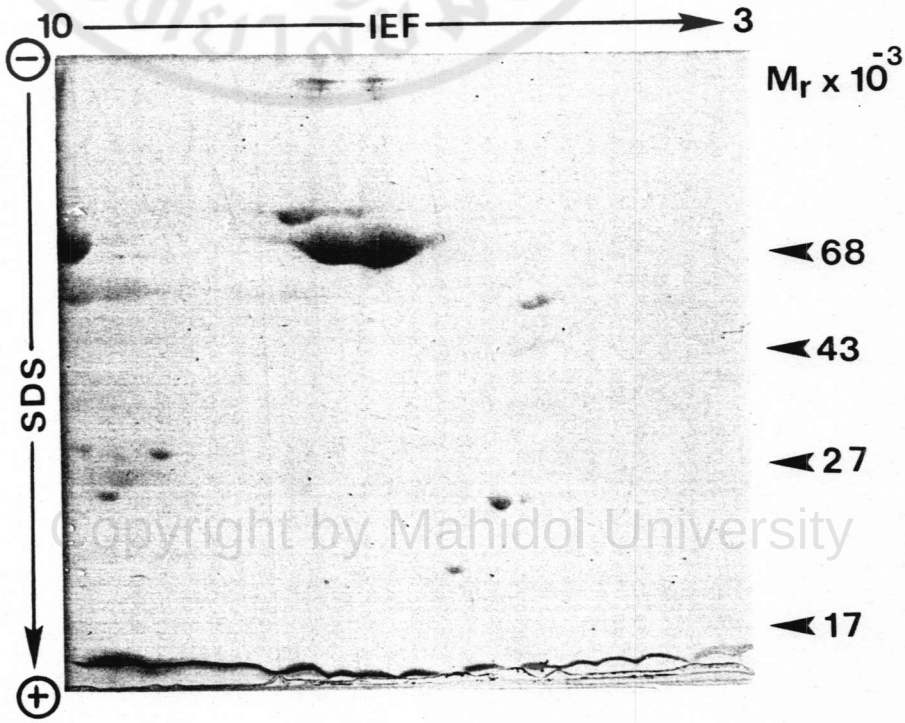
Fig. 14 Coomassie blue-stained, two dimensional gel electrophoretic protein pattern of P. falciparum (K_1)-infected erythrocytes, maintained in the absence (A) and presence of $1x10^{-4}$ M cycloheximide (B), $1x10^{-5}$ M tetracycline (C), and $1x10^{-4}$ M cycloheximide plus $1x10^{-5}$ M tetracycline (D)

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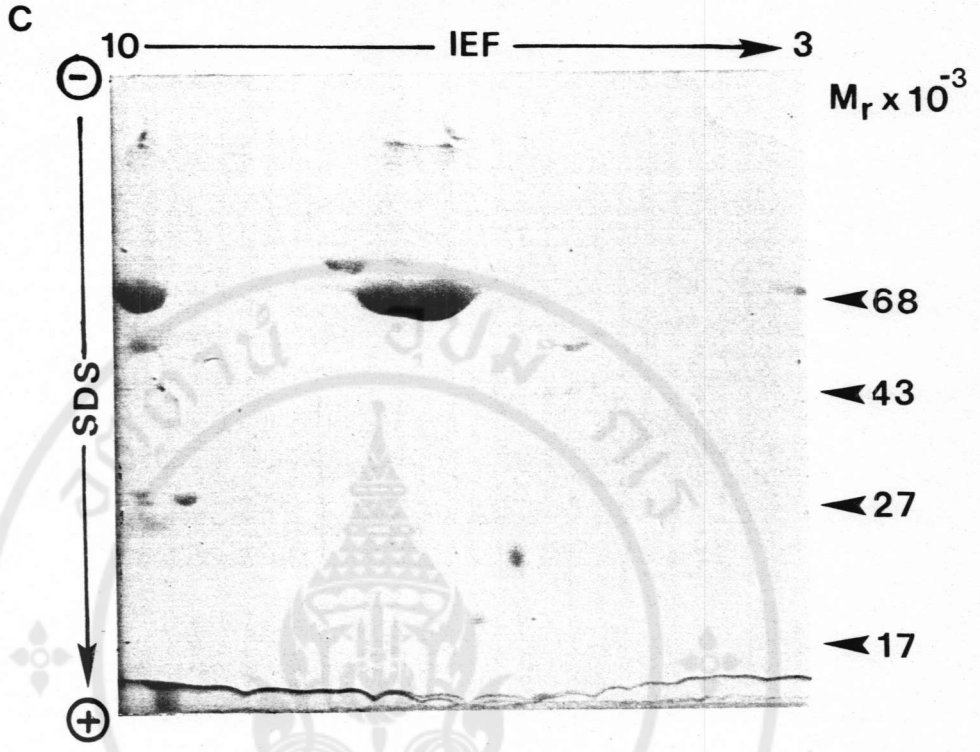
A

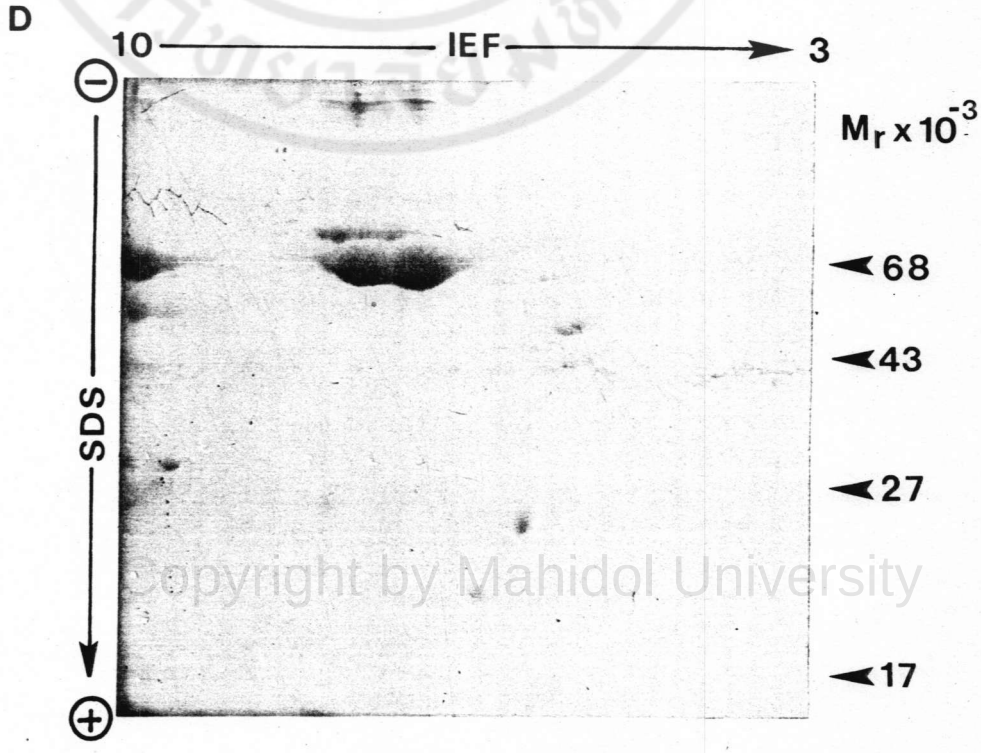


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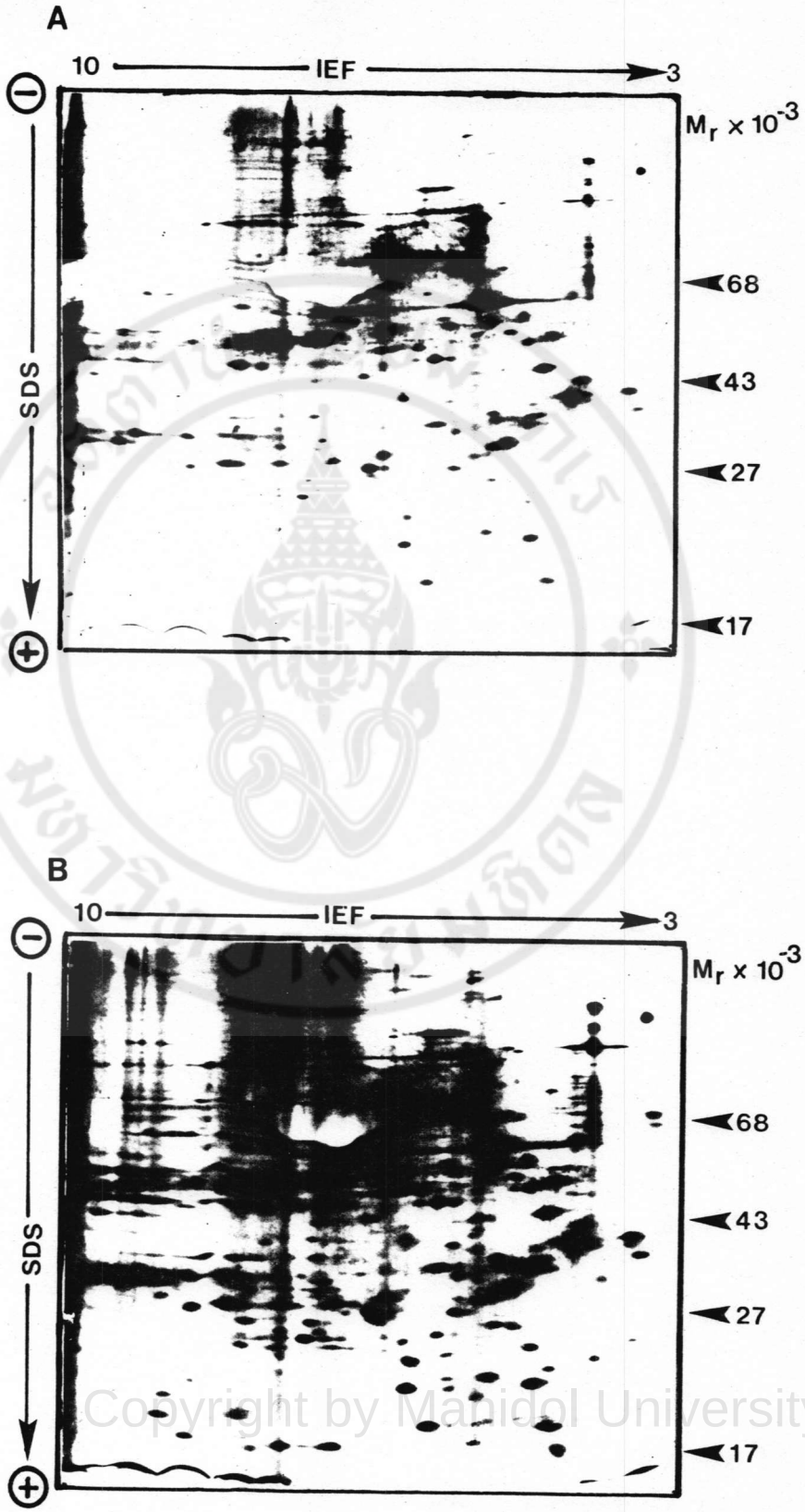




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Fig. 15 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine. Culture at 18.90% parasitemia (2.19% ring form, 14.10% trophozoite, 2.61% schizont) were metabolically labeled for 7 h. 50 µl of labeled sample (34531 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B)

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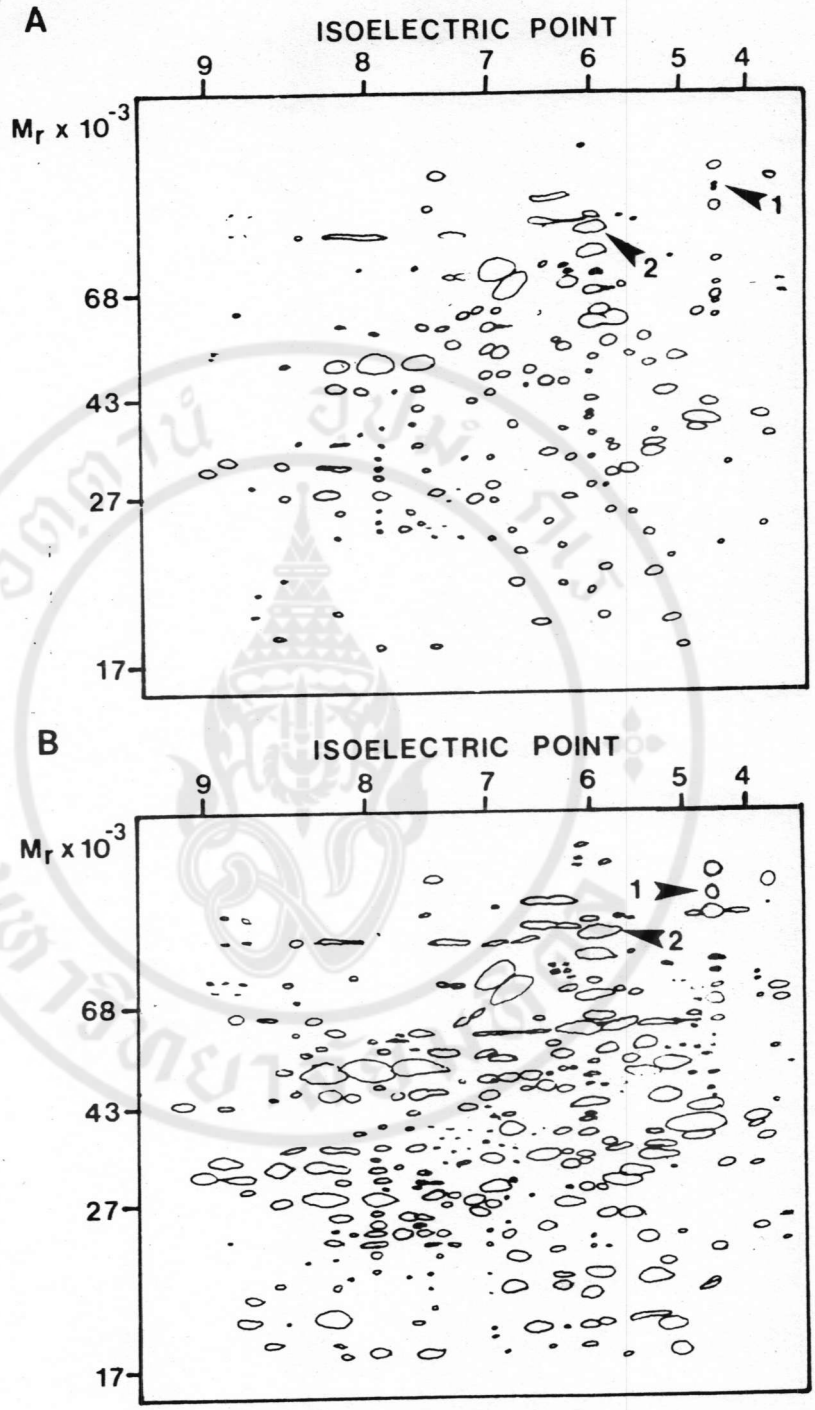
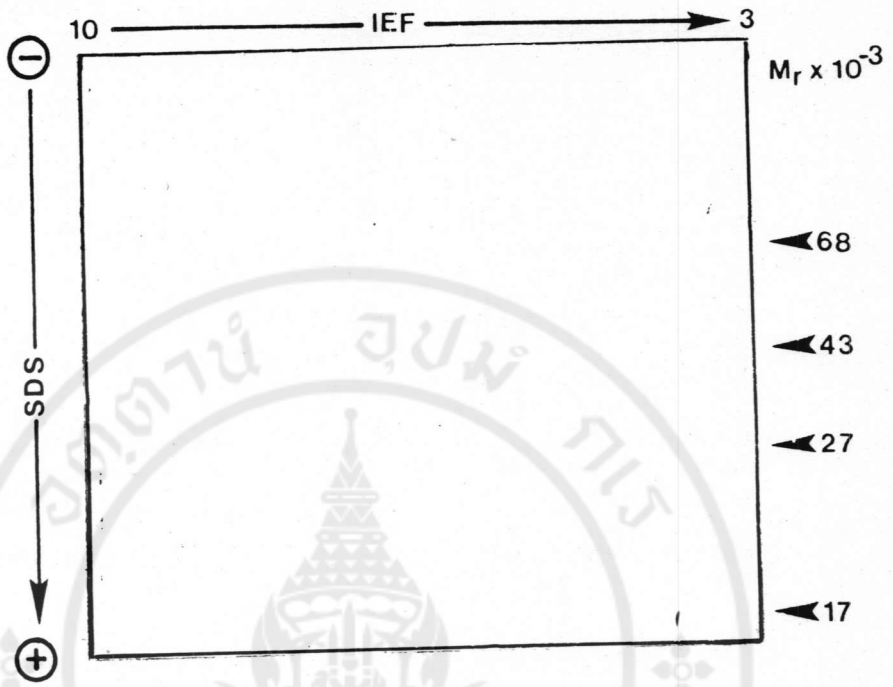


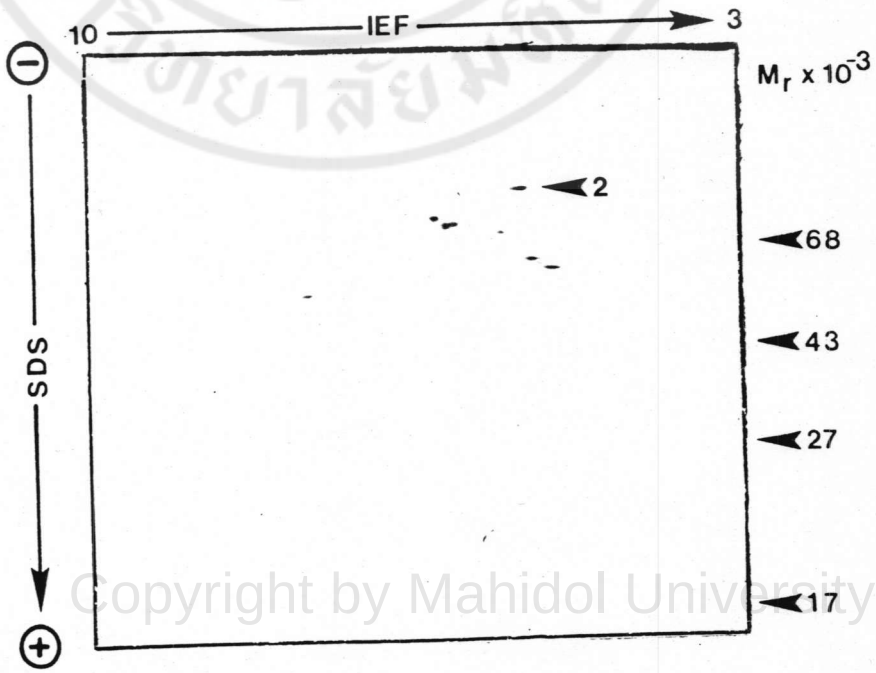
Fig. 16 Diagrams of fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1) exposed for 10 days (A) 2 months (B)

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Fig. 17 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S] -methionine in the presence of $1x10^{-3}$ M cycloheximide for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% Schizont). 50 µl of labeled sample (200 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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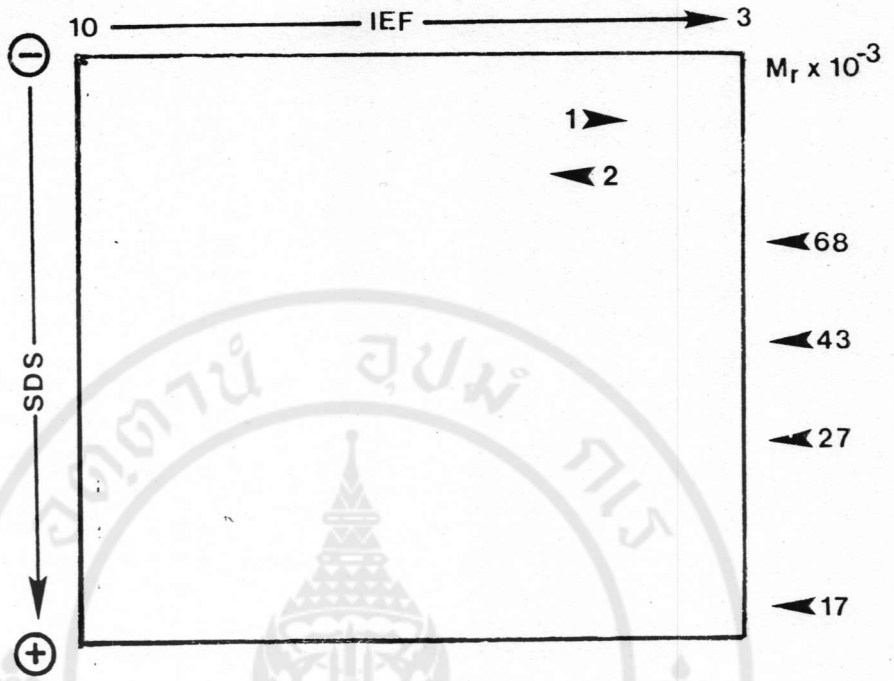


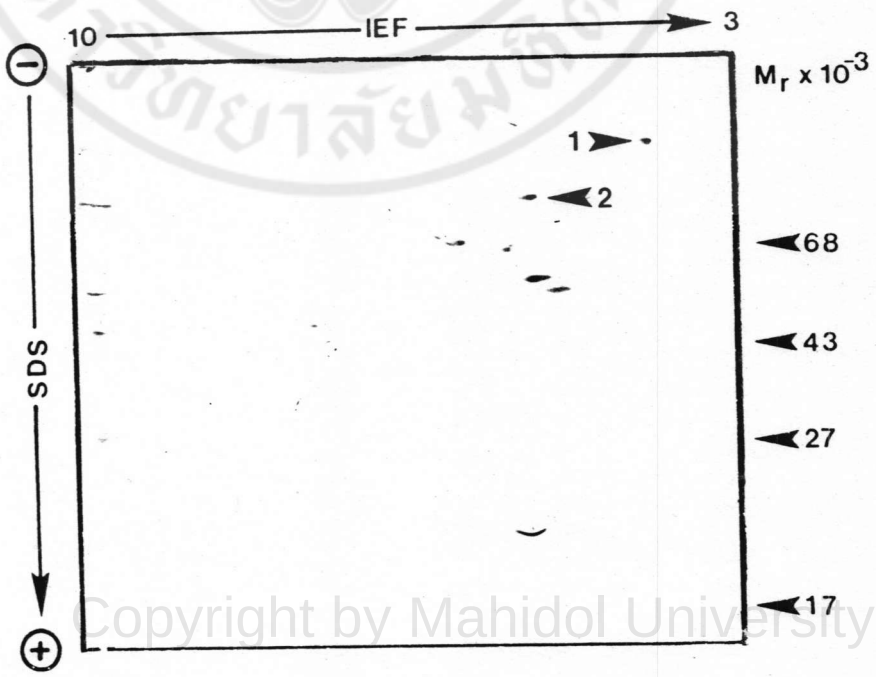


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Fig. 18 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S] -methionine in the presence of $1x10^{-4}$M cycloheximide for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (1066 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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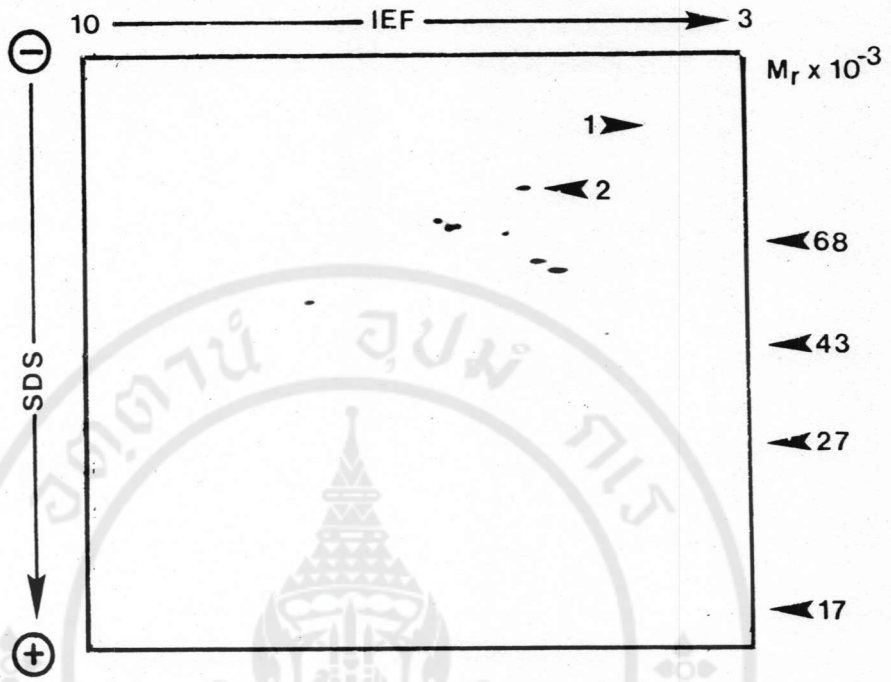


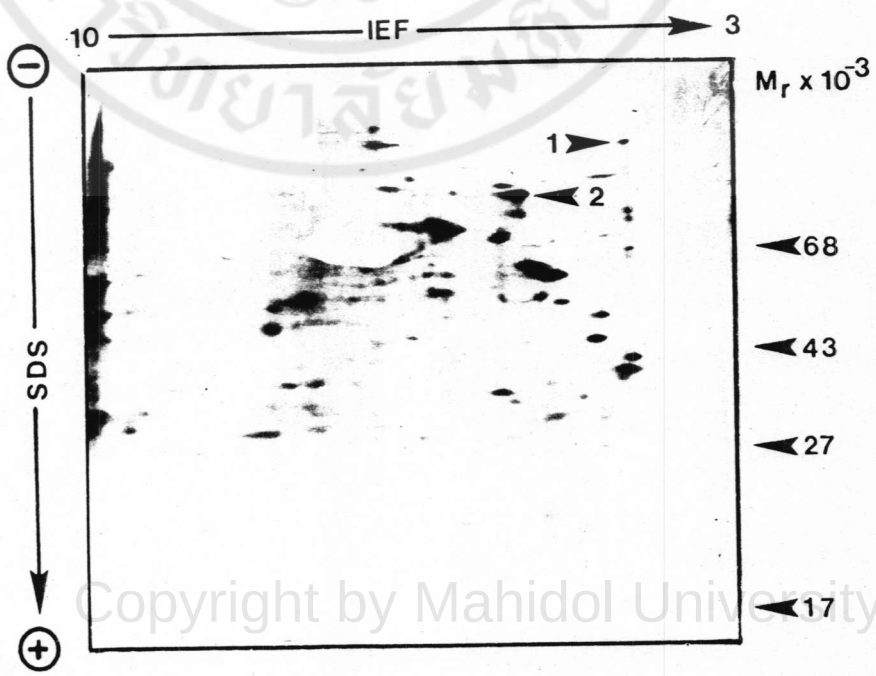


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Fig. 19 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-5}$ M cycloheximide for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (2677 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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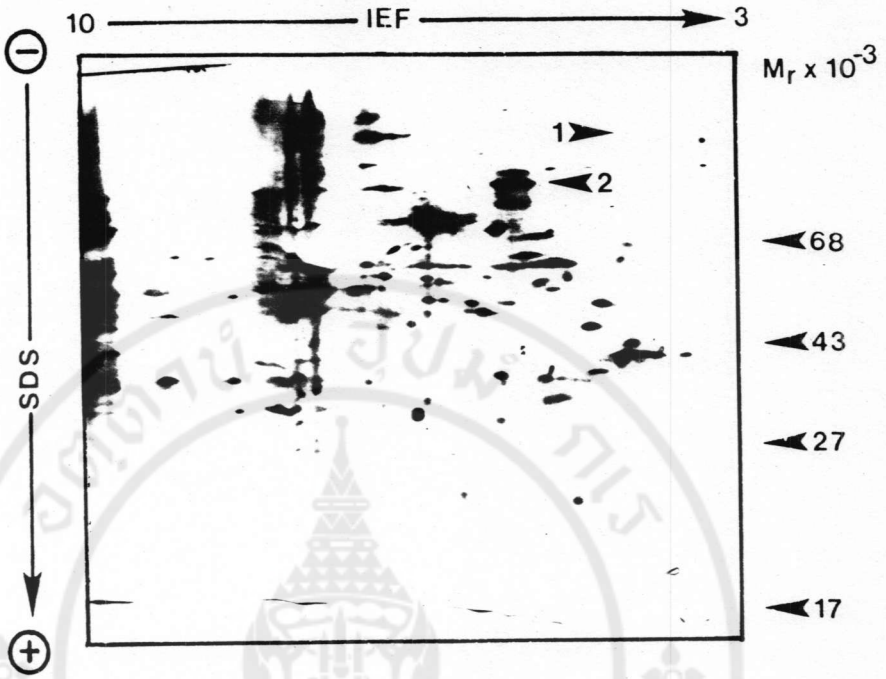


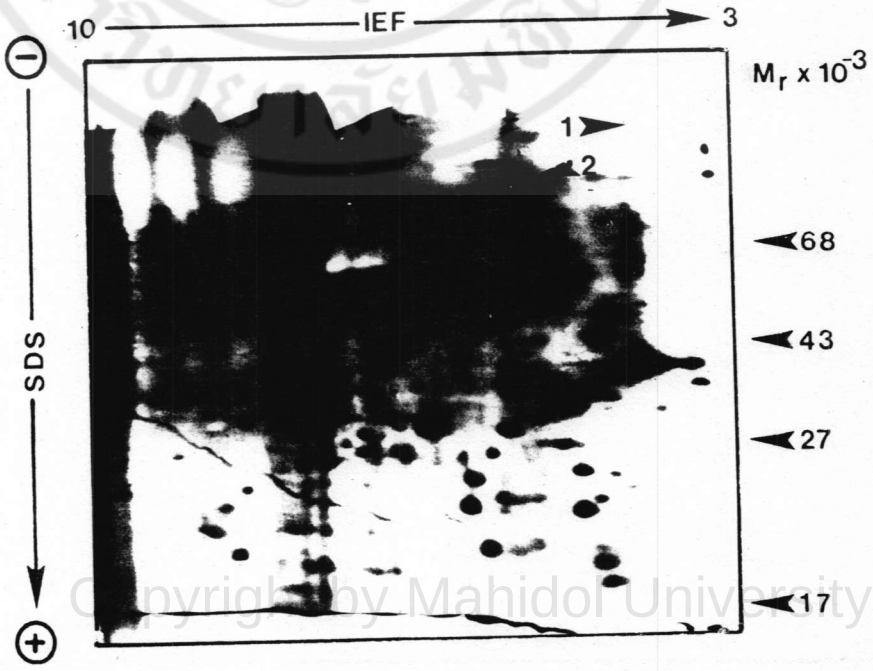


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Fig. 20 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-4}$ M tetracycline for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (22271 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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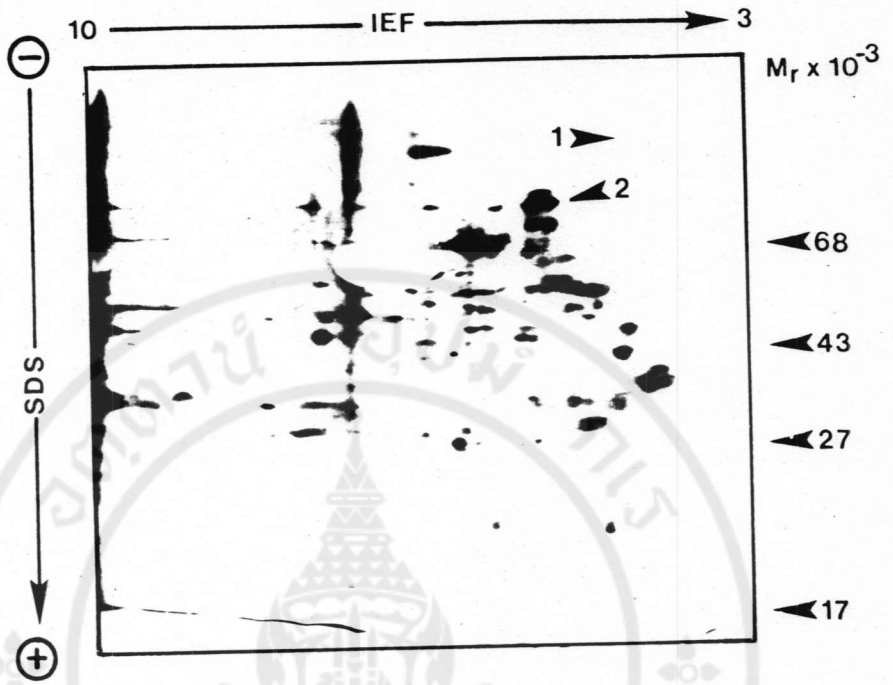


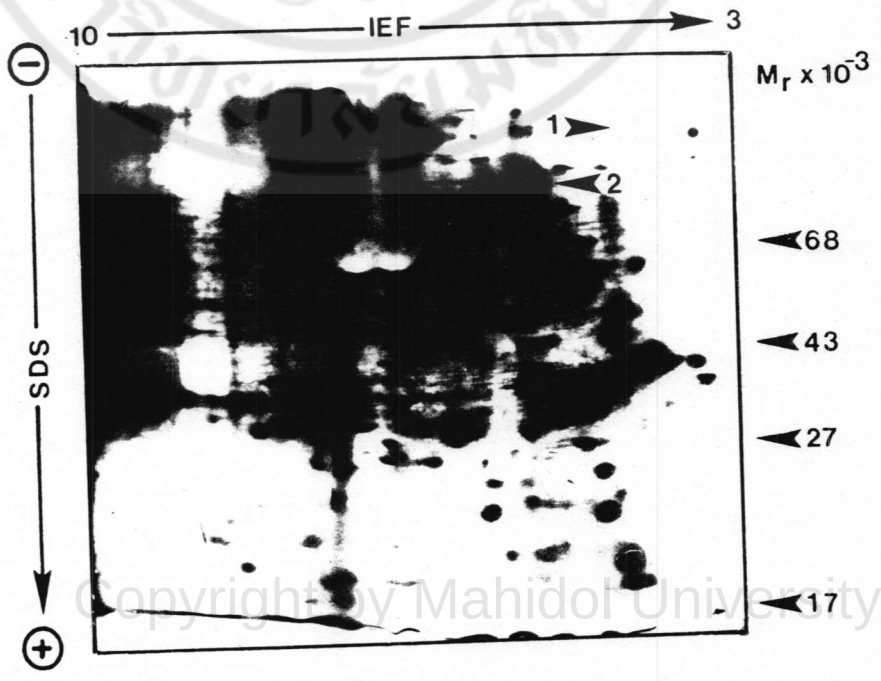


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Fig. 21 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-5}$ M tetracycline for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (31445 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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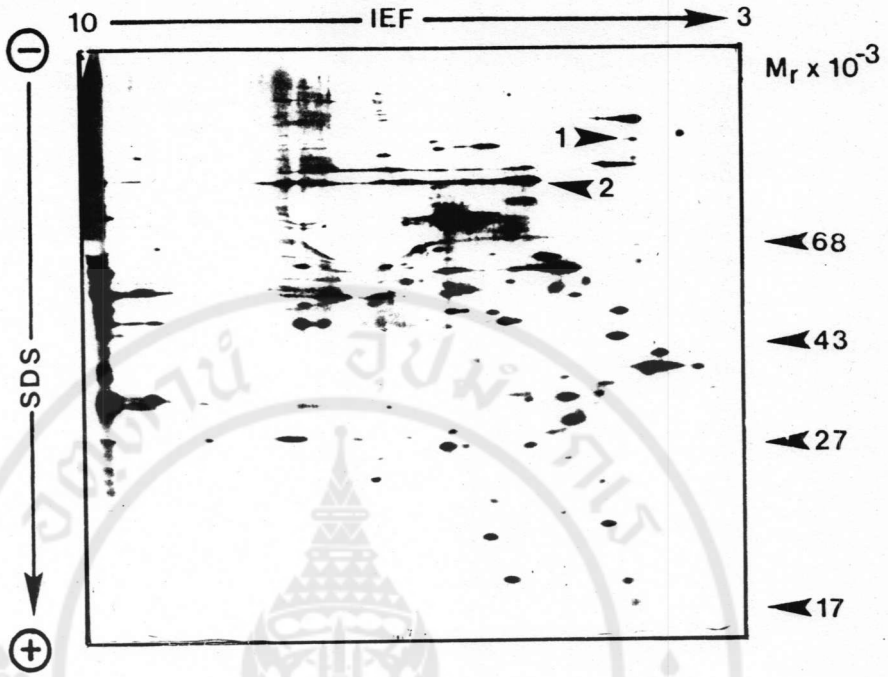


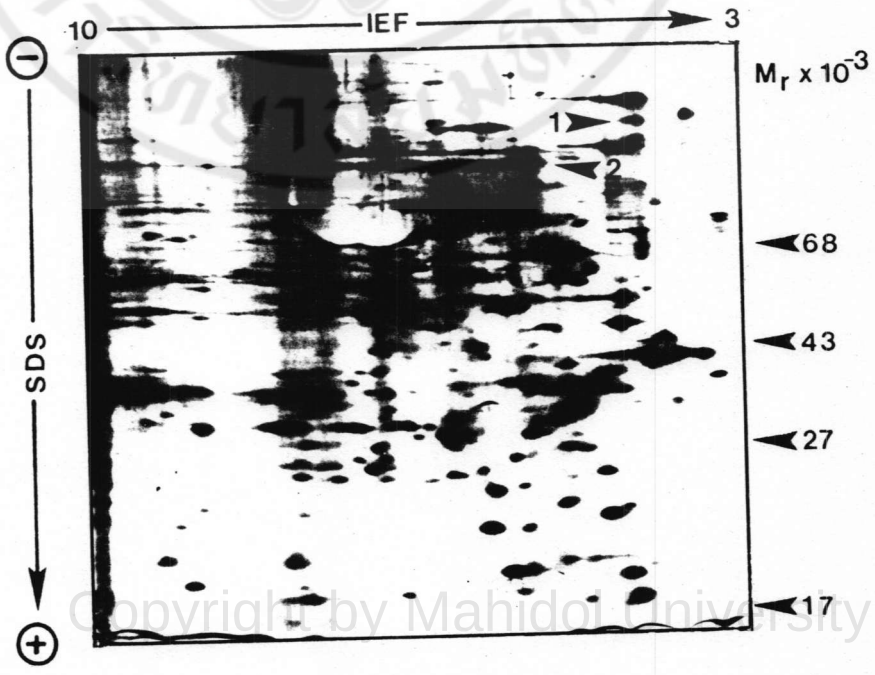


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Fig. 22 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-6}$ M tetracycline for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (35646 cpm/µl) was analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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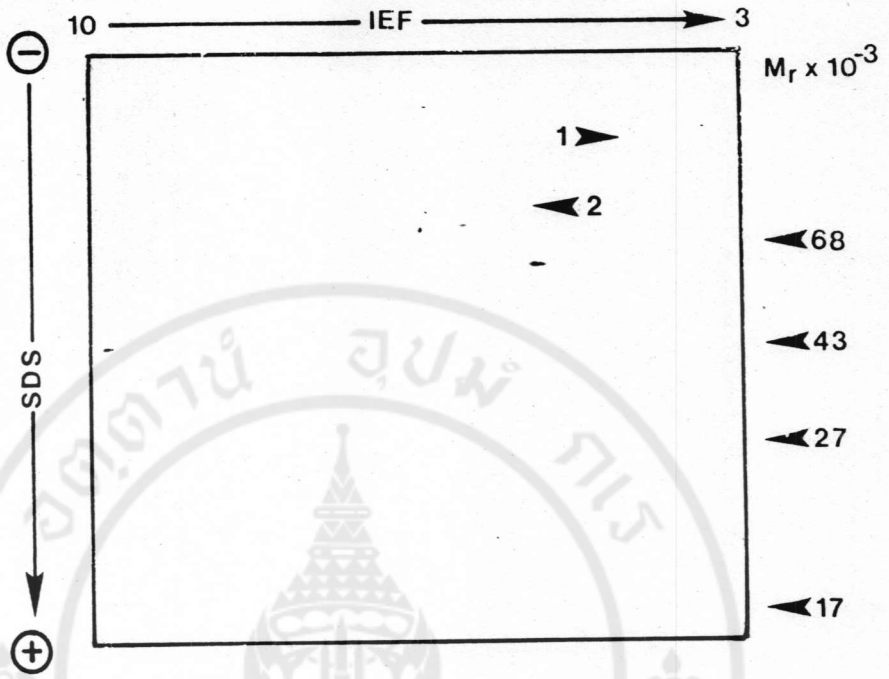


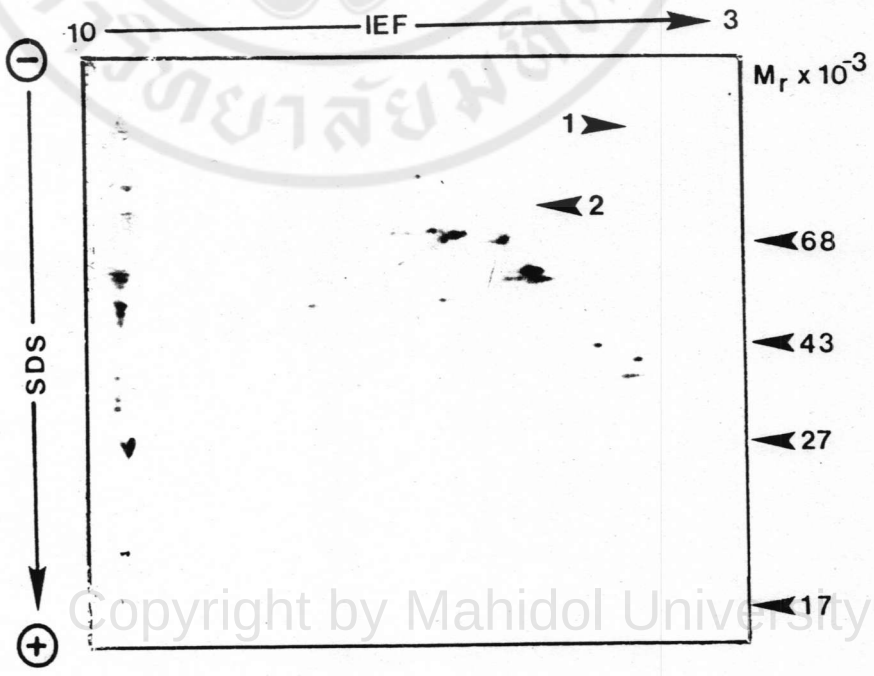


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Fig. 23 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-5}$ M tetracycline plus $1x10^{-4}$ M cycloheximide for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (728 cpm/µl) were analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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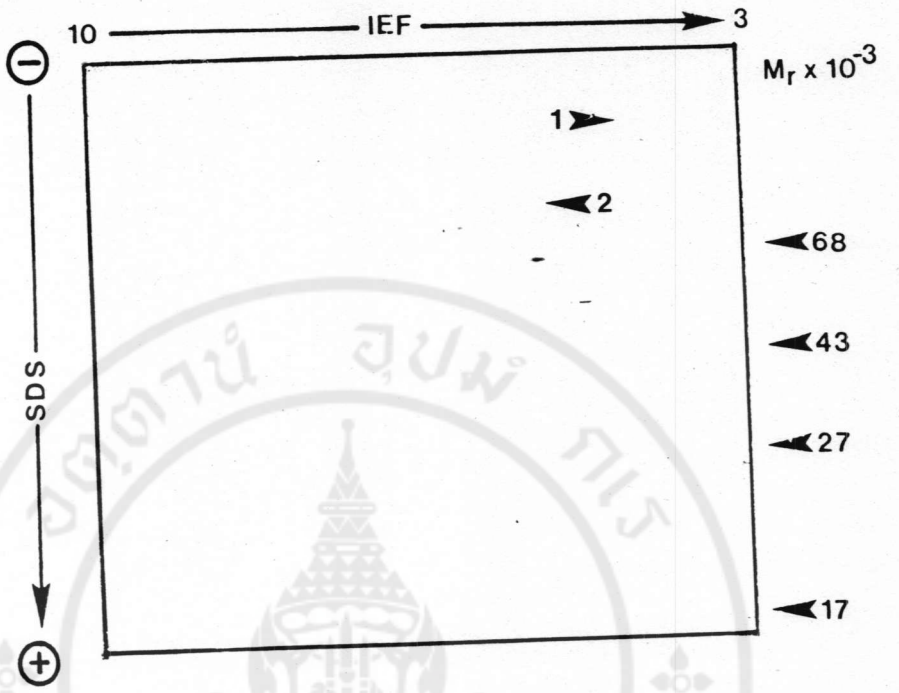


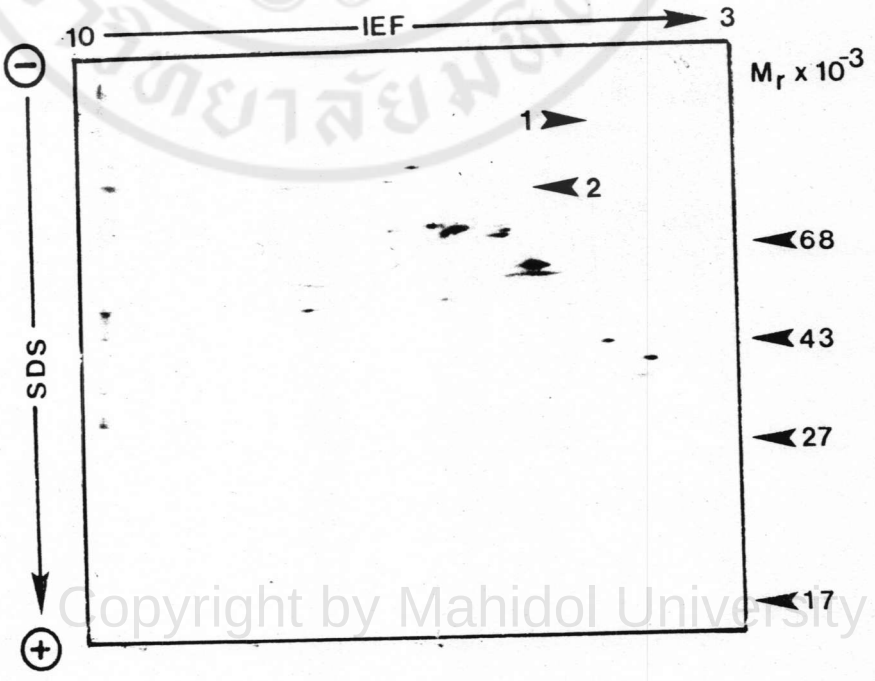


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Fig. 24 Fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine in the presence of $1x10^{-6}$ M tetracycline plus $1x10^{-4}$ M cycloheximide for 7 h. Parasitemia was 18.90% (2.19% ring form, 14.10% trophozoite, 2.61% schizont). 50 µl of labeled sample (1057 cpm/µl) were analysed by 2D-PAGE and fluorography for 10 days (A) and 2 months (B).

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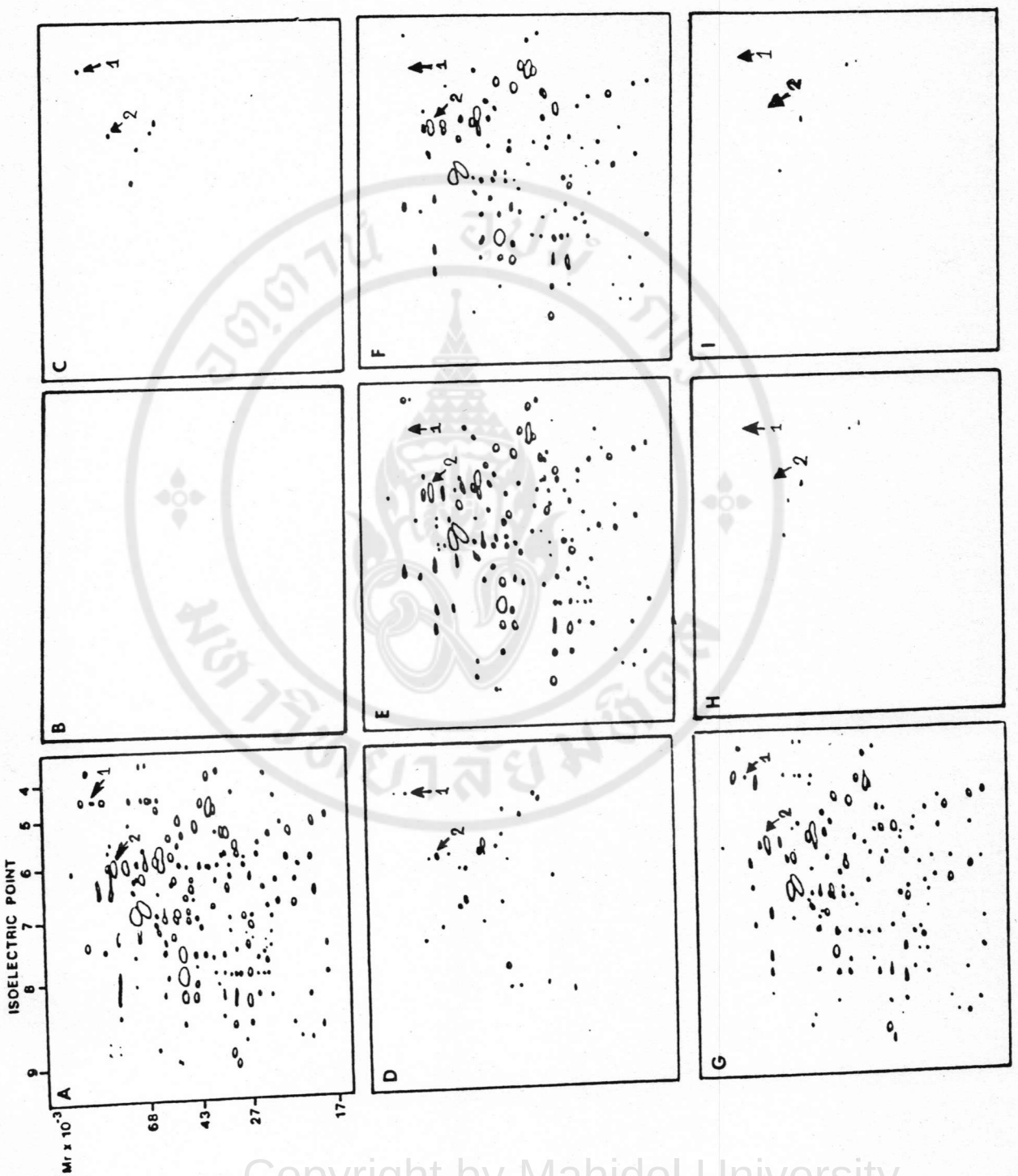




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Fig. 25 Diagrams of 10 days exposed-fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum (K_1), metabolically labeled with [^{35}S]-methionine. Parasites were labeled in the absence (A) and presence of cycloheximide at concentrations of $1x10^{-3}$ M (B), $1x10^{-4}$ M (C), $1x10^{-5}$ M (D), tetracycline at concentrations of $1x10^{-4}$ M (E), $1x10^{-5}$ M (F), $1x10^{-6}$ M (G), and in the presence of $1x10^{-4}$ M cycloheximide plus $1x10^{-5}$ M (H) or $1x10^{-6}$ M (I) of tetracycline.

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Fig. 26 Diagrams of 2 months exposed-fluorograms of two dimensional gel electrophoretic protein pattern of P. falciparum, metabolically labeled with [^{35}S]-methionine. Parasites were labeled in the absence (A) and presence of cycloheximide at concentrations of $1x10^{-3}$ M (B), $1x10^{-4}$ M (C), $1x10^{-5}$ M (D), tetracycline of $1x10^{-4}$ M (E), $1x10^{-5}$ M (F), $1x10^{-6}$ M (G) and in the presence of $1x10^{-4}$ M cycloheximide plus $1x10^{-5}$ (H) or $1x10^{-6}$ (I) of tetracycline.

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5. $^{45}Ca^{2+}$ uptake by P. falciparum (K_1)-infected erythrocytes at different stage of development

P. falciparum-infected erythrocytes exhibited higher $^{45}Ca^{2+}$ uptake than normal erythrocytes (Fig. 27,28,29). $^{45}Ca^{2+}$ uptake was markedly increased in schizont stage (1.28±0.12 nmol $^{45}Ca^{2+}/10^9$ cells/ 4 h for 12% parasitemia) when compared to ring and trophozoite stages (0.46±0.03 nmol $^{45}Ca^{2+}/10^9$ cells/4 h) (Fig. 27).

6. Effect of tetracycline on $^{45}Ca^{2+}$ uptake by P. falciparum (K_1)-infected erythrocytes

Tetracycline at the concentration of $0.68x10^{-5}$ M, had no effect on $^{45}Ca^{2+}$ uptake by both normal and all stages of P. falciparum-infected erythrocytes after incubation for 4 h in the serum-free, bicarbonate-supplemented RPMI-1640 medium (Fig. 27). After incubation at the same drug concentration in same medium, $^{45}Ca^{2+}$ uptake by infected erythrocytes was enhanced at 16 h and markedly enhanced at 20 h, while little effect on $^{45}Ca^{2+}$ uptake was observed for normal erythrocytes (Fig. 28). When infected erythrocytes were maintained in the presence of the same drug concentration in serum and bicarbonate-supplemented medium and further exposed to $^{45}Ca^{2+}$ in the presence of drug for 4 h, tetracycline did not showed any effect on initial $^{45}Ca^{2+}$ uptake rate of infected erythrocytes even at 24 h of preexposure (Fig. 29).

7. [^{3}H]-tetracycline uptake by P. falciparum (K_1) infected erythrocytes

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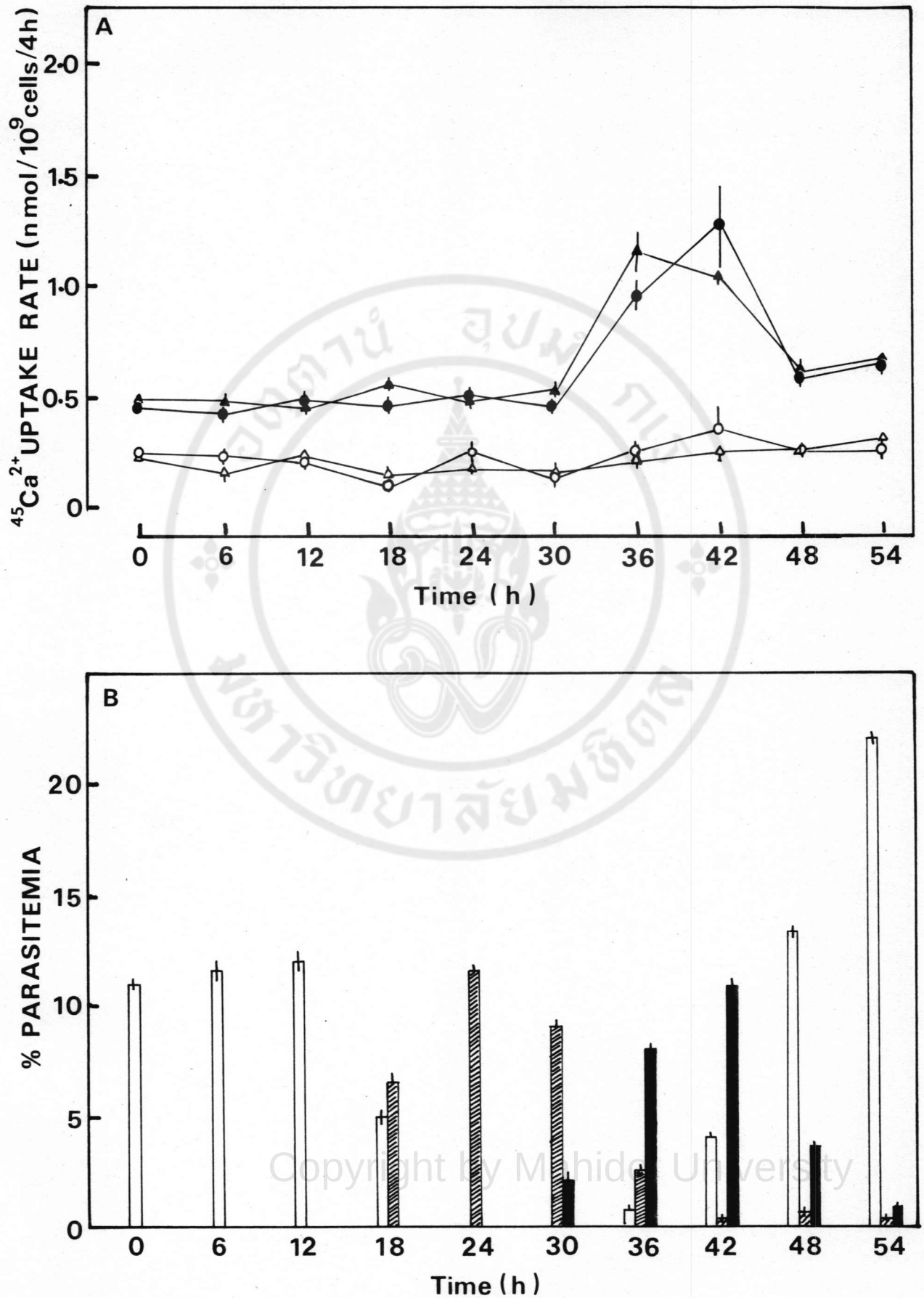
Both normal and infected erythrocytes exhibited a time-saturable [^{3}H]-tetracycline uptake. They rapidly accumulated [^{3}H]-tetracycline

Fig. 27 Effect of tetracycline on $^{45}Ca^{2+}$ uptake in incomplete RPMI 1640 medium by erythrocytes carrying P. falciparum (K_1) at different stages of development.

A) Profile of $^{45}Ca^{2+}$ uptake by P. falciparum-infected erythrocytes after 4 h incubation in the absence (●—●) and presence of 0.68×10^{-5} M tetracycline (3 µg/ml) (■—■) Normal age-matched erythrocytes in the absence (○—○) and presence of 0.68×10^{-5} M tetracycline (3 µg/ml) (□—□) were used as controls. Each point was the mean of triplicate values.

B) Kinetics of development of parasites during experiment □-ring form, ▨ -trophozoite, ■ -schizont.

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Fig. 28 Effect of tetracycline on $^{45}Ca^{2+}$ uptake in incomplete RPMI 1640 medium by P. falciparum (K_1)–infected erythrocytes. The parasitemia at the begining of the experiment was 16.66% (9.83% ring form, 4.46% trophozoite, 2.37% schizont). Each point was the mean of triplicate values. $^{45}Ca^{2+}$ uptake by P. falciparum-infected erythrocytes in the absence (■—■) and presence (●—●) of 0.68×10^{-5} M tetracycline; and by normal age-matched erythrocytes in the absence (□—□) and presence (○—○) of 0.68×10^{-5} M tetracycline.

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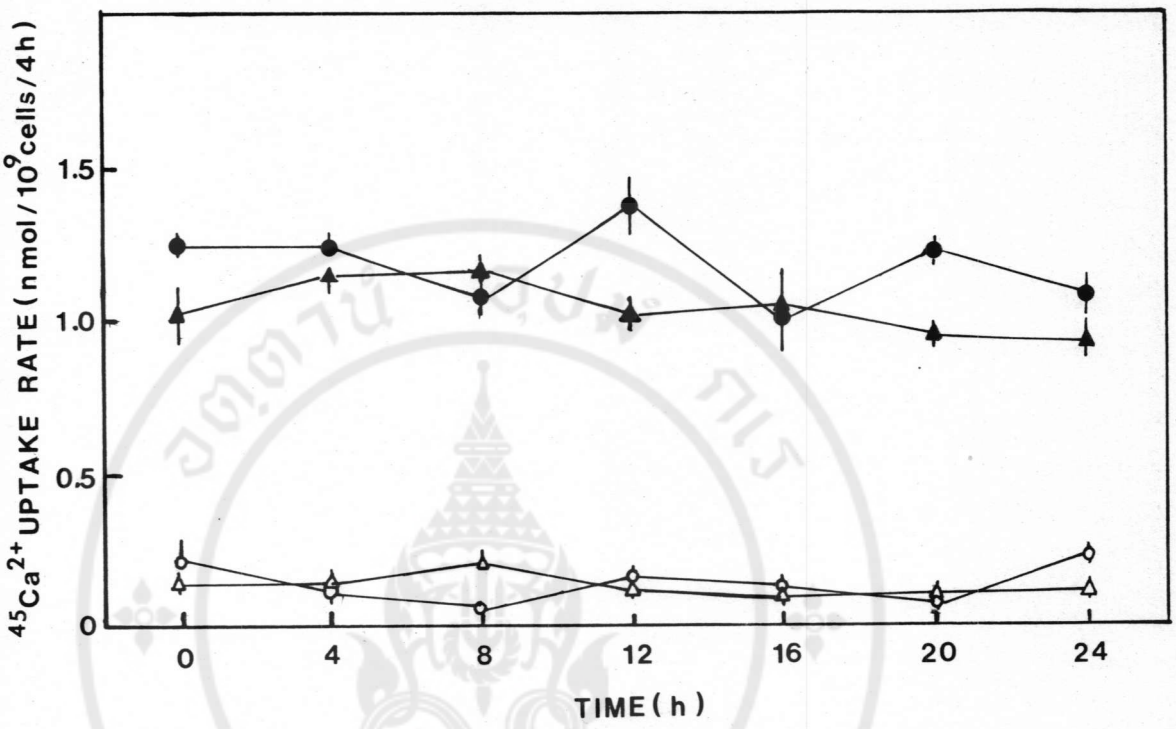


Fig. 29 Effect of tetracycline-pretreatment of $^{45}Ca^{2+}$ uptake rate of P. falciparum (K_1)-infected erythrocytes in complete RPMI 1640 medium. $^{45}Ca^{2+}$ uptake in the absence of tetracycline by : untreated P. falciparum-infected erythrocytes (●—●); untreated normal age-matched erythrocytes (○—○). $^{45}Ca^{2+}$ uptake in the presence of 0.68 x 10^{-5} M tetracycline by : tetracycline-pretreated P. falciparum-infected erythrocytes (▲—▲); tetracycline-pretreated normal age-matched erythrocytes (Δ—Δ). Parasitemia at 0, 4, 8, 12, 16, 20 and 24 h of incubation were 19.02%, 20.00%, 19.51%, 19.71%, 21.12%, 20.11% and 21.14%, respectively. Each point was the mean of triplicate values.

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Fig. 30 $[^3H]$-tetracycline uptake by P. falciparum (K_1)-infected erythrocytes. Parasitemia at the begining of the experiment was 13.20% (6.43% ring form, 4.20% trophozoite, 2.37% schizont). $[^3H]$-tetracycline uptake by P. falciparum-infected erythrocytes, ●–●; normal age-matched erythrocytes, ○—○. Each point was the mean of triplicate values.

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and reached a steady state within the first 30 min of incubation (Fig.30). Infected erythrocytes showed higher uptake than normal ones. At the concentration of 0.68×10^{-5}M (3 µg/ml) tetracycline, [^{3}H]-tetracycline uptake values were 0.34 nmol/10^9 cells (0.15 µg/10^9 cells) and 0.5 nmol/10^9 cells (0.22 µg/10^9 cells) for normal and infected erythrocytes at the percentage of 13.20, respectively.

8. Autoradiochromatogram of dihydroorotate dehydrogenase products

Fig. 31 shows autoradiochromatogram of DHO-DHase products. Three areas of different R_f were detected. The areas containing dihydroorotic acid and orotic acid had R_f 0.42-0.52 (mean R_f = 0.47) and R_f 0.25-0.33 (mean R_f = 0.29). Another minor component had R_f 0.55-0.59 (mean R_f = 0.57). The major compound detected from the assay of DHO-DHase from untreated P. falciparum had a mean R_f of 0.29, assigned as orotic acid and from 72 h tetracycline-treated parasites, a mean R_f of 0.47, assigned as dihydroorotic acid. The major compound detected from the assay of DHO-DHase from 72 h tetracycline-treated parasites in the presence of 0.3 mM menadione plus 0.1 mM cytochrome c had a mean R_f of 0.29, and was assigned as orotic acid.

9. Locations of dihydroorotate dehydrogenase (DHO-DHase) and glutamate dehydrogenase (Glu-DHase) in P. falciparum (K_1)

DHO-DHase was shown to be located mainly on 10,000xg pellet, whereas Glu-DHase was located on 10,000xg supernatant fraction of P. falciparum, since the major activity (≃80%) of DHO-DHase was in 10,000xg pellet (Table 3) and Glu-DHase activity was detected only in the 10,000xg

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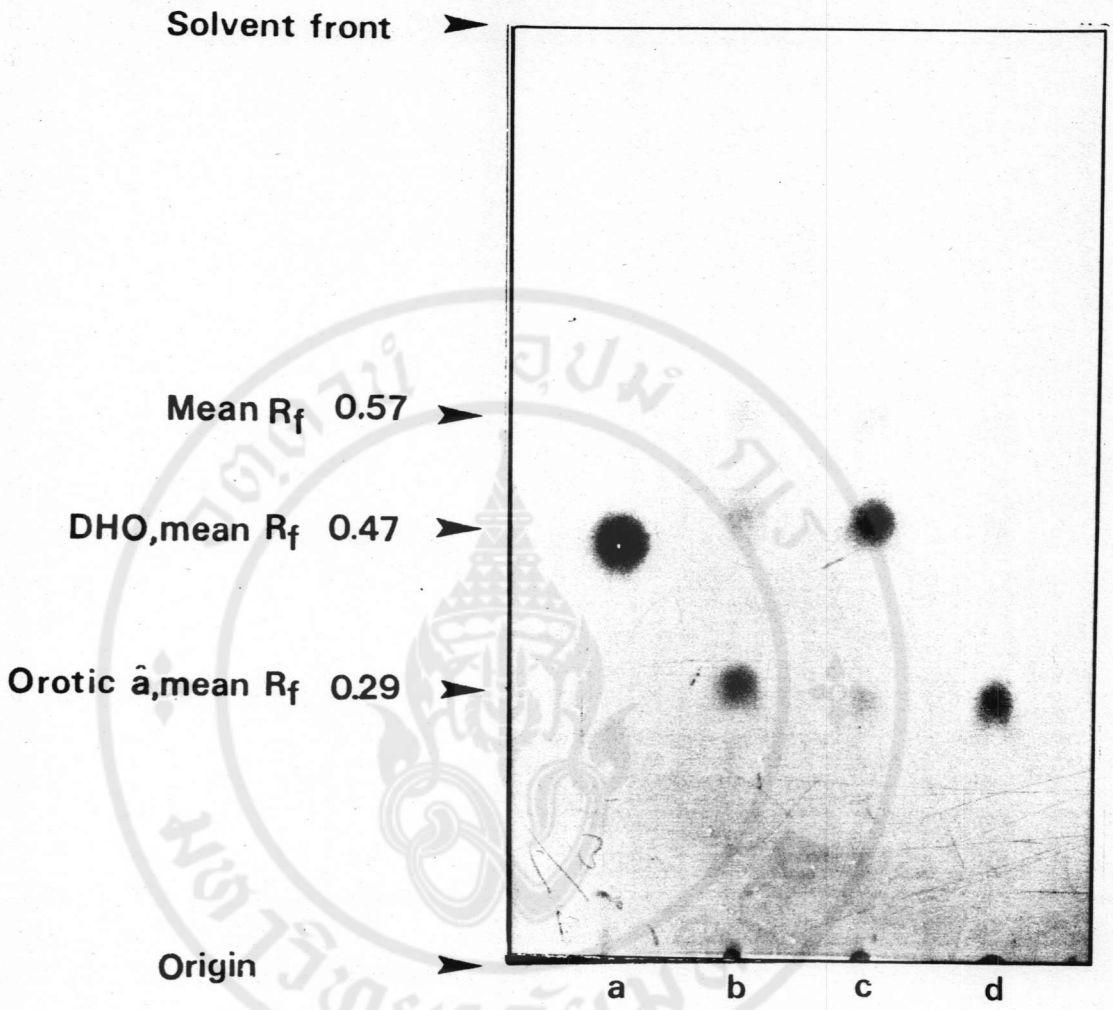


Fig. 31 Autoradiograms of paper chromatographic patterns of DHO-DHase product

Lane a) Stock of [carboxy-^{14}C]-DHO

b) [Carboxy-^{14}C]-DHO incubated with 10,000xg pellet of P. falciparum

c) [Carboxy-^{14}C]-DHO incubated with 10,000xg pellet of 72 h-tetracycline-pretreated P. falciparum

d) [Carboxy-^{14}C]-DHO incubated with 10,000xg pellet of 72 h-tetracycline-pretreated P. falciparum in the presence of 0.1 mM cytochrome c plus 0.3 mM menadione.

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Table 3 Location of DHO-DHase in P. falciparum[a]

	% of recovered activity[b]	
	10,000 x g supernatant	10,000 x g pellet
I	18	82
II	21	79
III	15	85
Mean	18	82

a) Isolated P. falciparum after sonication

b) Calculated as (total activity recovered in each fraction / sum of activity in both fractions) x 100

Table 4 Effect of various concentrations of tetracycline on DHO-DHase activity of P. falciparum

Concentration(M)	% relative activity		
	I	II	Mean
Control	100	100	100
10^{-3}	98	96	97
10^{-4}	116	94	105
10^{-5}	108	90	99
10^{-6}	111	97	104
10^{-7}	104	94	99

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supernatant (Table 6). Both DHO-DHase and Glu-DHase activity could not be detected in normal erythrocytes (Fig. 32, Table 6).

10. Effect of tetracycline on dihydroorotate dehydrogenase activity of P. falciparum (K_1)

Tetracycline did not show any direct effect on DHO-DHase activity even at concentration as high as 10^{-3} M (Table 4). When parasites were maintained in the presence of $1x10^{-5}$ M of drug prior to enzyme assay, however, the DHO-DHase activity was markedly decreased within 36 h of exposure (Fig. 32). This decrease in enzyme activity was observed prior to reduce in parasitemia.

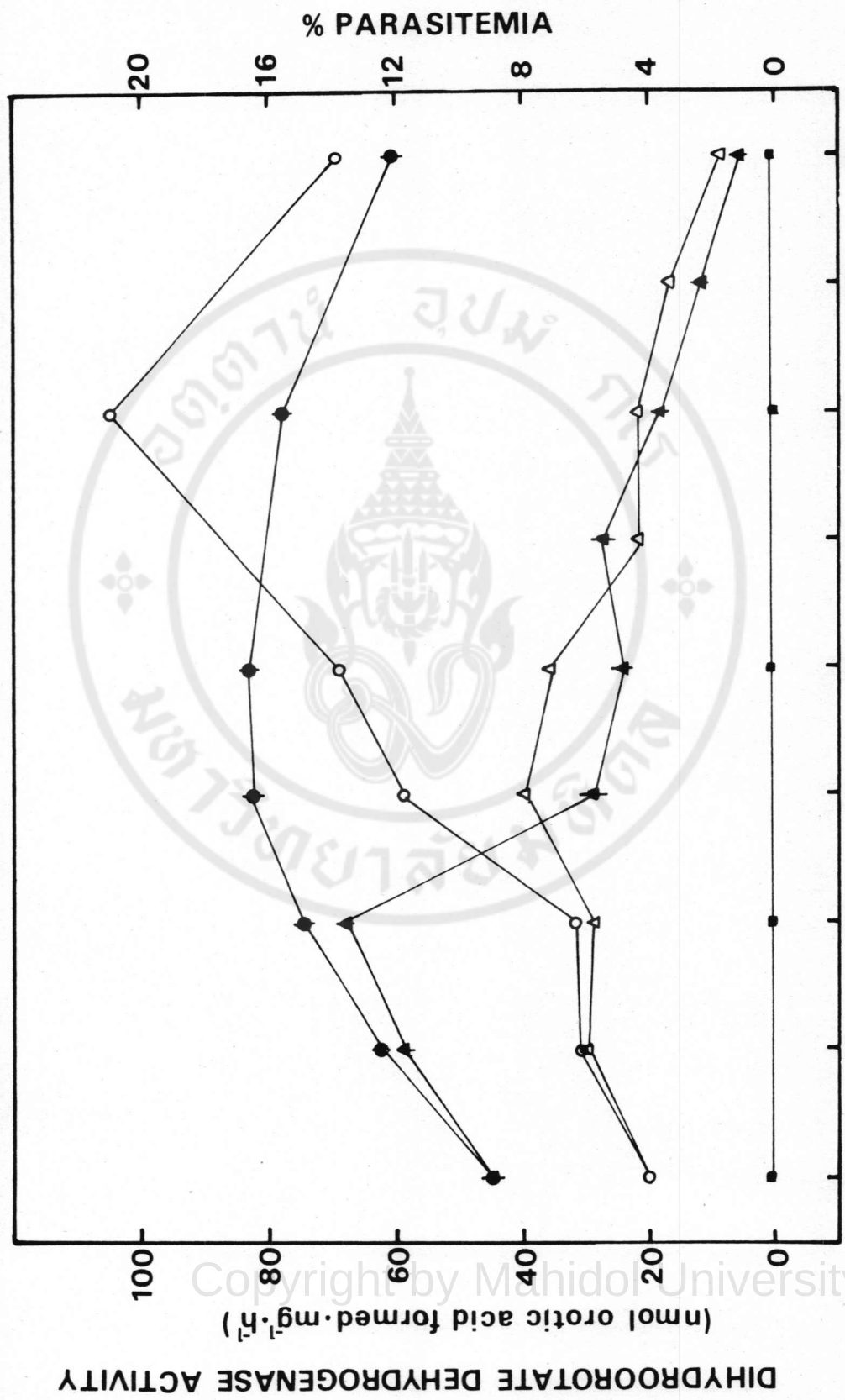
11. Effect of additional electron carriers on dihydroorotate dehydrogenase activity from tetracycline-treated P. falciparum (K_1)

In order to see whether the decrease in DHO-DHase activity of tetracycline-treated parasites was the result of the defect in enzyme itself or in the respiratory chain linked to it, the enzyme activity of drug-treated parasite was assayed in the presence of additional electron carriers : 0.3 mM menadione, 0.1 mM cytochrome c or both (Fig. 33). These electron carriers could stimulate the enzyme activity to different degrees. 0.1 mM cytochrome c was less effective than 0.3 mM menadione and did not enhance the efficiency of the latter (Table 5). The additional electron carriers could stimulate latent activity of DHO-DHase from tetracycline-treated parasite and the degree of stimulation was found to increase with time of drug pretreatment (Table 5).

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Fig. 32 Dihydroorotate dehydrogenase (DHO-DHase) activity from tetracycline-treated P. falciparum for various periods. DHO-DHase activity of normal erythrocyte lysate (■—■), 10,000xg pellet of P. falciparum maintained in the absence (●—●) and presence (▲—▲) of $1x10^{-5}$ M tetracycline. % Parasitemia of P. falciparum maintained in the absence (○—○) and presence (△—△) of $1x10^{-5}$ M tetracycline.

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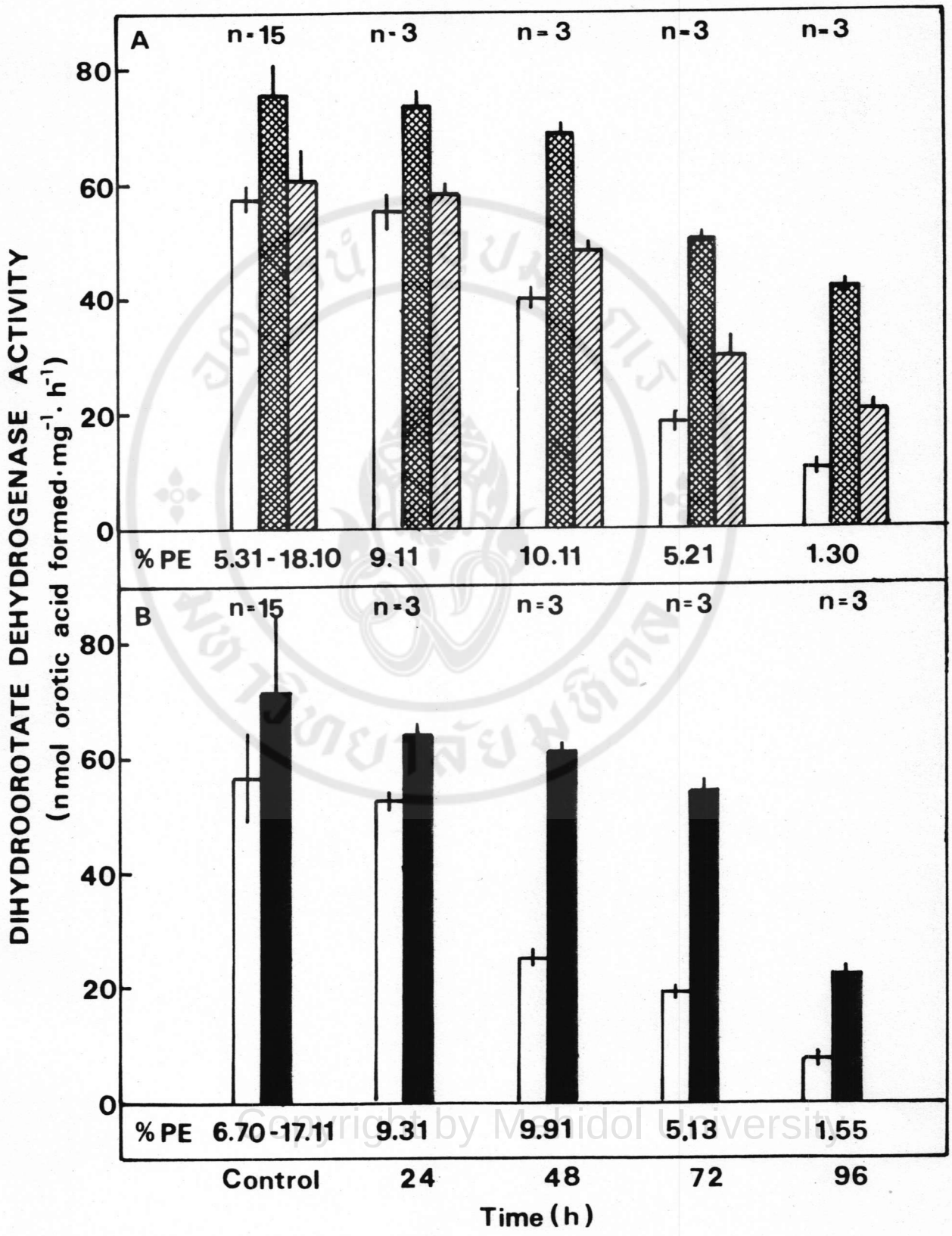


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Fig. 33 Effect of additional electron carriers on dihydroorotate dehydrogenase activity from tetracycline-treated P. falciparum (K_1). P. falciparum was maintained in the presence of $1x10^{-5}$M tetracycline and DHO-DHase activity in 10,000xg pellet was assayed in the absence of additional electron carrier (□) and presence of 0.1 mM cytochrome c (▨), 0.3 mM menadione (▩), or in the presence of 0.1 mM cytochrome c plus 0.3 mM menadione (■).

(%PE – Percent parasitemia)

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Table 5 Effect of additional electron carriers on DHO-DHase from tetracycine treated P. falciparum

Additional electron carrier	Relative activity (Drug exposure time(h))				
	Control	24	48	72	96
None	1	1	1	1	1
Cytochrome c (0.1 mM)	1.06	1.05	1.33	1.64	1.93
Menadione (0.3 mM)	1.31	1.32	1.72	3.02	3.93
Cytochrome c (0.1 mM) + Menadione (0.3 mM)	1.28	1.24	2.44	2.84	3.60

Table 6 Distribution of Glu-DHase in P. falciparum fractions

	Enzyme activity ($n mol \cdot mg^{-1} \cdot min^{-1}$)		
	10,000 x g supernatant	10,000 x g pellet	NE
I	32.34 ± 1.79 (n=3)	UD	UD
II	31.00 ± 5.41 (n=3)	UD	UD
III	28.69 ± 1.76 (n=3)	UD	UD
IV	26.29 ± 0.18 (n=3)	UD	UD
V	27.08 ± 0.65 (n=3)	UD	UD
Mean	29.91 ± 3.96 (n=15)	UD	UD

UD = Undetectable
NE = Normal erythrocyte

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Fig. 33, like Fig. 32, shows that DHO-DHase activity of tetracycline-treated parasite was markedly decreased prior to decrease in parasitemia. When the enzyme was assayed in the presence of 0.3 mM menadione, 0.1 mM cytochrome c or both, enzyme activity was decreased along with or after the reduction in parasitemia.

12. Glutamate dehydrogenase activity (Glu-DHase) from tetracycline-treated P. falciparum (K_1)

In order to see whether the decrease in DHO-DHase activity of tetracycline-treated P. falciparum observed prior to reduce in parasitemia (Fig. 32) is not due to the general effect of drug treatment, the activity of another enzyme located only in the supernatant fraction, Glu-DHase, from drug-treated parasite was studied. Fig. 32 shows that, unlike the decreasing pattern of DHO-DHase activity, Glu-DHase activity of tetracycline-treated P. falciparum was not decreased until after parasitemia was well reduced.

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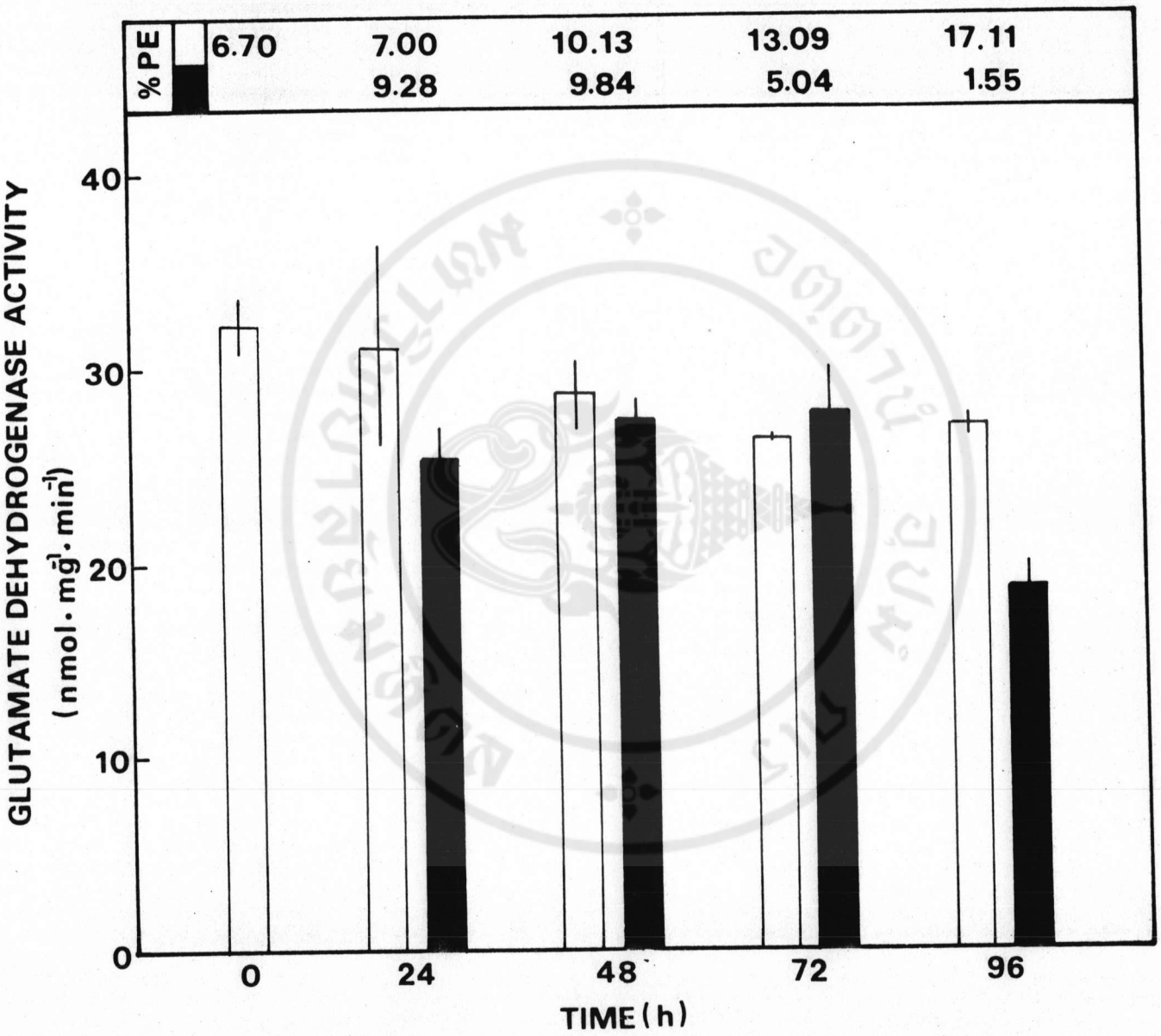
Fig. 34 Glutamate dehydrogenase (Glu-DHase) activity from tetracycline-treated P. falciparum (K_1)

Glu-DHase of P. falciparum maintained in the absence (□) and presence (■) of 10^{-5} M tetracycline. Each bar was the mean of triplicate values.

Activity for normal erythrocytes was undetectable.

(%PE – Percent parasitemia)

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DISCUSSION

1. Effect of tetracycline on P. falciparum (K_1) growth and development in vitro

Tetracycline has been used as one of the drugs in clinical treatment of malaria for a long time. The therapy with this drug requires extended treatment for success (Rieckmann, 1984). This indicates the slow action of this drug on this parasite. Recent observation from in vitro experiments (Geary and Jensen, 1983; Divo et al, 1985), consistent with this clinical indication, showed increase in antiparasitic activity of tetracycline with time of exposure. Results from in vitro experiments shown here (Fig. 4-6) confirm previous results. The 50% effective concentration values (EC_{50}) for both 48 h and 96 h obtained from these experiments (Fig. 5) were similar to those reported previously and were within the therapeutic range for antimalarial and antibacterial activity, normally about 3-5 μg/ml (Sande and Mandell, 1980). The antimalarial potency of other 70S ribosome specific antibiotics (other tetracycline derivatives, clindamycin series, chloramphenicol series and macrolide series of drugs),mitochondrial inhibitors (Janus Green, rhodamine 123, antimycin A), and antimetabolites (analogs of riboflavin, nicotinamide, pyridoxin and thiamin) was also shown to be increased with time (Divo et al, 1985a; Geary et al, 1985; Gershon and Howells, 1986b; Izumo and Tanabe, 1986). By contrast, 80S ribosome specific inhibitors (cycloheximide,

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anisomycin), and conventional antimalarial drugs (chloroquine, quinine, mefloquine, amodiaquine) showed only minor or no increase in potency in prolonged incubation (Divo et al, 1985a; Geary et al, 1985).

Both tetracycline-pretreated and untreated normal erythrocytes could support parasite reinvasion to the same extent, either in the absence and presence of drug (Table 2). By contrast, drug-treated parasites reinvaded new erythrocytes far less effectively than the untreated ones. Therefore, the decrease in ring stage, observed at 48 h of tetracycline-treatment, due to the reduction in merozoite reinvasion, was not the result of the direct effect of this drug on this process or the effect on normal erythrocytes, but due to the effect on parasitized cells or parasites.

The invasion of an erythrocytes by a malarial merozoite follows a definite sequence involving a number of erythrocyte membrane components, properties and processes (Hadley et al, 1986b). Interfering with these components, properties or processes can alter the invasion process. For example, the increase in mechanical rigidity (decrease in deformibility) of the erythrocyte membrane has been shown to lead to decrease in merozoite reinvasion (Dluzewski et al, 1985; Hadley et al, 1986a). Our results suggest that tetracycline did not alter membrane components and properties of normal erythrocytes required for merozoite invasion. On the other hand, tetracycline may affect the invasion capacity of the merozoites through toxic effect on the schizonts. An example of such toxic effect interfering with merozoite reinvasion was shown recently for conjugated N-acetyl glucosamine (Hadley et al, 1986a).

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Possible explanations for the slow action of tetracycline on the parasite may be raised as follows :

a) The drug penetrates slowly into the parasite,

b) The action of the drug requires a duration of time to become significant for inhibition of parasite growth and maturation. The first explanation is less likely than the second one by the following reasons. The drug entered the parasitized cells to a greater extent than the normal ones, and reached the saturable level within the first 30 min of incubation (Fig. 30). At the concentration of 0.68×10^{-5} M tetracycline, [^{3}H]-tetracycline uptake value were 0.34 nmol/10^{9} cells and 0.5 nmol/10^{9}cells for normal and infected erythrocytes at 13.20% parasitemia, respectively. If the level of parasitemia of infected cells is extrapolated to 100%, the [^{3}H]-tetracycline uptake value will be equal to 1.55 nmol/10^{9} cells. Furthermore, this drug at the concentration of 10^{-5} M (4.44 µg/ml), which is in the therapeutic level, could begin to inhibit protein synthesis of the parasite within 7 h of incubation, although to a very small extent (Fig. 10). This evidence indicates that this drug enters the parasite long before the apparent effect on parasite growth could be observed. The second explanation, therefore, is more plausible. Tetracycline may inhibit some metabolic processes of the parasite, resulting in a general and much more pronounced effected later in the cell cycles. Furthermore, only in the later stages of parasite development did the effect of tetracycline inhibition begin to be noticeable, since abnormal morphology could be observed at the stages of trophozoite and schizont

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but not at ring stage, after drug-treatment was prolonged to 48 h or more. Therefore, tetracycline may act on such anabolic processes as nucleic acid or protein synthesis which are relatively active and more indispensable in the later stages of parasite development (Gritzmacher and Reese, 1984; Inselburg and Banyal, 1984; De Rojas and Wasserman, 1985; Waki et al, 1985). Tetracycline action on eukaryotic cells was shown to be specifically on protein synthesis (Kwan et al, 1972; Gale et al, 1981). Tetracycline can inhibit both cytoplasmic and mitochondrial protein synthesis (see 'Introduction'). A number of reports (Warhurst et al, 1972; Kaddu et al, 1974) implied that the tetracyclines had no effect on cytoplasmic protein synthesis, since they did not inhibit chloroquine-induced pigment clumping in trophozoites in vitro, a process which requires protein synthesis. The antimalarial activity pattern of this drug resembled only other 70S ribosome specific inhibitors, but not any 80S ribosome specific inhibitors (Divo et al, 1985a; Geary et al, 1985; Gershon and Howells, 1986b). Mitochondria are the only organelle in eukaryotic cells that contain 70S ribosome system (Tzagoloff and Myers, 1986). Blood-stage malaria parasites do possess mitochondria (Langreth et al, 1978; Divo et al, 1985b; Slomianny and Prensier, 1986). Like 70S inhibitors, mitochondrial inhibitors also showed the increase in the antimalarial potency with time (Divo et al, 1985a; Geary et al, 1985). Furthermore, at the therapeutic level, tetracycline showed very little effect on total protein synthesis (Fig. 10). Therefore, the increasing antimalarial potency of this drug with time can be explained by the possibility that this

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drug inhibits mitochondrial protein synthesis, and it requires a duration of time before the consequence of a loss of newly synthesized proteins become significant for inhibition of parasite growth and maturation. Apparently, up to 1 day, the mitochondria originally present can still continue to function. It is only when many new parasites are being formed during schizogony, that a failure to synthesize new fully equipped mitochondria interferes with further growth and maturation. The results of the effect of this drug on activity of dihydroorotate dehydrogenase and glutamate dehydrogenase (to be discussed later) support this postulation. The failure to synthesize new mitochondria may lead to appearance of abnormal schizonts containing fewer number of merozoites.

2. Analysis of P. falciparum (K_1) proteins with cycloheximide-resistant, tetracycline-sensitive synthesis

Tetracycline is a 70S ribosome specific inhibitor (Gale et al, 1981). Cycloheximide, in contrast with tetracycline, specifically inhibits the function of 80S ribosomes but has no action on 70S ribosomes (Frankin and Snow, 1981). Mitochondrial protein synthesis is a 70S ribosome system. In order to prove that antimalarial activity of tetracycline is through its action on parasite mitochondrial protein synthesis, the approach was the attempt to demonstrate the parasite proteins, the synthesis of which were cycloheximide-resistant and tetracycline-sensitive with the assumption that they are synthesized from 70S ribosome system of parasite mitochondria. Two ways of the study were done to achieve this approach :

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a) Analysis by investigating the effect of tetracycline and cycloheximide on $[^{3}H]$-isoleucine incorporation by P. falciparum (K_1)

The different action of cycloheximide and chloramphenicol on 80S and 70S ribosomes, respectively, has been utilized in several investigations of lower eukaryotes to identify mitochondrial (70S ribosomal) protein synthesis (Kroon and De Jong, 1979). Gershon and Howells (1986a) used this approach to identify mitochondrial protein synthesis of P. falciparum by investigating the effect of these drugs on $[^{14}C]$-isoleucine incorporation. By the method employed, they could not demonstrate the cycloheximide-resistant, chloromphenicol-sensitive protein synthesis presumed to be due to mitochondrial system. The same method was used here with tetracycline instead of chloramphenicol (Fig. 10). Like the Gershon and Howells' report, the same observation, that the major fraction of parasite proteins was synthesized from the 80S ribosome system in the cytoplasm, was obtained. The cycloheximide-resistant, tetracycline-sensitive fraction of protein synthesis could not be detected. In Neurospora crassa, 2.15% of total protein synthesis have been shown to be mitochondrial in origin (Hawley and Greenwalt, 1970). In Saccharomyces cerevisiae, 8.9% of the protein of whole mitochondrial proteins were synthesized by the cycloheximide-resistant system (Schweyen and Kaudewitz, 1970), and in Trypanosoma brucei brucei. an average of 5.7% of total protein synthesis, was cycloheximide-resistant (Spithill et al, 1981) The results of this study, therefore, suggest that, in P. falciparum, mitochondrial protein synthesis is a considerably smaller fraction of overall protein synthesis than that found in a variety of other lower eukaryotes.

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b) Analysis by two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

It is well known that 2D-PAGE technique has particular advantages of facilitating comparison of a large number of proteins. This technique has been used to demonstrate variations among isolates of P. falciparum (Tait, 1981; Fenton et al, 1985), and to study mitochondrial translation products of mammalian cells (Bhat et al, 1982). This method was used here to demonstrate P. falciparum mitochondrial proteins with the assumption that their synthesis would be cycloheximide-resistant, tetracycline-sensitive. The results of 2D-PAGE analysis (Fig. 15-26), again, showed that most of parasite proteins were cytoplasmic in origin, since most of the spots were shown to be sensitive to cycloheximide and resistant to tetracycline. Some of these protein spots which still appeared in the presence of high concentration of cycloheximide might be synthesized by the 80S ribosome system at a high level and are incompletely inhibited by this drug. The fluorograms (Fig. 15) showed that these spots were more intense than most of the other spots.

A small number of spots were however shown to be different from most in drug sensitivity. One spot of 95 K, pI 4.5, appeared to be synthesized from cycloheximide-resistant, tetracycline-sensitive system which may be the 70S ribosome system of parasite mitochondria. Most of the studies on the analysis of mitochondrial products of other eukaryotes indicated that mitochondrially synthesized proteins were in the size range of 6-55 K and were hydrophobic in nature (Schatz and

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Mason, 1974; Tzagoloff et al, 1979). In view of this, the spot of 95 K, pI 4.5 which was detected as a possible mitochondrially synthesized protein in our studies may (a) serve as a precursor of some lower molecular weight proteins, or (b) in view of the hydrophobic nature of mitochondrially synthesized proteins (Tzagoloff et al, 1979) and reports showing the covalent joining of mitochondrially synthesized proteins (Beattie and Clejan, 1980), this high molecular weight protein may consist of aggregates of smaller subunits (Downer et al, 1976). Nevertheless, the synthesis of high molecular weight components in the range of 80-100 K has been shown to occur in other mitochondrial systems (Beattie et al, 1979; Alziari et al, 1981; Bhat et al, 1982).

Another spot of 85 K, pI 6 was also different from others in drug sensitivity. It could be detected when both drugs were used singly and disappeared only with combination of drugs. However, the marked reduction in intensity of this spot in the presence of cycloheximide used singly indicates that this protein is also sensitive to this drug. Since this 2D-PAGE technique defines proteins by their isoelectric points and molecular weights only (O'Farrell, 1975), it is possible that different proteins with the same or nearly the same isoelectric point and molecular weight can migrate to the same position. Consequently, the spot of 85 K, pI 6 might contain two or more different proteins, from both 80S ribosome system sensitive to cycloheximide, and 70S ribosome system sensitive to tetracycline. When the parasite was maintained in the presence of either drug, only the protein(s) sensitive to that drug was (were) inhibited. When the

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parasite was maintained in the presence of both drugs, both protein synthesizing systems were inhibited and no spot was detected.

The results from both methods above showed that mitochondrial protein synthesis is a very small fraction of overall protein synthesis. The explanation may be raised as follows:

(a) Cycloheximide is not absolutely specific but may affect a variety of other cellular functions including mitochondrial RNA polymerase (Schatz and Mason, 1974),

(b) Cytoplasmically synthesized enzymes and proteins inhibited by cycloheximide may usually play an important role in controlling mitochondrial protein synthesis (Tzagoloff and Myers, 1986), or

(c) Mitochondrial protein synthesis in P. falciparum is, indeed, at a very small level. This could result from either the presence of only a single mitochondrion (Langreth et al, 1978; Divo et al, 1985b; Slomianny and Prensier, 1986) or a low rate of synthesis per mitochondrion. The latter could be attributable either to a minimal level of expression of the mitochondrial genome or to a low turnover rate of mitochondrially synthesized proteins.

These reasons can also explain the work of Gershon and Howells (1986) that no constant cycloheximide-resistant fraction of total protein synthesis was observed at the concentrations of this drug between 10^{-7} and 10^{-2} M.

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3. The effect of tetracycline on Ca^{2+} uptake by P. falciparum (K_1)-infected erythrocytes

The calcium ion has an important role in regulating the activity of many cellular processes. It was also shown to be indispensable for parasite growth, maturation and reinvasion (Wasserman et al, 1982). Reports from various laboratories (Bookchin et al, 1981; Tanabe et al, 1982; Krungkrai and Yuthavong, 1983) have shown marked disturbances in Ca^{2+} status of malarial infected erythrocytes. Increased rates of $^{45}Ca^{2+}$ uptake were observed as well as increased Ca^{2+} content. Our results shown here (Fig. 27-29) confirm and extend those previous reports. Enhanced $^{45}Ca^{2+}$ uptake was observed for all stages of parasite development. Like previous reports (Tanabe et al, 1982; Krungkrai and Yuthavong, 1983), our results also showed that the greatest enhancement occured at schizont stage. The work of Krungkrai and Yuthavong (1981) showed enhanced $^{45}Ca^{2+}$ uptake accompanied by greatly enhanced lysis at schizont stage, and this result led them to suggest that calcium may have a role in the regulation of merozoite release. Enhanced host membrane permeability to calcium, probably combined with reduced efficiency of the Ca^{2+}-ATPase driven calcium pump, could lead to an increase in intracellular calcium. The parasite, furthermore, may have its own Ca^{2+} transport processes to accumulate calcium : Ca^{2+} influx, down the electrical component of the parasite membrane potential generated in part by an electrogenic H^+-ATPase and Ca^{2+} efflux, gated through a $Ca^{2+}+H^+$ antiport system (Mikkelsen et al, 1982; Tanabe et al, 1982, 1983).

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Most of the calcium (>90%) was shown to be localized within the parasites (Leida et al, 1981; Tanabe et al, 1982). Mitochondria are the organelle known as 'calcium sink' in eukaryotic cells (Carafoli, 1982). Malaria parasite was shown to possess mitochondria capable to maintain a high transmembrane potential (negative inside) (Divo et al, 1985b). Mitochondria of parasite may, therefore, be involved in Ca^{2+} accumulation and regulation. It is possible that if tetracycline has its action on parasite mitochondria, it may alter Ca^{2+} regulation in the parasite and lead to reduction in Ca^{2+} uptake by infected cells. Tetracycline was previously shown to be capable of reducing calcium accumulation and uptake by chick chondrocyte mitochondria, leading to increase in the serum Ca^{2+} level and defect of mineralized tissue (Shapiro et al, 1977).

The results reported here show that tetracycline, at therapeutic level, has no effect on initial Ca^{2+} uptake rate of all stages of development (Fig. 27). Surprisingly, tetracycline had an effect of enhancing further the Ca^{2+} uptake by P. falciparum-infected erythrocytes after 16 h of drug exposure, when the serum was omitted from the medium (Fig. 28). When the serum was present, however, no difference was observed (Fig. 29). This phenomenon cannot be fully explained at present but may be related to degradative changes in the parasite maintained in serum-free medium and to binding of tetracycline to serum protein (Jun and Lee, 1980). Energy-dependent extrusion Ca^{2+} from the cells (Schatzmann, 1982) may also be affected by tetracycline, but remains to be proven.

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It is also possible that, in view of Ca^{2+} chelating properties of tetracycline, a higher Ca^{2+} uptake was the result of a higher tetracycline uptake. However, this was not so, since no stoichiometry between [^{3}H]-tetracycline uptake (Fig. 30) and $^{45}Ca^{2+}$ uptake (Fig. 28) was observed. A plausible explanation for tetracycline-enhanced $^{45}Ca^{2+}$ uptake by P. falciparum-infected erythrocytes may be raised as follows. Tetracycline may act at parasite mitochondria, decreasing the Ca^{2+} accumulation by this organelle, as in the case of tetracycline-treated chick chondrocytes (Shapiro et al, 1977), and hence lead to releasing Ca^{2+} out off the parasite. Since the parasite is surrounded by erythrocyte cytosol, the released Ca^{2+} may be in the host cytosol. It was reported that Ca^{2+} at high concentration (>1 μM) could inhibit the activity of Ca^{2+}-ATPase, which drive Ca^{2+} pump, about 20-40% (Mikkelsen et al, 1984). The activity of Ca^{2+}-ATPase driven Ca^{2+} pump requires ATP (Schatzman, 1982). Tanabe et al (1983) had reported that, under optimal assay condition, the Ca^{2+}-ATPase activity of erythrocyte membranes from schizont-infected cells of P. chabaudi was reduced by approximately 30% relative to that of membranes from uninfected cells. ATP levels and calmodulin content required for the Ca^{2+}-ATPase were also reduced. They suggested that, at that condition in infected cells, Ca^{2+}-ATPase driven Ca^{2+} pump of schizont-infected cells functions at only about 15-20% of normal ones. Therefore, the higher of Ca^{2+} level, released from the parasite, in host cytosol, combined with the effect of ATP depletion after infected erythrocytes were maintained in the serum-free RPMI medium for a long period, may

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result in further reduction of the efficiency of the Ca^{2+} pump to force Ca^{2+} out off the infected cells, leading to a higher Ca^{2+} accumulation in the presence of tetracycline. However, this hypothesis requires further investigation for support.

4. Inhibition of pyrimidine biosynthesis of P. falciparum (K_1) by tetracycline

Pyrimidines play a fundamental role in nucleic acid structure and function in all living cells. One possible role of mitochondria of the malaria parasite is to serve for pyrimidine de novo biosynthesis by providing electron disposal mechanism (Gutteridge et al, 1979; Gero et al, 1981a, 1984). From the particulate nature of the enzyme and its linkage with a respiratory chain (Gutteridge et al, 1979; Gero et al, 1981a,b, 1984), it may be assumed that, like in mammalian system (Keppler and Holstege, 1982), dihydroorotate dehydrogenase (DHO-DHase), one of the enzyme in pyrimidine de novo biosynthetic pathway, is located on mitochondria. The experiments on the effect of tetracycline on DHO-DHase activity were designed to find out whether tetracycline acts at the mitochondrial level through the inhibition of pyrimidine biosynthesis of this parasite.

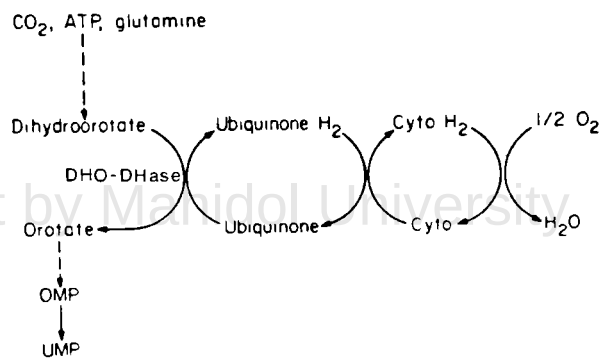


Fig.35 Proposed conversion of dihydroorotate to orotate (Sherman, 1984)

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There are many methods for activity assay of this enzyme, based on spectrophotometry (Yates and Pardee, 1957; Miller et al, 1968) or radioassay procedure (Shafritz and Senior, 1967; Herrmann et al, 1973; Shoaf and Jones, 1973; Smithers et al, 1978). The method used here was radioassay method based on conversion of L-[carboxy-^{14}C] DHO into [carboxy-^{14}C] orotic acid catalysed by DHO-DHase, and subsequent isolation by DEAE ion exchange chromatographic procedure (Herrmann et al, 1973). The stopped reaction mixture could be separated by this method into three areas of orotic acid, DHO, and an unknown compound (Fig. 31). The R_f value of this unknown compound was the same as reported, by using this method, for cabamoyl aspartate (CAA) (Herrmann et al, 1973). This unknown product, therefore, may be CAA, converted in reverse direction from DHO by dihydroorotase (DHOase), another enzyme of cytoplasmic origin in pyrimidine de novo biosynthetic pathway (Keppler and Holstege, 1982). This enzyme had been detected in soluble fraction of many species of Plasmodium including P. falciparum (Gero et al, 1981a; Hill et al, 1981a,b; Gero et al, 1984). Furthermore, Gero et al (1981a) also reported the presence of dihydroorotase, but a very low level in the particulate fraction of P. berghei due to contamination of this enzyme from soluble fraction.

DHO-DHase, catalysed the interconversion of DHO into orotic acid, was found predominantly in the particulate fraction of the extract of P. falciparum (Table 3). This observation complements other previous reports on this enzyme of the same and other species of Plasmodium (Gutteridge et al, 1979; Gero et al, 1981a, 1984). This result and those previous reports showed that the activity could not be

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solubilized by freezing and thawing or sonication, suggesting that it is a membrane-bound enzyme. This enzyme has been shown to be similar in mechanism and properties to the mammalian enzyme (Gutteridge et al, 1979; Gero et al, 1981a). In the mammalian system, this enzyme is also particulate and its location is on the outer surface of the innermembrane of mitochondria (Keppler and Holstege, 1982). DHO-DHase of P. falciparum may, therefore, be located on mitochondrial membrane as suggested by previous reports, since the parasite does possess mitochondria (Langreth et al, 1978; Divo et al, 1985b; Slomianny and Prensier, 1986). By contrast, the enzyme capable of catalysing the conversion of DHO into orotic acid in some protozoa, e.g., the Kinetoplastida (Trypanosoma spp., Leishmania spp.) and Crithidia spp. was shown to be localized in soluble fraction and was different with respect to mechanism and properties from mammalian enzyme (Gutteridge et al, 1979; Hammond and Gutteridge, 1982; Pascal et al, 1983; Tampitag and O'Sullivan, 1986). In Giardia lamblia), the enzyme involved in conversion of DHO into orotic acid, as well as other enzyme in pyrimidine de novo biosynthetic pathway, could not be detected (Lindmark and Jarroll, 1982).

The activity of DHO-DHase shown here was much higher than reported previously for P. falciparum (Gero et al, 1981b, 1984) and also for other species (Gutteridge et al, 1979). In those previous reports, the crude homogenate of the parasite was used as the source for enzyme assay, whereas 10,000xg pellet of parasite extract was used here. However, strain variation, the method of preparation and assay, and the relative distribution of the morphological forms of

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the parasite could also be contributing factors to the observed differences.

Besides DHO-DHase, the malaria parasite also contains other enzymes for completing the pyrimidine de novo biosynthetic pathway (Walsh and Sherman, 1968; Van Dyke et al, 1970; O'Sullivan and Ketly, 1980; Gero et al, 1981a,b; Reyes et al, 1982; Gero et al, 1984), but no enzyme of pyrimidine salvage pathway could be detected (Reyes et al, 1982). It is now well established that the intraerythrocytic malaria parasite is unable to salvage preformed pyrimidine from its environment and relies exclusively on de novo pathway for its pyrimidine requirement (Sherman, 1984). Tetracycline treatment led to marked reduction of DHO-DHase activity before the apparent effect on parasitemia could be seen (Fig. 32). As the mature mammalian erythrocyte lacks the pyrimidine de novo pathway (Keppler and Holstege, 1982), inhibition of this enzyme activity by tetracycline treatment could be responsible for subsequent lethal effect on this parasite. The previous reports also showed that inhibition of pyrimidine de novo biosynthetic enzymes resulted in highly effective antimalarial activity, such as inhibition of DHO-DHase by agents in the hydroxynaphthoquinone series (analogs of ubiquinone) (Hudson, 1984; Hudson et al, 1984; Hammond et al, 1985) and inhibition of orotate phosphoribosyltransferase and orotidylate decarboxylase, the last two enzymes in pyrimidine de novo pathway, by pyrazofurin (Scott et al, 1986). Thus, all of these results can be explained by the inability of malaria parasite to take up preformed pyrimidines to relieve the blockade.

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It is rather unlikely that such an effect would cause an almost immediate arrest of nucleic acid, and even more so, of protein synthesis. In the latter case, excisting cellular pools of m-RNA and t-RNA should suffice, at least temporarily, for protein synthesis before the shortage of pyrimidines would affect this process. This is especially true for the ring stage with low levels of nucleic acid and protein synthesis (Gritzmacher and Reese, 1984; Inselburg and Banyal, 1984; De Rojas and Wasserman, 1985; Waki et al, 1985). The observation that : (a) tetracy cline activity was more pronounced at longer time of exposure (Fig. 4-6), (b) DHO-DHase activity decreased before the reduction in parasitemia (Fig. 32), and (c) the abnormal morphology was noted at the stage of trophozoite and schizont, but not at ring stage (Fig. 7-9), support this postulation.

Like in the mammalian system (Keppler and Holstege, 1982), DHO-DHase of this parasite is linked to a respiratory chain, probably via ubiquinone (Fig. 35) (Gutteridge et al, 1979; Gero et al, 1981a, 1984; Sherman, 1984). Hydroxynaphthoquinones (ubiquinone analogs) could inhibit the activity of this enzyme directly in vitro by interfering with the respiratory chain linked to it (Gero et al, 1981a; 1984; Hammond et al, 1985). By contrast, like other conventional antimalarial drugs (Gutteridge et al, 1979), tetracycline could not inhibit in vitro activity of this enzyme (Table 4). This indicates that this drug did not directly act at both the enzyme itself and respiratory chain linked to it. The reduction in enzyme activity after tetracycline pretreatment was probably due to a defect in the respiratory chain since enzyme activity from pretreated parasites assayed in the

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presence of additional electron carriers was not affected until after the parasitemia was markedly reduced (Fig. 33). These indicate the defect in biosynthesis of some respiratory chain component. Our possible target is cytochrome c oxidase (cytochrome aa_3), one of the enzyme in respiratory chain. This enzyme is mitochondrially synthesized in other eukaryotes (Tzagoloff and Myers, 1986). The activity of this enzyme could be detected in many species of Plasmodium including P. falciparum (Scheibel and Miller, 1969a,b; Scheibel and Pflaum, 1970). Its formation could be inhibited by therapeutic concentration of tetracycline (De Vries and Kroon, 1970, De Jonge and Hulsmann, 1973; Van Den Bogert and Kroon, 1981; Van Den Bogert et al, 1983). The reduction in enzyme activity may be explained by the fact that parasite mitochondria are equipped with proteins or enzymes, some including DHO-DHase are cytoplasmic in origin and still synthesized during tetracycline treatment, but need to act in conjunction with mitochondrially synthesized proteins or enzymes.

Little reduction in DHO-DHase activity was found when the enzyme was assayed in the presence of additional electron carriers, even at after the parasitemia was markedly reduced (72 h) and abnormal parasites appeared. It is possible that, only after blockade of pyrimidine synthesis and exhaustion of the excisting cellular pool of nucleic acid and proteins, would total protein synthesis of the parasite be affected. Hence a relatively constant enzyme concentration was maintained.

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Cytochrome c is a natural electron acceptor in respiratory chain and may already be present in the parasite material. Therefore

exogenous cytochrome c was found to have only a little stimulating effect on the DHO-DHase activity (Fig. 33A, Table 5). Menadione, an artificial electron acceptor, provides a new alternative way and was found to be more effective in carrying electrons than cytochrome c (Fig. 33A, Table 5). The addition of 0.3 mM menadione was enough to stimulate a full enzyme activity, and further addition of cytochrome c did not increase the enzyme activity further (Fig. 33, Table 5).

5. Effect of tetracycline on glutamate dehydrogenase activity of P. falciparum (K_1)

Glutamate dehydrogenase (Glu-DHase) has been demonstrated in several species of Plasmodium including P. falciparum (Sherman et al, 1971; Walter et al, 1974; Carter and Waliker, 1977; Roth et al, 1982; Vander Jagt et al, 1982; Krungkrai et al, 1985, Ling et al, 1986). The predominant reaction is the oxidation of glutamate to α-ketoglutarate (Fisher, 1985). This reaction generates reduced NADP (NADPH) suggested as important for maintaining glutathione in the reduced form in the malaria parasite (Roth et al, 1982). The Glu-DHase of animal cells are in the mitochondria, probably matrix, whereas in fungal cells the enzyme is in the soluble phase of the cytosol (Smith et al, 1975). As in the case of fungi, the Glu-DHase of P. falciparum was shown to be a cytosolic protein by using fluorescent antibody technique (Ling et al, 1986). The activity of the enzyme was shown to be unaffected by tetracycline treatment (Fig. 34). The decrease in enzyme activity after 96 h of drug treatment may be due to a secondary effect. It is clear that this enzyme was much less affected by drug treatment than

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dihydroorotate dehydrogenase, The results indicate that the anti-parasitic activity of this drug is through its primary action on the mitochondria.

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SUMMARY

1. The antiparasitic activity against P. falciparum (K_1) in vitro of tetracycline was increased with time of exposure. This phenomenon may be explained by the possibility that the drug inhibits mitochondrial protein synthesis of the parasite, and it requires a duration of time to be significant for inhibition of parasite growth and maturation.

2. The reduction in merozoite reinvasion by tetracycline treatment was not due to the direct effect of the drug on merozoite invasion processes and the effect on normal erythrocytes, but due to the effect of the drug on parasitized cells or parasites.

3. The synthesis of a protein of 95 K, pI 4.5 of P. falciparum (K_1) was cycloheximide-resistant, tetracycline-sensitive, indicating that it may be synthesized from 70S ribosome system, probably of parasite mitochondria.

4. Dihydroorotate dehydrogenase, an enzyme involved in de novo pyrimidine biosynthesis, was found mainly in the particulate fraction of P. falciparum extract and presumed to be mitochondrial The activity of this enzyme was inhibited within 36 h when parasitized erythrocytes were cultured in presence of therapeutic concentration of tetracycline prior to assay. The decrease in enzyme activity was not due to the direct interaction of the drug with this enzyme as indicated by the lack of the effect on

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enzyme activity in its presence, but due to the defect in respiratory chain linked to it, caused by tetracycline treatment.

5. Glutamate dehydrogenase, a cytoplasmic enzyme, found in 10,000xg supernatant of P. falciparum extract, was not affected by tetracycline treatment, suggesting that tetracycline has no effect on cytoplasmic enzymes.

6. Increase in $^{45}Ca^{2+}$ uptake was observed for all stages of development of P. falciparum with the greatest enhancement at schizont stage.

7. Tetracycline at therapeutic level has no effect on initial $^{45}Ca^{2+}$ uptake of all stages of development. However, tetracycline at the same concentration further enhanced $^{45}Ca^{2+}$ uptake by P. falciparum-infected erythrocytes after incubation for 16 h, when the serum was omitted from the medium.

8. $[^{3}H]$-tetracycline uptake was greater in P. falciparum-infected erythrocytes (0.5 nmol/10^9 cells of 13.20% parasitemia) than in normal ones (0.34 nmol/10^9 cells).

9. Tetracycline-enhanced $^{45}Ca^{2+}$ uptake may not be due to the uptake of tetracycline-Ca^{2+} chelating complexes, as indicated by the lack of stoichiometry between $[^{3}H]$-tetracycline uptake and $^{45}Ca^{2+}$ uptake.

Based on the above observation, it is concluded that the antimalarial action of tetracycline is at mitochondrial level and the lethal effect is due to inability of the dormant stages of the parasite to replace the damaged mitochondria.

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APPENDIX I

Composition of RPMI 1640 medium

Component	mg/L
Inorganic salts	
$Ca(NO_3)_2 \cdot 4 H_2O$	100.00
KCl	400.00
$MgSO_4$	48.84
NaCl	6000.00
Na_2HPO_4 (anhy.)	800.00
Other components	
Glucose	2000.00
Glutathione (reduced)	1.00
Phenol red	5.00
Amino acids	
L-Arginine (free base)	200.00
L-Asparagine	50.00
L-Aspartic acid	20.00
L-Cysteine	65.00 (2HCl)
L-Glutamic acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine (Allo free)	50.00

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Component	mg/L
L-Leucine (Methionine free)	50.00
L-Lysine HCl	40.00
L-Methionine	15.00
L-Phenylalamine	15.00
L-Proline (Hydroxy L-Proline free)	20.00
L-Serine	30.00
L-Threonine (Allo free)	20.00
L-Tryptophan	5.00
L-Tyrosine	28.94 (Na Salt)
L-Valine	20.00
Vitamins	
Biotin	0.20
D-Ca Pantothenate	0.25
Choline Cl	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
Para-aminobenzoic acid	1.00
Pyridoxine-HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B_{12}	0.005

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APPENDIX II

Giemsa's stain

Giemsa powder, CP	0.6 g
Glycerol	50 ml
Absolute methanol, acetone free	50 ml

A small amount of dry stain was placed in a mortar and a small amount of glycerol added.The dye was ground thoroughly, and then poured off into a clean bottle. The grinding was repeated in this manner until all the stain has been mixed with glycerol. The glycerol-dye mixture was incubated in a water bath at 55-60^{o}C for 6-8 h with periodic shaking and then cooled to room temperature. 50 ml methanol was added, and the dye mixture kept stoppered at 37^{o}C for 2 weeks. The dye mixture was then filtered and stored in a dark bottle until use.

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