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THE PARTIAL PURIFICATION OF HUMAN ERYTHROCYTE
ADENYLATE KINASE AND THE EFFECT OF SOME COMMON
ANTIMALARIAL DRUGS ON THIS ENZYME

BY

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DEPARTMENT OF BIOCHEMISTRY,
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LEGON.

JUNE, 1976.

THIS WORK HAS BEEN DEDICATED TO

..... My Parents

..... My Husband

..... My Daughter



D E C L A R A T I O N

THE EXPERIMENTAL WORK DESCRIBED IN THIS THESIS

was carried out by me


AT THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON

UNDER THE SUPERVISION OF

Professor G.S. Asante

I CERTIFY THAT THIS WORK HAS NOT PREVIOUSLY
BEEN ACCEPTED FOR ANY DEGREE AND IS NOT BEING
CURRENTLY SUBMITTED IN CANDIDATURE FOR ANY DEGREE.

SIGNED:  CANDIDATE


..... SUPERVISOR

JUNE, 1976

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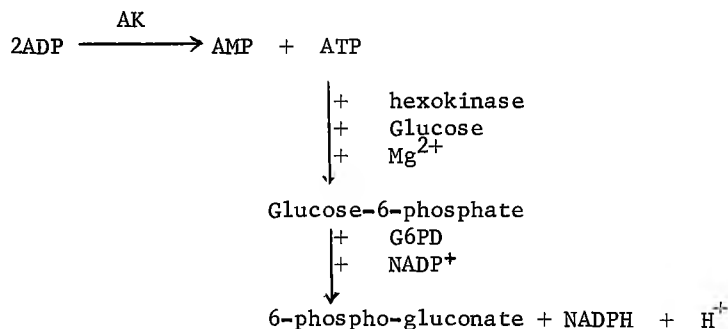
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ABSTRACT

The enzyme adenylate kinase (AK) was partially purified from human erythrocytes. The purification process involved the separation of the erythrocytes from whole human blood, rinsing of the cells in saline solution and finally lysing them in two volumes of water containing dithiothreitol. The crude haemolysate was dialysed against 0.01M sodium citrate buffer pH 5.0 and then treated with a suspension of CM-sephadex C-50, previously equilibrated in 0.01M sodium citrate buffer pH 5.0 containing 0.25M sodium chloride to reduce amount of haemoglobin present to effect better separation on column. After centrifuging at 30,000g the supernatant solution from this treatment was further subjected to sephadex G-100 column chromatography to obtain a partially purified enzyme preparation.

The activity of adenylate kinase was measured using the coupled assay system by Oliver (1957), which is



Absorbance at 340nm was measured for the NADPH produced over a period of 6 minutes using an SP 500 spectrophotometer with a recorder attached to it. This corresponded to ATP produced over the same period.

It was found that AK was unstable at 70°C and optimum activity was observed around 37°C- 40°C. Dilute solutions of AK were found to be unstable, but the enzyme was stable at 4°C in a concentrated form.

Adenylate kinase activity was also found to be inhibited at high substrate (ADP) concentration, and it is known that AMP, one of the products of the reaction inhibits AK activity, competitively. From substrate concentration of 3-10mM ADP, the activity decreased. Optimum activity was obtained at substrate concentration of 1-2mM ADP.

The effect of some common antimalarial drugs on the enzyme activity was also looked at. The antimalarial drugs used included chloroquine diphosphate, chloroquine sulphate, quinine hydrochloride, quinine sulphate, proguanil hydrochloride, primaquine phosphate and mepacrine hydrochloride. All the drugs at a concentration of 10^{-4} M inhibited the enzyme activity to various extent. The degree of inhibition depended on the incubation period of the enzyme with the particular drug. Quinine was found to inhibit the enzyme activity most. Inhibition of up to 62.0% was observed with quinine after 3 hours incubation period. For the same incubation period, primaquine phosphate inhibited the enzyme activity up to 42.0%, mepacrine up to 40.7%, chloroquine up to 30.3% and proguanil hydrochloride up to 28.0%. The type of inhibition, observed for chloroquine diphosphate, the most common antimalarial drug in the tropics, was non-competitive inhibition.

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LIST OF ABBREVIATIONS

ACD	Acid-citrate dextrose
ADP	Adenosine 5'diphosphate
AK	Adenylate Kinase
AMP	Adenosine 5'monophosphate
ATP	Adenosine 5'triphosphate
ATPase	Adenosine 5'triphosphatase
CM-Sephadex	Carboxymethyl sephadex
CoA	Coenzyme A
DEAE-Cellulose	Diethylamineethyl-cellulose
1,3,di PGA	1,3, diphospho-glyceric acid
2,3 d-PGA	2,3, diphospho-glyceric acid
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FH ₂ , FH ₄	Dihydro and tetrahydro folic acid
G3P	Glyceraldehyde-3-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Hb	Haemoglobin
HMS	Hexosemonophosphate shunt

NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NAD ⁺ P	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PABA	para-amino benzoic acid
PFK	phosphofructokinase
6PG	6-phosphogluconate
PEG	polyethylene glycol
RBC	Red blood cell
RNA	Ribonucleic acid
TCA	Tricarboxylic acid cycle
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER 1: INTRODUCTION

Adenylate Kinase (AK) has been found to be performing an important role in the overall regulation of the cellular energy metabolism.

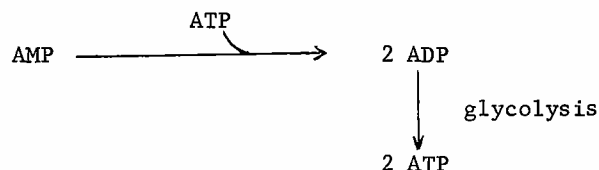
Experiments were carried out by Brewer (1967) to find the levels of Adenosine triphosphate (ATP) in American Negros and American Caucasians. In these experiments, the ATP levels of American Negros were found to be 3.17 moles/gHb, while those in the Caucasians were found to be 3.47 moles/gHb (Brewer, 1967). The level of ATP in the red blood cell (RBC) appears to be intimately related to the capacity of the red cell to resist certain types of stresses.

In the case of malaria infection, low levels of ATP may cause premature rupture of RBC before mature schizonts are formed, thus liberating premature parasites into the plasma, which might lead to the retardation of rate of increase in parasitaemia. From these studies, Brewer and Powell (1965) suggested that this low level of ATP in American Negros might be affording some means of protection against P. falciparum infection.

Experiments on the cultivation of malaria parasites have shown that ATP is essential for the extracellular cultivation of Plasmodium (Brewer and Powell, 1965; Peters, 1970). In the development of asexual erythrocytic forms of malaria parasites, the parasites are dependent on the host's erythrocytic ATP. This suggests that any efforts aimed at blocking or decreasing synthesis or regeneration of ATP in the host cell may afford a means of controlling malaria infection.

The host red cell ATP which is a source of energy, may also be used to provide purine bases for the parasite nucleic acid synthesis. This is very important for the parasite which cannot synthesize its own purine bases.

In most tissues, the generation of ATP occurs through oxidative phosphorylation, utilizing energy from NADH generated in the Tricarboxylic acid (TCA) cycle. However, this mechanism of energy production is absent in the non-nucleated mature mammalian red blood cell due to lack of mitochondria and must therefore depend on other enzyme systems and glycolysis for the generation of ATP from ADP and AMP. One of the enzymes involved in ATP generation from AMP in the red cell is adenylate kinase. The reaction proceeds as follows:-



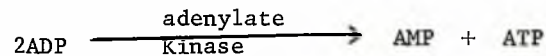
Secondly, the rate of glycolysis depends on the adenylate charge, i.e. the ratio of ATP, ADP and AMP present in the cell and adenylate kinase has been shown to be responsible for maintaining the suitable adenylate charge (Atkinson, 1968).

In the experiment carried out in this thesis, an attempt was made to purify adenylate kinase from human red blood cell and to find out if antimalarial drugs which are widely used in the prevention and treatment of malaria have any effect on the activity of the enzyme

and if so, the way in which they do so.

The enzyme was obtained from the red cell, by first using centrifugation to precipitate the red cells from the whole blood in acid-citrate dextrose (ACD) solution and then rinsing the cells several times in saline solution. The rinsed red blood cells were lysed in water and the cell membrane removed by high speed centrifugation.

The supernatant solution from this treatment containing adenylate kinase activity was subjected to sephadex and DEAE-cellulose column chromatography to obtain a partially purified enzyme. The adenylate kinase reaction followed in the experiment was in the direction:-

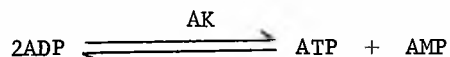


In order to bring out the importance of such a study, a brief review of the enzyme, adenylate kinase, has been given to show the importance of the enzyme in the body. The mature human red blood cell has been extensively reviewed. Malaria and malaria parasite together with antimalarial drugs and their effects on the parasite have been similarly reviewed. Efforts have been made to stress the dependence of the parasite on the host's red blood cell for certain important substances.

ADENYLATE KINASE

Adenylate Kinase (AK) also known as myokinase or ATP-AMP phosphotransferase, is present in many different tissues and has very high activity in some tissues, especially, the human red blood cell and muscles (Altman, 1959; Rapley and Harris, 1970; Tsuboi et al, 1975). The enzyme catalyses the equilibrium reaction of adenine nucleotides, thus providing a unique buffering role against rapid concentration changes in any of the components of this nucleotide pool. Adenylate kinase also serves as a primary regulatory enzyme for those reactions requiring the participation of adenine nucleotides; for example ATP as co-substrate for hexokinase reaction, ATP as substrate and inhibitor of phosphofructo kinase (PFK) and AMP as activator for PFK reaction (Tsuboi et al, 1975).

The reversible reaction involving AK can be represented as follows:-



This interconversion of adenine nucleotides seems to be of particular importance in organelles which have high turnover of chemical energy, for example chloroplasts and mitochondria (Mazelis, 1956; Markland et al, 1966).

Several isoenzymes of AK have been shown to occur in the human red blood cell (Fildes and Harris, 1966; Rapley and Harris, 1970; Modiano et al, 1970; Brownson and Spencer, 1972A; Tsuboi et al, 1975).

Three types of AK isoenzymes were discovered in the human red blood cell (Fildes and Harris, 1966), and these were designated as AK1, AK2-1, and AK2. These isoenzymes were shown to be genetically determined variations (Fildes and Harris, 1966; Rapley and Harris, 1970). Several isoenzymes have also been found in the human muscle (Noda and Kuby, 1957). Khoo and Russel (1972), also showed that adenylate kinase isoenzymes in the human red blood cell and muscle, exhibited different electrophoretic behaviour from AK of other tissues.

Russel et al (1974), showed that adenylate kinase existed in several other organs of the human body such as the liver, kidney, brain and the heart. The adenylate kinase isoenzymes in these organs were shown to be organ specific.

All adenylate kinase isoenzymes are low molecular weight proteins ranging from 20,000 - 31,000 (Brownson and Spencer, 1972a; Russel et al, 1973). The muscle adenylate kinase with a molecular weight of 22,000 is one of the smallest known phosphoryl transferases (Morrison et al, 1972).

Criss (1970), using rat liver, showed that adenylate kinase was present in subcellular fractions, with the mitochondrion showing the highest AK activity followed by cystol, then nuclei and finally microsomes. Four isoenzymes of AK were discovered in rat liver and were designated as AKI, AKII, AKIII and AKIV. Criss (1970), also discovered that the activities of AK isoenzymes I, II, and IV remained reasonably constant in the liver but the activity of AKIII was responsive to

dietary changes.

Further work by Filler and Criss (1971), involving the use of foetal rat liver, showed that as the foetus developed, the AK activity in the tissues increased and continued to increase immediately after birth linearly at a rate of about 0.15 units/day/mg protein till adult concentration of 2.2 units/day/mg protein was reached between 10-14 days after birth. This means that as energy supply changes as at birth, the adenylate kinase activity increases to meet the high energy requirement and dietary change of the young mammal.

All the above observations seem to implicate the importance of AK in energy metabolism. The localization of different isoenzymes in the mitochondrion and cytoplasm could be extremely important in terms of the ability of the liver cell to regulate its energy system involving ATP, ADP and AMP.

It is believed that adenylate kinase in addition to other components of the body, is involved in shifting the adenylate charge (Atkinson et al, 1968). This adenylate charge ratio is represented as follows by Atkinson:-

$$\frac{(ATP) + \frac{1}{2}(ADP)}{(ATP) + (ADP) + (AMP)}$$

Atkinson et al (1968), postulated that the adenylate charge acts as the energy mediating system of the cell and is capable of switching

metabolic pathways on and off. The energy charge of the adenylate system, has been defined by Atkinson (1966, 1970), as the chemical energy change of the cell and to be equal to the average number of anhydride-bound phosphate groups per adenosine moiety. Several enzymes of glycolysis and the TCA cycle are inhibited by an adenylate charge of one i.e. high ATP or stimulated by adenylate charge of zero, (high AMP). Examples of such enzymes inhibited by high ATP and stimulated by high AMP are PFK and citrate synthetase. The energy charge of the adenylate system provides the cell with a fine intracellular regulatory control which is capable of regulating the entire metabolic pathway. Another observation which points out to the fact that adenylate kinase is involved in the maintenance of the adenylate charge is the fact that the observed concentrations of adenine nucleotides, ATP, ADP and AMP, change in such a way as to affect the equilibrium constant of the adenylate kinase reaction (Markland and Wadkins, 1966; Start and Newsholme, 1968; Ballard, 1970).

The following properties of adenylate kinase help it to regulate the energy flow of the cell:-

- a) its dual localization inside and outside the mitochondria;
- b) its existence in multiple isomeric forms;
- c) its response to dietary and hormonal stimuli.

Comparing the activities of various enzymes of the human RBC, Altman (1959), showed that adenylate kinase has the highest enzyme activity in the human RBC.

The table below gives the comparison of some of the RBC enzyme activities.

TABLE 1

TABLE COMPARING ACTIVITIES OF SOME HUMAN RED BLOOD CELL ENZYMES

ENZYME	ACTIVITY*	SUBSTRATE	CONC.OF SUBSTRATE**
Adenylate Kinase	328,000	ADP	1.7-2.2
Phosphofructo kinase	384	Fructose-6-phosphate	-
Glucose-6-phosphate dehydrogenase	848	Glucose-6-phosphate	0.73-0.91
ATPase	5.6	ADP, ATP	1.7-2.2; 9.9-10.9
Glutathione reductase	670	Oxidized glutathione (GSSG)	5,000
Glucokinase	20	Glucose	37.0
Glyceraldehyde-3-phosphate dehydrogenase	2,610	Glyceraldehyde 3-phosphate	-

* enzyme activity expressed as $\mu\text{moles substrate utilized/hr/10}^{11}$ RBC

** The endogenous substrate concentration expressed as $\mu\text{moles/10}^{11}$ RBC.

The presence of such a high adenylate kinase activity in the red cell suggests the importance of this enzyme in the cell.

Some other properties of adenylate kinase include the following:- It has a pH optimum of 7.5. Partially purified rabbit muscle adenylate kinase is fairly stable and loses 21% activity after 10 minutes at 100°C in 0.1N hydrochloric acid (Colowick and Kalckar, 1943). The pure enzyme is denatured by glass or cellophane surfaces, but may be stored in polythene tubes (Callaghan, 1956). As a kinase, it requires Mg^{2+} ions as cofactor and is inhibited by EDTA (Bowen and Kernin, 1955). Muscle Adenylate kinase is inhibited by AMP, H_2O_2 (Colowick and Kalckar, 1943), fluoride and adenosine-5-monosulphate (Callaghan and Weber, 1959). The crystalline enzyme contains two sulphhydryl (SH) groups per mole of molecular weight of 21,000 (Noda and Kuby, 1957).

Most of the work done on muscle adenylate kinase involved the use of purified enzyme from muscle using centrifugation and chromatography. but in the case of red blood cell adenylate kinase, most of the work involved the use of the crude enzyme preparation in the haemolysate. (Fildes and Harris, 1966; Modiano et al, 1970; Russel et al, 1974).

THE HUMAN RED BLOOD CELL/ERYTHROCYTE

The mature human red blood cell (RBC), unlike other cells of the body, has no nucleus, endoplasmic reticulum or mitochondria (Yunis and Yasmineh, 1969; Altman, 1959). In spite of this, its cellular integrity is preserved and the mature RBC has become adapted to a cellular metabolism depended almost entirely on glycolysis and hexose-monophosphate shunt (HMS). It retains only atrophied remnants of the tricarboxylic acid cycle, oxidative phosphorylation and biosynthetic activities (Altman, 1959).

The human RBC normally has a discoid biconcave shape and is considered to be composed of two interdependent units, the membrane (Stroma) and the cytoplasm. The membrane consists of glycolipid, glycoprotein and lipoprotein and is permeable to certain substances, for example, glucose, which enters the RBC instantaneously, independent of extracellular concentration, within limits (Klinghoffer, 1940).

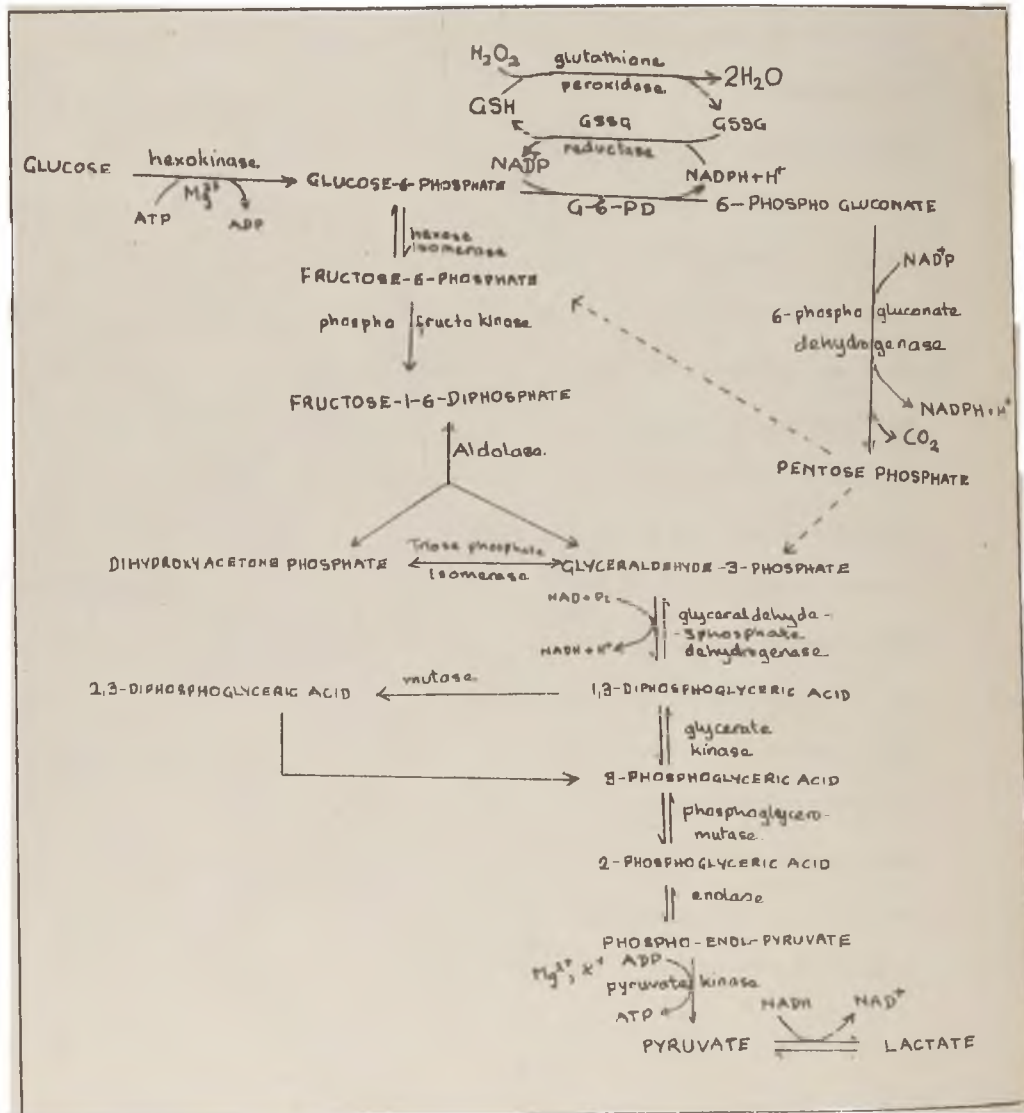
The membrane contains firmly bound enzymes e.g. adenosine triphosphatase (ATPase) and certain metabolites. Because of the affinity of certain enzymes for the membrane, there is a differential distribution of enzymes between the interior and exterior of the cell membrane. This is of considerable importance in the regulation of movement of metabolites across the RBC membrane. The membrane-bound enzymes include a variety of ATPases, and acetylcholinesterase (Altman, 1959). These enzymes are often inactive in the intact cell because they are tightly bound to the lipoprotein matrix of the stroma. Their enzymatic activities however become detectable when the cell is damaged as during haemolysis or ageing of the cell.

The cytoplasm of the mature human RBC contains all the enzymes of the glycolytic and HMS pathways, adenylate kinase, glutathione reductase, methaemoglobin reductase and several other enzymes which do not form complete pathways. One other important component of the RBC cytoplasm is haemoglobin (Hb) which is involved in the gaseous transport in the body.

Metabolism of the Mature Human RBC:

The mature human RBC depends entirely on glycolysis for its energy supply. About 90-95% of glucose in the RBC is metabolised in this way to provide energy for the cell's energy-requiring processes. The other 5-10% is metabolised through the HMS pathway to generate reducing power in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Murphy, 1960). The cell utilizes the energy produced from glycolysis and the reducing power from HMS to maintain itself for approximately 120 days in vivo (Harris et al, 1970). During this time, the red cell is subjected to several physical and chemical stresses such as (i) hydrostatic and osmotic pressure during passage through the capillaries, (ii) the degradative effects of oxidative substances such as hydrogen peroxide, (iii) toxic products resulting from drugs and their metabolites and (iv) diseases like malaria (Szienberg and Marks, 1961). As the cell cannot replace its constituents rapidly to meet the depletion rate during ageing, the cell eventually dies.

FIGURE 1



a. Glycolysis in Human Red Blood Cell:

The glycolytic pathway in the red cell, is the same as in other tissues of the body e.g. muscle. The process is represented in Figure 1. There is also a shunt pathway which produces 2,3 diphosphoglycerate (2,3 di PGA) from 1,3 di PGA through the action of a mutase. The function of 2,3 di PGA was not known, but its concentration in the cell was found to be high and increased when a person was anoxic. Later in 1967, Benesch and Benesch, found that 2,3 di PGA binds deoxyhaemoglobin thus facilitating the liberation of oxygen from oxy-haemoglobin at low oxygen tension; thus oxygen supply to various tissues can still go on at low oxygen tension.

b. The Hexose monophosphate shunt pathway in human RBC:

About 5-10% of the glucose in the cell is metabolized through this pathway (Figure 1). This process generates NADPH which is utilized in the following reactions:- (a) The reduction of met-Hb to Hb, (b) The reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). GSH produced is used to keep the sulphhydryl groups on enzymes and proteins of the cell membrane reduced, thus keeping the membrane structure stable and intact. If there is a deficiency of the enzyme G6PD which is involved in the generation of NADPH, the cell is deprived of enough supply of GSH, especially if it is overloaded with oxidants. This condition will bring about the distortion of the cell membrane leading to eventual lysis of the cell. This is observed in G6PD deficient individuals after primaquine administration.

MALARIA AND THE MALARIA PARASITE

The causative agents of malaria in man belong to the genus Plasmodium and class Sporozoa (Moulder, 1962).

These parasites are obligate intracellular parasites i.e. they have to develop inside the host cell (Moulder, 1962).

They have two hosts, man and the mosquito.

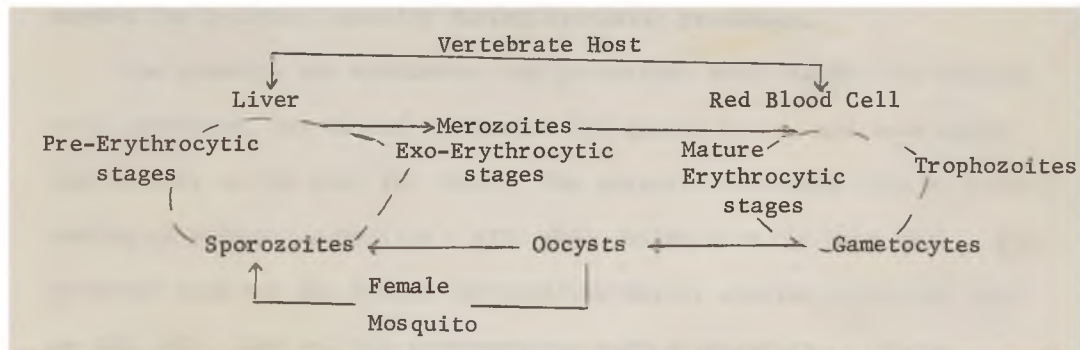
The four common species of Plasmodia that cause malaria in man include the following:- P.falciparum, P.malariae, P. ovale and P. vivax, with the most common species being P. falciparum (WHO bulletin, 1969; Rollo, 1965). In West Africa P. falciparum causes up to 80% of all the malaria cases while P. malariae causes up to 25%, P. ovale up to 5% and P. vivax up to less than 1% (WHO bulletin, 1969).

Malaria parasites are injected as sporozoites into man through the salivary fluid of the female mosquito during the process of feeding (Moulder, 1962; Rollo, 1965). The sporozoites then enter into the circulation and rapidly disappear into the parenchymal cells of the liver, where they grow and multiply. When the parasites mature, the liver cells rupture and the merozoites are released. Some of these enter the RBCs to start the blood cycle while others re-enter new liver cells. Those merozoites which invade the RBCs, grow and multiply using the host Hb as nitrogen source (Maegraith and Deegan, 1956; Rollo, 1965). The parasite gets the full complement of all amino acids except methionine, from the Hb and it obtains a supplement of this from the blood plasma (McKee and Gieman, 1948; Fulton and Grant, 1956).

The parasite uses other substances in the host cells for growth and multiplication and at maturity, the RBC ruptures to release mature merozoites. During lysis of the cell, the host develops a fever.

For some reason some of the merozoites invading the RBCs do not follow the asexual pattern of reproduction. They differentiate into male and female gametocytes and no further development of these occurs until the blood is ingested by a female mosquito. In the gut of the mosquito, the female gametocyte is fertilized and the resulting zygote develops in the gut wall and finally harbours in the salivary gland of the mosquito, ready to be transferred into man for further development. A schematic representation of plasmodial life-cycle is shown in Fig.2 below.

FIGURE 2
SCHEMATIC REPRESENTATION OF PLASMODIAL LIFE-CYCLE



Because of its high rate of multiplication, the parasite needs more energy, and experiments have shown that the parasitized RBCs use glucose 25-100 times faster than the normal red cell (Moulder, 1962). Malaria parasite obtains energy by breaking down glucose which it obtains from

the host cell. Glucose is metabolized through the glycolytic pathway (Speck and Evans, 1945) and the TCA cycle (Marshall, 1948). TCA cycle intermediates are obtained from the plasma since the host red cells virtually lack these.

The parasite has some metabolic lesions for the production of certain important substances and as a result of this, has to rely on the host RBC for those substances (Moulder, 1962). One of such lesions occurs in the synthesis of co-enzyme A (CoA), which is needed to acylate acetate in order to enter into the TCA cycle. It is also deficient in the enzyme, dihydrofolate reductase, so although it can synthesize folic acid from para-aminobenzoic acid (PABA), the folic acid cannot be utilized directly unless it has been reduced to tetrahydrofolate (FH₄). This conversion is largely done by the host red cell reductase. FH₄ is needed for 1-carbon transfer during synthetic processes.

The parasite can synthesize its pyrimidine bases needed for nucleic acid synthesis, but cannot synthesize its purine bases, and once again has to rely on the host for these. The parasite overcomes this by readily taking up purine nucleotides - ATP, NADH, NADPH from the host cell. The parasite membrane has become permeable to highly charged molecules such as COA, ATP, NADH and TCA intermediates such as succinate. It is believed that there are special transport systems for these compounds and that these systems may require energy.

Advantage could be taken of some of these metabolic lesions in the destruction of the malaria parasite. Since the parasite grows and

multiplies rapidly there should be a plentiful supply of energy and nucleic acids, RNA and DNA. Any measures taken to cut off or reduce the supply of these substances will affect the development and multiplication of the parasite.

In the extracellular cultivation of malaria parasite, additional ATP has to be provided to the culture medium to achieve the extracellular survival of Plasmodium (Trager, 1967; Peters, 1970; Weidner, 1973). Any measures to cut off or reduce the supply of ATP in the host cell will affect the development of the parasite.

ANTIMALARIAL DRUGS

In spite of wide spread control and eradication campaigns, malaria constitutes one of the leading causes of mortality and morbidity in the tropics. Malaria can be eradicated by destroying the mosquito vectors, but such a control is expensive and difficult to achieve. Therefore the prevention and treatment of malaria is largely by the administration of antimalarial drugs.

The chief antimalarial drugs employed in malaria therapy include:-

- (i) quinacrine
- (ii) chloroquine and related compounds
- (iii) chloroguanidine
- (iv) pyrimethamine
- (v) primaquine
- (iv) quinine

Sulphonamides are also used together with some of these drugs (Rollo, 1970).

Dapsone, an antileprotic drug, has also been used for treating malaria; the acetyl derivative of dapsone is normally used (Segal et al, 1973).

Antimalarial drugs can be divided roughly into three groups:-

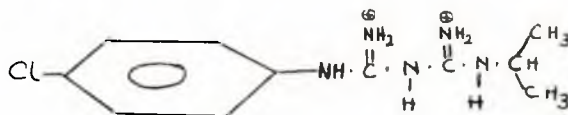
- Group I - Sulphonamides, Biguanidines, Diamino-pyridines
- Group II - Mepacrine and 4-amino-quinolines
- Group III - 8-amino-quinolines

Members of group I include:- Sulphonamide, Biguanidine e.g. chloroguanidine (trade name Paludrine), diaminopyridine e.g. Pyrimethamine.



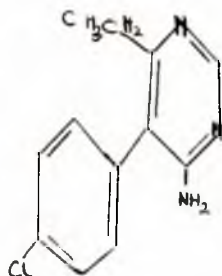
a.

Sulphonamide



b.

Chloroguanidine (paludrine)



c.

Pyrimethamine (Daraprim)

The drugs in this group attack the parasites only when they are in the RBC, i.e. they have schizonticidal effect (Rollo, 1965). Their effect is very slow and resistance to these drugs is easily developed by the parasite. They however have a marked degree of potentiation in their effect when given together (Hurly, 1959). The biochemical actions of some of these drugs have been investigated. Sulphonamides act as competitive inhibitor of para-amino benzoic acid (PABA) because of structural similarity of sulphonamide and PABA. As a result of this, the synthesis of folic acid

from PABA is inhibited (Cohen and Yielding, 1965; Ferone, 1970).

STRUCTURES OF p-AMINOBENZOIC ACID AND SULPHANILAMIDE
SHOWING STRUCTURAL SIMILARITY



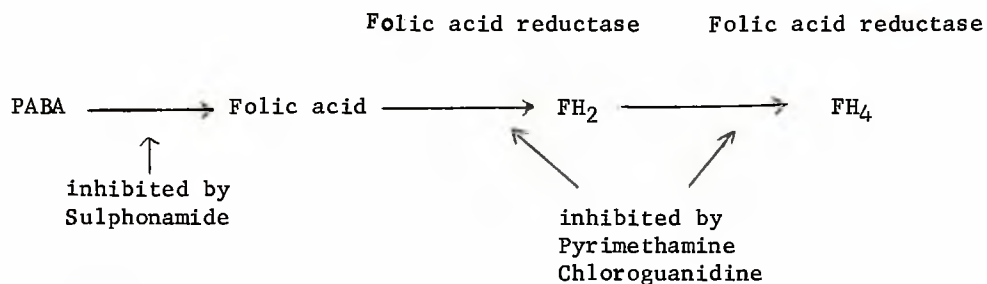
p-amino benzoic acid
(PABA)



sulphanilamide

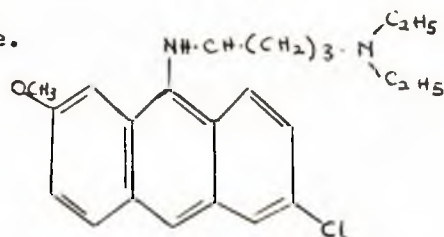
Pyrimethamine and chloroguanidine act as inhibitors of folic acid reductase (Doctor, 1956; Wood and Hitchings, 1959). This enzyme is involved in the conversion of folic acid to tetrahydrofolate (FH₄) or Folinic acid. This means that if sulphonamides and pyrimethamine are given together, the production of FH₄ from PABA is completely inhibited. This explains why members of this group have potentiation effect on each other.

The steps in the synthesis of FH₄ that are inhibited by antimalarial drugs in Group I are shown in the following reactions:

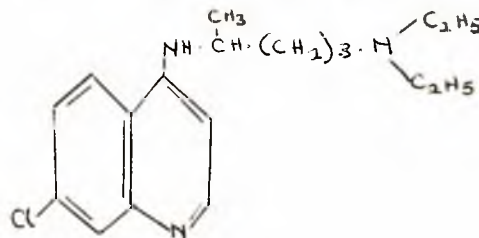


Members of Group II include:- Quinacrine and 4-aminoquinolines

e.g. chloroquine.



Quinacrine (e.g. Mepacrine/atebrin)

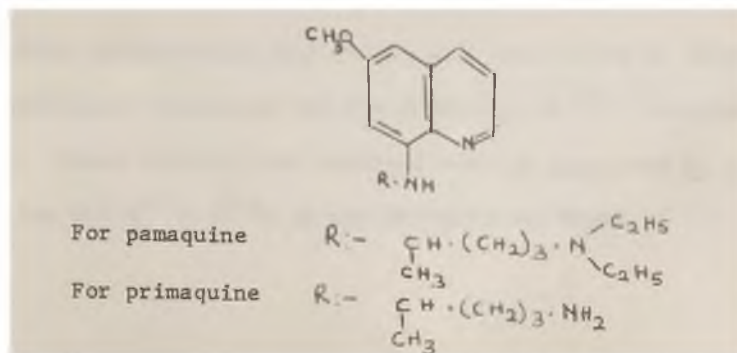


Chloroquine

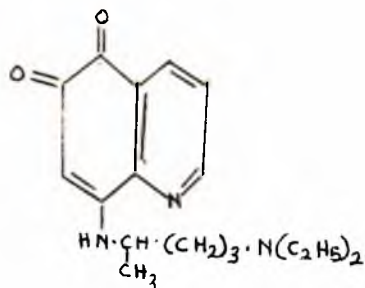
There are two types of chloroquine, chloroquine phosphate and chloroquine sulphate. Some of the trade names for chloroquine phosphate include:- Aralen, Avolcar, Resochin and Malarex; and for chloroquine sulphate, Nivaquine.

Members of Group II also possess schizonticidal effect (Olantunde, 1971), but unlike the members of Group I, their effect is fast and independent of multiplication of the parasite. They can attack the parasite at all stages of the erythrocytic cycle and resistance of the parasite to these drugs is developed with difficulty (Rollo, 1970), although it has been shown to occur. Chloroquine especially is very widely used and in West Africa, it has been proved the most effective drug against falciparum malaria (Young et al, 1963). The drugs in this group do not have any potentiation effect when administered together, and they also have the disadvantage of not being able to attack the parasite in the tissue stages. Unlike members of the first group, their mechanism of action appears to be largely completely non-specific.

Members of Group III include the 8-amino quinoline derivatives, namely, primaquine and pamaquine.



These drugs are active against the tissue stages of plasmodium. Their antimalarial effect is thought not to be given by the quinoline drugs themselves, but by their metabolic products such as 5,6-quinoline quinone.



5, 6, quinoline quinone

Like the drugs of Group II, the drugs in Group III do not have any specific action, but they have been shown together with drugs in Group II, to bind DNA and RNA molecules, by intercalating with the molecule (Kurnick and Radcliffe, 1962; Cohen and Yielding, 1965; Hunsicker, 1969).

Schellenberg and Coatney (1961), using chick blood infected with P. gallinaceum found that quinine, chloroquine and quinacrine inhibited the incorporation of ³²P - labeled phosphate into both DNA and RNA, whereas pyrimethamine and triazine, a metabolite of chloroguanidine specifically inhibited the incorporation of ³²P - labelled phosphate into DNA. These effects were observed both in vitro and in vivo, using as low as 10⁻⁴ - 10⁻⁶M of the antimalarial drugs.

It was found that double stranded DNA produced marked changes in the absorption spectrum of chloroquine which is optically dense at 365nm, but minor changes occurred with single stranded DNA. Chloroquine elevated the thermal dissociation temperature (T_m) of DNA, i.e. it stabilized the helix. It was concluded that chloroquine forms a complex with DNA by ionic interactions (Allison et al, 1965; Irvin et al, 1949). Several workers have implicated guanine residues as being specifically involved in the interaction between chloroquine and DNA (Stollar and Levine, 1963; Alisson et al, 1965; O'Brien et al, 1966; Ciack, et al, 1966). Because of their interaction with DNA and RNA molecules, quinacrine, chloroquine and quinine inhibit DNA-and RNA- polymerase activities.

The antimalarial drugs are given as either preventive drugs or for treatment, depending on the dosage given. In the case of chloroquine, for example, an oral dose of 0.5g is given on the same day of each week as preventive dose, but for treatment of acute attack, of either P.vivax or P. falciparum, an initial dose of 1.0g is given, followed by 0.5g of dose after 6-8 hours on each of two consecutive days to attain a total of 3.0g in three days (Brit. Pharm. Codex, 1968).

Chloroquine as well as the other antimalarial drugs are usually given orally since evidence shows very rapid and high level of absorption from the alimentary canal (Olatunde, 1971). Chloroquine is absorbed almost completely from the gut when taken by mouth, with less than 10% excretion (Berliner et al, 1948; Olatunde, 1971).

Parasitized RBCs have been shown to absorb antimalarial drugs more

than unparasitized cells and for chloroquine, parasitized cells can concentrate it 300-600 times more than the levels found in non-parasitized cells and also plasma level which is 10^{-6} M (Berliner et al, 1948; Polet and Barr, 1968; Macomber et al, 1966).

CHAPTER II: EXPERIMENTAL PROCEDURES

Materials

Outdated human blood from the Military Hospital Blood Bank, Accra served as the source of red blood cell adenylate kinase.

Chemicals included:-

Hexokinase (from yeast type III), Glucose-6-phosphate dehydrogenase, chloroquine di-phosphate, quinine hydrochloride and quinine sulphate from Sigma chemicals.

Sephadexes, G-100 and G-200, CM-C25, CM-C-50, from Pharmacia Fine chemicals; DEAE-cellulose from Whatman Biochemicals Limited, Maidstone, Kent.

Primaquine phosphate and chloroguanidine from Imperial Chemical Industries Limited, Pharmaceutical Division, Macclesfield, Great Britain.

Mepacrine hydrochloride from the Pharmaceutical Division, Ghana Industrial Holding Corporation, Accra.

Chloroquine sulphate from May and Baker Limited, Dagenham, England.
Dithiothreitol from Koch Light Chemicals, England.

All other chemicals used were purchased from the British Drug House (BDH) Chemicals. All reagents used were of AnalaR grade or the best grade available.

Methods

A. Purification of adenylate kinase:

Three different methods were employed to purify adenylate kinase from whole human blood.

a. The use of CM-sephadex, C-25 and C-50 column chromatography and salt gradient

The expired blood which had been collected by intravenous puncture into Acid-Citrate-dextrose (ACD) was used. The blood was centrifuged at 1,000g for 10 minutes at 4°C to pellet cells. The supernatant liquid with the buffy layer was removed by aspiration using a water-pump. The RBCs were resuspended in 0.9% sodium chloride solution and re-centrifuged at 1,000g for 10 minutes at 4°C to pellet the cells again, with the supernatant solution removed. This process was repeated three times. The buffy layer had to be removed as thoroughly as possible since it consists of white blood cells and platelets which contain proteolytic enzymes and will therefore digest the enzyme protein if present. After rinsing the RBCs, the cells were lysed by the addition of two volumes of distilled water containing 100 µM Dithiothreitol (DTT) to protect the enzyme. Lysis continued for 15-20 minutes in the cold (approx. 4°C), with stirring. The haemolysate resulting from this process was centrifuged at 4°C for one hour at 100,000g using MSE 50 centrifuge to precipitate the stroma. Previously, the stroma was precipitated by the addition of 1.0N hydrochloric acid to the haemolysate till its pH was 6.0 prior to centrifugation as indicated in the method being followed (Brownson and Spencer, 1972a), but this step was omitted after several trials as it was found to cause precipitation of the haemoglobin in the crude enzyme preparation during storage. The stroma

was therefore precipitated by centrifugation only. The supernatant solution from the centrifugation served as the crude enzyme for all subsequent experiments.

The crude enzyme was dialysed for 24 hours in the cold against frequent changes (4 times) of 0.01M Sodium citrate buffer pH 5.0, containing 100 μ MDTT. Exactly 50ml of the dialysed fraction was applied to CM-sephadex c-25 column, (3.5 x 55 cm) previously equilibrated in the same buffer. The column was eluted with a sodium chloride gradient (100-250mM) in the same buffer, and 15.0ml fractions were collected using an automatic fraction collector. The chromatography was carried out at 4°C. The material for chromatography was changed from cm-sephadex c-25, to cm-sephadex c-50.

b. DEAE-Cellulose Chromatography:

The whole blood was treated as in Method I to obtain a crude enzyme preparation. The dialysed fraction was applied to DEAE-cellulose column, previously equilibrated in sodium phosphate buffer pH 7.0. The column was eluted with a sodium chloride gradient (100-250mM) in the same buffer. The first set of tubes, (15ml fractions) were pooled together, concentrated using 20% polyethylene glycol (PEG) 6,000 solution in phosphate buffer pH 7.0 and re-applied to sephadex G-75 column.

c. CM-Sephadex, C-50 was equilibrated in sodium citrate buffer pH 5.0 containing 100 μ MDTT and sodium chloride at the following concentrations:

- i) No sodium chloride
- ii) 0.25M sodium chloride
- iii) 0.50M sodium chloride
- iv) 0.75M sodium chloride
- v) 1.0M sodium chloride

Equal volumes of CM-sephadex suspension equilibrated in the above solutions were added separately to the same volumes of dialysed crude enzyme fraction and allowed to stand at 4^oC overnight. These were then centrifuged to remove the sephadex. Supernatant solutions (SN) obtained were assayed for adenylate kinase activity. The SN from (i) above, was colourless and showed no adenylate kinase activity. The SN from (ii) to (v) showed the presence of Hb but had adenylate kinase activity, with both the Hb concentration and the AK activity increasing from (ii) to (v). The supernatant solution from (ii) which contained the least amount of Hb but had a substantial amount of enzyme activity was concentrated using 20% PEG solution in sodium citrate buffer pH 5.0 and applied to sephadex G-100 column (3.5 x 55 cm) and eluted with citrate buffer pH 5.0 containing 100 μ MDTT. Ten millilitre fractions were collected.

Fractions showing AK activity were pooled, concentrated and reapplied to a second sephadex G-100 column (3.5 x 55cm) and eluted with

the same buffer. Fractions from this second chromatography which showed AK activity were again pooled, concentrated and stored at 4°C in plastic tubes as glass surfaces and freezing could cause denaturation of the enzyme (Callaghan, 1956). This enzyme preparation served as the partially purified enzyme for subsequent determinations.

B. Determination of the Activities of Enzymes used in Adenylate kinase

Assay system:

The enzyme activities of hexokinase and glucose-6-phosphate dehydrogenase were determined using the following reaction mixtures:-

TABLE 2

HEXOKINASE

TABLE 3

GLUCOSE-6-PHOSPHATE DENYDROGENASE

REAGENTS	CONCENTRATION IN 1.0 ml REACTION MIXTURE	REAGENTS	CONCENTRATION IN 1.0 ml REACTION MIXTURE
Glucose	40.0mM	Glucose-6- phosphate	40.0mM
ATP	1.0mM	G6PD	0.25 Units
MgCl ₂ 6H ₂ O	2.0mM	NADP ⁺	180 μM
NADP ⁺	180 μM	Tris-HCl buffer pH8.0	100mM
G6 PD	0.25 units		
Hexokinase	0.5 units		
Tris-HCl buffer pH 8.0	100mM		

The activities were determined by measuring the increase in absorbance at 340nm over 6 minutes due to the formation of NADPH.

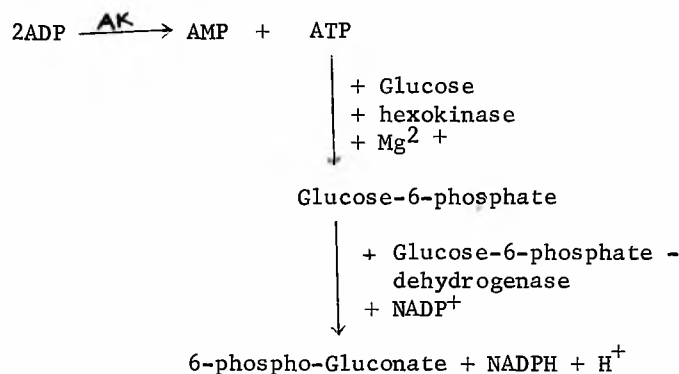
C. Enzyme Assay for Adenylate Kinase Activity:

The following reaction mixture was used for the assay of the enzyme activity. Final concentrations of the reaction components were as indicated in Table 4 below:

TABLE 4
REACTION MIXTURE FOR THE DETERMINATION OF ADENYLATE
KINASE ACTIVITY

REAGENTS	FINAL CONCENTRATION IN A TOTAL VOLUME OF 1.0 ml
ADP (trilithium salt)	1.0mM
Glucose	40.0mM
NADP ⁺ (sodium salt)	180 μM
Magnesium Chloride (MgCl ₂ · 6H ₂ O) ...	2.0mM
Hexokinase	0.5 units
Glucose-6-phosphate dehydrogenase ..	0.25 units
Tris-HCl buffer pH 8.0	100mM
Enzyme preparation	19.0 μg

Adenylate kinase activity was measured as the rate of ATP formation determined in the forward reaction: $2\text{ADP} \longrightarrow \text{ATP} + \text{AMP}$, by using the coupled assay system of Oliver (1959) which is :-



All the reagents were prepared by dissolving weighed amounts in 0.1M tris-HCl buffer pH 8.0. Measurements were carried out in 1.0ml cuvette with 0.2cm light path at 340nm using the Unicam SP500 spectrophotometer with the temperature maintained at 30°C. The instrument was set at zero, 30 seconds after the initiation of the reaction and the increase in absorbance due to the formation of NADPH was recorded over a period of 6-8 minutes using a recorder attached to the spectrophotometer. The allowance of 30 seconds after the initiation of the reaction and before readings were recorded allowed enough time to mix the contents of the cuvette and insert it in the cuvette chamber; it also cancelled out the interference due to haemoglobin and some of the antimalarial drugs which also absorbed at 340nm. The initial rate of the reaction was determined by taking the reaction rate of the first 3 minutes of the curve which was linear. Activity was expressed as μ mole ATP produced/mg protein.

D. Protein Determination:-

Protein determinations were carried out using the method of Lowry (1953) and measurements were taken at 750nm on Unicam SP500 spectrophotometer. Bovine Serum albumin was used as the standard. For fractions from the column chromatography, the absorption at 280nm was used as an approximate measure for estimating protein concentration.

E. Effect of Temperature on the stability of adenylate kinase:-

The enzyme preparation was incubated for 10 minutes in a water bath at the following temperatures:- 25^o, 30^o, 37^o, 40^o, 45^o, 50^o, 60^o, 70^o, 80^o, 90^o, and 100^oC cooled after the incubation period before being added to the assay mixture for the assay of adenylate kinase activity at 30^oC and pH 8.0. Only the enzyme preparation containing adenylate kinase was incubated at the different temperatures and not the whole reaction mixture.

F. The Effect of Substrate concentration on Adenylate Kinase Activity:-

Different concentrations of ADP, the substrate for adenylate kinase reaction, were prepared. The following millimolar concentrations were present in the reaction mixture (Table 4) of a total volume of 1.0ml:- 0.1, 0.3, 0.5, 0.7, 1.0, 3.0, 5.0, 7.0 and 10. The rate of the reaction was measured at 340nm due to the formation of NADPH.

G. The Effect of Antimalarial Drugs on Adenylate Kinase Activity:-

This was determined by incubating the antimalarial drugs with the

enzyme preparation for 5 minutes, 30 minutes, 1 hour and 3 hours before adding it to the rest of the reaction mixture. The antimalarial drugs were at a concentration of 10^{-4} M in a total volume of 1.0ml of the reaction mixture. The absorbance at 340nm due to the production of NADPH, was recorded over a period of 6 minutes. The control had no antimalarial drug, but was incubated for the same period of time.

The absorption spectra of the various antimalarial drugs used were determined over the range of 250-500nm, to find out if they absorb at 340nm, the wavelength at which the enzyme activity was measured.

The absorption spectra obtained are shown in Figs. 3 and 4. All the antimalarial drugs except proguanil hydrochloride absorb at 340nm with chloroquine having the highest absorbance. This interference on the assay system was cancelled out by setting the spectrophotometer initially at zero after the initiation of the reaction with the antimalarial drug present as well.

FIG. 3ABSORPTION SPECTRA OF ANTIMALARIAL DRUGS (I)

Absorption was measured for each antimalarial drug over 250-500nm at a concentration of 10^{-4} M in 100mM Tris-HCl buffer, pH 8.0, using Unicam SP500 spectrophotometer. The blank solution was 100mM Tris-HCl buffer pH 8.0

For Fig. 3

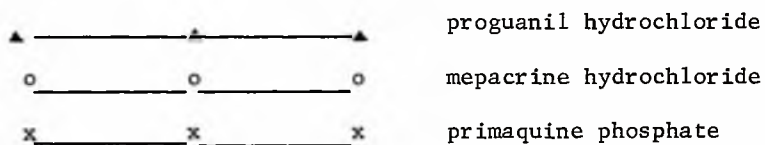


FIGURE 3

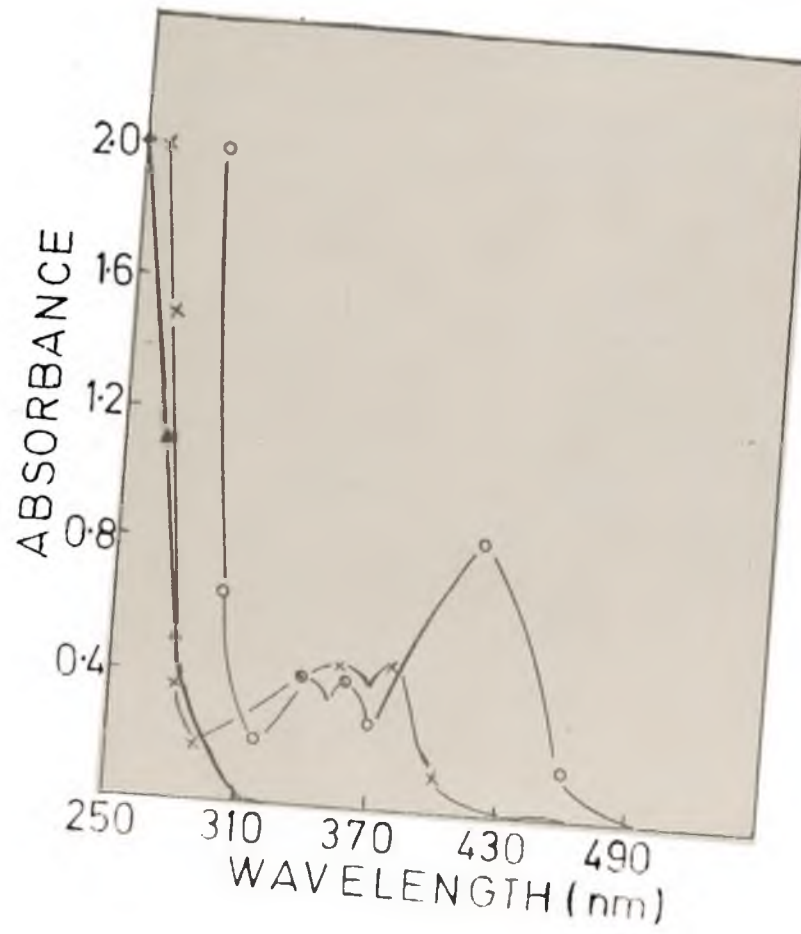


FIG. 4ABSORPTION SPECTRA OF ANTIMILARIAL DRUGS (II)

Absorption was measured for each antimalarial drug over 250-500nm at a concentration of 10^{-4} M in 100mM Tris-HCl buffer, pH 8.0 using Unicam SP500 spectrophotometer. The blank solution was 100mM Tris-HCl buffer pH 8.0.

For Fig. 4

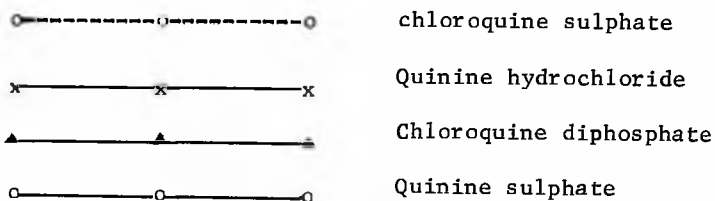
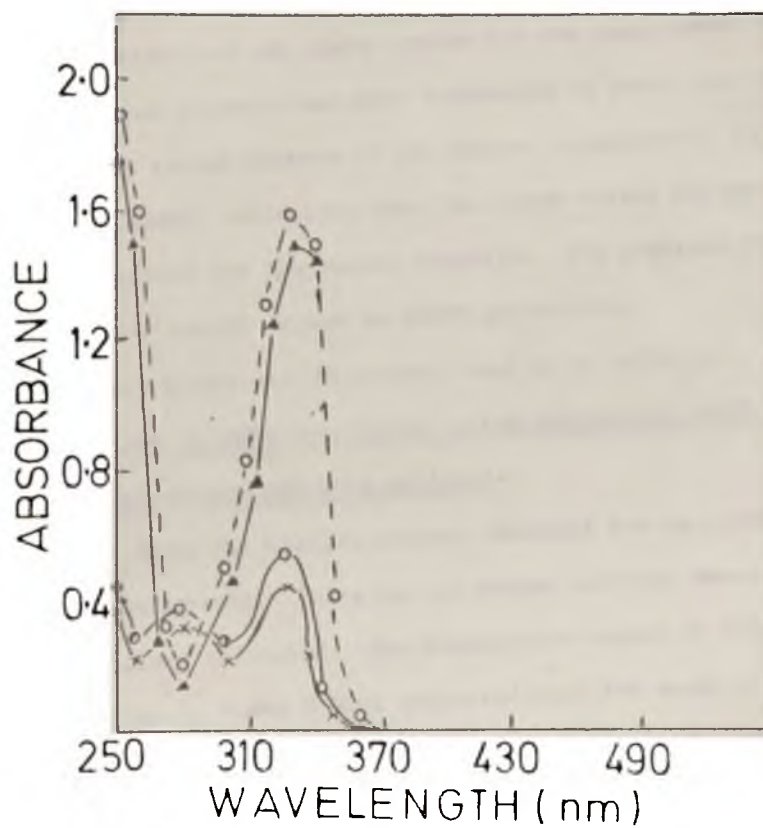


FIGURE 4



CHAPTER II: RESULTS

a. Determination of the Activities of Enzymes used in Adenylate

Kinase Assay System:-

Figs 5 and 6 show the activity curves for hexokinase and glucose-6-phosphate dehydrogenase respectively using the methods described in Page 30. Both enzymes were active at the concentrations used.

The efficiency of the assay system for the measurement of adenylate kinase activity was also determined by using pure myokinase with the assay system instead of the enzyme preparation. Fig. 7 shows the curve obtained, indicating that the assay system did not lack any cofactors required for the enzyme activity. The controls which had no myokinase or no ADP showed no NADPH production.

The assay system was therefore found to be efficient.

b. Purification of Adenylate Kinase using cm-sephadex c-25 and c-50 column chromatography and salt gradient:-

Fig. 8 shows the elution pattern obtained for cm-sephadex c-25 column chromatography. There was no enzyme activity detected in any of the fractions collected. The haemoglobin stayed on top of the column. Figs 5, 6 and 7 have indicated that the assay system was efficient. Therefore the results obtained with the fractions collected indicated that the enzyme had either remained on the column or had been destroyed.

The next step followed was to increase the sodium chloride gradient

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The next step followed was to increase the sodium chloride gradient

for eluting the column from 100 - 250mM to 250 - 500mM and finally to 1.0M. With increased salt gradient, the haemoglobin was washed down around the gradient of about 500mM, but quite a lot of Hb still remained bound to the column material and could not be flushed down with a mixture of 1.0M NaCl and 1.0M NaOH. Fig. 9 shows the elution profile with increased sodium chloride gradient. Fractions collected were tested for AK activity; fractions showing AK activity, unfortunately had a lot of haemoglobin as well, (approx. protein determination at 280nm - reading was off scale). This meant that the enzyme had not been lost, but was still coming down with the haemoglobin. Also, at such a high sodium chloride concentration, the column shrunk to almost a third of its original length; also haemoglobin started clumping down and precipitating out of solution. The shrinkage was partially corrected by previously equilibrating the sephadex with buffer containing sodium chloride (0.2M). Fractions which showed AK activity but had less haemoglobin were pooled together and concentrated using 20% PEG in sodium citrate buffer pH 5.0. Concentrated samples obtained showed AK activity - meaning that PEG did not cause any loss of AK activity. The concentrated sample was applied to sephadex G-200 column previously equilibrated in sodium citrate buffer pH 5.0. Fractions from this column chromatography were collected and tested for AK activity.

Fractions showing AK activity still contained some amount of haemoglobin. Fig. 10 shows the elution profile obtained. The bulk of the enzyme was still in the fraction with haemoglobin. This method was therefore found to be unsatisfactory.

Going back to literature, (Fischer, 1971) it was found that haemoglobin behaved abnormally on sephadex columns. Haemoglobin was eluted very late in spite of its high molecular weight (mw) of about 60,000 (cf MW of adenylate kinase, 20-30,000); the conclusions drawn from the above observations were that (a) Hb was coming down at the same time as the enzyme which has a lower molecular weight and as a result of this Hb was masking the enzyme or (b) a complex might have been formed between Hb and the enzyme.

One of the ways of getting rid of Hb earlier from sephadex columns was to complex it with haptoglobin (an α -globulin of plasma) with molecular weight of 100,000, but this haptoglobin could not be easily obtained. Other methods were therefore sought.

c. DEAE-Cellulose Column (Chromatography):

Elution of column with sodium chloride gradient, 100-500 mM in 0.01M phosphate buffer pH 7.0 was carried out. The first set of fractions collected (15.0ml each) showed AK activity but had a lot of Hb. Application of the pooled concentrated sample from DEAE-

cellulose chromatography to sephadex G-75 column still yielded fractions with AK activity, but with haemoglobin as well. This second method proved unsatisfactory too. Other methods were therefore sought to reduce the amount of Hb present in the haemolysate before its application to the sephadex column.

d. The third method employed involved the addition of the haemolysate to an equal volume of CM-sephadex c-50 suspension equilibrated in 0.01M sodium citrate buffer, pH 5.0, containing different concentrations of sodium chloride. The samples were treated as described in the method on Page 29. Fig. 11 shows the elution pattern. There was an initial protein peak which had no Hb and showed no AK activity. The bulk of Hb came before the fractions with AK activity. Fractions showing AK activity were pooled, concentrated and reappplied to a second sephadex G-100 column. The column was eluted with 0.01M sodium citrate buffer pH 5.0. Fig. 12 shows the elution pattern. A single protein peak which corresponded to AK activity peak was observed. Fractions showing AK activity were pooled, concentrated and stored in plastic tubes at 4°C. This sample served as partially purified enzyme for subsequent determinations.

Table 5 shows the purification table.

TABLE 5
PURIFICATION OF ADENYLATE KINASE FROM HUMAN RED BLOOD CELL

FRACTION	Volume (ml)	Total Protein (mg)	Specific Activity μ mole ATP/min/mg. Protein	Total Yield Activity %	Purification Factor
Crude	120	12,000	2.98×10^{-1}	3.67×10^3 100	1
Dialysed	120	12,000	2.98×10^{-1}	3.67×10^3 100	1
Concentrated sample from cm-sephadex	25	250	4.74	1.19×10^3 33.2	16.0
1st Chromatography on G-100 sephadex (conc.)	25	26.25	4.06×10^1	1.07×10^3 29.8	136.4
2nd Chromatography on G-100 sephadex (conc.)	20	19.0	4.57×10^1	8.68×10^2 24.3	153.7

Calculation:-

$$\text{Molar Extinction coefficient (E)} = \frac{\text{absorbance}}{\text{concentration} \times \text{path length}}$$

$$E(340) \text{ for NADPH} = 6.22 \times 10^3$$

$$\text{NADPH produced/min} = \text{ATP produced/min}$$

$$\text{Specific activity} = \frac{\mu \text{ mole ATP produced/min}}{\text{mg protein}}$$

$$\text{Total activity} = \text{specific activity} \times \text{total protein}$$

$$\% \text{ yield} = \frac{\text{total activity (n)} \times 100}{\text{total activity (initial)}}$$

$$\text{Purification factor} = \frac{\text{specific activity (n)}}{\text{specific activity (initial)}}$$

The increase in absorbance due to the production of NADPH at 340nm was measured over a period of 6 minutes. Two control tubes were set up; (i) the tube had the enzyme and all the other components of the reaction mixture except the substrate (ADP) (ii) tube had all the components of the reaction mixture but lacked the enzyme. Fig 13 shows the type of curves obtained.

e. The Effect of Temperature on the stability of adenylate kinase:

Fig 14 shows the type of curve obtained when the enzyme was incubated for 10 minutes at different temperatures from 25 - 100°C before being used for the assay. Quite a high activity was observed after the enzyme had been incubated at 25 - 40°C with a peak around 37°C. The enzyme activity progressively decreased from 45°C to 60°C and was completely lost after incubating it at temperatures from 70-100°C.

f. The Effect of Substrate Concentration of adenylate kinase activity:

At high substrate concentrations from 3-10mM ADP the rate of the reaction progressively decreased, while at lower substrate concentrations from 0.1-1.0mM ADP the rate of reaction increased as substrate concentration increased. Fig 15 shows the curve obtained for the activity of the enzyme at the different substrate concentrations. The peak of the curve is at substrate concentration of 2-3mM ADP.

The Lineweaver-Burk plot of the reciprocal of substrate

concentration against the reciprocal of initial velocity of the reaction ($1/(s)$ vrs $1/V$) was made. Fig. 16 shows the plot with

$$V_{\max} = 1.11 \times 10^2 \mu \text{ mole/min}$$

$$K_m = 3.45 \times 10^{-1} \text{mM}$$

g The Effect of Antimalarial Drugs on AK activity:

The following antimalarial drugs were used with the enzyme:

- i) Chloroquine diphosphate
- ii) Chloroquine sulphate
- iii) Quinine hydrochloride
- iv) Quinine sulphate
- v) Mepacrine hydrochloride
- vi) Primaquine phosphate
- vii) Proguanil Hydrochloride

These antimalarial drugs were prepared in 0.1M tris-HCl buffer pH 8.0. All antimalarial drugs had a final concentration of $10^{-4}M$ in the assay system. The enzyme was incubated at $37^{\circ}C$ for 5 minutes, 30 minutes, 1 hour and 3 hours with each antimalarial drug before being added to the reaction mixture Table 4 for the assay at $30^{\circ}C$. For the control, there was no antimalarial drug, but the enzyme was incubated at $37^{\circ}C$ for the same period of time as the test samples. This was done to cancel out any effect on the enzyme due to its incubation at $37^{\circ}C$ over the period of time involved.

Table 6 shows the average percentage inhibition of the initial activity of the enzyme over the period of 3 hours.

TABLE 6

TABLE SHOWING PERCENTAGE (%) INHIBITION OF INITIAL
ACTIVITY OF ADENYLATE KINASE BY ANTIMALARIAL DRUGS

ANTIMALARIAL DRUG (10^{-4} M)	* AVERAGE % INHIBITION			
	5 min	30 min	1 hr.	3 hr.
Chloroquine diphosphate	6.8	12.5	16.6	28.3
Chloroquine sulphate	16.5	19.5	23.5	30.0
Quinine hydrochloride	8.82	24.5	30.0	50.0
Quinine sulphate	13.1	18.1	30.0	62.0
Mepacrine hydrochloride	12.7	16.0	18.5	40.7
Proguanil hydrochloride	10.1	15.4	16.7	28.0
Primaquine phosphate	13.8	14.8	16.0	42.0

All the antimalarial drugs used inhibited the initial activity of AK. The extent of inhibition normally increased with the increase in the incubation period of the enzyme with the drug.

* Each average % inhibition represents the average of 10 readings.

Figs 17, 18 and 19 show the percentage inhibition of AK activity over the period of 3 hours for the various antimalarial drugs. Both quinine hydrochloride and quinine sulphate inhibited AK activity to a higher degree, 62.0%; followed by primaquine phosphate, 42.0%; mepacrine, 40.0%; then chloroquine up to 30.0% and finally proguanil hydrochloride 28.0% respectively after the 3 hour incubation period.

The effect of chloroquine diphosphate on the initial rate of reaction at different substrate concentrations was also determined. Fig 20 shows the Lineweaver-Burk plots of $1/(S)$ vs $1/V$ with and without chloroquine diphosphate.

$$V_{\max} \text{ (without drug)} = 1.11 \times 10^2 \mu \text{ mole/min}$$

$$V_{\max} \text{ (in presence of drug)} = 1.0 \times 10^2 \mu \text{ moles/min}$$

$$K_m = K_i = 3.45 \times 10^{-1} \text{ mM}$$

The type of curves obtained show that the inhibition by chloroquine diphosphate was non-competitive.

Fig. 5

ACTIVITY OF HEXOKINASE USED IN ASSAY SYSTEM

The reaction mixture used contained:-

2.0mM MgCl₂

40.0mM Glucose

180 μM NADP⁺

0.25 units G6PD

0.5 units Hexokinase

1.0mM ATP

100 mM Tris HCl buffer pH 8.0

The reaction was initiated by the addition of ATP and the enzyme activity was followed by measuring the increase in absorbance of NADPH produced, at 340nm over a period of 6 minutes.

FIGURE 5

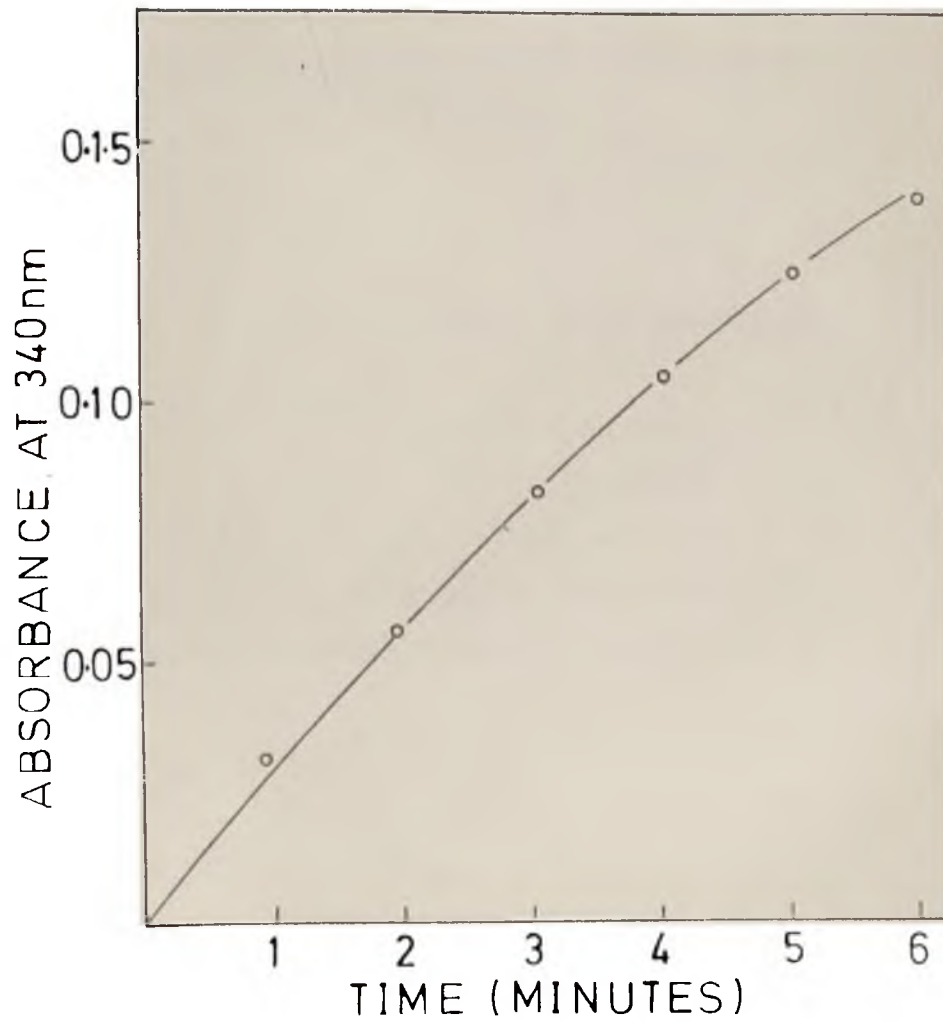


FIG. 6

THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE USED
IN ASSAY SYSTEM

The reaction mixture (1.0ml) contained:-

40.0mM Glucose-6-phosphate

180 μ M NADP⁺

0.25 units G6PD

100mM Tris HCl buffer pH 8.0

Activity measurements were carried out as in Fig. 5.

FIGURE 6

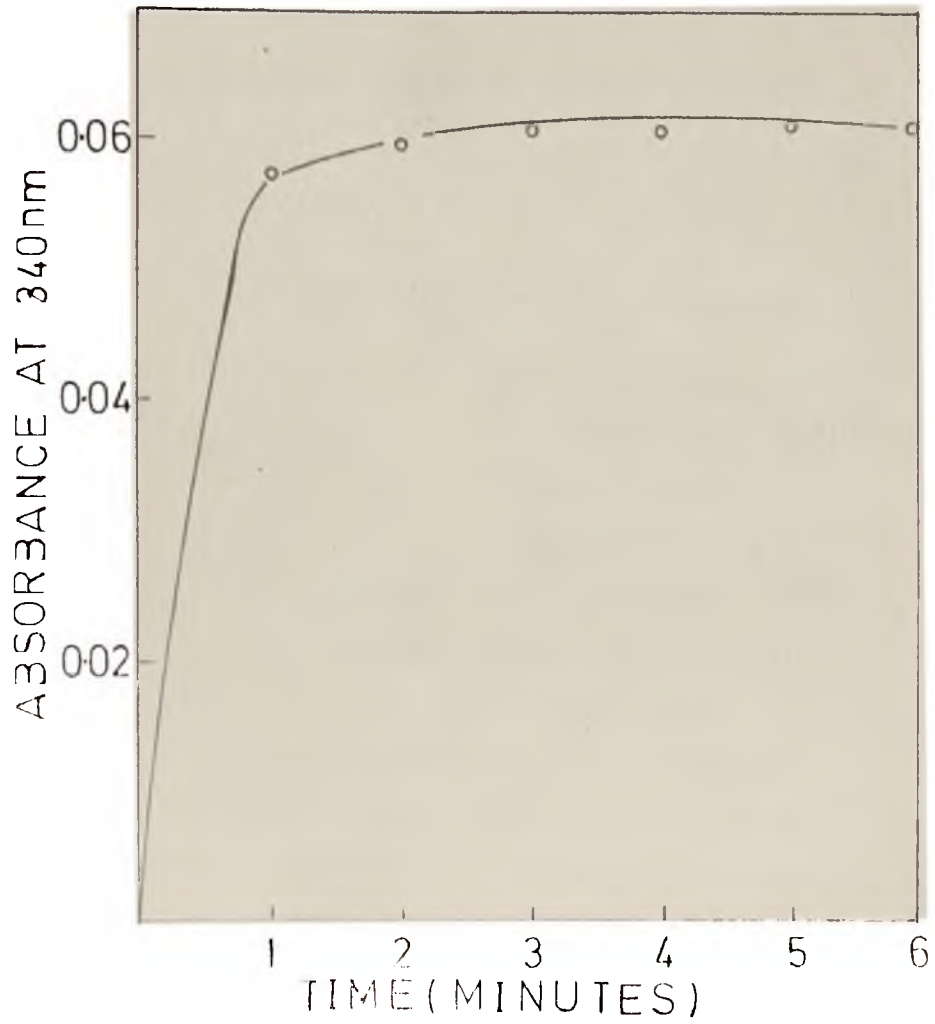


FIG. 7ACTIVITY MEASUREMENTS OF COMMERCIAL MYOKINASE FROM RABBIT
MUSCLE

The assay mixture (1.0ml) contained:-

2.0mM MgCl₂

40.0mM Glucose

0.25 units G6PD

0.5 units Hexokinase

180 μM NADP⁺ (sodium salt)

1.0mM ADP (trilithium salt)

100mM Tris-HCl buffer pH 8.0

Approximately 0.36 units per assay of myokinase was used. All the solutions were prepared in 100mM tris HCl buffer pH 8.0. Absorption of NADPH produced was measured at 340nm using Unicam SP500 spectrophotometer, over 7 minutes. Absorbance corresponds to myokinase activity.

Assay system same as for adenylate kinase activity measurements.

FIGURE 7

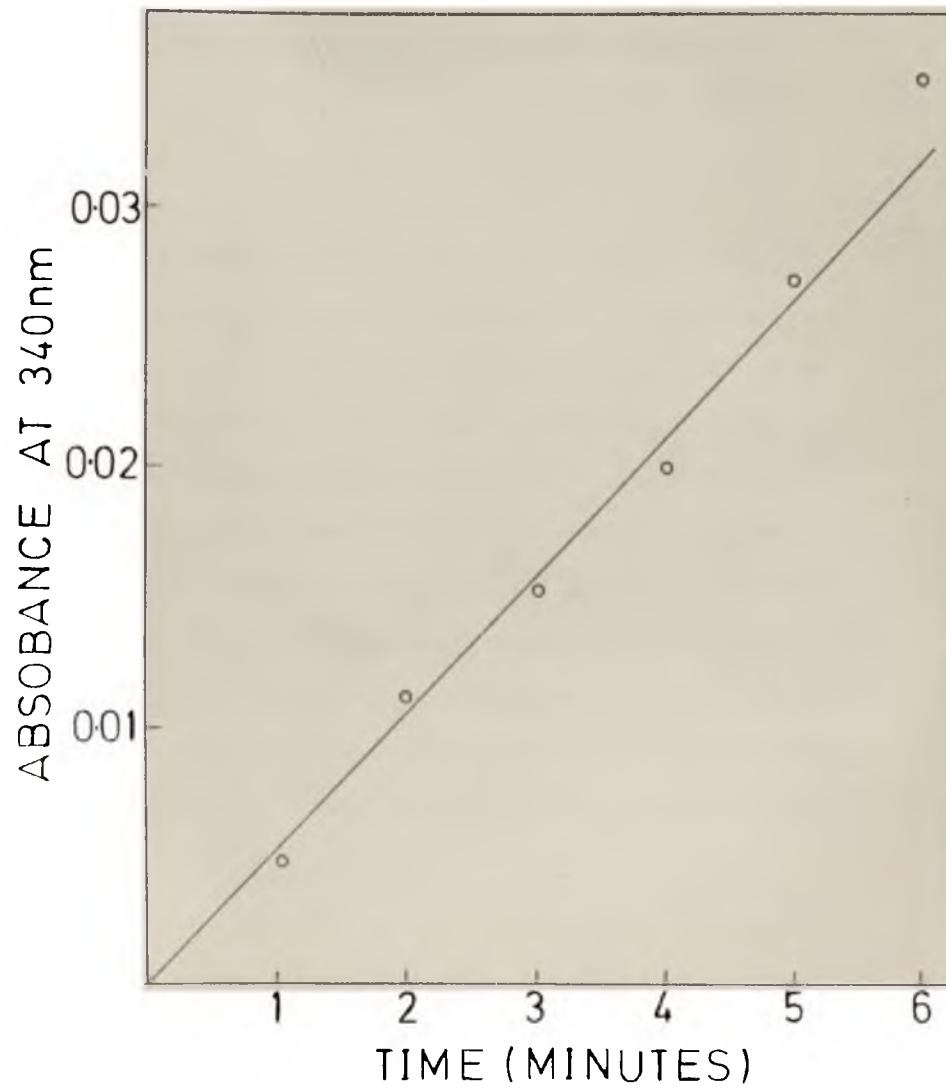


FIG. 8ELUTION PROFILE OF HAEMOLYSATE ON CM-SEPHADEX C-25 COLUMN
(3.5 x 55cm)

Crude haemolysate was dialysed for 24 hours at 4°C against changes (4 times) of 10mM sodium citrate buffer, pH 5.0, containing 100 µM dithiothreitol. Dialysed fraction (50 ml) was applied to CM-sephadex, C-25 column (3.5 x 55cm) previously equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride (100-250mM) in the same buffer. 15ml fractions were collected using LKB automatic fraction collector. Absorbance at 280nm on SP 500 spectrophotometer was used to estimate protein concentrations in the fractions. Adenylate kinase activity was determined in the fractions. None of the fractions showed adenylate kinase activity. The haemoglobin stayed on top of the column.

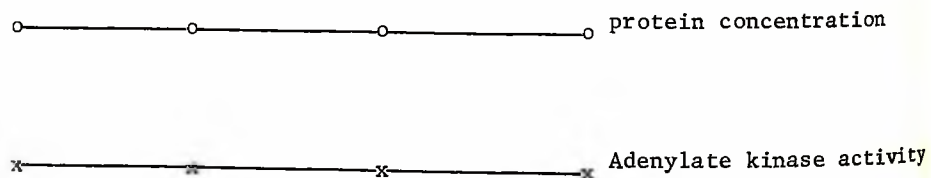


FIGURE 8

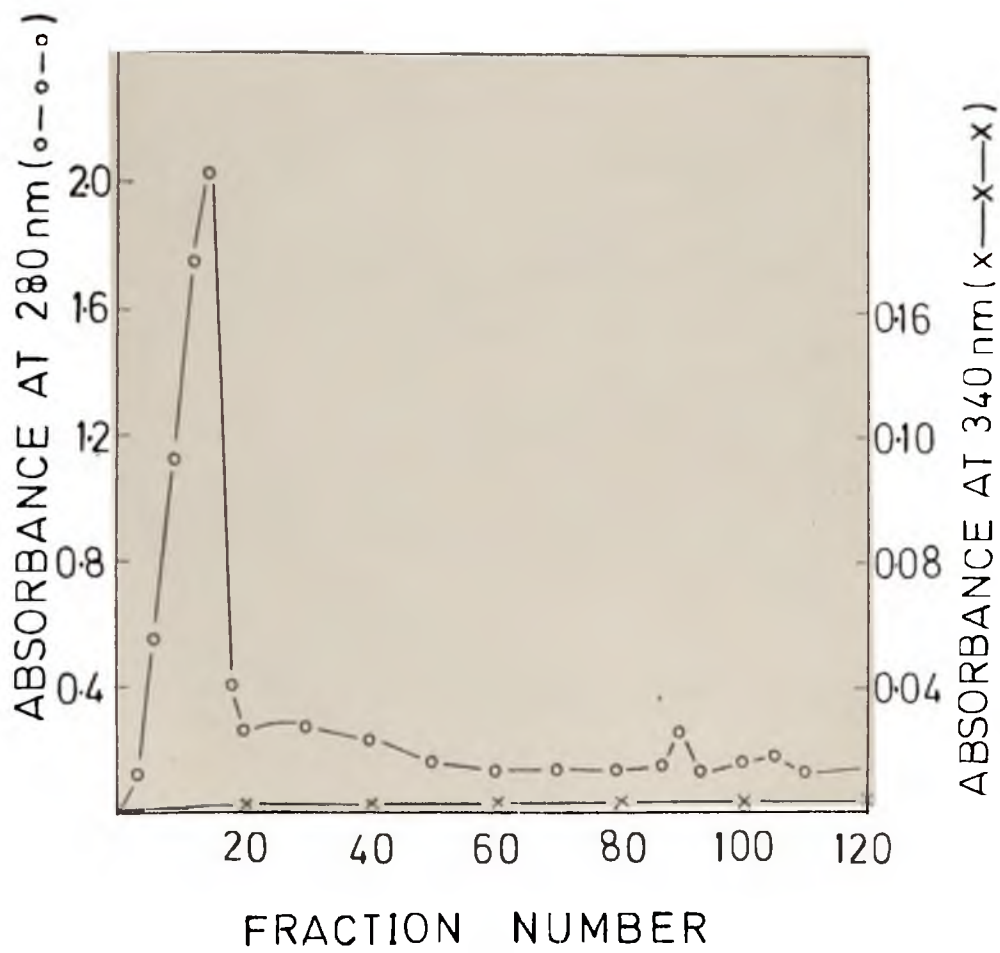


FIG. 9

ELUTION PROFILE OF HAEMOLYSATE ON CM-SEPHADEX, C-50 COLUMN
(3.5 x 55 cm), WITH SODIUM CHLORIDE GRADIENT PROGRESSIVELY
INCREASED FROM 100mM - 1.0M

Chromatography was set up as in Fig. 8, using CM-sephadex C-50, instead of C-25. Sodium chloride gradient was increased linearly from 100mM - 1.0M. Haemoglobin stayed on top of the column and started coming down at sodium chloride concentration of 500mM.

○————○————○
Adenylate kinase activity
as measured at 340nm

×————×————×
Approximate protein concentration,
measured at 280nm.

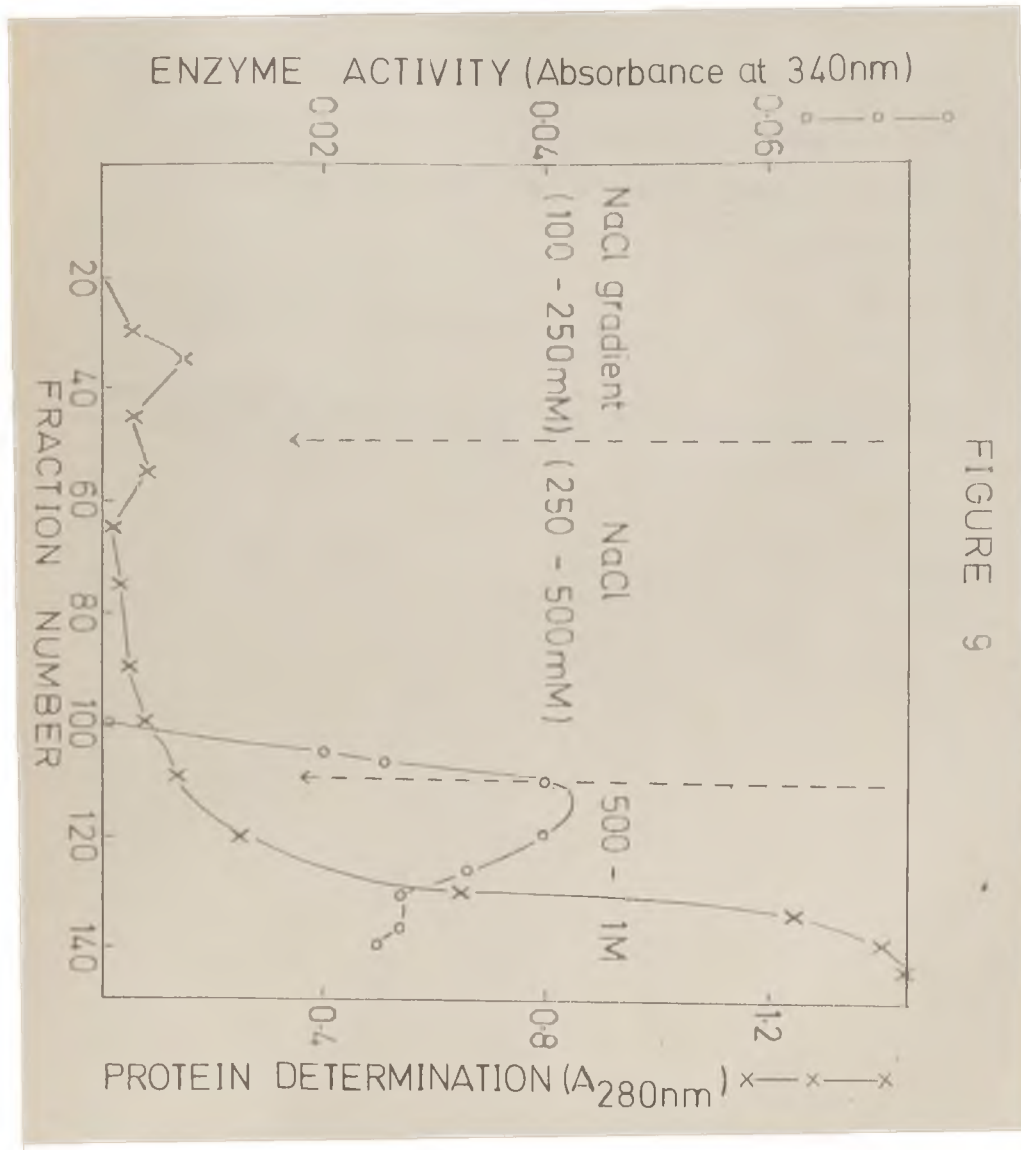


FIGURE 9

FIG. 10ELUTION PROFILE ON SEPHADEX G-200 CHROMATOGRAPHY

Fractions from Fig 9, showing adenylate kinase activity were pooled, concentrated and applied to sephadex G-200, column. Haemoglobin was still present in the fractions showing adenylate kinase activity.

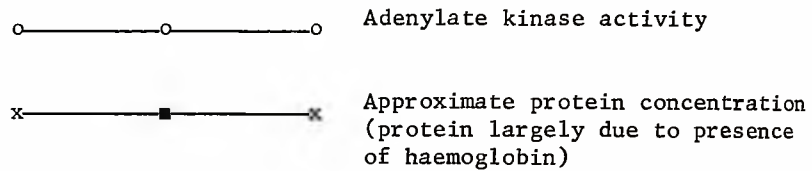


FIGURE 10

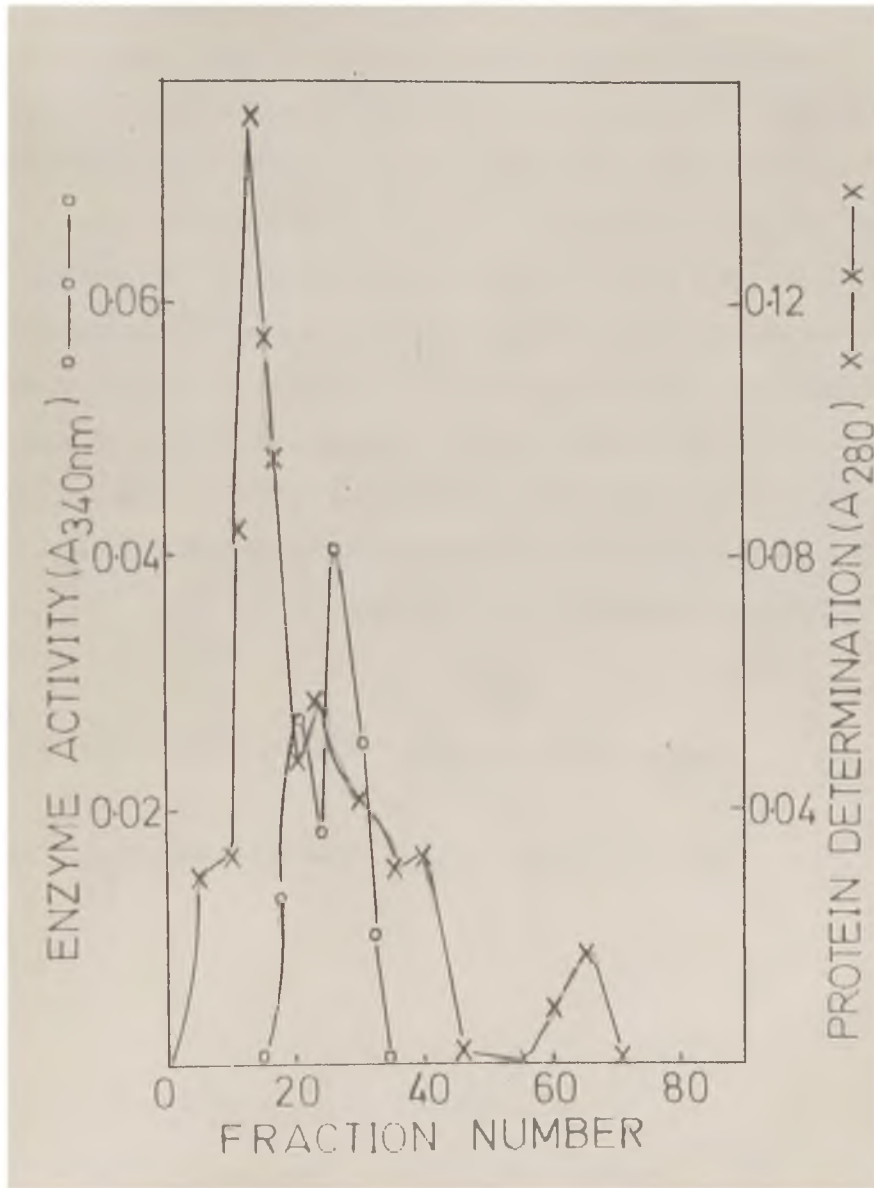


FIG 11ELUTION PROFILE FOR 1ST SEPHADEX G-100 COLUMN CHROMATOGRAPHY

The dialysed haemolysate was added to an equal volume of cm-sephadex, C-50 suspension equilibrated with 0.25M sodium chloride in 10mM sodium citrate buffer pH 5.0, containing 100 μ M dithiothreitol. This was left overnight at 4^oC and centrifuged to remove the sephadex. This process removed some of the haemoglobin present. Supernatant solution obtained was concentrated using 20% PEG 6000 in 10mM sodium citrate buffer pH 5.0 containing 100 μ M DTT. The concentrated sample was applied to sephadex G-100 column (3.5x55cm) and eluted with 10mM sodium citrate buffer pH 5.0 . Fractions were collected using LKB automatic fraction collector and the fractions were tested for adenylate kinase activity.

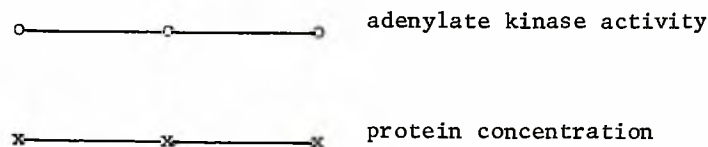


FIGURE 11

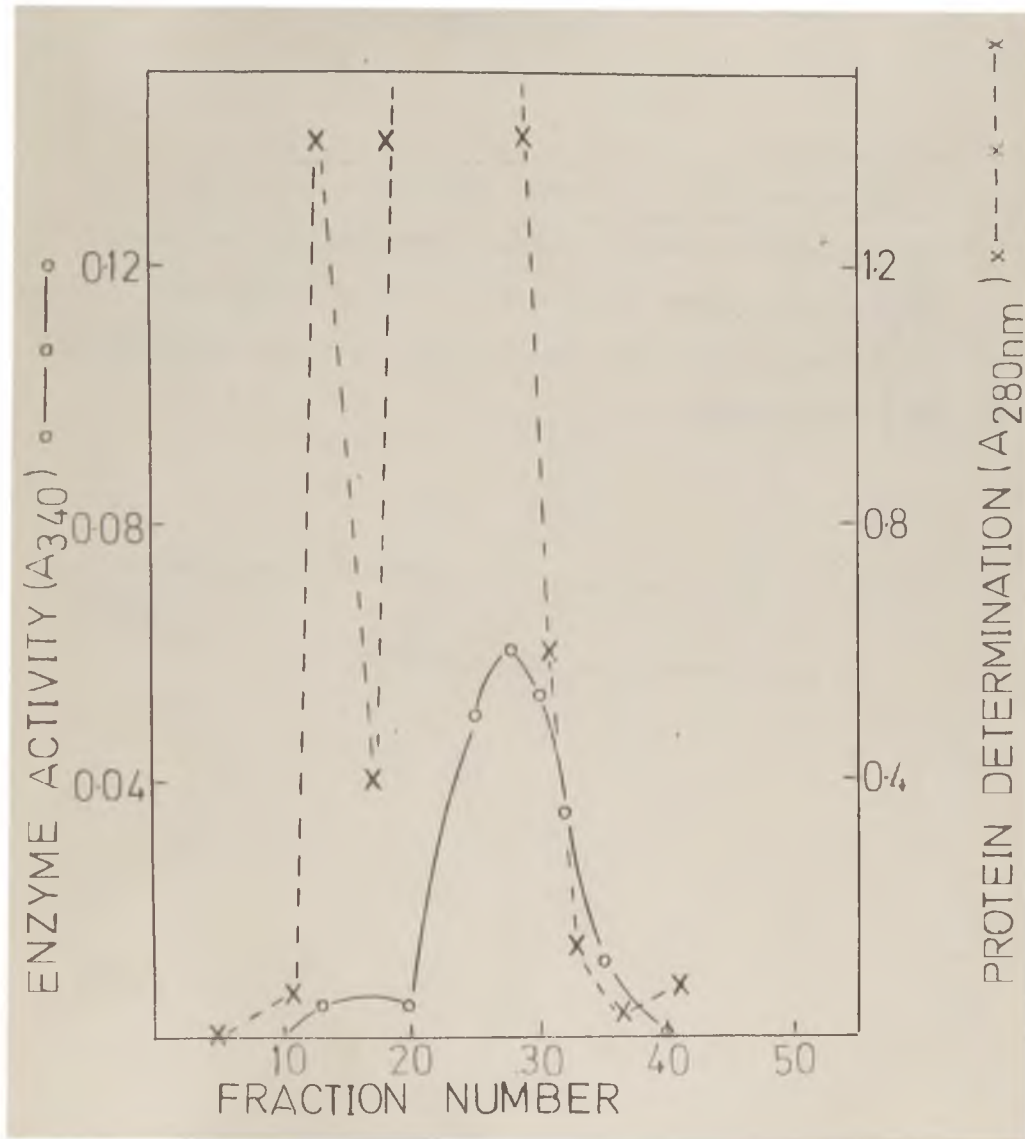


FIG. 12ELUTION PROFILE FOR 2ND SEPHADEX G-100 COLUMN
CHROMATOGRAPHY

Fractions from Fig. 11 which showed AK activity were pooled together, concentrated as in Fig. 11, and reappplied to a second sephadex G-100 column (3.5 x 55cm). 15.0ml fractions were collected.

A single protein peak was obtained. This peak corresponded to the adenylate kinase peak.

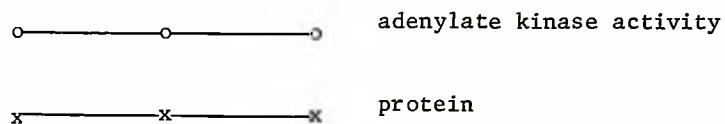


FIGURE 12

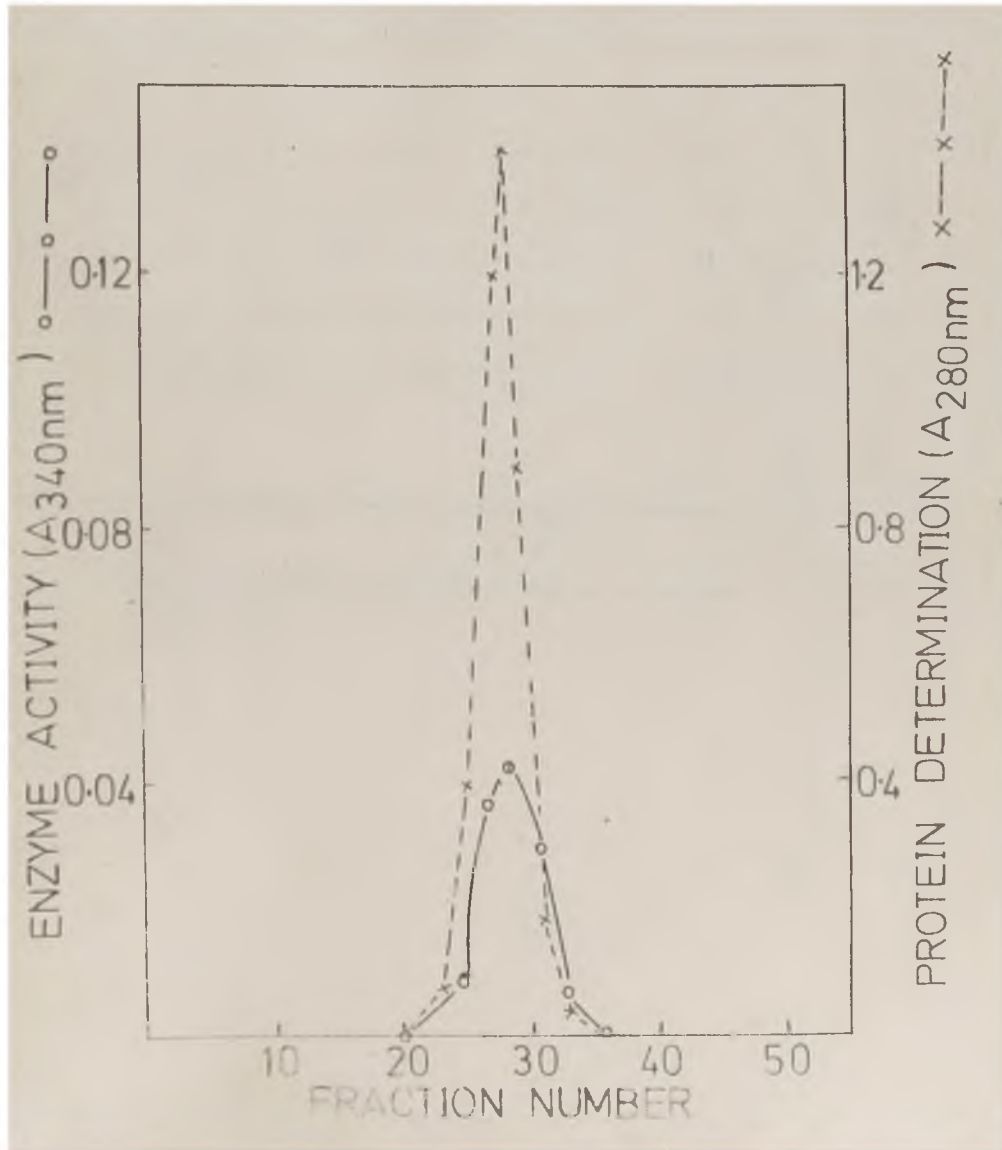


FIG. 13

DETERMINATION OF ADENYLATE KINASE ACTIVITY USING
COUPLED-ASSAY SYSTEM (PAGE 31)

Absorption of NADPH produced was read at 340nm using Unicam SP500 spectrophotometer. Two control tubes were set up.

Control 1:- contained all the components of the reaction mixture except the substrate (ADP).

Control 2:- contained all components of the reaction mixture except the adenylate kinase preparation. The curve obtained showed very little endogenous production of NADPH

○—————○—————○ complete assay mixture
●-----○-----○ control 1 and 2

FIGURE 13

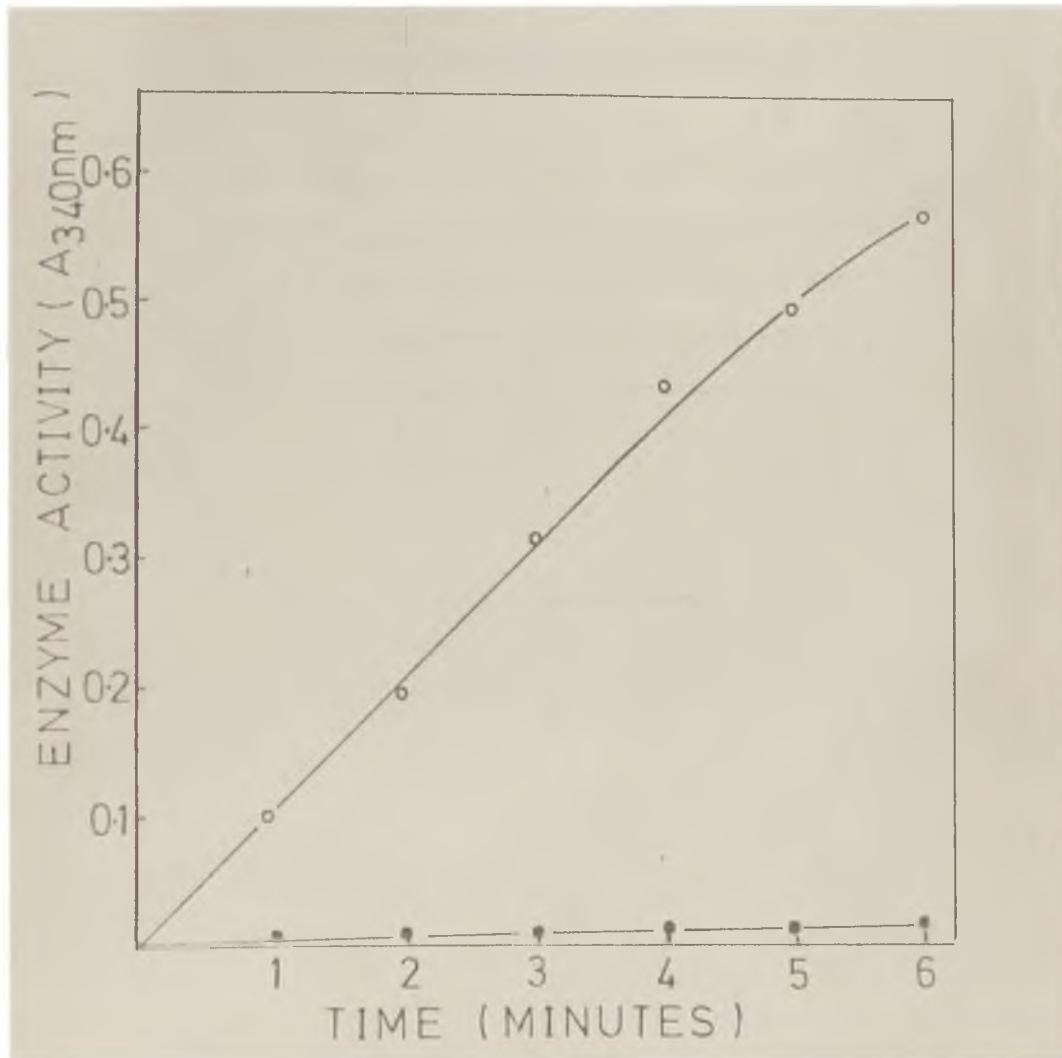


FIG. 14THE EFFECT OF TEMPERATURE ON STABILITY OF ADENYLATE KINASE

Adenylate kinase preparation, was incubated in a water bath at different temperatures for 10 minutes. The enzyme was then cooled and used in assay of the enzyme activity.

Optimum activity for the enzyme observed between 37° - 40°C while activity was completely lost at 70°C.

FIGURE 14

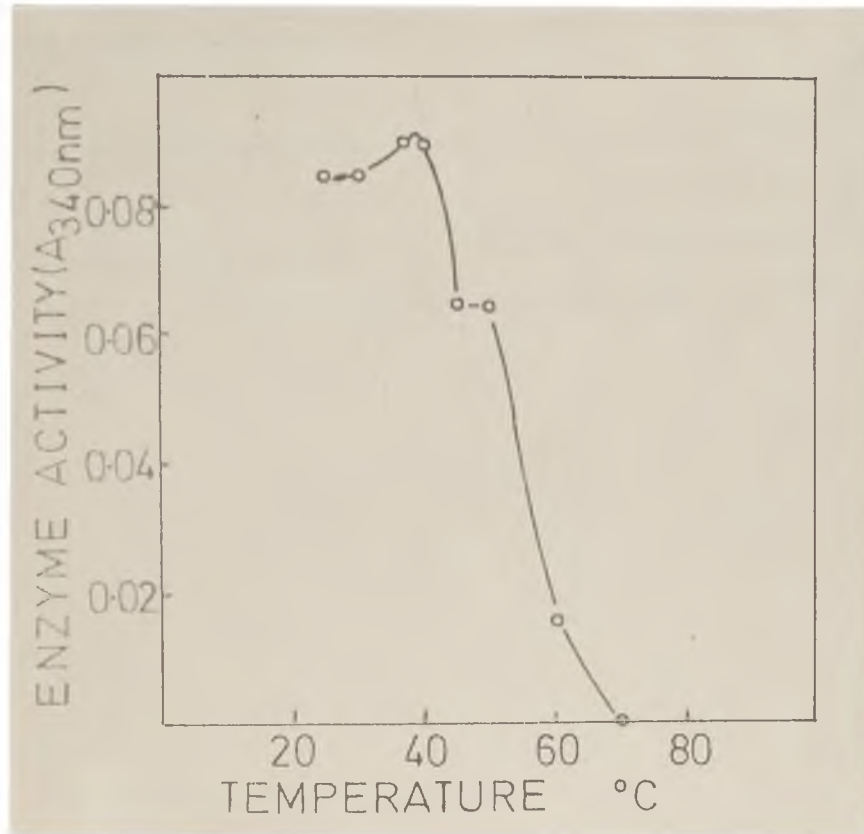


FIG. 15THE EFFECT OF SUBSTRATE CONCENTRATION ON ADENYLATE KINASE
ACTIVITY

Substrate concentration was varied ranging from 0.1 - 10mM ADP in the reaction mixture.

Optimum activity was obtained with substrate concentration of 1.0 - 2.0mM ADP.

Enzyme activity declined from 3 - 10mM ADP; with the activity decreasing as substrate concentration increased.

FIGURE 15

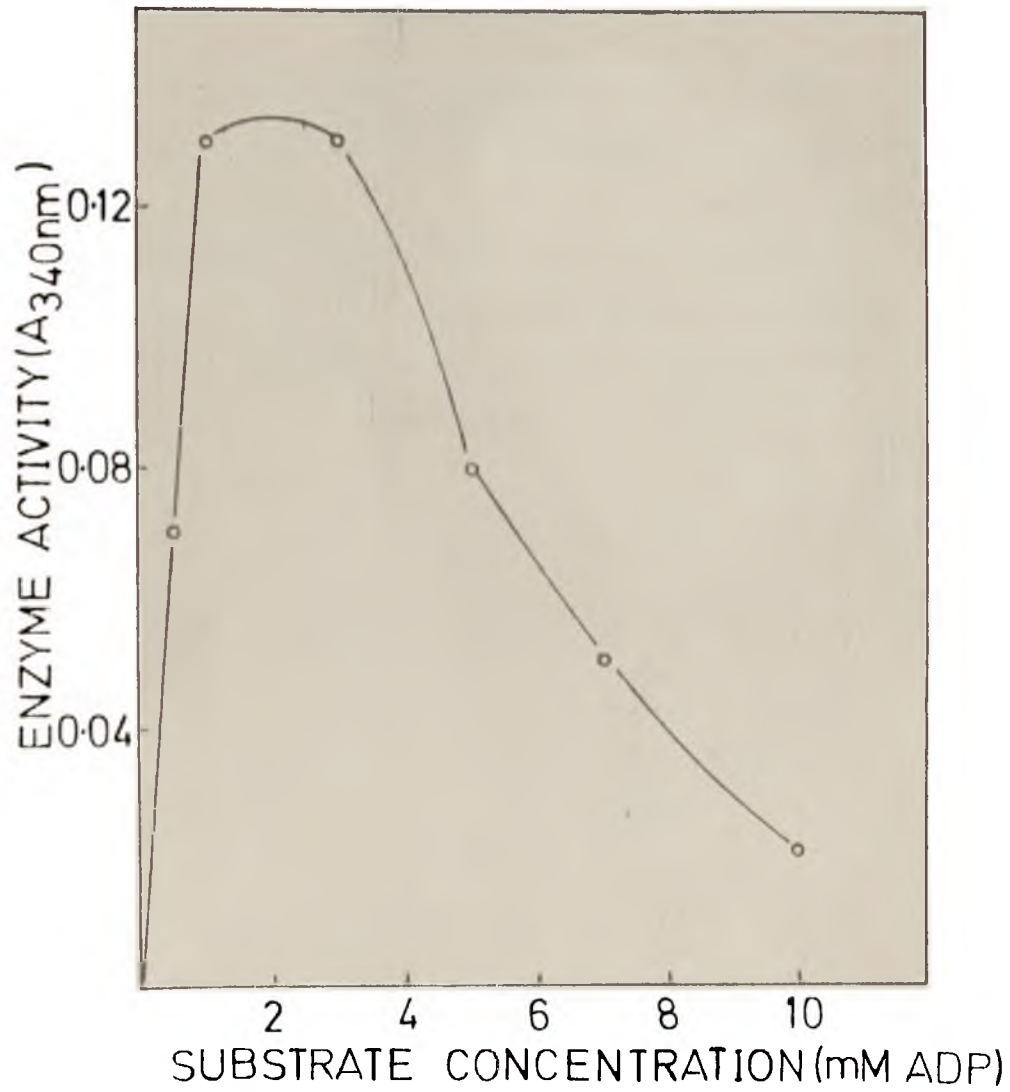


FIG. 16

THE LINEWEAVER-BURK PLOT OF THE RECIPROCAL OF INITIAL
VELOCITY (1/V) VERSUS THE RECIPROCAL OF SUBSTRATE CON-
CENTRATION 1/(S)

Different substrate concentration ranging from 0.1 - 1.0M ADP was used in the assay mixture. The initial velocity was calculated for each assay.

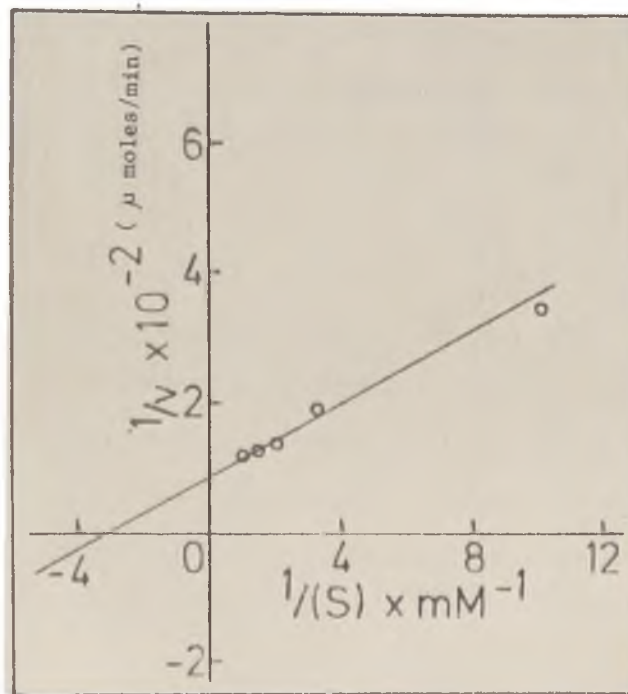
Initial velocity (v) was expressed as:- μ moles ATP/min

Substrate concentration as - mM ADP

From plot $V_{max} = 1.11 \times 10^2 \mu$ moles/min

$K_m = 3.45 \times 10^{-1} \text{mM}$

FIGURE 16



FIGS 17, 18, 19PLOTS OF PERCENTAGE INHIBITION OF ADENYLATE KINASE
ACTIVITY BY ANTIMALARIAL DRUGS

Antimalarial drugs were incubated with adenylate kinase preparation at 37°C over 5 minutes, 30 minutes, 1 hour and 3 hours before addition to the assay mixture.

The reaction rate was measured over 6 minutes by measuring the rate of production of NADPH at 340nm. Control tube had no antimalarial but incubated over the same period of time.

○ ————— ○ ————— ○	Quinine sulphate
x - - - - x - - - - x	Quinine hydrochloride
x ————— x ————— x	Chloroquine sulphate
▲ — - - - ▲ — - - - ▲	Primaquine phosphate
⊙ ————— ⊙ ————— ⊙	Chloroquine diphosphate
⊗ ————— ⊗ ————— ⊗	Mepacrine hydrochloride
▲ ————— ▲ ————— ▲	Proguanil hydrochloride

FIGURE 17

Percentage Inhibition of enzyme by Quinine Sulphate and Quinine Hydrochloride

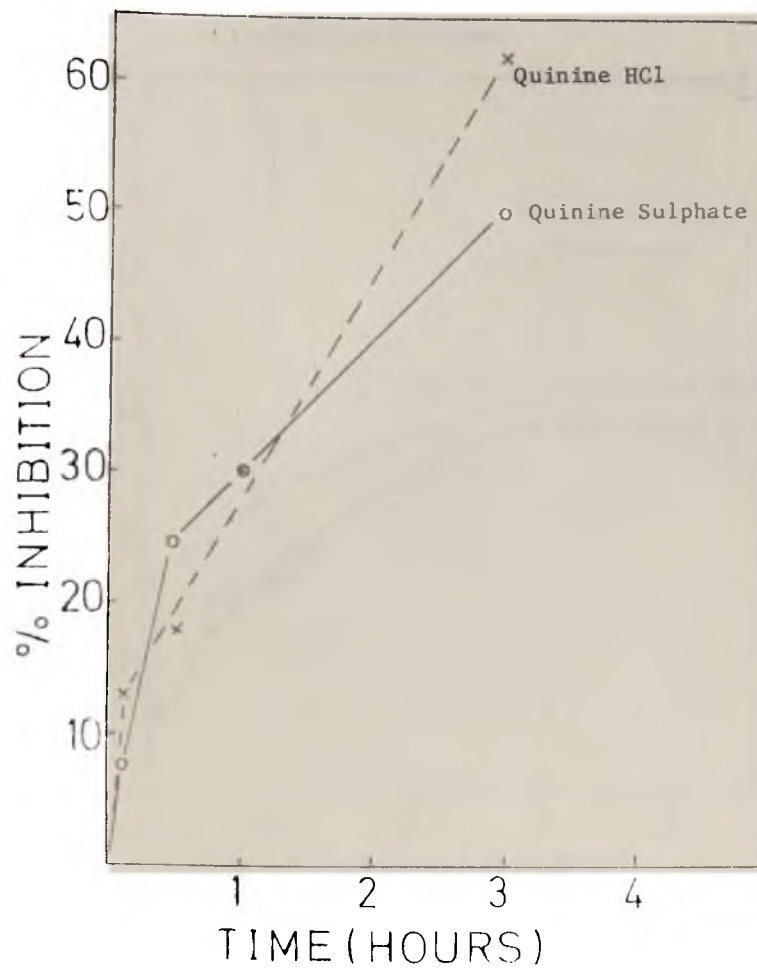


FIGURE 18

Percentage Inhibition of enzyme by Chloroquine and Primaquine Phosphate

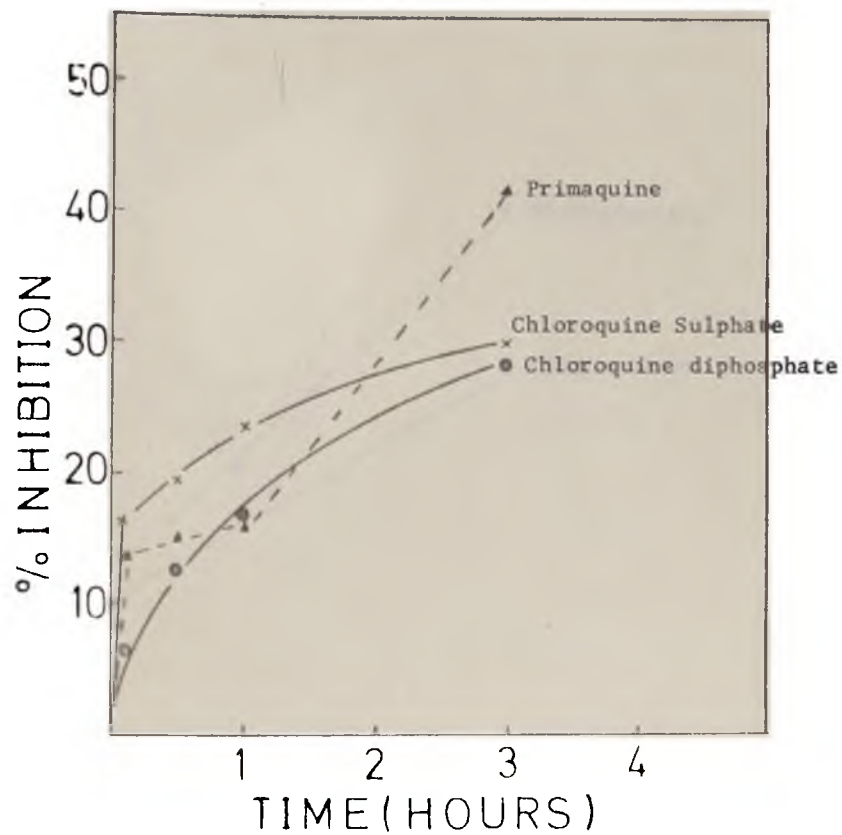


FIGURE 19

Percentage Inhibition of enzyme by Mepacrine Hydrochloride and Proguanil Hydrochloride

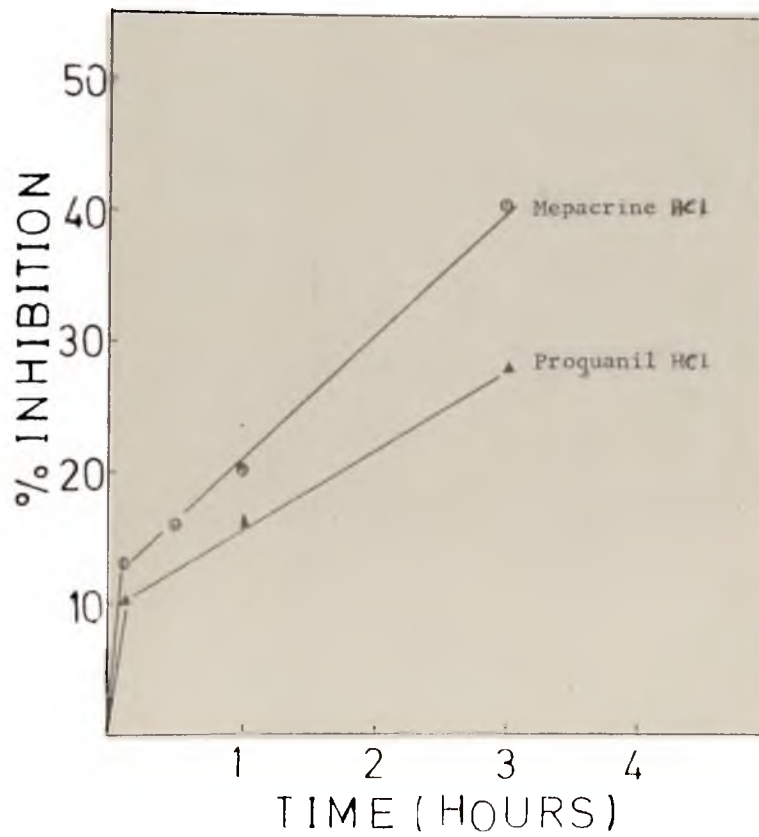


FIG. 20LINEWEAVER-BURK PLOT OF 1/V VERSUS 1/(S) WITH
AND WITHOUT CHLOROQUINE DIPHOSPHATE

Chloroquine concentration used was 10^{-4} M substrate concentration ranging from 0.1 - 1.0mM ADP.

* o-----o-----o without chloroquine

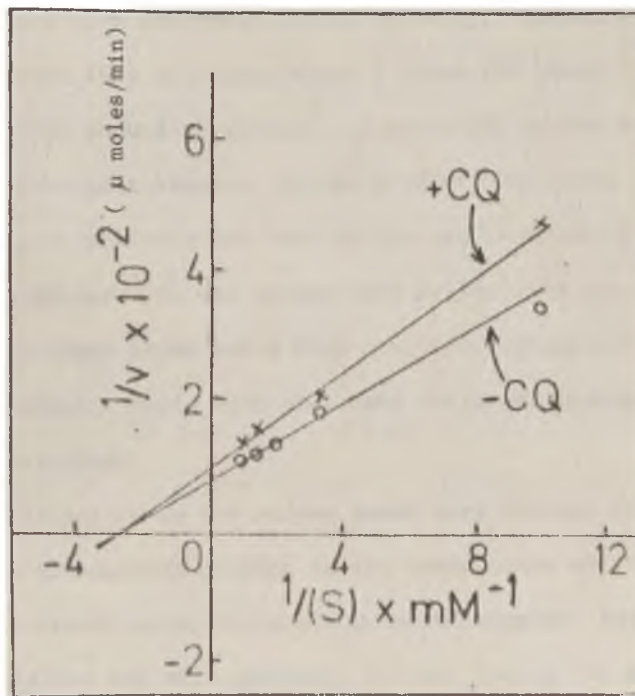
x-----x-----x with chloroquine

V_{max} (in presence of drug) = 1.0×10^2 μ moles/min

* V_{max} = 1.11×10^2 μ mole/min

$K_m = K_i$ = 3.45×10^{-1} mM

FIGURE 20



CHAPTER IV: DISCUSSION

Purification of Adenylate Kinase:-

Outdated human blood contained a high adenylate kinase activity and blood which had expired for over 2-3 weeks still showed very high adenylate kinase activity. These findings confirmed to those observed by Brownson and Spencer (1972a) and Tsuboi et al (1975), who observed that outdated human blood had high adenylate kinase activity. Brownson and Spencer (1972a) observed that old human blood (stored for about 2 weeks in ACD solution at 5°C) showed an increase of about 20% of the total enzyme activity of adenylate kinase. Rapley et al (1970) using rats, found that adenylate kinase activity was less in the red blood cells of newborn infants than adults. It was indeed very helpful and convenient to observe that outdated human blood had a high adenylate kinase activity, as fresh human blood samples would have been very difficult to come by in the quantities required.

The purification of the enzyme posed very serious problems. There was a lot of haemoglobin present in the haemolysate which was extremely difficult to remove using ion-exchange chromatography involving CM-sephadex or DEAE-cellulose and salt gradient, without losing the enzyme activity. The purification process was further complicated by the abnormal behaviour of haemoglobin on sephadexes (Fischer, 1971).

According to the method described by Brownson and Spencer, (1972a), the enzyme could be partially purified using CM-sephadex ion-exchange chromatography and salt gradient from 0.1 - 0.25M sodium chloride. In this

study such a system did not work at all. Fig 8 shows the results obtained with none of the fractions collected giving any adenylate kinase activity. The haemoglobin stayed on top of the column and only started coming down when the salt gradient was increased from 0.25 - 1.0M sodium chloride (Fig 9). With the increase in salt gradient, adenylate kinase activity was detected in some of the fractions, but haemoglobin was present as well. This observation seems to suggest that the enzyme was either bound to the haemoglobin or that it was coming down at the same time along the column as the haemoglobin without necessarily being bound to it.

With the exception of recent work done by Tsuboi et al (1975) which involved several steps in the complete purification of adenylate kinase, a lot of other work carried out on human RBC adenylate kinase, involved the use of the crude enzyme in the haemolysate (Fildes and Harris, 1966; Modiano et al, 1970; Rapley and Harris, 1970; Russel et al (1974). Perhaps this is due to the difficulty in separating the enzyme from haemoglobin. Other methods were therefore sought to remove most of the haemoglobin from the haemolysate before its application to the column. The use of DEAE-cellulose column chromatography also gave unsatisfactory results, with haemoglobin present in the fractions showing adenylate kinase activity. Ammonium sulphate precipitation was employed, using different concentrations of ammonium sulphate solution. The haemoglobin was precipitated and the supernatant solution left showed no adenylate kinase activity.

The final method used to purify the enzyme involved the use of CM-sephadex suspension and sephadex G-100. An equal volume of CM-sephadex suspension equilibrated in different concentrations of sodium chloride solution from 0-1.0M was added to the haemolysate. Like the results

obtained for ammonium sulphate precipitation, the total removal by the CM-sephadex suspension with no sodium chloride, gave a colourless solution which showed no adenylate kinase activity. The other supernatant solutions showed the presence of both haemoglobin and adenylate kinase activity with the enzyme activity and haemoglobin concentration increasing in the supernatant solution as the sodium chloride concentration in the suspension increased. Sephadex G-100 column chromatography was carried out with the supernatant solution with the least haemoglobin concentration. A second sephadex G-100 column chromatography of the sample gave some fractions with adenylate kinase activity but completely devoid of any haemoglobin while few other fractions showed the presence of both haemoglobin and adenylate kinase and others had only haemoglobin. This means the initial removal of some of the haemoglobin from the haemolysate, helped in getting a better separation on the column. The haemoglobin was therefore not necessarily bound to the enzyme. It must have been its abnormal behaviour on sephadexes (Fischer, 1971) which was complicating the purification process.

Table 5 gives the detailed account of purification. The final enzyme preparation had a purification factor of 153.7 with a yield of 24.3%. The yield was quite low as the process used to purify the enzyme resulted in the loss of some of the enzyme as fractions obtained from the column which showed the presence of both enzyme and haemoglobin were discarded before the second column chromatography. The specific activity increased with each purification step, with the final enzyme preparation having a

specific activity of 4.57×10^1 μ moles ATP/min/mg protein compared to that of the initial crude preparation with the specific activity of 2.98×10^{-1} μ moles ATP/min/mg protein.

Enzyme Properties :-

a. The Effect of Temperature on the Stability of the Enzyme:- Optimum enzyme activity was observed around 37°C - 40°C after the enzyme preparation had been incubated for 10 minutes in a water bath at different temperatures. The enzyme activity progressively decreased from 45°C and was completely lost at 70°C . Tsuboi et al (1975), showed that the impure enzyme was less stable while only minimal losses were observed for the pure enzyme even at 100°C when there are protective agents like dithiothreitol and Triton-X. The temperature instability observed in this present study seem to implicate the impurity of the enzyme preparation. Also enzyme instability was encountered in dilute solution even in the crude haemolysate, but relatively stable in the concentrated form. This observation conforms to that of Tsuboi et al (1975). The enzyme was therefore stored at 4°C in a concentrated form and diluted as and when needed. Polyethylene glycol-6000 used for concentrating the enzyme preparation caused no loss in enzyme activity.

In this experiment, only the adenylate kinase preparation was incubated at the different temperatures, cooled immediately before its addition to the reaction mixture and not the whole reaction mixture was incubated at the various temperatures. This was done so that the other enzymes present in the assay mixture would not be exposed to the same treatment, thus making it possible to observe the effect of temperature

on the adenylate kinase stability alone.

b. The Effect of Substrate concentration on the initial activity of adenylate kinase:- At higher substrate concentration (3-10mM ADP), there was a decrease in adenylate kinase (AK) activity. AMP, one of the products of adenylate kinase reaction, has been shown to inhibit adenylate kinase reaction (Noda, 1958; Brownson and Spencer, 1972b; Tsuboi et al, 1975). There was apparently competitive inhibition of the enzyme by AMP because of the structural similarity between ADP (the substrate) and the AMP. In the assay system used to measure AK activity, ATP produced was progressively removed in the presence of glucose and hexokinase to form glucose-6-phosphate, so that more of the AMP was formed. Therefore as the reaction proceeded the concentration of AMP increased, thus effecting more inhibition. Optimum activity was observed between 1-2mM ADP concentration. The K_m for ADP and V_{max} of the partially purified enzyme were $3.45 \times 10^{-1} \text{ mM}$ and $1.11 \times 10^2 \mu \text{ moles/min}$ respectively.

The Effect of Antimalarial Drugs on adenylate kinase activity:- All antimalarial drugs used had a concentration of 10^{-4} M . This concentration of antimalarial drugs was chosen by considering that the plasma level of chloroquine diphosphate was 10^{-6} M and that it can be concentrated 300-600 times in the red blood cell (Berliner et al, 1948; Polet and Barr, 1968). Secondly, at higher concentration of chloroquine diphosphate ($5 \times 10^{-4} \text{ M}$), the spectrophotometer could not be set at zero as the absorbance is high at 340nm at this concentration. Thus the concentration of the antimalarial drugs used was chosen by considering both the physiological levels of chloroquine and also the limitations in the assay method. The adenylate kinase preparation was incubated with the antimalarial drugs at 37°C for 5 minutes, 30 minutes, 1 hour and 3 hours, and cooled immediately before being added to the reaction mixture for the assay at 30°C . The control for

this experiment had no antimalarial drug but had the enzyme preparation which had been treated as above before its addition to the reaction mixture. This was done to dispel any doubt that the inhibition of the enzyme activity was due to the incubation as well and not the antimalarial drug alone. By setting the spectrophotometer at zero initially, before the enzyme activity was recorded over 6 minutes, the absorbance due to the antimalarial drugs at 340nm, (Figs 3 and 4) was removed and did not affect the readings recorded. Secondly, by using this method, it was possible to expose only the adenylate kinase preparation to the antimalarial drugs during the incubation period thus minimizing the effect of the drugs if any, on the other enzymes present in the reaction mixture to just the period over which the activity of AK was measured. Though Cotton and Suttorius (1971) have shown that chloroquine phosphate inhibited G-6PD, no inhibition was observed at concentration of 10^{-4} M chloroquine phosphate over the period of 6 minutes using the assay mixture outlined in table 4 (Page 31). Little or no effect was observed on the enzyme activities using the other antimalarial drugs over the period of 6 minutes. At a concentration of 10^{-4} M all the antimalarial drugs used inhibited AK activity. The degree of inhibition depended on the incubation period of the enzyme with a particular drug.

Results obtained in Table 6 shows that chloroquine diphosphate inhibited the activity of AK by 28.3% after 3 hour incubation period. There was a percentage inhibition of 6.5% after 5 minutes incubation, compared to 12.5% and 16.6% after 30 minutes and 1 hour respectively.

Percentage inhibition therefore increased with the increase of the incubation period.

With quinine, percentage inhibition of up to 50.0% and 62.0% were observed for quinine hydrochloride and quinine sulphate respectively after 3 hours incubation. With mepacrine hydrochloride, proguanil hydrochloride and primaquine phosphate, percentage inhibition of up to 40.7%, 28.0% and 42.0% respectively were observed. Like the previous observations, percentage inhibition increased with the increase in incubation period.

Comparing the effects of the various drugs on AK activity, it could be seen that the enzyme is inhibited most by quinine (up to 62.0%) followed by primaquine (42.0%), mepacrine (40.7%) chloroquine sulphate, (30.0%) chloroquine diphosphate(28.3%)and finally proguanil hydrochloride (28.0%). Looking at the structures of all the antimalarial drugs used, it is seen that there is no structural similarity between the drugs and the substrate for the enzyme activity i.e. ADP, therefore non-competitive inhibition was likely to be the type of inhibition being observed.

The type of inhibition for chloroquine diphosphate, the most common antimalarial drug in the tropics was looked at by using 10^{-4} M chloroquine diphosphate at different ADP concentrations. From Fig 20, the Lineweaver-Burk plots of the reciprocal of the initial velocity ($1/v$) versus the reciprocal of the substrate concentration $1/(s)$ gave curves in which the K_m for ADP in presence of the drug was the same as K_m for ADP alone.

Adenylate kinase has been shown to be involved in regulating the cell's energy metabolism by regulating the adenylate charge i.e. the ratio of ATP, ADP and AMP in the system (Atkinson, 1968). It has also been shown by Brewer and Powell,(1965)and Peters,(1970)that for the extracellular cultivation of malaria parasite ATP is required. This ATP may serve as a source of energy and of purine bases to the parasite. Since the antimalarial drugs have been shown in the present study to affect adenylate kinase which is responsible for regulating adenine nucleotides in the host cell, these drugs might ultimately affect the growth and development of the parasites as well. It is possible that these drugs exert their antimalarial effect by affecting ATP production as well as the other effects such as inhibition of DNA and RNA polymerases activities by chloroquine (Cohen and Yielding, 1965; Hunsicker, 1969); since all the antimalarial drugs used in this experiment have been shown to have non-specific mode of action on the parasite, the type of inhibition observed in this study may be contributing to their mode of action.

CONCLUSION

In conclusion, the following results were obtained:-

1. Outdated human blood contained a high adenylate kinase activity. Adenylate kinase could be purified using sephadex chromatography. The purification process was complicated by the presence of high concentration of haemoglobin, so the haemoglobin concentration was reduced by using a suspension of CM-sephadex C-50 in 0.25M sodium chloride with the crude haemolysate, before its application to sephadex column chromatography.
2. The enzyme stability was affected by temperature. Optimum activity was observed between 37^o-40^oC and was completely lost at 70^oC after 10 minutes incubation at that temperature. Enzyme instability was encountered in dilute solutions and therefore the enzyme had to be concentrated before storage, and diluted when needed.
3. The initial enzyme activity was proportional to substrate concentration. There was an increase in activity using substrate concentration of 0.1mM - 1.0mM. However as substrate concentration increased from 3.0 -10mM the activity decreased. AMP, one of the products of the reaction apparently inhibits the enzyme activity (i.e. product inhibition).
4. The initial enzyme activity was also inhibited up to 62.0% by some common antimalarial drugs after 3 hours incubation. Inhibition was found to increase with the increase of incubation period. The degree of inhibition also depended on the type of drug involved. Quinine was found to inhibit the enzyme activity to the highest degree, followed by primaquine phosphate, mepacrine hydrochloride, chloroquine and finally proguanil hydrochloride in that order.

The type of inhibition observed for chloroquine diphosphate was non-competitive inhibition.

5. The inhibition of adenylate kinase by these drugs might be one of the ways in which they exert their antimalarial effect, by cutting down the supply of adenine nucleotides in the form of ATP to the parasite.

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