A STUDY OF GLYCEROPHOSPHATE OXIDASE IN TRYPANOSOMA BRUCEI

by

Alan H. Fairlamb, BSc. (Med.Sci.), M.B., Ch.B.

Thesis submitted for the Degree of

Doctor of Philosophy

Department of Biochemistry

University of Edinburgh

August 1975.

I hereby declare that this Thesis has been composed myself and has not been submitted in any previous application for a Degree. The work of which this is a record has been done by myself and all sources of information have been specifically acknowledged by means of references.

TABLE OF CONTENTS

PAGE

2. MATERIALS AND METHODS

ACKNOWLEDGEMENTS

I would like to thank Professor Piet Borst and Mr. F. Opperdoes of the University of Amsterdam for their exchange of experimental findings on the glycerophosphate oxidase and for allowing me to visit their laboratory to gain experience in density gradient centrifugation techniques. I am grateful to Dr. Miklos Muller of Rockefeller University, New York, for his critical advice on the presentation of the results of subcellular fractionation studies. I would also like to thank Miss Marion Thomson, Miss Marlene Walker and Miss Elizabeth Fletcher for their excellent technical assistance in the handling of the experimental animals and the routine isolation of the trypanosomes. The continued interest and support of Professors G.S. Boyd and R.B. Fisher is also gratefully acknowledged. But most of all, I am indebted to Dr. I.B.R. Bowman for his unceasing encouragement, advice and stimulating discussion in support of this research project.

The author was in receipt of an award from the Faculty of Medicine Graduate Scholarship Fund, University of Edinburgh.

ABSTRACT OF THESIS

1. After pretreatment with saponin, bloodstream trypanosomes are easily disrupted by gentle homogenisation. Glycerophosphate oxidase has been purified 10 fold by differential centrifugation after prior filtration through CM23 cellulose. Further purification to 24 fold has been achieved by sucrose density gradient centrifugation. The glycerophosphate oxidase is a particulate enzyme located possibly in micro-bodylike structures 0.3 - 0.5 μ diameter which have an equilibrium density of d = 1.169 in sucrose density gradients.

2. Conditions for optimal activity of the glycerophosphate oxidase have been determined. The K_m for glycerophosphate was found to be 1.72 ± 0.08 and 6.5 ± 0.7 mM in tris buffer, pH 8.0 in the presence or absence of bovine serum albumin. Bovine serum albumin stimulates the glycerophosphate oxidase activity 2 to 3 fold.

3. Hydrogen peroxide is not a product of the glycerophosphate oxidase reaction, but the possibility that it is present as an enzyme-bound reaction intermediate can not be excluded.

4. Inhibitor studies of the glycerophosphate oxidase using the artificial electron acceptor phenazine methosulphate lead to the conclusion that the enzyme is composed of at least two components: a glycerophosphate dehydrogenase component linked to a terminal oxidase component capable of catalysing a 4 electron transfer to molecular oxygen.

5. FAD has been identified as a coenzyme for the glycerophosphate oxidase. Flavin, iron and copper are present in a ratio of 1: 8: 4 in partially purified preparations. Acid-labile sulphide was not

detected, but thiol groups are essential for activity.

6. EPR studies revealed a cuproprotein with a signal at $g = 2.05$ which was released from the glycerophosphate oxidase by freezing and thawing. The significance of this "non-blue" cuproprotein has been discussed. The iron and copper present in glycerophosphate oxidase prepared free of the cuproprotein could not be detected by EPR.

7. Two models of the glycerophosphate oxidase have been proposed and discussed on the basis of the analytical findings, EPR spectra and the mode of action of the various inhibitors.

8. Suramin was found to be a potent inhibitor of the glycerophosphate oxidase. Analogues of suramin with little or no chemotherapeutic action are not effective inhibitors of the oxidase and a close correlation was found between therapeutic action and inhibitor action against the oxidase. These findings suggest that the glycerophosphate oxidase is at least one of the principal sites of action of this drug.

ABBREVIATIONS

GP	D,L-glycerol-3-phosphate
L-α glycerophosphate	L-glycerol-3-phosphate
GP oxidase	L-glycerol-3-phosphate oxidase
GP dehydrogenase	L-glycerol-3-phosphate: (acceptor) oxidoreductase (E.C. 1.1.99.5).
NAD^+-dependent GPDH	L-glycerol-3-phosphate: NAD^+ 2-oxidoreductase (E.C. 1.1.1.8)
DHAP	Dihydroxyacetone phosphate
PES	Phenazine ethosulphate
PMS	Phenazine methosulphate
BSA	Bovine serum albumin
DMF	Dimethylformamide
SHAM	Salicylhydroxamic acid
CLAM	3-Chlorobenzhydroxamic acid
TTFA	2-Thenoyltrifluoroacetone
EDTA	Ethylenediaminetetra-acetic acid
TCA	Trichloroacetic Acid
pOMB	p-Hydroxymercuribenzoate
EPR	Electron paramagnetic resonance
Tiron	1,2-Dihydroxybenzene-3,5-disulphonic acid
PCV	Packed cell volume
RSA	Relative specific activity
TMPD	NNN'N'-Tetramethyl-p-phenylenediamine dihydrochloride

LIST OF TABLES

FACING PAGE

LIST OF FIGURES

FACING PAGE

FACING PAGE

FACING PAGE

CHAPTER 1

A. GENERAL SURVEY OF THE LITERATURE ON THE GLYCEROPHOSPHATE OXIDASE OF BLOODSTREAM TRYPANOSOMES

1. Biology and life cycle

As a consequence of evolution to a parasitic mode of existence, the Trypanosoma genus has undergone considerable biochemical adaptation to such diverse hosts as insects, fish, birds and mammals. The morphological differences found not only between individual monogenetic species, but also between different stages of the life cycle of the digenetic trypanosome can be correlated with biochemical changes in their abilities to metabolise carbohydrate and in the enzymes involved in electron transport of reducing equivalents to oxygen. During the life cycle of the digenetic African trypanosomes, respiration undergoes marked changes in its sensitivity to cyanide. Cyanide sensitive respiration is cytochrome-dependent, albeit sensitive only at high concentrations of cyanide, whereas cyanide insensitive respiration is mediated by a non-mitochondrial, cytochrome-independent L-glycerol-3-phosphate oxidase (GP oxidase), which is unique to the bloodstream forms of the subgenus Trypanozoon. Electron transport systems in Trypanosomatidae have been the subject of a number of reviews (Baernstein, 1963; Grant, 1966; von Brand, 1966; Newton, 1968; Hill and Anderson, 1970; Newton et al., 1973; Bowman, 1974; Bowman and Flynn, 1975). A recent comprehensive review of cyanide insensitive respiration in the plant and animal kingdoms including protozoa has recently been published (Henry and Nyns, 1975). It is intended to limit this present review to the GP oxidase of the African bloodstream forms of the trypanosome.

The trypanosomes of human sleeping sickness, T.rhodesiense and T.gambiense and animal sleeping sickness, T.brucei, which are morphologically indistinguishable from each other, undergo a complex life cycle involving changes in form in the tsetse fly (Glossina spp.)

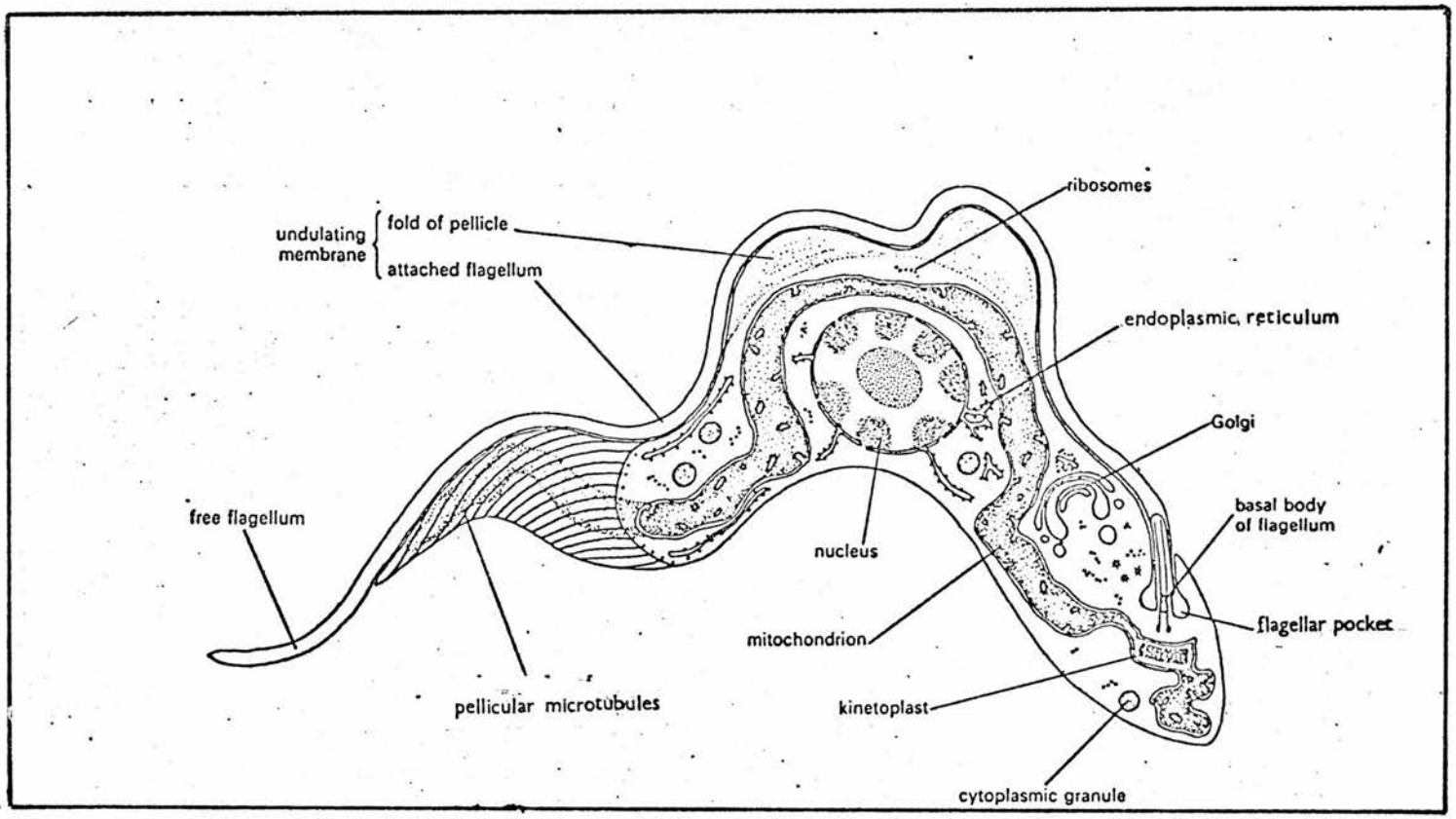


Fig. 1.1. Diagram of trypomastigote bloodstream form as seen by electron microscopy. Pellicular microtubules are shown only at the anterior end of the flagellate. Note the single mitochondrial tubule. (After Vickerman, 1970).

and the mammalian host. Strains of the Trypanozoon subgenus that are infective to the insect vector show a wide and continuous variation in form, or pleomorphism, ranging from long slender (LS) flagellates with a free flagellum at the anterior end, through intermediate forms (ISS), to short stumpy forms (SS) with no free flagellum. The transformation from LS to ISS and SS forms is accompanied by the development of the single mitochondrial tubule containing an increased number of internal mitochondrial cristae and the acquisition of mitochondrial NADH tetrazolium reductase activity (Vickerman, 1965). The GP oxidase is the principal terminal oxidase of these forms which do not contain haem or cytochromes. Monomorphic strains of the Trypanozoon subgenus are morphologically identical to the long slender forms and are monogenetic i.e. are non infective to the tsetse fly and cannot be cultured in vitro. A diagram of the intermediate bloodstream form (ISS) is illustrated in fig. 1.1. The monomorphic LS form differs from the ISS form only in the almost total lack of mitochondrial cristae.

Upon ingestion of the SS form by the insect vector in a blood meal from an infected mammal, the short stumpy form undergoes a biochemical and morphological transformation into the insect midgut form. The trypanosome becomes elongated with a free flagellum once more, the kinetoplast migrates to lie posterior to the nucleus and the mitochondrion develops into an extensive branching network with prominent cristae. Terminal oxidation is now cytochrome mediated and sensitive to cyanide at mM concentrations. The culture form is often regarded as biochemically and morphologically identical to the insect midgut form (promastigote), although this

may not be the case. The promastigote form migrates to the salivary glands of the fly where transformation into epimastigote and then metatrypanosome occurs. Involution of the mitochondrion occurs at the metatrypanosome stage and it is this form that infects the mammalian host when the insect bites an uninfected animal, thus completing the cycle.

2. Carbohydrate metabolism

The bloodstream forms of the African trypanosome show an absolute dependence on a source of exogenous carbohydrate. Glycerol, glucose, fructose and mannose will support normal motility and respiration of the long slender (monomorphic) form of T.rhodesiense (Ryley, 1962) and in addition α-oxoglutarate will support motility (Vickerman, 1965) and respiration (Flynn and Bowman, 1973) of the intermediate and short stumpy forms of a pleomorphic strain of T.rhodesiense. In all morphological types of the bloodstream trypanosome, the metabolism of carbohydrate is incomplete, little if any carbon dioxide is formed and the main end product of glycolysis is pyruvate with small amounts of glycerol (Bowman and Flynn, 1975). In the case of one morphological form of T.rhodesiense in which all the enzymes of the tricarboxylic acid cycle were present in varying amounts, the TCA cycle was shown to operate at an insignificant rate (if at all) in vivo (Flynn and Bowman, 1973).

The relationship between glycolysis and the GP oxidase in monomorphic strains of the Trypanozoon subgenus is illustrated in

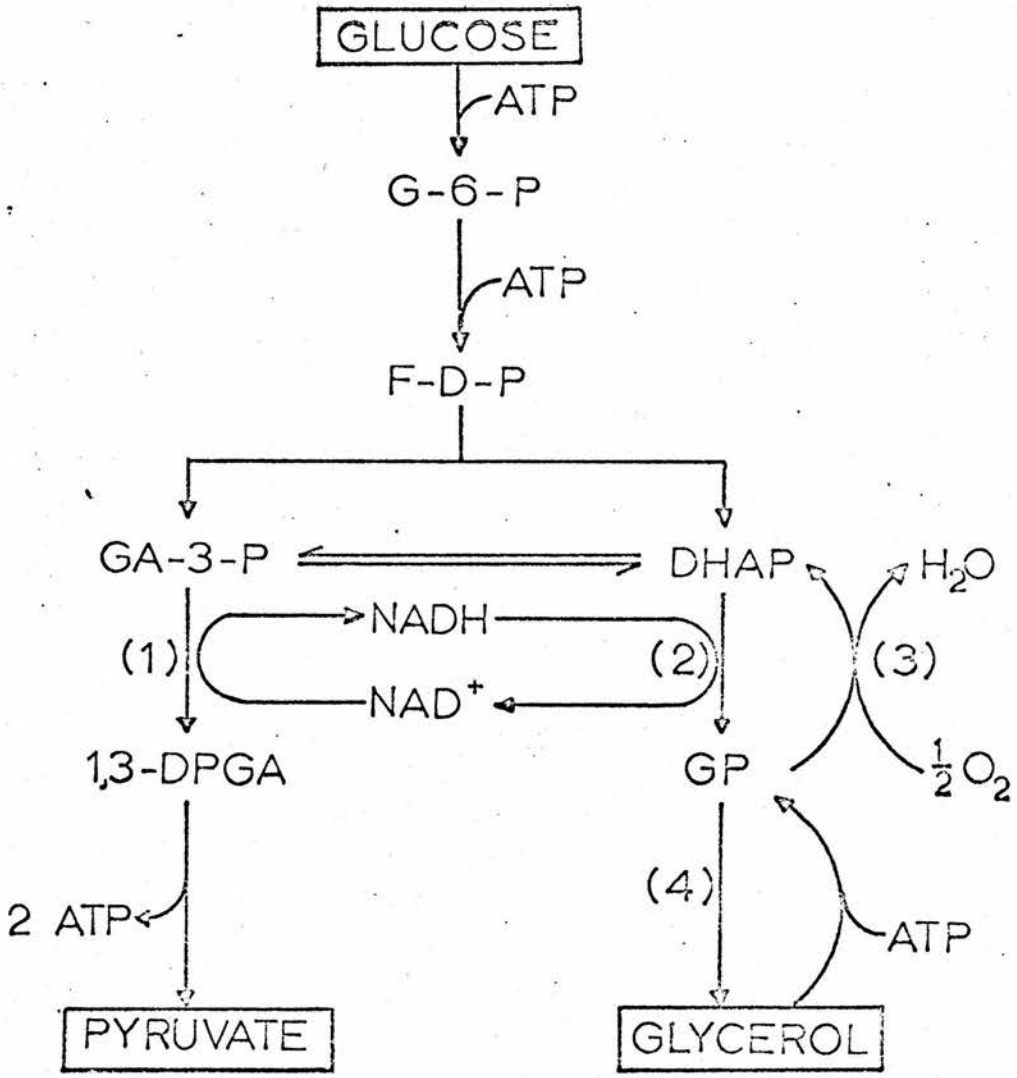


Fig. 1.2. The glycolytic pathway in monomorphic (LS) bloodstream forms of the trypanosome.

fig. 1.2 . Glyceraldehyde-3-phosphate: NAD^+ oxidoreductase generates NADH (reaction 1) which is reoxidised by L-glycerol-3-phosphate: NAD^+ oxidoreductase (reaction 2), dihydroxyacetone phosphate (DHAP) being reduced to L-glycerol-3-phosphate (GP) which is in turn reoxidised by the GP oxidase (reaction 3). Thus the oxidation of NADH which is necessary for the maintenance of glycolysis is accomplished by the sum of the coupled reactions 2 and 3:

$$DHAP + NADH+H^+ \longrightarrow GP + NAD^+ \quad \text{(reaction 2)}$$

$$GP + \tfrac{1}{2}O_2 \longrightarrow DHAP + H_2O \quad \text{(reaction 3)}$$

Sum: $NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD^+ + H_2O$.

Only catalytic amounts of DHAP are required for operation of the cycle. With the theoretical scheme of metabolism proposed in fig. 1.2 glucose is quantitatively converted to pyruvate under aerobic conditions with a net yield of 2ATP per mol glucose utilised; glycerol is similarly converted to pyruvate aerobically with a net yield of 1ATP per mol glycerol utilised.

Under anaerobic conditions, Grant and Fulton (1957) and Ryley (1956; 1962) showed that one mol of glycerol and one mol of pyruvate accumulated per mol of glucose metabolised. Glycerol is not utilised under anaerobic conditions. These observations can be accounted for by the glycolytic sequence outlined in fig. 1.2 . Under anaerobic conditions, the GP oxidase is inoperative (reaction 3) and DHAP is the terminal electron acceptor for NADH produced by glycolysis. For each mol of triose phosphate metabolised to pyruvate, a mol of NADH is reoxidised by the reduction of DHAP to

GP (reaction 3) and hence to glycerol (reaction 4). Phosphatase activity has been identified in Trypanosoma (Harvey, 1949; Gerzeli, 1955) and has been presumed to catalyse reaction 4; under these conditions no net yield of ATP would result (Bowman, 1974; Bowman and Flynn, 1975). Yet there is some evidence to suggest that this assumption may be incorrect. Fulton and Spooner (1958) argued that a drug inhibiting the GP oxidase may be of limited value chemotherapeutically as it had been shown (Fulton and Spooner, 1956) that T.rhodesiense can survive, at least for some hours, anaerobically. Glucose utilisation of bloodstream forms of the Trypanozoon subgenus under anaerobic conditions proceeds at approximately the same rate obtained aerobically over an interval of 30 to 90 minutes (Ryley, 1956) - with the exception of T.brucei where the anaerobic rate is 40% of aerobic glycolysis. In the bloodstream form of T.rhodesiense, Ryley (1962) found that anaerobic glycolysis is markedly reduced when 5%CO_2 is absent from the N_2 gas phase, yet CO_2 had no effect on aerobic utilisation of glucose or glycerol. Moreover, with a N_2 5%CO_2 gas phase anaerobic utilisation of glucose is virtually abolished by the presence of glycerol. It would appear that these observations have never been satisfactorily explained. Bloodstream trypanosomes of the Trypanozoon subgenus do not appear to have any endogenous stores of carbohydrate or "high energy phosphate" as in the absence of substrate the trypanosomes rapidly lose their motility and disintegrate (Ryley, 1956). One theoretical explanation that has been suggested (Opperdoes and Borst, 1975, private communication) involves the transphosphorylation of glucose or of hexose phosphate by the GP produced, thereby allowing glycolysis to operate anaerobically with a yield of one mol ATP per mol glucose

utilised. Further research into this intriguing problem is required to establish the relative importance of aerobic and anaerobic glycolysis in supplying the energy requirements of the cell.

3. Occurrence of glycerophosphate oxidase in Trypanosomatidae

Blood-stream trypanosomes of the Trypanozoon subgenus are characterised by an extremely rapid, but incomplete oxidation of glucose, about 50-100% of their own dry weight per hour (von Brand, 1951). The rapid aerobic glycolysis is associated with exceptionally high rates of respiration, whole cell suspensions using 70-140 nmol O_2/min/mg protein with glucose as substrate, which is about 50 times the rate of respiration of mammalian tissues (von Brand, 1951). High rates of cytochrome independent glycerophosphate oxidation have been demonstrated in broken cell preparations of bloodstream forms of T.rhodesiense (monomorphic strain), T.gambiense, T.evansi, T.equinum (Grant et al., 1961), T.rhodesiense (pleomorphic strain), T.brucei (monomorphic strain) (Flynn and Bowman, 1973) and T.equiperdum (Bayne et al., 1969a). Much lower rates of oxygen consumption in the presence of glycerophosphate are observed with homogenates of culture forms of T.rhodesiense, T.gambiense, T.cruzi, C.oncopelti, L.donovani (Grant et al., 1961) and T.conorhini (Bayne et al., 1969a). Moreover, the rate of oxygen consumption by homogenates of culture forms with succinate or NADH is about the same as or greater than that with glycerophosphate (Grant et al., 1961; Bayne et al., 1969b). This is in contrast to the finding that neither succinate nor NADH are oxidised appreciably by bloodstream forms. These observations,

together with the fact that they are cyanide (0.5 mM) sensitive suggest that the GP oxidase is replaced in the culture form by a cytochrome mediated electron transfer to oxygen. Srivastava and Bowman (1971, 1972) and Bowman et al. (1972) found that the rate of oxygen uptake with glycerophosphate stayed constant in all forms of T.rhodesiense. Nevertheless, these workers found succinate and NADH oxidation rates to be about the same as GP oxidation rates in the culture form and to be cyanide (3 mM) sensitive within 3 days of transfer into culture, again suggesting that the GP oxidase has been superseded by a cytochrome oxidase. The bloodstream forms of T.vivax, T.congolense and T.lewisi have one fifth of the rate of GP supported oxygen utilisation found in the Trypanozoon spp. (Grant et al., 1961). The bloodstream form of T.lewisi is similar to the culture forms of other species in that it possesses cytochromes and is cyanide sensitive (Fulton and Spooner, 1959) suggesting that the oxidation of GP is cytochrome mediated. Cytochromes have not been identified in T.congolense and T.vivax, but several conflicting reports as to these organisms' sensitivity to cyanide have been reported (see Fulton and Spooner, 1959 for a further discussion). It remains possible that GP oxidase is present in these two organisms.

4. Properties of glycerophosphate oxidase

Trypanosomal GP oxidase unlike that of mammalian brain mitochondria (Sacktor et al., 1959; Ringler and Singer, 1959) and insect flight muscle (see Sacktor, 1961) reacts with oxygen without the intervention of pyridine nucleotide coenzymes or of cytochromes.

The high activity is sufficient to account for the respiration of T.rhodesiense in vitro. The enzyme complex is insensitive to the classical inhibitors of the mammalian cytochrome system such as cyanide, azide, amytal and antimycin A (Grant and Sargent, 1960; Flynn and Bowman, 1973) and does not appear to be coupled to the phosphorylation of ADP (Grant and Sargent, 1960; Bide, 1964). The enzyme shows specificity for L-glycerol-3-phosphate as substrate; glycerol, D,L-glycerol-2-phosphate, D-glycerol-3-phosphate and propane 1:2-diol-1-phosphate neither served as substrates for the enzyme nor did they inhibit the oxidation of L-glycerol-3-phosphate when added separately in equimolar amounts (Grant and Sargent, 1960, 1961).

Grant and Sargent (1961) identified L-glycerol-3-phosphate dehydrogenase as a component of the GP oxidase. Although the activity of the GP oxidase was lost after freeze drying or by treatment with acetone at $-15^{\circ}C$, the residual material was capable of oxidising GP to DHAP in the presence of a suitable electron acceptor such as phenazine methosulphate, 2,6-dichlorophenol-indophenol or methylene blue. The dehydrogenase activity remained essentially unchanged after 1 year's storage at $2^{\circ}C$. Attempts to solubilise particulate dehydrogenase component were unsuccessful; prolonged sonication, treatment with salt solutions of various pH values and ionic strengths, trypsin, phospholipase A, butan-1-ol and deoxycholate were not effective.

Thus the GP oxidase consists of at least two enzyme components, an L-glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate: (acceptor) oxido-reductase) (E.C. 1.1.99.5) and a second unknown

component involved in the transfer of electrons to oxygen. These two components together comprise the GP oxidase (E.C.-unclassified). It is important to distinguish between the glycerol-3-phosphate dehydrogenase (which will be subsequently referred to as the glycerophosphate dehydrogenase component of the oxidase) and the "cytoplasmic" L-glycerol-3-phosphate: NAD^+ 2-oxidoreductase (E.C.1.1.1.8) which catalyses reaction 2 in fig. (1.2), and will be referred to as the NAD^+-dependent glycerophosphate dehydrogenase (NAD^+- GPDH).

A number of inhibitors have been tested on the GP oxidase and its dehydrogenase component. Although unaffected by thiol-alkylating agents such as iodoacetate and iodoacetamide, both the GP oxidase and its dehydrogenase component are inhibited by N-ethylmaleimide and by other thiol inhibitors including p-hydroxymercuribenzoate, organic tervalent arsenicals and hydrogen peroxide (Grant and Sargent, 1960, 1961). The heavy metal ions Cu^{2+} and Zn^{2+} inhibited the dehydrogenase component, but Cd^{2+} and arsenite were without effect (Grant and Sargent, 1961). The inhibition by p-hydroxymercuribenzoate of the dehydrogenase component could be completely reversed by incubation of the treated enzyme with 1 mM-glutathione at 37°C for 10 min and some protection against inhibition was noted when the enzyme was incubated with p-hydroxymercuribenzoate in the presence of the substrate or the electron acceptor, phenazine methosulphate (Grant and Sargent, 1961). Bide and Grant (1964) reported that the GP oxidase was inhibited by metal chelating agents. In the presence of increasing amounts of o-phenanthroline, about 50% of the iron in the preparation was removed before the activity of the enzyme system was affected. The synergistic effect of p-hydroxymercuribenzoate

on the inhibitory effect of o-phenanthroline suggested that a thiol group is part of or near to the binding site of the metal essential to the enzyme. Nevertheless, this does not necessarily imply that SH groups are involved in the binding of the substrate or in the transfer of electrons to the terminal electron acceptor.

The GP oxidase contained no detectable haem pigment, 0.13 nmol FAD/mg of N and 7.7 nmol iron/mg of N together with traces of other metals. The molar ratio of FAD to iron was therefore 1:60 which, even after allowing for the observation that 50% of the iron could be removed by o-phenanthroline without inhibiting the GP oxidase, is much higher than that reported for any other metalloflavoprotein enzyme. Thus, in summary, the presence of iron, thiol groups and possibly FAD have been implicated as components of the oxidase.

Two further groups of inhibitors, aromatic hydroxamic acids and diphenylamine, have been found active against the GP oxidase by Evans and Brown (1972, 1973a,b). Diphenylamine was found to inhibit the cyanide insensitive oxidation of glycerophosphate and NADH in cell free homogenates of bloodstream and culture forms of T.brucei and also of succinate and ascorbate/TMPD in culture forms. The NADH oxidase activity of bloodstream forms was at least twice as sensitive to inhibition by diphenylamine as the corresponding activity in culture forms, suggesting different routes of NADH oxidation in the 2 forms. The oxidation of glycerophosphate was inhibited to a similar degree in both bloodstream and culture forms, but the bloodstream form differed from the culture form in that it was unable to donate electrons to mammalian cytochrome c, suggesting that the GP oxidase activity in the two forms was mediated by

different enzymes (Evans and Brown, 1972). Diphenylamine is an inhibitor of cyanide insensitive respiration in various plant systems (Baker, 1963; Sharpless and Butow, 1970). Baker (1963) concluded that the site of inhibition of NADH and succinate oxidation in mitochondria isolated from sweet potato was a region in the electron transport chain between the flavoprotein and cytochrome components. Two other antioxidants, N-phenyl-β-naphthylamine and N-p-tolyl-1-naphthylamine, were found even more effective in inhibiting NADH:cytochrome c reductase activity, but their effects on the GP oxidase have not been tested. Sharpless and Butow (1970) also have used diphenylamine in the study of cyanide insensitive NADH and succinoxidases, inducible in Euglena gracilis grown in the presence of antimycin A. These authors found that the alternative oxidases were markedly stimulated by AMP and preferentially sensitive to diphenylamine and the chelating agent thenoyltrifluoracetone (TTFA).

3-Chlorobenzhydroxamic acid (CLAM) and 13 other aromatic hydroxamic acids (no details) were reported to inhibit the cyanide insensitive respiration of bloodstream and culture forms of T.brucei (Evans and Brown, 1973a). The same authors, in a more detailed study of the effect of CLAM, reported that the GP oxidase of the monomorphic bloodstream form of T.rhodesiense and pleomorphic bloodstream form of T.brucei were inhibited 100% and 95%, respectively in the presence of 0.1 mM CLAM. This compound was found to inhibit motility of bloodstream forms of T.brucei totally after 10 and 60 min at a concentration of 3.0 and 0.3 mM respectively (Evans and Brown, 1973b). Hydroxamic acids are weak acids, forming bidentate ligand complexes in which the metal ion is bound to both oxygen

atoms of the hydroxamate moiety and showing a propensity for hydrogen bonding (Smith, 1965). The mode of action of these substituted benzhydroxamic acids is unknown, but Henry and Nyns (1975) point out in a review that "up to now, the cyanide insensitive mitochondrial respiration has been proved in every case to be sensitive to hydroxamic acid derivatives."

5. Intracellular localisation of the glycerophosphate oxidase

As noted earlier, the GP oxidase and its dehydrogenase component are firmly particle bound and attempts by Grant and Sargent (1960, 1961) to solubilise the GP dehydrogenase component were unsuccessful. Location of the GP oxidase by histochemical methods has been reported by a number of authors (Vickerman, 1965; Ryley, 1966; Bayne et al. 1969a). Vickerman (1965) examined the NADH diaphorase activity in several bloodstream forms of African trypanosomes. In bloodstream forms of T.vivax, T.congolense and intermediate and short stumpy forms of T.brucei intense staining of the mitochondrial tubule was noted. However, old monomorphic laboratory strains or long slender forms of a recently isolated pleomorphic strain of T.brucei or T.evansi showed no staining of the mitochondrion, but granules of formazan were scattered throughout the cytoplasm, which were presumed to represent the location of the GP oxidase. Ryley (1966) reported that washed blood forms of trypanosomes incubated with glycerophosphate, vitamin K_3 and a tetrazolium salt under aerobic or anaerobic conditions develop large numbers of formazan granules. Electron microscopic observations indicated that the formazan granules arise

in clusters in unidentified spherical structures quite distinct from the mitochondrial system. In contrast, Vickerman, in a discussion following a review by Bowman (1974, p.272) stated that he had been unable to locate formazan deposits in electron micrographs using conditions similar to Ryley. Bayne et al. (1969a) found that cells and fractions obtained from T.equiperdum suspended in 0.25 M sucrose containing glycerophosphate, phenazine methosulphate and tetranitroblue tetrazolium developed microscopically observable formazan deposits. The cells developed numerous spherical clusters of the formazan in the cytoplasm and the fractions concentrated the formazan in spherical bodies. In contrast, T.conorhini which does not contain a cyanide insensitive GP oxidase does not reduce the tetrazolium salt unless cyanide was included in the incubation medium. Localisation of enzymic activities by histochemical techniques using tetrazolium salts is fraught with difficulties. Permeability of the tetrazolium salt to the site of reduction, and non-specific binding to tissue protein, together with the tendency of some formazans to crystallize or dissolve in lipid deposits can make interpretation difficult (Altman, 1972).

Bide and Grant (1964) found that after disruption of cells by ultrasonication the GP oxidase could be located in two fractions obtained by differential centrifugation with a specific activity of 6-7 times that obtained previously by Grant and Sargent (1960). It should be noted, however, that the specific activity of both fractions was only some 2-3 fold higher than the specific activity of the whole cell homogenate (Bide, 1964). On electron microscopic examination, the denser fraction contained granules associated with

cell membranes and fragments of flagella while the less dense fraction contained similar granules of 0.01 - 0.1 μ diameter. Bayne et al. (1969a) obtained homogenates from T.equiperdum using a more gentle method of disruption by repeated homogenisation in a glass homogeniser fitted with a motor driven Teflon pestle. The majority of the GP oxidase activity was found to sediment in a mitochondrial (12,000g x 10 min) fraction and had a specific activity about 3 times greater than the homogenate. The GP oxidase in the mitochondrial fraction was further purified by density gradient centrifugation in linear sucrose gradients (0.8 - 2.0 M sucrose, 16,000g x 20 min). The oxidase activity under these conditions was found in two main fractions, a "heavy" fraction and a "light" fraction sedimenting to specific gravities of 1.22 and 1.10, respectively. It should be emphasised that this is not an isopycnic separation, but rather a velocity sedimentation method because the centrifugal force applied would be inadequate for the "light" fraction to achieve its equilibrium density in 20 min. The "light" fraction on electron microscopic examination was found to contain spherical bodies 0.3 - 0.5 μ diameter, flagella, pellicular membrane and endoplasmic reticulum. Some of the bodies in the fraction appeared to have swollen during the isolation process since the matrices were ~~matrix was~~ no longer homogeneous.

6. Problems of cell disruption and subcellular fractionation

Kinetoplastida in general are quite resistant to cell rupture in isotonic media by conventional techniques and the various species

can be arranged in terms of decreasing ease of rupture in the sequence: Crithidia, Leishmania, and Trypanosoma culture forms, Trypanosoma bloodstream forms (Simpson, 1972). The same author suggests that resistance to disruption is most likely a function of the pellicular microtubules running the entire length of the trypanosome (fig. 1.1). However, the differences in ease of disruption between individual species cannot be related to the number of microtubules per trypanosome as all species have in general 60 - 120/pellicle (Angelopoulos, 1970). Sections through the widest part of the cell were examined by electron microscopy and an estimate of the microtubules/pellicle of 77-93 was made for this strain of T.brucei which does not differ from the values reported above from Crithidia, Leishmania and Trypanosoma culture forms. The most striking difference between the pellicle of the bloodstream and culture forms of Trypanosoma spp. is the absence of the surface coat in the culture forms (Brown et al., 1973; Vickerman, 1969). There is some disagreement as to the strength of attachment of the surface coat to the plasma membrane of the trypanosome. Taylor et al. (1974) found that, contrary to other reports (Vickerman, 1969, 1972; Vickerman and Luckins, 1969), extensive washing of T.brucei in saline and other media did not remove the surface coat. They suggested that trypanosomes reported to be without a surface coat after washing were moribund and probably beginning to disintegrate. Cross (1973) found that the surface coat is absent from isolated plasma membranes of T.brucei, and can no longer be visualised on the external surface of the plasma membrane once the cells have been rendered leaky, supporting the view of Taylor et al. (1974). The immunologically variable antigens of bloodstream trypanosomes

are located in the surface coat (Vickerman and Luckins, 1969) and these antigens are most likely the so-called soluble 4S glycoprotein antigens isolated from homogenates of bloodstream trypanosomes by several workers (Williamson and Brown, 1964; Cross, 1973; Allsop and Njogu, 1974). It is not known whether the surface coat contributes to the trypanosome's resistance to disruption. The surface coat can be removed by incubation with pronase (Vickerman, 1969), but the effect of homogenising such a preparation has not been investigated.

The methods employed for disrupting trypanosomes have been in general rather severe, resulting in extensive fragmentation of flagella, pellicle and subcellular organelles and making subsequent subcellular fractionation difficult. For example both methods so far used to prepare the GP oxidase have yielded particulate fractions of low relative specific activity heavily contaminated by flagellar and pellicle fragments (Bide, 1964; Bayne et al., 1969a). Two methods used for disruption of trypanosomes have been reported in which the cells are pretreated with detergents in order to facilitate homogenisation. Kusel and Storey (1972) pretreated C.fasciculata with digitonin and after disruption with a Polytron blender isolated a mitochondrial fraction showing respiratory control. Simpson and Simpson (1974) isolated Kinetoplast DNA networks and minicircles from C.fasciculata after preincubation with pronase and Sarkosyl NL in 0.15M NaCl and 0.5M EDTA at 56°C. Neither of these methods are suitable for the isolation of GP oxidase and therefore part of the work presented in this thesis has been devoted to the development of a suitable method to render the bloodstream forms of

the trypanosome sensitive to disruption by gentle homogenisation.

Subcellular fractionation in Kinetoplastida has not been extensively studied and is hampered by the unsatisfactory methods of disruption used. In no case has a well-characterised, pure subcellular fraction been obtained. For a review of cell disruption and subcellular fractionation in Kinetoplastida, see Simpson (1972).

7. Mechanism of action

It has been proposed (Grant and Bowman, 1963; Bide and Grant, 1964; Grant, 1966) that the GP oxidase may consist of an L-glycerol-3-phosphate: oxygen oxidoreductase, (reaction 1) and a substrate specific peroxidase (L-glycerol-3-phosphate: hydrogen peroxide oxidoreductase, reaction 2) catalysing:

$$GP + O_2 \longrightarrow DHAP + H_2O_2 \quad (1)$$

$$GP + H_2O_2 \longrightarrow DHAP + 2H_2O \quad (2)$$

$$2\,GP + O_2 \longrightarrow 2DHAP + 2H_2O \quad (3)$$

These coupled reactions would account for the overall stoichiometry of reaction 3. Although hydrogen peroxide is not a detectable product of the GP oxidase (Fulton and Spooner, 1959), Bide and Grant (1964) reported that particulate preparations of the oxidase rapidly reduced H_2O_2 in the presence of glycerophosphate. If this coupled reaction sequence were to operate, reaction (2) must not be rate limiting as bloodstream trypanosomes are sensitive to hydrogen peroxide ($> 10^{-4}$ M) as they contain no catalase or peroxidase

(Fulton and Spooner, 1956, 1959). Fulton and Spooner (1959) also note that the addition of peroxide does not increase the rate of oxidation of NADH by homogenates of the parasite, neither does excess catalase slow it. However, Ryley (1962) found that blood forms showed some catalatic activity at high concentrations of homogenate, but at lower concentrations, oxygen evolution never reached the theoretical maximum, indicating inactivation of the enzyme system responsible.

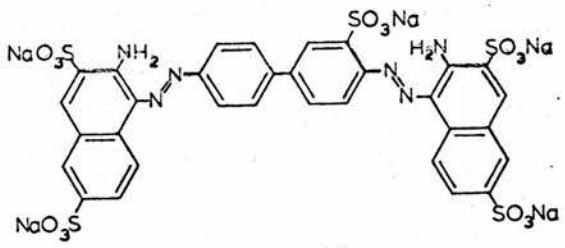
B. GENERAL SURVEY ON THE EFFECTS OF SURAMIN

The two most recent and comprehensive reviews on this compound open with the following statements: "The mode of action of suramin remains enigmatic even after more than half a century of use" (Williamson, 1970); "Among compounds that have significance as chemotherapeutic agents, few has as devious and interesting history as suramin sodium" (Steck 1971). The review presented below is in complete agreement with these observations. However, despite the comparative ignorance surrounding the mode of action of this drug suramin is a highly potent trypanocidal agent which has marked clinical value both in the prophylaxis against the African trypanosomiases and in the treatment of the early stages of infection in man and is still in widespread use today (Apted, 1970; Waddy, 1970; Faust et al., 1970). Suramin is also recommended for the treatment of trypanosomiases in animals infected with T.evansi and T.brucei and as a prophylactic in combination with quinapyramine against T.simiae and T.evansi in pigs, horses and camels (Finelle, 1974).

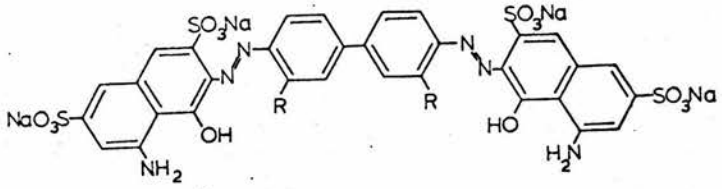
1. Trypanocidal activity of compounds related to suramin

The development of suramin* began with Erhlich's interest in the bis-azo dyes as potential chemotherapeutic agents. Trypan Red (fig. 1.3a) was the first of these compounds found to be both curative and prophylactic against laboratory infections of T.equinum in mice. Nichol and Mesnil (1906) discovered that Trypan Blue,

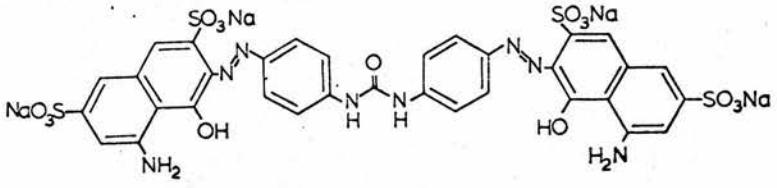
* compound VIII, Table 1.1.



a) TRYPAN RED



b) TRYPAN BLUE (R = CH_3) c) AFRIDOL BLUE (R = Cl)



d) AFRIDOL VIOLET

Fig. 1.3. Structure of bis-azo dyes with trypanocidal activity.

Afridol Blue and Afridol Violet (fig. 1.3 b,c,d) were more active than Ehrlich's Trypan Red, but none of these dyes came into field use as a trypanocide. Reference to fig. 1.3 shows that all these compounds carry sulphonic acid substituents in the 3,6 position of the naphthylamine residues. Trypan Red, Trypan Blue and Afridol Blue contain a central benzidine portion diazotised and coupled symmetrically to two substituted naphthylamine sulphonate residues. Afridol Violet, however, differs from the three benzidine drugs in being a symmetrical urea derivative, with diphenyl urea in place of diphenyl as the central nucleus of the compound. Over the period 1913-1916, therapeutic activity was found to be markedly favoured by two further major changes in the molecule: the introduction of 1-naphthylamine-4,6,8-trisulphonic acid group, and of m- instead of p-amino-benzoyl moieties. The synthesis of suramin was accomplished in 1916, but testing and the chemical constitution remained secret until after World War I when the French workers, Fourneau, Trefouel and Vallee elucidated the structure in 1924, through vigorous research into the extensive patent literature surrounding suramin and its analogues, culminating in the synthesis of some 21 compounds related to suramin.

2. Distribution of suramin in the host's tissues

Suramin is normally administered intravenously in man (hence its less extensive use in the treatment of animal trypanosomiases), because intramuscular or subcutaeneous injections cause severe local irritation and necrosis, and it is not absorbed when given orally. Its value as a prophylactic drug lies in its persistence in the

blood stream bound firmly to plasma proteins (Dewey and Wormall, 1946; Town et al., 1950). Plasma concentrations of suramin greater than the minimum prophylactic concentration of 1.3 mg/100 ml (Vierthaler and Boselli, 1939) can be found 3 months after a single intravenous dose of suramin (Dangerfield et al., 1938). Spinks (1948) demonstrated that (1) many symmetrical compounds of high molecular weight with polysulphonated naphthylamine end-groups had a prolonged survival in the plasma, (2) the structural requirements for persistence are much less specific than those for trypanocidal activity, and (3) suramin is probably not hydrolysed in vivo. In general, the tissue concentrations of suramin are about the same as the plasma concentration (Zeiss and Utkina-Ljubovtzova, 1930; Boursnell and Wormall, 1939). The two notable exceptions to this are the brain which contains little or no suramin and kidney which contains approximately double that of other tissues per gm weight. The strongly anionic nature of suramin accounts for its inability to enter erythrocytes, to pass the "blood-brain barrier" into the cerebrospinal fluid or to be absorbed from the gastro-intestinal tract after oral administration.

3. Distribution of suramin within the cell

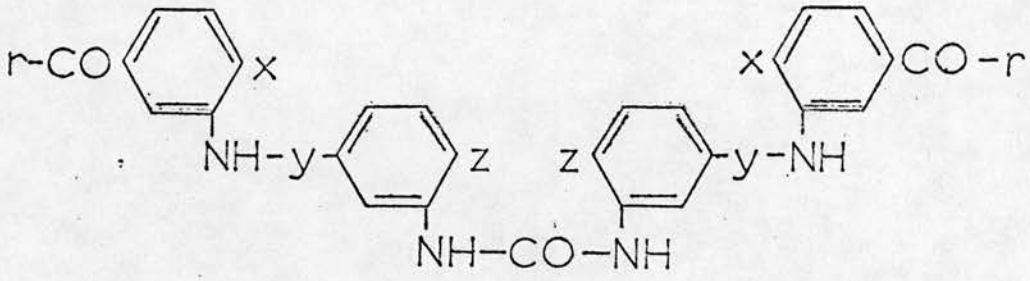
Suramin is known to be localised in basophilic inclusion bodies in the cytoplasm of the trypanosome (Ormerod, 1951; Ormerod and Shaw, 1963) and current evidence suggests that suramin is localised at least in part in the lysosomes of the parenchymal cells of rat liver (Davies et al., 1971; Jacques et al., 1975) and the Kupffer cells

of the hepatic sinusoids (Buys et al., 1973). Suramin retards proteolysis of I^{125}-albumin in lysosomal fractions from rat liver homogenates by partial inhibition of lysosomal proteinases (Davies et al., 1971). Buys et al.(1973), using an autoradiographic technique, demonstrated that the rate of disappearance of formaldehyde-treated I^{131}-albumin from lysosomes in the Kupffer cells of the liver was similarly retarded by suramin. However, the localisation of the majority of the suramin in cells is not known, but these studies would support the proposal that suramin is primarily taken up into secondary lysosomes of cells as a protein-bound complex, then released from the lysosome by proteolysis of the suramin-protein complex to reach secondary unknown sites within the cell.

4. Toxicity of suramin to the host

Suramin produces many toxic effects in animals, the most common being some degree of damage to the kidneys, ranging from mild albuminuria to severe nephritis. For a review of this subject see Williamson (1970). The author notes that the potential teratogenic properties of suramin have not been investigated. Since then, Mercier - Parot and Tuchmann - Duplessis (1973) have reported that foetal abortion and resorption occurs in pregnant rats and that foetal abnormalities occur when pregnant mice are treated with suramin between the 8th - 12th day of gestation. As suramin does not pass the placental barrier, teratogenicity is thought to occur by some indirect effect.

TABLE 1.1. Trypanocidal activity of some substituted sym-bis (m-amino-benzoyl-m-amino-benzoic acid) carbamides.



COMPOUND	SUBSTITUENTS r	x	y	z	TOXICITY(mice) mg/kg	Trypanocidal activity in vivo, mg/kg a)relapse	b)complete cure
I	NTSA(3,6,8)	H	CO	H	500(LETHAL)	125	250
II	NTSA(3,6,8)	CH_3	CO	CH_3	750(TOXIC)	-	-
III	NTSA(3,6,8)	CH_3	CO	H	500-600(TOXIC)	12.5	50
IV	NTSA(4,6,8)	CH_3	CO	CH_3	500(TOXIC)	25	250
V	NTSA(4,6,8)	OH	CO	CH_3	1250(NON TOXIC)	-	-
VI	NTSA(4,6,8)	H	CO	H	600(NON TOXIC)	12.5	50
VII	NTSA(4,6,8)	H	CO	CH_3	200-500(LETHAL)	-	-
VIII	NTSA(4,6,8)	CH_3	CO	H	500(NON TOXIC)	1.55	3.1
IX	NTSA(4,6,8)	CH_3	SO_2	H	900(NON TOXIC)	-	-
X	NTSA(4,6,8)	CH_3	CO	-NH-CO-NH-	*500(TOXIC)	-	-

Data adapted from Fourneau et al. (1924). Compound VIII is suramin and compound VI is desmethyl suramin. NTSA, naphthylamine trisulphonic acid. * Compound X is an isomer of suramin with the central ureido linkage at para (position z) instead of meta.

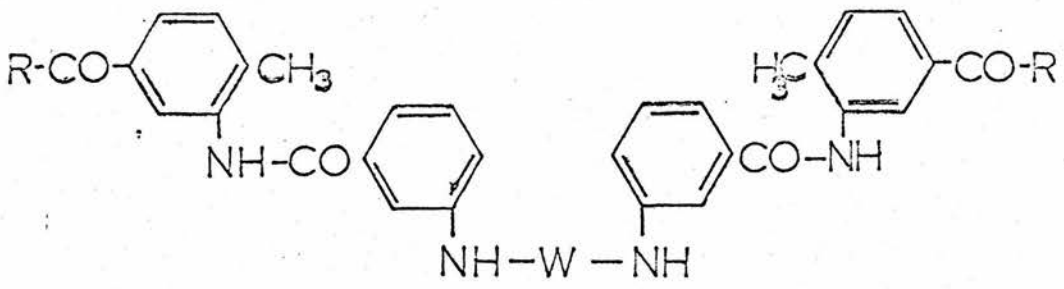
In spite of the high incidence of toxic reactions, suramin has remained in constant use for some 45 years and is still usually the drug of choice in early infections of humans by T.rhodesiense or T.gambiense despite the advent of newer drugs (Apted, 1970).

5. Specificity of the structure of suramin in relation to trypanocidal activity and host toxicity

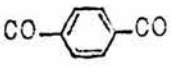
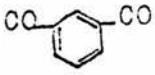
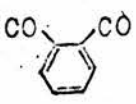
Since the synthesis of suramin, several attempts have been made to improve suramin's trypanocidal activity and to lessen its toxicity to the host animal, but without success. Some of the compounds that have been tested that are closely related to suramin are given in tables 1.1, 1.2, 1.3. The toxicity of these compounds can be markedly influenced by the substituents at positions x and y. Toxicity to the host decreases with the introduction of one or two methyl groups into compounds containing naphylamine-3,6,8-trisulphonic acid substituents (compounds I, II, III, table 1.1). But with naphylamine-4,6,8-trisulphonic acid substituents in position r (compounds IV, to X) the effect of substituting one or two methyl groups is more complex. With one methyl group at position z and x = H (compound III) one obtains the most toxic compound of the series, yet substitution of CH_3 at x lowers the toxicity (compound IV) and substitution of OH at x yields the least toxic compound of the series (compound V).

Trypanocidal activity is markedly altered depending on the substituents at r, x, y, z. Naphthylamine-4,6,8-trisulphonic acid is superior to the 3,6,8 derivative in all cases, cf. compound I with VI, II with IV, II with VIII. Also, the introduction of one methyl

TABLE 1.2. Trypanocidal activity of some compounds related to suramin

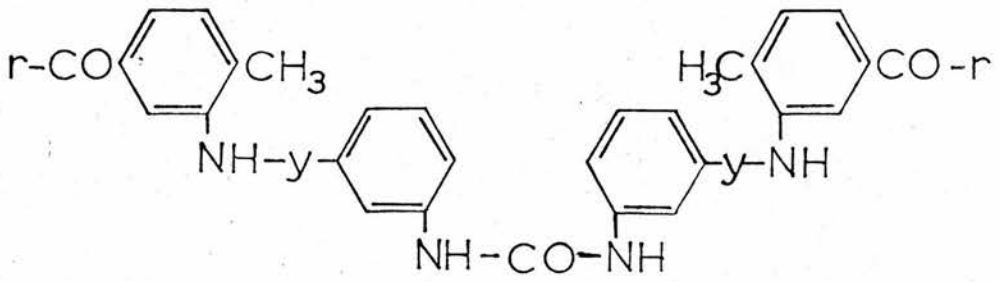


R = naphthylamine (4,6,8) trisulphonic acid.

SUBSTITUENT W	TRYPANOCIDAL ACTIVITY
CO (SURAMIN)	++++
CS	-
CO-CO	-
$CO-CH_2-CO$	-
$CO-C(C_2H_5)_2-CO$	-
	-
	-
	-

Replacement of carbamido (ureido) linkage of suramin for any of the above substituents results in loss of activity. Adapted from Bauer and Becker (1928).

TABLE 1.3. Trypanocidal activity of some substituted sym-bis (m-aminobenzoyl-m-amino-p-methylbenzoic acid) carbamides (y-CO) and the related sulphonamides sym-bis (m-aminobenzenesulphonyl-m-amino-p-methylbenzoic acid) carbamides, ($y=SO_2$).



COMPOUND	SUBSTITUENTS r	y	Trypanocidal Activity	Reference
III	NTSA (3,6,8)	CO	++	Fourneau et al(1924)
VIII	NTSA (4,6,8)	CO	++++	" " " "
IX	" "	SO_2	-	" " " "
	Thiazol-2-yl	CO	-	Adams et al.(1956)
	" " "	SO_2	-	" " " "
	2-pyridyl	CO	-	" " " "
	" "	SO_2	-	" " " "
	5-sulpho-2-pyridyl	CO	-	" " " "
	" " " "	SO_2	-	" " " "
	4-carboxy-3-hydroxy-phenyl	CO	-	" " " "
	" " " "	SO_2	-	" " " "
	ADSA	SO_2	+	Personal communication to Spinks (1948)

NTSA : Naphthylamine trisulphonic acid. ADSA : aminonaphth-8-ol-4,6--disulphonic acid

group at x increases the trypanocidal activity, yet the introduction of a second methyl group at z decreases or abolishes it (I < III > II and VI < VIII > IV). Bauer and Becker (1928) have shown that substitution at x by Cl, OCH_3 or C_3H_7 (where Y = H, CH_3, OCH_3, C_3H_7) all resulted in loss of trypanocidal activity. These workers also demonstrated that the spacing between the pair of 4,6,8, naphthylamine trisulphonic acid groups is critically important. Substitution of the carbonyl function of the ureido linkage by any of the residues defined in table 1.2 results in loss of trypanocidal activity *in vivo*. Moreover, changing the carbamide linkage from meta (as in suramin, compound VIII) to para (compound X) abolishes the trypanocidal activity. Substitution of the carboxyl function at y for a sulphonamide linkage also abolishes activity (compounds VIII, IX, Tables 1.1, 1.3). Further examples of substitutions at y and r diminishing the trypanocidal activity in comparison with suramin (VIII) are given in table 1.3.

In summary then, it would appear that the structural requirements for trypanocidal activity are remarkably specific; changes in any part of the molecule can effect the toxicity to the animal and to the trypanosome. This specificity, however, could conceivably be turned to good use in investigations as to the site of action of suramin in the trypanosome. By testing a series of analogues on isolated enzymes or on whole organisms it may be possible to detect parallel sensitivities *in vivo* and *in vitro*. All these compounds would be expected to have similar half-lives *in vivo* as Spinks (1948) demonstrated that many symmetrical compounds with poly-sulphonated naphthylamine end groups had a prolonged survival in the plasma.

6. Mode of action of suramin

The mode of action of suramin remains a mystery even after 50 years of intermittent attention by several workers. One reason for this failure to identify a biochemical site of action stems from the comparative lack of knowledge of the metabolism of the trypanosome until this last decade. Many early studies have employed enzymes of a mammalian origin most of which, not surprisingly, are inhibited only at relatively high concentrations of the drug. The most sensitive enzymes examined appear to be hyaluronidase, inhibited at 10^{-5} - 10^{-6}M (Beiler and Martin, 1948), fumarase, inhibited at about 10^{-7}M (Quastel 1931), urease at pH 5 (ca. 10^{-4}M), hexokinase (10^{-4} - 10^{-5}M) Town, et al., 1949; Wills and Wormall 1949), RNA polymerase (10^{-5}M, stimulation at 10^{-6}M) (Waring, 1965), calcium transport in sarcoplasmic reticulum (10^{-5}M) (Layton and Azzi, 1974), Na^+/K^+ ATPase of red cells (10^{-4} - 10^{-5}M) (Fortes et al., 1973) and various esterases involved in blood clotting and fibrinolysis (ca. 10^{-4}M) (Elsen and Loveday, 1973).

The inhibitory effect of suramin on enzymes is antagonised in many instances by additional protein, especially serum proteins (Quastel, 1931; Town et al., 1950; Wills and Wormall, 1950a). Some enzymes e.g. urease, amylase, are inhibited on the acid side of their isoelectric points, but inhibition falls sharply at about the isoelectric point as the pH is increased (Wills and Wormall, 1950a; Wills, 1954). A method of determining isoelectric points of some enzymes using this phenomenon has been published (Wills and Wormall, 1950b; Wills 1952) and a hypothetical mode of action proposed (Wills and Wormall, 1950a). These authors suggested that suramin,

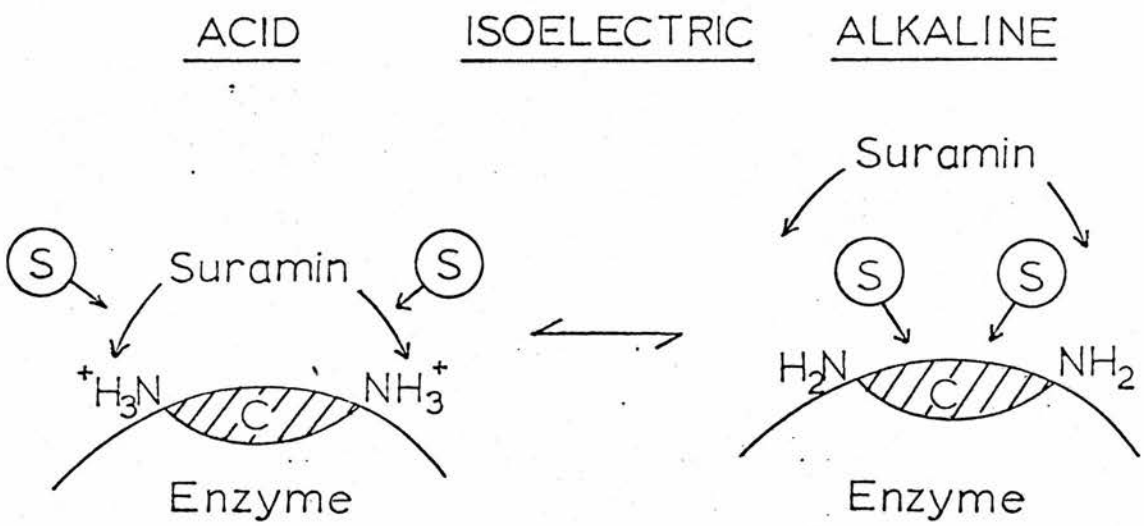


Fig. 1.4. Diagrammatic representation of pH-dependent inhibition of some enzymes by suramin as proposed in the 'bridge' hypothesis of Wills and Wormall (1950a).

(S) = substrate.

a competitive inhibitor of urease, forms a bridge across the active centre of the enzyme by the association of the negatively charged sulphonic acid groups of suramin with basic groups lying near the active centre of the enzyme (fig. 1.4). However, pH dependent inhibition of enzymes by suramin occurs at about pH5 which could not be due to the loss of ionisation of free amino groups as suggested in their illustration (fig. 1.4) because amino groups would remain fully protonated at this pH. Moreover, inhibition of urease can not be reversed by changing the pH, suggesting once suramin binds to the enzyme irreversible denaturation occurs (Wills, 1954). However, the "bridge hypothesis" could explain suramin's inhibitory effect on all enzymes so far studied, but pH dependent inhibition must occur by some other mechanism.

Roehl (1926) summarised most of the important aspects of suramin's trypanocidal action. He showed that suramin (a) *in vitro* affected trypanosome infectivity, but not motility, (b) showed initially reversible binding to trypanosomes, (c) induced cytoplasmic granule formation, (d) combined with basic cellular proteins such as protamines and (e) depended on an intact host defence system for effective trypanocidal action. In marked contrast to the immediate trypanocidal action of trivalent arsenical drugs *in vitro*, prolonged exposure to suramin is required. It was at one time thought that some chemical change in the composition of suramin must occur in the body since its trypanocidal action *in vitro* is very slight whereas *in vivo* it is very high. Jancso and Jancso (1934) claimed that they were able to show that the trypanocidal action of suramin *in vitro* is of the same order as *in vivo* (0.017 mg/ml, i.e. ca. 10^{-5}M)

provided the drug is allowed to act on the trypanosomes for at least 24 hours. However, Hawking (1939) and Yorke, Murgatroyd and Hawking (1931) found that considerably greater concentrations of suramin were necessary to kill all the trypanosomes after 24 hours incubation *in vitro* (0.62 - 1.25 mg/ml vs. 0.017 - 0.025 mg/ml). The reason for this discrepancy is not clear; one of the many possible factors responsible could be the amount of serum protein present in the incubation mixture. In the experiments of Hawking *et al.* the concentration of serum used in the culture medium was approximately 7 fold higher than those of Jancso and Jancso. If the suramin is absorbed as a protein bound complex as discussed earlier, then the amount of suramin bound per mol of serum protein would be an important factor in determining the intracellular concentration that would have accumulated after 24 hours exposure to the drug.

The chemotherapeutic action of suramin is also dependent to some extent on an intact reticulo-endothelial system. Although suramin can produce a cure in mice that have been splenectomised and have had their reticulo-endothelial cells blockaded by injections of a colloidal copper preparation, the minimum curative dose is increased two fold. Also the time required for the clearance of parasites from the blood stream is prolonged to 25-44h, the same period as is necessary *in vitro*, compared with 15 - 24h in the normal animal. Moreover, the same degenerative changes can be seen in the trypanosomes in the blood of a blocked animal as in the *in vitro* observations. In striking contrast, the normal infected animal when treated with suramin shows few abnormal forms because as soon

as the parasite is damaged by the drug it is removed from the circulation by the phagocytes (Jancso and Jancso, 1934; 1935).

Suramin is absorbed in small quantities by the trypanosome in vivo and in vitro and the concentration of the drug would appear to be higher in the serum than in the parasite (Issekutz, 1933b; Hawking, 1939). Trypanosomes isolated from rats 4-10 hours after the administration of suramin contained 5 - 10.5 mg% (0.03 - 0.07 mM) compared with 24 - 42 mg% (0.17 - 0.29 mM) found in the serum (Issekutz, 1933b). Hawking (1939) studied the uptake of suramin by intact blood stream trypanosomes in vitro and found that after 40 minutes exposure to 100 mg% (0.7 mM) suramin, the feebly motile trypanosomes contained 9 mg% (0.07 mM) [recalculated from Hawking (1939), assuming 10^{10} cells ≡ 1 ml packed cells.] Dead trypanosomes incubated under similar conditions contained 5-10 fold higher concentration of the drug. From these incomplete data it would seem that the in vivo and in vitro situations are similar in that the suramin is not actively concentrated within the trypanosome as opposed to the absorption of arsenical drugs and acriflavine which are actively concentrated within the cell (Hawking 1937, 1938). It is of interest to note that Hoffman - Berling (1955) found that suramin is an extremely effective inhibitor of the ATP-dependent motility of isolated trypanosomal flagella prepared by glycerol extraction of the whole cells. This author found that motility was affected by as little as 0.002 mM suramin. Both Hawking and Issekutz have found mean intracellular concentrations of suramin 15-35 fold higher than this, yet motility was still present to some extent. Clearly further work on the mode of uptake and the subcellular distribution of the drug is required.

7. Effect of suramin on trypanosomal enzymes

In a recent review by Williamson (1970), he states that "suramin at concentrations up to 10^{-2} - 10^{-3} M has little or no effect, even after several hours exposure, on the motility, respiration or glycolysis of blood forms of African trypanosomes." There is little evidence in the literature in support of this statement. Von Fenyvessy and Reiner (1928) and Issekutz (1933a) found respiration and glucose metabolism also initially unaffected by suramin in vitro. However, Issekutz (1933a) and Glowazky (1937) reported that trypanosomes isolated after several hours exposure to suramin in vivo were still motile, but that respiration and glycolysis were depressed. No recent studies have been reported to confirm these findings and as these preparations were probably contaminated with blood elements, their interpretation is open to doubt. The effect of suramin on glycolysis and oxygen utilisation by broken cell preparations has never been studied, yet, as will be demonstrated in this thesis, suramin is an extremely potent inhibitor of the GP oxidase of blood stream trypanosomes (Fairlamb and Bowman, 1975). However, the effect of suramin on the respiration of intact and broken cell preparations of the trypanosomatids Crithidia and Leptomonas has been studied . Hill and Hutner (1968) showed that suramin had no effect on the respiration of intact cells of Crithidia fasciculata after 4 hours exposure to suramin even at 10^{-2}M. Succinoxidase activity in mitochondrial preparations from this organism was inhibited 80% by 1 mM suramin. Bacchi et al. (1968) also found that succinoxidase activity in mitochondrial preparations from C.fasciculata was inhibited 86% at 1 mM suramin; similar inhibitions

were obtained with glycerophosphate, malate or NADH as substrates. Studies by Goldberg et al.(1974) on Leptomonas sp.showed that suramin was a poor inhibitor of growth and had little effect on oxygen utilisation by whole cells. In mitochondrial fractions, suramin (1 mM) was found to inhibit succinate and glycerophosphate oxidase by 90% and proline oxidase by 60%.

As noted earlier, Waring (1965) reported that 10^{-5}M suramin was an effective inhibitor of DNA dependent RNA polymerase from Escherichia coli. Hill and Bonilla (1974) found that suramin is active against RNA polymerase II from Crithidia fasciculata using K-DNA as template (80% inhibition at 0.06 mM). Similar results were obtained with RNA polymerase I (83% inhibition at 0.25 mM). Kahan et al. (1968), studying protein synthesis in cell free preparations of Crithidia fasciculata, found poly U-directed incorporation of leu-C^{14} and phenylalanine - C^{14} into protein was inhibited by stilbamidine and hydroxystilbamidine isethionate, antrycide and suramin all in the range 0.1 - 1 mM. These trypanocides were found to inhibit protein synthesis both at the levels of leucyl-tRNA synthesis and the peptidyl transfer reaction.

The dihydrofolate reductases of T.(T.)brucei rhodesiense, Schistosoma mansoni and several filarial worms are sensitive to suramin, the trypanosome enzyme being inhibited by 50 per cent in the presence of 0.03 mM of this drug (Jaffe et al., 1972). Thymidine kinase from culture and blood stream forms of T.(T.)brucei rhodesiense is inhibited 50% by 0.4 mM suramin (Chello and Jaffe, 1972). It should be pointed out, however, that suramin is not selectively toxic against the trypanosome dihydrofolate reductase or thymidine

kinase in comparison with the analogous mammalian enzymes.

In summary, it can be seen that the mode of trypanocidal action of suramin has received little attention. Partly this is due to the pronounced delay in suramin's trypanocidal action with intact cells both *in vivo* and *in vitro*. Studies on the bloodstream trypanosome *in vitro* are also hampered by the difficulty in maintaining this form *in vitro* for longer than 24 hours. Moreover, the use of related trypanosome species such as culture forms of the *Trypanozoon* subgenus or *Crithidia fasciculata* is unsatisfactory for a number of reasons. Hawking (1963) showed that culture forms of *T.congolense* and *T.rhodesiense* were much less sensitive to suramin than the bloodstream forms. Lehmann(1964) obtained similar results on cultures of *T.brucei* and *T.rhodesiense*. It is tempting to ascribe these differences in sensitivity to the absence of the GP oxidase in the culture forms. However it should be noted that: i) permeability factors may be involved as the culture forms were in general less sensitive to trivalent arsenicals, Berenil, Antrycide, Prothidium or Samorin, ii) sequestration of suramin by binding to protein present in the culture medium may affect the results as discussed earlier, iii) other metabolic differences are present in culture as opposed to bloodstream forms. The suggestion that *C.fasciculata* and *C.oncopelti* were suitable model organisms to study the effects and mode of action of trypanocidal drugs for members of the genus *Trypanosoma* (see e.g. Newton, 1962) has been strongly criticised by Gottlieb *et al*. (1972) for several reasons, including differences in carbohydrate metabolism, terminal oxidation and sensitivities to trypanocidal drugs compared to the bloodstream

trypanosomes. If the mode of action of suramin is to be fully elucidated, then studies should be carried out specifically and directly on the bloodstream forms of T.brucei, T.rhodesiense and T.gambiense which, as can be seen from this review, is something that is sadly lacking.

CHAPTER 2

MATERIALS AND METHODS

1. Strain maintenance

A monomorphic strain of Trypanosoma brucei TREU 55, was used throughout these experiments. Strain TREU 55 was derived from a wild type T.brucei that was established first by passage through laboratory mice (TREU 1), and then laboratory rats. Stabilates in 7.5% (w/v) glycerol were stored in a Revco freezer at - 70°C and the contents of two such ampoules were diluted in citrate saline and injected intraperitoneally into 2 rats. Once the infection was established the strain was maintained by syringe passage every 2 or 3 days. Infected tail blood from a parasitised animal was diluted with citrated saline and 0.2 ml was inoculated intraperitoneally into animals under ether anaesthesia. The number of trypanosomes used to infect a rat was adjusted such that approximately 10 - 15 organisms were visible per high power field on microscopic examination. Under these conditions the peak of parasitaemia occurs on the third day after infection, the animal dying at about $3\frac{1}{2}$ days. Wistar strain male white rats and specific pathogen free Wistars were used for the first two years of this investigation and subsequently SPF male Hooded rats were used. No difference in the course of the trypanosomal infection was noted other than the total yield of trypanosomes was reduced by approximately half from 1 to 0.5 ml packed cells per rat. As the levels of parasitaemia were the same in these two strains, it is presumed that the poorer yields from the Hooded rats can be ascribed to their smaller average size.

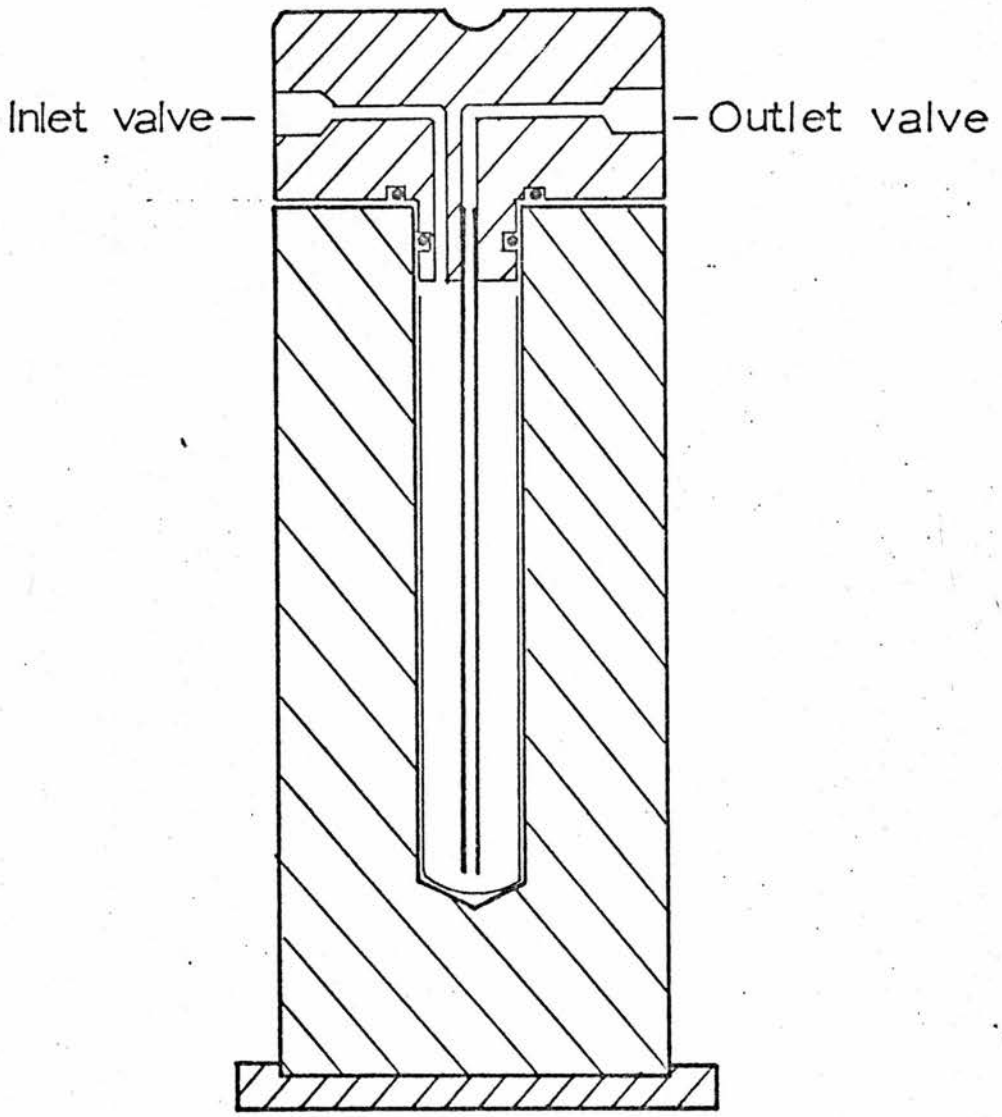


Fig. 2.1. Cross-section of nitrogen cavitation apparatus.

2. Production of antibody to rat erythrocytes from a rabbit

A washed suspension of rat erythrocytes in ~~Krebs~~ Krebs' saline was injected intravenously into a marginal ear vein of a rabbit to give a total of 6 injections over a period of 3 weeks. When the serum titre against rat erythrocytes gave a titre of about $^{1}/_{1000}$ the rabbit was anaesthetised and exsanguinated and the serum obtained was stored in 2 ml aliquots at -20°C. The activity of the preparation was not observed to decrease up to 1 year after storage at -20°C.

3. Disruption of trypanosomes by explosive decompression

This method is based on the fact that when a cell suspension is exposed to a high pressure under a gas, a relatively large amount of gas dissolves in the medium and diffuses into the cells until equilibrium is achieved. On suddenly releasing the external pressure, the gas that has diffused into the cells rapidly comes out of solution rupturing the walls and membranes of the cells. A diagram of the apparatus used is given in fig. 2.1. The apparatus was constructed in the departmental workshop based on a design from the National Institute of Medical Research, Mill Hill, London. Volumes of 20-40 ml of trypanosomal suspension in SEP buffer, pH 7.4, were placed in a 50 ml pyrex glass tube and inserted into the chamber of the device. The outlet valve on the lid of the apparatus was closed and fitted onto the steel body. The lid was secured firmly in place by means of a steel framework fitted with a large

threaded steel rod which can be screwed down into a depression in the top of the cavitation apparatus (fig. 2.1). After connecting the inlet valve to a nitrogen cylinder fitted with a high pressure reducing valve, the nitrogen gas pressure was raised to 1,000 p.s.i. After a suitable time interval (usually 5 min), the inlet valve was closed and the cell suspension was forcibly expelled into a measuring cylinder by opening the outlet valve. All operations were performed at 1-5°C in a cold room.

4. Disruption of trypanosomes by a French pressure cell

The instrument used was manufactured by the American Instrument Co., Silver Springs, Maryland, U.S.A. Up to 40 ml of whole trypanosomes could be disrupted at one time in the chamber of the cell. The ratio of the area of the hydraulic piston to the area of the compression piston of the pressure cell is 5:1, so that pressures used to disrupt the trypanosomes were 5 times that indicated on the hydraulic pressure guage. The pressure reported in this thesis were the final pressures applied in disrupting the cells. All operations were carried out in a cold room. Complete disruption of the organisms (as estimated by phase contrast microscopy) was achieved at a final pressure of 2,500 p.s.i.

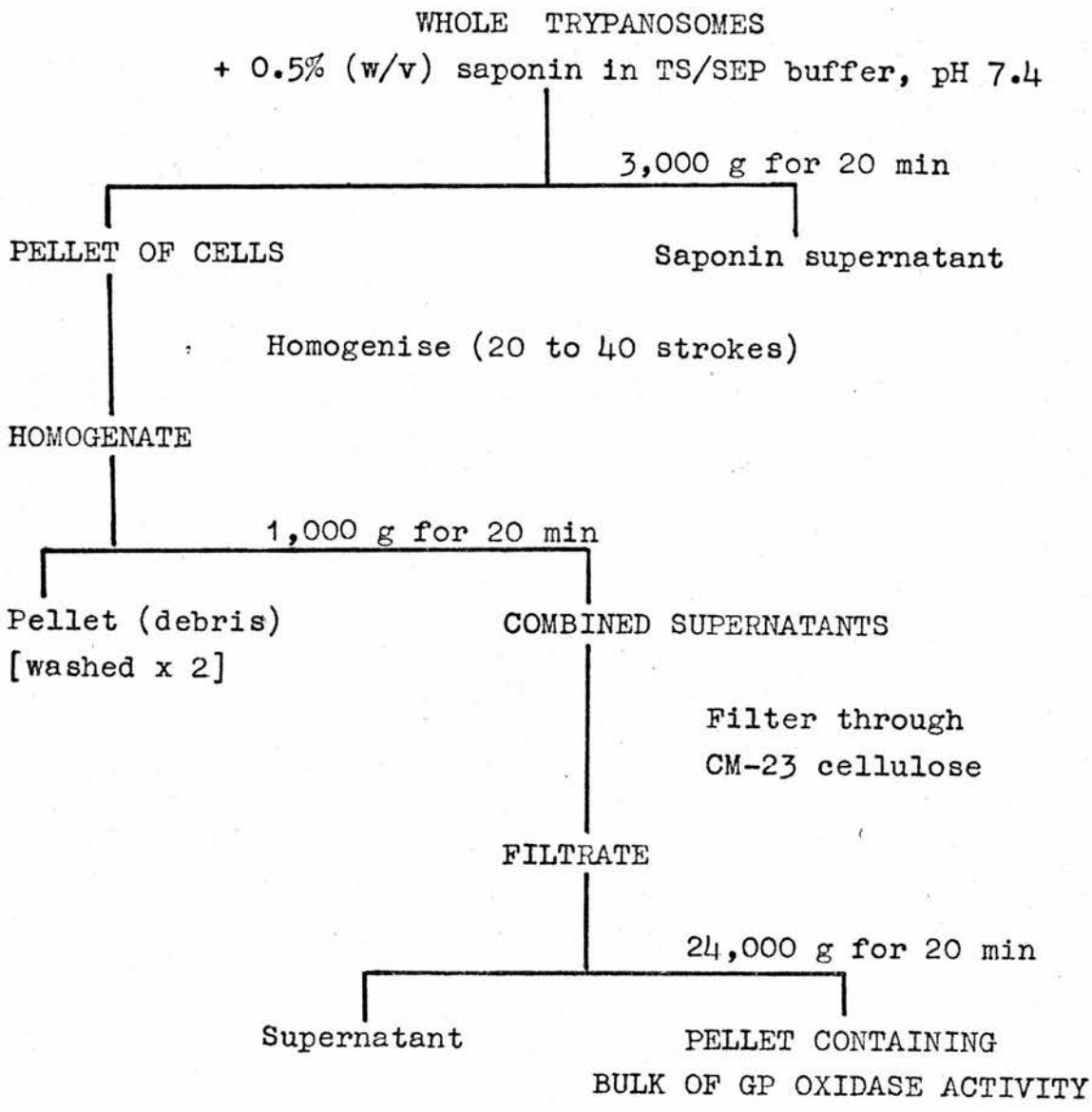


Fig 2.2. Standard procedure for the isolation of partially purified glycerophosphate oxidase.

5. Standard procedure for isolation of the glycerophosphate oxidase

a) Treatment of the whole cells with saponin

Trypanosomes were isolated free of contaminating blood elements by the method of Flynn and Bowman (1973), but omitting the final wash in glucose-free ~~Krebs~~ Krebs' saline, then resuspended in preparative buffer (either SEP buffer, pH 7.4 on T.S buffer, pH 7.4) containing 0.5% (w/v) saponin to give a ratio of 1 ml of packed cells to 10 ml saponin-buffer. The immotile whole trypanosomes were sedimented after 5 min in an MSE Mistral 4L centrifuge (8 x 50 ml swing out bucket rotor) at 3,000 g max for 20 min. The brake was not applied at the end of the run to prevent stirring of the sedimented trypanosomes.

b) Preparation of the homogenate

The supernatant was removed by aspiration and the pellet resuspended in preparative buffer (1 ml packed cells to 10 ml buffer) and homogenised by 20-40 strokes of a hand-operated Dounce homogeniser equipped with a tight fitting Perspex ball-type pestle (Wesley Coe, [Cambridge], Ltd.). The homogenate was routinely examined under phase-contrast microscopy to ensure that breakage of the cells was complete.

c) Fractionation of the whole homogenate

The homogenate was diluted with an equal volume of either SEP buffer, pH 7.4 which was used for experiments on sucrose density gradients, or TS buffer, pH 7.4 which was used in experiments for

metal analysis, and centrifuged at 1,000 g max for 20 min in an 8 x 50 ml fixed angle rotor in an MSE 18 centrifuge. The rotor was decelerated without applying the brake to minimise stirring of the tube contents. The pellet contained a few whole cells, many nuclei and a mesh of flagella entrapping other smaller subcellular particles.

The supernatant was carefully decanted and stored in ice. The pellet was resuspended in buffer (1 ml PCV to 10 ml buffer) using either a Pasteur pipette or not more than 2 strokes with the homogeniser and centrifuged as before. This washing procedure was repeated twice in all and the three combined 1,000 g supernatants containing approximately 70% of the total G P oxidase were passed through a 10-15 cm x 3.14 cm^2 column of CM23 cellulose prepared and equilibrated according to the manufacturers instructions (Whatman Biochemicals Ltd.). Slight negative pressure up to 15 cm of mercury may be applied to increase the flow rate. The column was washed with a small volume of buffer and the eluant containing the G P oxidase activity was centrifuged at 24,000 g max for 20 min in an MSE 18 centrifuge. The supernatants were discarded and the pellets resuspended and homogenised in a small volume of preparative buffer. Samples of the saponin-treated whole cells and final oxidase containing fractions were analysed for activity and protein content and the remainder of the 24,000 g pellet stored in 2 ml capped tubes (Sterilin) in a liquid nitrogen refrigerator until required or used within 24 h for kinetic and inhibitor studies.

All operations were carried out at 0-4°C. The centrifugal forces given throughout this thesis were the values calculated for

TABLE 2.1. Proportions of solutions used in making linear sucrose density gradients

		Volume ml	Sucrose* molarity	Volume/ tube ml	Sucrose density d_4^{20}
Gradient :	mixing chamber	80	0.8	50	1.11
formation :	reservoir	80	2.5		1.31
Cushion :	mixing chamber	25	2.5	6	1.31
Sample layer :	24,000g pellet	-	0.25	1-2	1.03
Overlay :		-	0.25	2-3	1.03
Total volume per gradient				60	

* All sucrose solutions contained 0.1 mM EDTA, 10 mM (K^+) phosphate buffer, pH 7.4.

the bottom of the centrifuge tube (g_{max}) unless specified otherwise.

The subcellular distribution of enzymic activities are given as relative specific activity (RSA) as a function of percentage cumulative protein recovered. Relative specific activity is calculated as the ratio of the specific activity of the fraction concerned to the starting specific activity. The percentage of the total activity recovered for any fraction is given by the area of each histogram block (i.e. RSA x per cent protein). The percentage recoveries and relative specific activity of each enzymic activity of the homogenate (or saponin treated cells) are given in each figure legend.

6. Isopycnic sucrose density gradient centrifugation of partially purified glycerophosphate oxidase

Triplicate linear density gradients from 0.8 - 2.5 M sucrose containing 0.1 mM EDTA, 10 mM potassium phosphate buffer, pH 7.4, were prepared at room temperature in 60 ml cellulose nitrate centrifuge tubes suitable for a Beckman SW 25.2 swing-out bucket rotor and then cooled to 0° in an ice bath. The gradients were prepared simultaneously by means of a three way flow device attached to the outflow from the mixing chamber of a conventional 2 x 100 ml gradient maker. Table 2.1 gives details of the volumes of solutions used. The 24,000 g pellet was loaded onto the precooled gradient with 2-3 ml of 0.25 M sucrose, 0.1 mM EDTA, 10 mM phosphate buffer as overlay. Each tube was weighed and balanced by the addition of 0.25 M sucrose, EDTA, phosphate buffer if necessary. The gradients

were centrifuged in a Beckman L2-65B ultracentrifuge at 23000 rpm for 120 min (38,000 g x 120 min at the sample zone). After centrifugation the gradients were fractionationated in a cold room by a bottom sampling method. A fine bore metal tube was carefully inserted to the bottom of the centrifuge tube and the gradient removed at either 40 or 80 ml/h by means of a peristaltic pump, connected to a Uvicord analyser (260 nm) and drop fractions collected by an LKB Ultrorac fraction collector. The refractive index of each fraction was determined by an Abbe refractometer and converted to sucrose density (d_4^{20}) using data obtained from International Critical Tables (1927). The protein content of each fraction was determined from ratios of E_{260}/E_{280} by the method of Warburg and Christian (1942) and also by the method of Lowry et al. (1951) for those fractions containing the majority of the GP oxidase activity. Enzymatic assays for GP oxidase, NAD^+ -dependent glycerophosphate dehydrogenase, GP tetrazolium reductase and acid phosphatase are described elsewhere.

The results of the density gradient experiments were calculated as fractional enzyme activity or fractional protein of the total recovered and plotted as a function of density for each sample essentially as described by Beaufay et al. (1959).

7. Acrylamide gel electrophoresis of purified GP oxidase

The method used was essentially that of Maizel (1969). Two fractions containing the peak of the oxidase activity from a density gradient separation (overall purification 13.9, S.A. 2100 nmol/O_2/min/mg

protein) which had been stored at -20° for 6 weeks, were thawed, combined and diluted with approximately 10 volumes of SEP buffer, pH 7.4, and sedimented by centrifugation at 24,000g for 30 min. The pellet containing 2.2 mg protein was homogenised in 1 ml of a solution containing 1% (w/v) sodium dodecyl sulphate (SDS), 4 M urea and 1% (w/v) mercaptoethanol and heated in a boiling water bath for 1 min. Duplicate samples of 0.02, 0.05 and 0.10 ml of the SDS denaturated fractions containing the GP oxidase were layered on 10 cm acrylamide gels containing 7% (w/v) acrylamide, 0.1% SDS and 0.1 M sodium phosphate buffer pH 7.4. The electrode buffer contained 0.1% (w/v) mercaptoethanol, 0.1% (w/v) SDS and 0.1 M sodium phosphate buffer, pH 7.4. Bromophenol blue was added to each gel as a marker. Electrophoresis, using a Shandon gel electrophoresis apparatus, was run for 5 h at a potential of 3v/cm, until the bromophenol blue marker reached the bottom of the gel. The extruded gels were fixed in 50% (w/v) trichloracetic acid overnight, stained with Coomassie blue and, after destaining of the gel by soaking in distilled water and several changes of 7% (w/v) acetic acid, scanned at 600 nm using a modified Pye Unicam SP500 spectrophotometer fitted with a gel scanning device.

8. Chromatography of GP oxidase preparations on Bio-Gel agarose

Bio-Gel agarose (Calbiochem Ltd., Bio-Rad Laboratories, Richmond, California) columns were prepared (50 cm in length, approx. 400 ml volume) and packed according to the manufacturers instructions and equilibrated with SEP buffer, pH 7.4 by washing with several column volumes of buffer. When not in use buffer containing 0.02% (w/v)

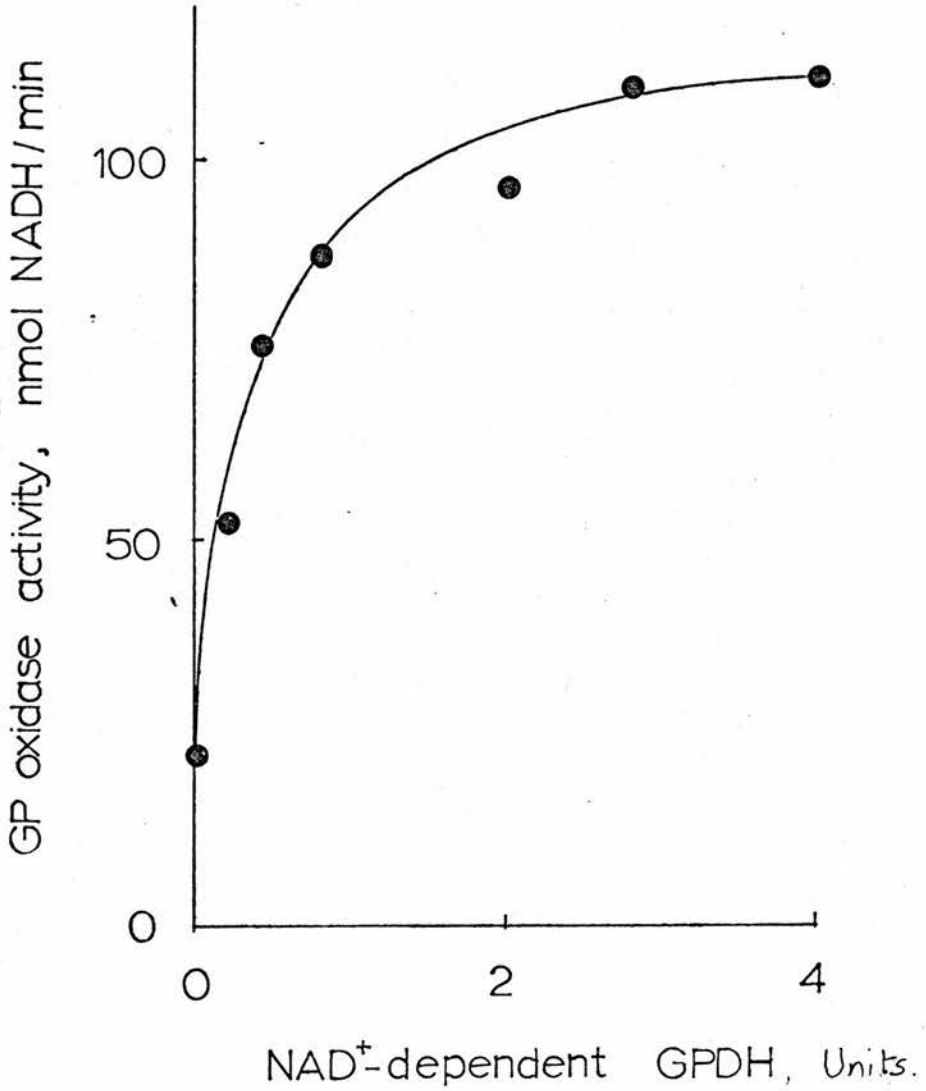


Fig. 2.3. Effect of exogenous NAD^+-dependent GPDH on the spectrophotometric assay for GP oxidase. Assays in TS buffer, pH 8.0 containing 16 mM LaGP, 0.12 mM NADH and 0.02 ml GP oxidase in a total volume of 2.5 ml, temperature 37°C.

sodium azide was equilibrated with the columns to prevent degradation of the agarose beads by microbial contaminants. Before use the columns were thoroughly washed with buffer to remove any sodium azide. All operations were carried out at 1-5°C. The exclusion volumes for the gels (0.5m, 1.5m and 5m) were determined using blue dextran as marker.

9. Methods used for assay of GP oxidase activity

a) Polarographic

GP oxidase activity was measured polarographically at 37°C with a Clark oxygen electrode (Yellow Spring Instrument Co.) polarised at -0.8 V, the electrode being adapted to fit a Perspex reaction chamber (3 ml capacity) equipped with a magnetic stirrer and connected to a potentiometric recorder. About 1-2 min were required for temperature equilibration. Calculation of O_2 uptake was based on a dissolved O_2 concentration of 217 μM in air-saturated medium at 37°C (Kielley, 1963). Activity of fractions was routinely measured in 0.02 M tris buffer, pH 8.0 containing BSA 3.3 mg/ml and 13.3 mM LαGP in a 3.0 ml total volume. For kinetic and inhibitor studies 0.25 M sucrose, 0.02 M tris buffer pH 8.0 containing 13.3 mM LαGP was used. c.f. Srivastava and Bowman (1971).

b) Spectrophotometric

GP oxidase activity was measured from the rate of production of dihydroxyacetone phosphate measured by the rate of oxidation of NADH in the presence of excess NAD^+- dependent GPDH (4 U/assay, see fig.2.3).

The assay mixture contains:

2.24 - 2.15 ml	0.25 M sucrose, 0.02 M tris buffer, pH 8.0	
0.10 ml	NAD^+-dependent GPDH, rabbit muscle,	4U/assay
0.05 ml	NADH (5 mg/ml)	0.12 mM
0.08 ml	DL GP (100 µmol/ml)	16 mM LαGP
0.01 - 0.10 ml	GP oxidase sample	

Under these conditions the rate of NADH oxidation is proportional to oxidase concentration between 30 - 100 nmol NADH/min (0.07 - 0.25 Δ A340 nm/min).

10. Assay of NAD^+- dependent glycerophosphate dehydrogenase activity

Activity was assayed in a recording spectrophotometer at 340 nm following the oxidation of NADH on addition of dihydroxyacetone phosphate (DHAP). DHAP was prepared enzymatically from fructose-1-6-diphosphate (FDP) in the presence of aldolase and triose phosphate isomerase (TIM). The assay mixture contained:

1.0 ml	0.06 tris buffer, pH 8.0,	0.02 M final concentration
0.05 ml	FDP, 103 mg/ml,	3.3 mM " "
0.05 ml	NADH, 5 mg/ml,	0.1 mM " "
0.05 ml	Aldolase	0.9 U per assay
0.01 ml	TIM	2.4 U " "
1.74 - 1.83 ml	distilled water	

The mixture was incubated for 10-15 min, until conversion of FDP to DHAP reached equilibrium and then 0.01-0.1 ml of enzyme added to start the reaction. Reaction rates were linear from 30 sec to 4 min after the addition of NAD^+-dependent GPDH.

Methods 9.b and 10 were developed by myself.

11. Assay for acid phosphatase activity

The method used was that of Linhardt and Walter (1963), using 5.5×10^{-3}M p-nitrophenyl phosphate as substrate in 0.05 M citrate buffer, pH 4.8. The rate of reaction was linear over 30 min and enzyme activity was proportional to product formed up to an absorbance at 400 nm of 1.2 absorbance units. Enzyme activities were converted to μmol of p-nitrophenol produced per h using the extinction coefficient $E^{400\ nm}_{mM} = 18.8 cm^2/mmol$.

12. Assays for GP and NADH tetrazolium reductase activities

A number of mono- and ditetrazolium salts were tested for reduction in this system. Of these, the monotetrazolium salt, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride, (INT, BDH Chemicals Ltd.) was the most actively reduced. The assay mixture contained:

0.5 ml 0.06M tris buffer, pH 8.0, containing 10 mg/ml BSA

0.5 ml INT (0.4% w/v)

0.5 ml DLαGP (12.5 mM LαGP final conc.) or NADH (1 mM final conc.)

0.07 - 0.1 ml enzyme

0.4 - 0.49 ml distilled water.

Reactions were started by the addition of an appropriate volume of enzyme and the reaction mixture incubated at 37°C for 15 min. The reaction was stopped by the addition of 2.5 ml acetone/HCl (acetone/5N HCl, 90: 10 v/v) and the absorbance measured at 480 nm. After corrections for reagent blanks and no substrate controls the formazan

produced is proportional to enzymic activity in the range 0 to 1 absorbance units.

13. Catalase activity

The spectrophotometric method of Beers and Sizer (1952) was used. This method has the disadvavtage in that it is suitable only for high concentrations of hydrogen peroxide (0.5 - 30.0 mM). An alternative method using the O_2 electrode was designed. Buffer, 0.25 M-sucrose 0.02 M tris pH 8.0, was bubbled vigorously with prepurified nitrogen gas for several minutes and a 3 ml sample transferred to the electrode chamber using a suitable needle and syringe. Hydrogen peroxide (50 - 1000 nmol) was added to the oxygen-depleted buffer and after 2-4 min equilibration enzyme samples were added. Corrections for back-diffusion of oxygen through the sample port were made. With this method final concentrations of 15 - 300 μM H_2O_2 could be used. No catalase activity in trypanosomal fractions was detected using either method.

14. Assay for superoxide dismutase

The method used was that of McCord and Fridovich (1969). Cytochrome c is reduced by xanthine oxidase and xanthine via superoxide (O_2^-) and superoxide dismutase is estimated by the decrease in rate of reduction of cytochrome c according to the following reactions:

$$\text{xanthine} + O_2 \longrightarrow \text{urate} + O_2^- \qquad \text{(xanthine oxidase)}$$

$$O_2^- + \text{cyt.}\underline{c}\ Fe^{3+} \longrightarrow O_2 + \text{cyt.}\underline{c}\ Fe^{2+}$$

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad \text{(superoxide dismutase)}$$

The reaction mixture contained 0.05 M (K^+) phosphate buffer, pH 7.8, 10^{-4} M EDTA, 1 x 10^{-5} M ferricytochrome c, 5 x 10^{-5} M xanthine and approximately 6 x 10^{-9} M xanthine oxidase to reduce cytochrome c at a rate of 0.025 absorbance units (550 nm)/min in 3 ml total volume, temperature 25°C. Horse heart cytochrome c (type III, Sigma Chemical Co. Ltd.) was used throughout as other less pure preparations can be contaminated with superoxide dismutase. Xanthine oxidase from B.D.H. Chemicals Ltd. or aged preparations of this enzyme were unsatisfactory and freshly prepared xanthine oxidase (Sigma Chemical Co. Ltd.) was used. Using the latter commercial enzyme preparation, double reciprocal plots of percentage inhibition of cytochrome c reduction against concentration of superoxide dismutase were linear, 50% inhibition occurring with 1 unit of superoxide dismutase as defined by McCord and Fridovich (1969).

15. Methods used for the estimation of protein

The method used was that of Lowry et al. (1951). Bovine serum albumin (BSA) standards were included in each assay. Calibration curves were linear from 0-200 µg of BSA per assay. Trypanosome protein samples were also linear over this range of absorbances (0-0.5 absorbance units). High concentrations of tris buffer and saponin were found to interfere with the method and suitable controls were included where necessary. High concentrations of sucrose have been reported to interfere with this method (Hinton et al., 1969). The

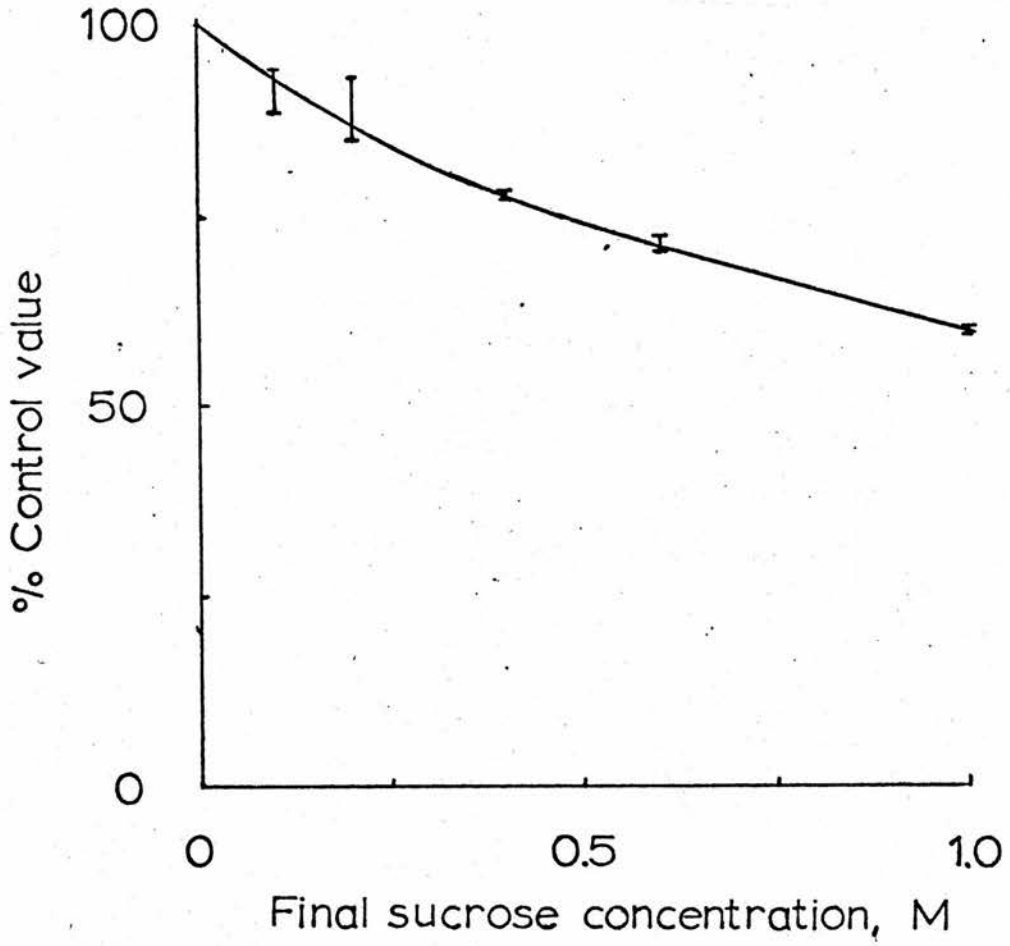


Fig. 2.4. Effect of sucrose on the estimation of protein by the method of Lowry et al. (1951). Vertical bars represent the standard deviation of duplicate samples.

effect of varying concentrations of sucrose on a standard (100 µg) amount of bovine serum albumin can be seen in fig. 2.4. Gradient fractions containing GP oxidase activity were corrected for the inhibitory effect of sucrose on colour production using this graph which is in good agreement with the results of Hinton et al. (1969).

For the estimation of protein in sucrose density gradient fractions the method of Warburg and Christian (1942) was used. A duplicate blank gradient was fractionated in the usual manner and used to correct for trace amounts of substances absorbing in the ultra-violet region that are contaminants of the high concentrations of sucrose employed. The standard curve obtained was used to correct all density gradient experiments.

16. Statistical methods

Mean, $(\bar{x})$, standard deviation and standard error of the mean were calculated using a Hewlett-Packard HP45 calculator, employing the following formulae:

$$\bar{x} = \frac{\Sigma x}{n}$$

$$\text{S.D.} = \sqrt{\frac{\Sigma x^2 - n\bar{x}^2}{n-1}}$$

$$\text{S.E.M. (S.D. of } \bar{x}) = \sqrt{\frac{\Sigma x^2 - n\bar{x}^2}{n(n-1)}}$$

Degrees of freedom = $n - 1$

Unweighted linear regression analyses of data were done with the aid of an Olivetti Programma 101 using programmes written for the department by Dr. I.A. Nimmo. Kinetic data was analysed using Wilkinson's method of regression using the same computer.

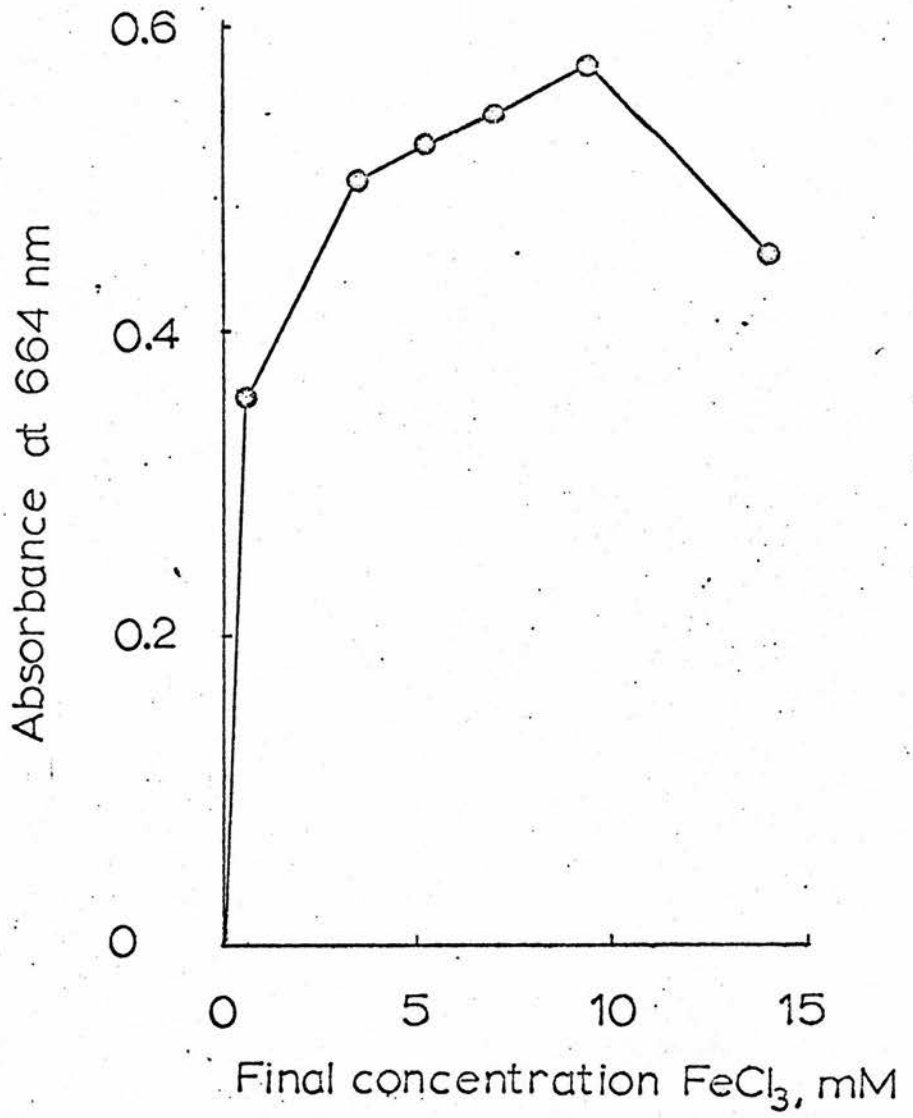


Fig. 2.5. The effect of ferric chloride on the production of methylene blue in the assay of acid-labile sulphide.

17. Estimation of acid-labile sulphide

This method was developed by Fogo and Popowsky (1949) for the spectrophotometric determination of hydrogen sulphide in gases and modified for biological samples by King and Morris (1967), Miller (1970a). In the original method, hydrogen sulphide was absorbed in a suspension formed by adding sodium hydroxide to a solution of zinc acetate. The zinc sulphide precipitate formed was allowed to react with p-aminodimethylaniline (N,N-dimethyl-p-phenylenediamine) in acidic solution in the presence of ferric chloride to give, presumably, methylene blue, which was then determined spectrophotometrically. For biological material, the sample was treated directly with NaOH - $Zn(CH_3COO)_2$ mixture and then coupled with p-aminodimethylaniline as in the original procedure. The precipitate of protein was removed by centrifugation, and the absorbance of the clear blue supernatant was measured at 670 nm. Different concentrations of $FeCl_3$ were used in these methods; Fogo and Popowsky and King and Morris used final concentrations of 0.68 and 0.70 mM respectively whereas Miller used 14 mM $FeCl_3$. Methylene blue production was found to vary with $FeCl_3$ concentration (Fig. 2.5) and maximal colour development was found at 9.3 mM $FeCl_3$. This concentration was therefore used in all assays. The following is an adaptation of the method by King and Morris (1967).

Reagents:

Zinc acetate, 2.6% in water

Sodium hydroxide, 6% in water

N,N-Dimethyl-p-phenylenediamine dihydrochloride, 0.1% in 5N HCl.

Ferric chloride, 0.153 M in 0.6 N HCl.

Standard sodium sulphide, 2 x 10^{-2}M in oxygen-free water, standardised by iodometry. This solution was carefully diluted with oxygen-free water to 1 x 10^{-4}M. Solutions of sodium sulphide were not stable and in the presence of oxygen, about 5% of the sulphide was oxidised in 1 hour (Miller, 1970a)

Oxygen-free water. Double distilled water was first boiled and then cooled with simultaneous vigorous bubbling of "prepurified" nitrogen gas.

Standardisation of sodium sulphide solution

Large crystals of $Na_2S.\ 9H_2O$ (about 2.4g) were washed quickly with distilled water to remove oxidation products such as sulphite and some unknown inhibitory compounds on the surface, then dissolved in 500 ml deoxygenated water and stored in a tightly stoppered 500 ml flask. After samples were withdrawn, the flask was flushed with oxygen-free nitrogen and immediately restoppered. Standardisation of the freshly prepared stock Na_2S soln. by titration against iodine using starch indicator (Vogel, 1961) gave a concentration of 0.01996 M. After storage for one month 4.3% loss of Na_2S had occurred.

Procedure:

0.5 ml zinc acetate, 0.1 ml NaOH, and 0.7 ml sample or standard were pipetted into glass-stoppered test tubes, with a small drop of water touched on the stopper as a seal. Each tube was shaken on a Vortex test tube shaker for one minute. Then 0.25 ml of the diamine reagent was added rapidly, gently swirled until the contents became clear and 0.1 ml ferric chloride added. The tube was immediately stoppered and shaken on a Vortex mixer for 1 minute.

The mixture was allowed to stand at room temperature for 30 minutes. The protein precipitate was removed by centrifugation at about 10,000g for 10 mins. The absorbance was measured at 664 nm against a reagent blank. The absorbance was linear with sulphide concentration within the range 5 to 40 nmoles of sulphide per tube. Under these conditions an extinction coefficient of 23,700 (23,640-23,750) was obtained at 664 nm. Values of 28,500 and 26,500 (25,000-27,750) have been reported for E at 670 nm by Lovenberg et al. (1963) and King and Morris (1967), respectively.

18. Wet ashing of protein samples for total metal analysis

Wet ashing of samples was used to liberate all metal from the organic material and to destroy any complexing agents that might interfere with the analysis. Acid digestion of samples by concentrated sulphuric and nitric acids followed by oxidation with hydrogen peroxide was used as recommended by the Analytical Methods Committee (1967).

Procedure: samples and standards were pipetted into 150 x 17 mm Pyrex test tubes (previously cleaned by mock ashing when first used, or by boiling briefly in 2N-HCl for reuse, finally flushing with water). The volume should not exceed 1 ml. Concentrated H_2SO_4 (0.1 ml) was added and the tubes were heated until charring occurred. After cooling, 0.1 ml of concentrated HNO_3 was added and the tube reheated until charring reoccurred. After cooling the additions of 0.1 ml HNO_3 were repeated until a pale brown solution was obtained on heating to fumes of sulphuric acid (usually 2-3 0.1 ml portions). After cooling, 1 drop of 30% w/v H_2O_2 was added and the tubes reheated to fumes of sulphuric acid. The addition of H_2O_2 was

repeated until the digest was colourless (usually 2 drops).

19. Estimation of total copper

This method is a microadaptation of that of Martens and Githens (1952), using zinc dibenzyldithiocarbamate as the complexing agent, which is superior to the more commonly used sodium diethyldithiocarbamate since the copper complex is more stable and interference by ferric iron is less. The procedure was an adaptation of that of Brumby and Massey (1967).

Procedure:

The acid-digested samples and standards were cooled and diluted with 3.5 ml water. 1.5 ml of zinc dibenzyldithiocarbamate, 0.01% (w/v) in reagent grade carbon tetrachloride was added and the tube agitated vigorously on a vortex mixer for 3 minutes. After allowing the layers to separate, the lower organic phase was withdrawn with a Pasteur pipette into a 1.5 ml cuvette and read the absorbance at 435 nm against a reagent blank. Spuriously high absorbance occurred if any of the aqueous phase was introduced into the cuvette. The same cuvette was used for all readings and was rinsed with absolute ethanol and air dried between samples. The calibration curve was linear in the range 5 - 50 nmoles of copper, using $CuSO_4$ $5H_2O$ in 0.1 N HCl as standard. Phosphate buffer and EDTA were found to interfere with the method and therefore sucrose - Tris buffer, pH 7.4 was used in preparation of the purified GP oxidase.

20. Estimation of total iron

a) After wet ashing

The method used was essentially that of Brumby and Massey (1967). The sample was ashed as described previously and the iron was reduced with thioglycolic acid, complexed with 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) and extracted into isoamyl alcohol. Bathophenanthroline is superior to o-phenanthroline in the estimation of iron because interference by copper is less (Brumby and Massey, 1967). Saturated solutions of sodium acetate pH 6.0, were made iron-free by the method of Van De Bogart and Beinert (1967). Freshly prepared thioglycolic acid was used. Bathophenanthroline obtained from B.D.H. Biochemicals Ltd., Poole, Dorset gave unacceptably high blank values compared with that from Sigma Chemicals Ltd. which was used subsequently for the assay of total and acid-extractable iron. Standard calibration curves were linear in the range 0-20 nmoles iron.

b) Without wet ashing

This method was developed by Doeg and Ziegler (1962) for the estimation of total iron in mitochondria using an extraction procedure in place of the acid digestion. Iron was released into solution by treatment of the sample with thioglycolic and acetic acids and extracted as the complex with 4,7-diphenyl-1,10-phenanthroline into isoamyl alcohol. The procedure used was taken from Brumby and Massey (1967). The calibration curve was linear from 0-20 nmoles standard iron solution.

21. Estimation of non-haem iron

The method used is that of Brumby and Massey (1967). Samples were made to 5% (w/v) trichloracetic acid and aliquots of the acid-extract analysed for iron in the presence of ascorbic acid as reductant and o-phenanthroline as complexing agent. Standard iron solutions in trichloracetic acid gave linear calibration curves in the range 0 - 200 nmol.

22. Estimation of Flavins

a) Fluorescent method

The method used was essentially as described by Udenfriend (1962a). The procedure differentiates between FAD and other flavins (riboflavin and FMN) extracted from biological material by cold trichloracetic acid. One aliquot (A_1) was neutralised immediately and a second sample (A_2) left to hydrolyse overnight and then neutralised. The fluorescence emission was measured at 535 nm, (excitation 450 nm) first in the oxidised state and again after reduction by dithionite to estimate residual nonflavin fluorescence. Appropriate standards of riboflavin and blanks were included in each experiment. Fluorescence was proportional to riboflavin concentration from 0-3 nmol per assay. The FAD concentration in each tube is then equivalent to $A_2 - A_1/0.85$.

b) By difference spectra

Reduced minus oxidised difference spectra of the bulk particulate oxidase preparation were measured using an Aminco - Chance split beam

recording spectrophotometer in 3 ml cuvettes, 1 cm path length, approximately 17 mg protein/ml. Samples were reduced by the addition of 100 µmol solid DL α GP and the difference spectrum recorded within one minute of mixing; no further change in absorbance was noted on repeated scanning.

23. EPR spectrophotometry

I am indebted to Dr. I. Mason for performing all the EPR scans presented in this thesis and for his advice in interpretation of some of the results.

EPR measurements were done on a Varian E - 4 EPR Spectrophotometer under the following conditions: microwave power, 50 mW; frequency 9.14 GHz; modulation amplitude 12.5 G. The temperature was maintained at -172°C by means of a flow of cold nitrogen gas.

24. Analytical methods for the determination of small amounts of hydrogen peroxide

a) Using scopoletin and horse radish peroxidase

This method was developed by Andreae (1955) for the estimation of hydrogen peroxide in pea juice and has been subsequently used for the estimation of hydrogen peroxide produced by irradiation of water by γ-rays (Perschke and Broda, 1961) and the production of hydrogen peroxide in pigeon heart mitochondria (Loschen et al., 1971). Scopoletin (6-methoxy-7-hydroxy-1,2-benzopyrone) is oxidised stoichiometrically (1:1) by hydrogen peroxide with loss of its fluorescence, in the presence of horse-radish peroxidase (HRP).

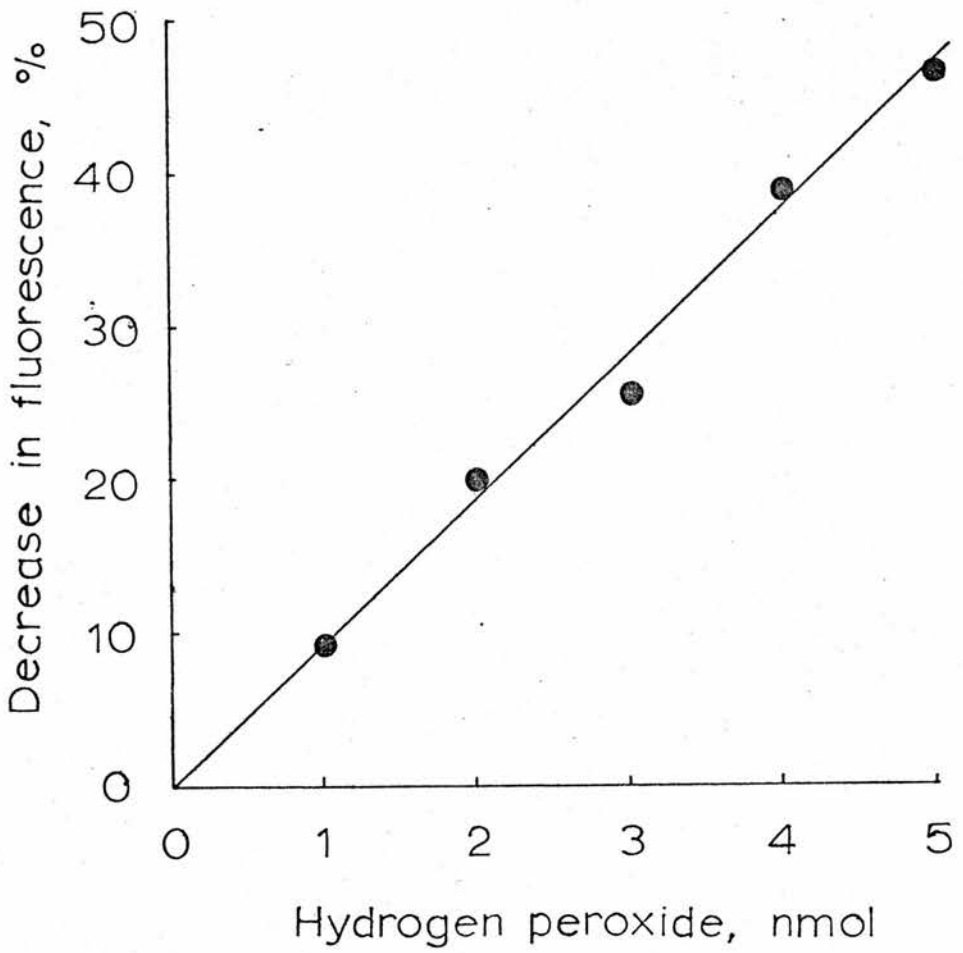


Fig. 2.6. Estimation of hydrogen peroxide by the oxidation of scopoletin in the presence of horseradish peroxidase (HRP). Each sample contained 0.1 mg HRP and 0.4 μM scopoletin in TS buffer, pH 8.0 in a total volume of 3.0 ml. The decrease in fluorescence was measured after the addition of standard amounts of hydrogen peroxide. Other experimental details are described in the Methods.

As little as 10^{-10} mol of hydrogen peroxide can be estimated. The method used was essentially that of Loschen et al. (1971). Fluorescence measurements were carried out in a Hitachi Perkin Elmer Spectrophotofluorometer (excitation 388 nm, emission 460 nm, slit widths 10 nm each, sensitivity x 1). All assays were carried out in 0.25 M sucrose, 0.02 M tris, pH 8.0. A stock solution of scopoletin (Sigma Chemical Co. Ltd.) was stored deep frozen without loss in fluorescence for 12 months. The stock solution was thawed and diluted to give approximately 90% full scale fluorescence with the instrument settings given above - a final concentration of 0.4 - 0.5 µM. Fluorescence assays were carried out observing the general precautions described by Udenfriend (1962b). Decrease in fluorescence was found to be proportional to the amount hydrogen peroxide added from 0-5 nmol (fig. 2.6), but did not give the expected stoichiometric ratio of 1 mol of scopoletin oxidised per mol peroxide utilised. In these experiments a stoichiometry of 1 nmol scopoletin oxidised per6mol peroxide utilised was obtained. Possibly the scopoletin or HRP (Sigma Chemical Co. Ltd., crude, approx. 10% pure) was contaminated with a non-fluorescent substrate competing with scopoletin for oxidation by HRP.

b) Using Diacetyldichlorofluorescin (LDADCF)

LDADCF is a stable non-fluorescent reduced fluorescein derivative which, when properly "activated" in the presence of peroxidase and micro amounts of hydrogen peroxide, is rapidly oxidised to a fluorescent compound. The method used is essentially that of Keston and Brandt (1965) and Keston et al. (1965). Diacetyldichlorofluorescin (LDADCF) was synthesised from dichlorofluorescein (DCF)

in a two stage procedure as described by Brandt and Keston (1965). DCF was reduced in the presence of zinc and glacial acetic acid to its colourless leuco derivative dichlorofluorescin (LDCF), and then after purification by recrystallisation was acetylated by acetic anhydride in pyridine to yield diacetyldichlorofluorescin (LDADCF). LDADCF was recrystallised twice from hot chloroform and dried in vacuo over phosphorous pentoxide. The white solid (76% yield) was judged pure after analysis by paper chromatography, fluorescent spectrophotometry and melting point estimations (208-210°C uncorr., versus 209-210°C reported by Keston and Brandt). After alkaline activation, i.e. hydrolysis, to LDCF, and dilution in phosphate buffer, the reagent was autoxidised at approximately 3% per 24 h (Brandt and Keston, 1965). The addition of 0.14 mM Zn^{++} was necessary to inhibit autoxidation of LDCF in the presence of horseradish peroxidase (HRP).

i)	Alkali activated LDADCF: (2 µM LDADCF in 0.025 M sodium phosphate buffer, pH 7.2)	3.0 ml
ii)	Sample containing 0-1 nmol H_2O_2	0 - 0.2 ml
iii)	H_2O to give a total volume of 3.2 ml	0.2 - 0.0 ml
iv)	Horseradish peroxidase (Crude, Sigma Chemical Co.Ltd. 0.9 mg/ml)	0.02ml

All reagents were stored at 0°C before use. Reagents i) ii) and iii) were mixed and preincubated for 5 min in a 37°C water bath and the reaction initiated by the addition of HRP. After a further incubation of 30 min at 37°C, the samples were chilled in ice and the fluorescence measured in a Hitachi Perkin Elmer Spectrophotofluorometer (excitation 496 nm, emission 522 nm, slit widths 4 nm

each, sensitivity x 1). The fluorescence increase was proportional to hydrogen peroxide added in the range 0 - 1.0 nmol. Quantities as low as 0.1 nmol of hydrogen peroxide could be reliably estimated.

25. Electron microscopy

Samples of pellets of whole trypanosomes, saponin-treated trypanosomes and 24,000g fractions containing the 8.3 fold purified GP oxidase were fixed in 10% glutaraldehyde in Palade-sucrose buffer, pH 7.4 for 24 h at 4°C and then post-fixed for 1 h in 1% osmium tetroxide in Palade-sucrose buffer. The procedure adopted for fixing samples obtained by isopycnic sucrose density gradient centrifugation was as follows: a 5 ml sample of the gradient was diluted to 20 ml with 10% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 containing 10% (w/v) sucrose, fixed overnight at 4°C and then sedimented at approximately 35,000g for 30 min. The pellet was stored in 0.1 M phosphate buffer, pH 7.4 containing 10% (w/v) sucrose for 48 h at 4°C and then post-fixed as described. The post-fixed pellets were washed in 10% ethanol, dehydrated in absolute ethanol, treated with propylene oxide and embedded in Araldite. Sections were cut on a Porter-Blum MT2 microtome, mounted on Athene 483 grids and stained with lead citrate and uranyl acetate. Grids were examined in an AEI EM6 electron microscope at an accelerating voltage of 50 kV.

I am most grateful to Mr. R. McDougall and Dr. C. Trotter of the Anatomy Department, University of Edinburgh for preparing and examining the sections.

26. Buffers

a) Preparative buffers

SEP buffer, pH 7.4: 0.25 M sucrose 0.1 mM EDTA, 10 mM (K^+) phosphate buffer, pH 7.4.

Tris-sucrose (TS) buffer, pH 7.4: 0.25 M sucrose, 10 mM tris-HCl, buffer, pH 7.4.

b) Analytical buffers

ENK buffer, pH 7.4: 66 mM (Na^+/K^+) phosphate buffer, pH 7.4 containing 0.3 mM EDTA, 25 mM nicotinamide, 50 mM KCl.

BSA/ENK buffer, pH 7.4: 10 mg bovine serum albumin per ml ENK buffer. 1 ml of this buffer was used in a 3 ml final volume in some early assays for GP oxidase activity giving final concentrations of BSA (3.3 mg/ml), phosphate (22 mM), EDTA (0.1 mM), nicotinamide (8.3 mM) and K^+ (38.6 mM).

BSA/Tris buffer, pH 8.0: 10 mg bovine serum albumin per ml 60 mM tris - HCl buffer, pH 8.0. 1 ml of this buffer was used in a 3 ml final volume for the assay of GP oxidase activity in fractionation studies giving final concentrations of BSA (3.3 mg/ml) and tris (20 mM).

Tris-sucrose (TS) buffer, pH 8.0: 0.25 M sucrose, 20 mM tris-HCl, pH 8.0. This buffer was used for inhibitor and kinetic studies on the GP oxidase. BSA was omitted because of its propensity for binding many of the inhibitory compounds used in these studies.

Palade-sucrose buffer, pH 7.4 (for electron microscopy): 1.47 g sodium barbitone, 0.97 g sodium acetate, 11.25 g sucrose and 0.05 g $CaCl_2$ were dissolved in 200 ml distilled water and the pH adjusted to 7.4 with 1 N HCl and the volume made up to 250 ml. 1% (w/v) osmium tetroxide was dissolved in this buffer immediately before use.

27. Chemicals

All chemicals were of the highest purity available ('Analar' grade or its equivalent) purchased from BDH Chemicals Ltd. or Sigma Chemical Co. Ltd. Salicylhydroxamic acid was purchased from Aldrich Chemical Co. Ltd., Wembley, Middlesex and 3-chlorobenzhydroxamic acid (CLAM) and 3,4,5,-trimethoxybenzhydroxamic acid were a gift from Dr. B.T. Storey, School of Medicine, University of Pennsylvania. Bovine serum albumin (fraction V) used in the routine assays for GP oxidase activity was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, England and DL- α -glycerophosphate (grade X) from Sigma Chemical Co. Ltd. Diphenylamine and catechol were recrystallised twice before use.

All enzymes were purchased from Boehringer Mannheim GmbH except for superoxide dismutase, xanthine oxidase and horseradish peroxidase from Sigma Chemical Co. Ltd.

The trypanocidal compounds were generous gifts from the pharmaceutical companies given in the text. Pyrrolnitrin and compound S72.8991 were gifts from E. Lilly and Co. Ltd., Windlesham, Surrey and Hoechst Pharmaceuticals Ltd., Hounslow, Middlesex, respectively.

CHAPTER 3

RESULTS OF CELL DISRUPTION AND SUBCELLULAR FRACTIONATION

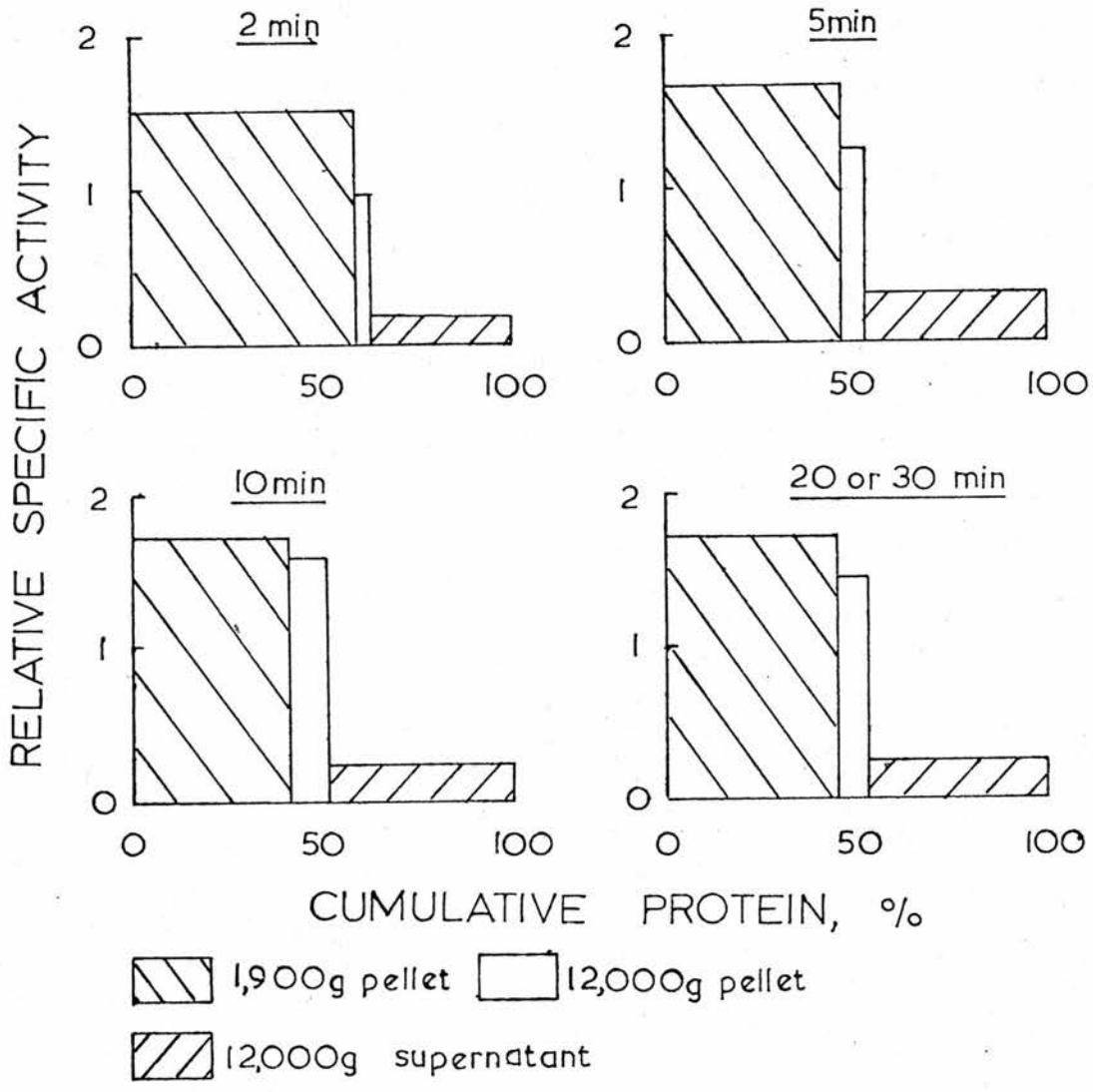


Fig. 3.1. Subcellular distribution of GP oxidase from trypanosomes disrupted by one exposure to nitrogen cavitation, after varying exposure times to 1,000 p.s.i. N_2 gas. Homogenates (0.9 mg protein/ml SEP buffer, pH 7.4) were fractionated by centrifugation in an MSE 18 centrifuge (8 x 50 ml fixed angle rotor) at 1,900 g for 15 min and the supernatant from this fraction was further centrifuged at 12,000 g for 15 min. Mean specific activity of the homogenates was 80 $\pm$ 5 nmol O_2/min/mg protein. Mean recoveries of GP oxidase and protein were 87 $\pm$ 6% and 89 $\pm$ 6%, respectively.

1. Disruption of bloodstream trypanosomes by nitrogen cavitation

Preliminary tests demonstrated that explosive decompression was essential for cell breakage. Exposure of the live organisms to 1,000 psi of nitrogen gas in the apparatus without extrusion through the outlet valve does not damage the cells; neither does instantaneous extrusion through the narrow orifice of the outlet valve without prior equilibration with the high gas pressure.

Fig. 3.1 shows the distribution of the GP oxidase in subcellular fractions after a single exposure to nitrogen gas at 1,000 psi for different time intervals. After 2 min, 90% of the GP oxidase activity is in the large particle fraction consisting mainly of non-motile unbroken cells. After 5 or more minutes, the amount of GP oxidase in the large particle fraction is decreased to about 75%, with the remainder of the activity approximately equally distributed between the 12,000g pellet and supernatant. Thus 2 minutes exposure is an inadequate time interval for the nitrogen gas to equilibrate in the cells. Time intervals greater than 5 minutes do not appreciably increase the amount of GP oxidase released from the 1,900g pellet. The yield and purification obtained in the 12,000g pellet is unsatisfactory in all cases. Hence, the effect of a second exposure of the cell suspension to nitrogen cavitation was investigated, using an equilibration time of 5 min.

Fig. 3.2 shows the results of a typical experiment. Again, after a single treatment by nitrogen cavitation the large particle fraction contains about 75% of the total GP oxidase activity

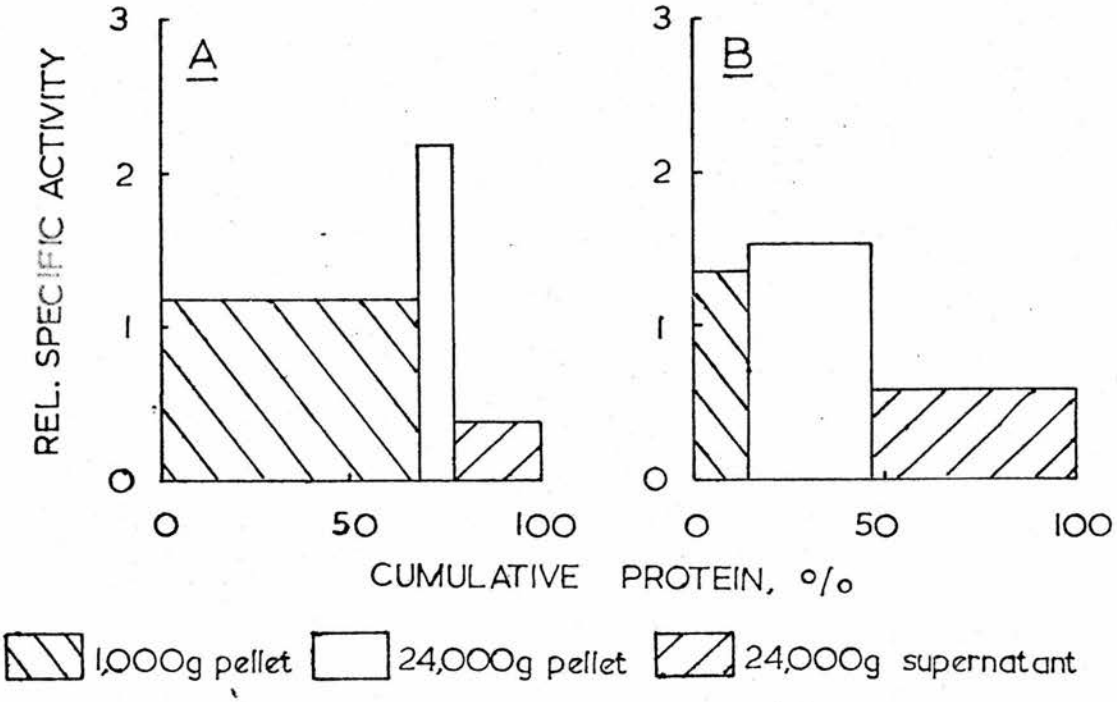


Fig. 3.2. Subcellular distribution of GP oxidase activity from trypanosomes disrupted by nitrogen cavitation. Sample A, one treatment, sample B, two treatments of nitrogen cavitation at 1,000 p.s.i. for 5 min. Homogenates were fractionated by differential centrifugation in an MSE 18 centrifuge (8 x 50 ml rotor) at 1,000 g for 20 min and the supernatant from this fraction was further centrifuged at 24,000 g for 20 min. The specific activity of the homogenate was 175 nmol O_2/min/mg protein. Recoveries of GP oxidase activity were 94% and 90%, respectively; recoveries of protein were 85% and 77% for A and B, respectively.

(cf. fig. 3.1, 5 min exposure). The yield and relative specific activity of the light particle fraction is about 2 fold higher when the centrifugal force applied is increased from 12,000 g x 15 min (fig. 3.1) to 24,000g x 20 min (fig. 3.2). When the cell preparation is exposed to nitrogen cavitation twice, cell disruption is complete and the bulk of the GP oxidase activity is transferred from the 1,000g pellet into the other two fractions. Homogenates obtained by a double exposure to nitrogen cavitation were fractionated by differential centrifugation under various conditions, but no discrete particulate fraction containing the majority of the GP oxidase activity could be isolated. Disruption of trypanosomes by nitrogen cavitation was abandoned as an unsatisfactory method for the isolation of GP oxidase particles because of the polydisperse distribution of the GP oxidase activity.

2. Disruption of bloodstream trypanosomes by a French pressure cell

The French pressure cell is an extremely effective method of disrupting bloodstream trypanosomes; even at the lowest pressure tested (2,500 psi) complete cell breakage occurrs. Fig. 3.3 shows the distribution of the GP oxidase in subcellular fractions prepared as in fig. 3.4. At the lowest applied pressure (2,500 psi) the GP oxidase activity is approximately evenly distributed throughout the particulate and soluble fractions. The "microsomal" fraction (110,000g pellet) is the only fraction in which an appreciable increase in specific activity is obtained compared to the specific activity of the homogenate.

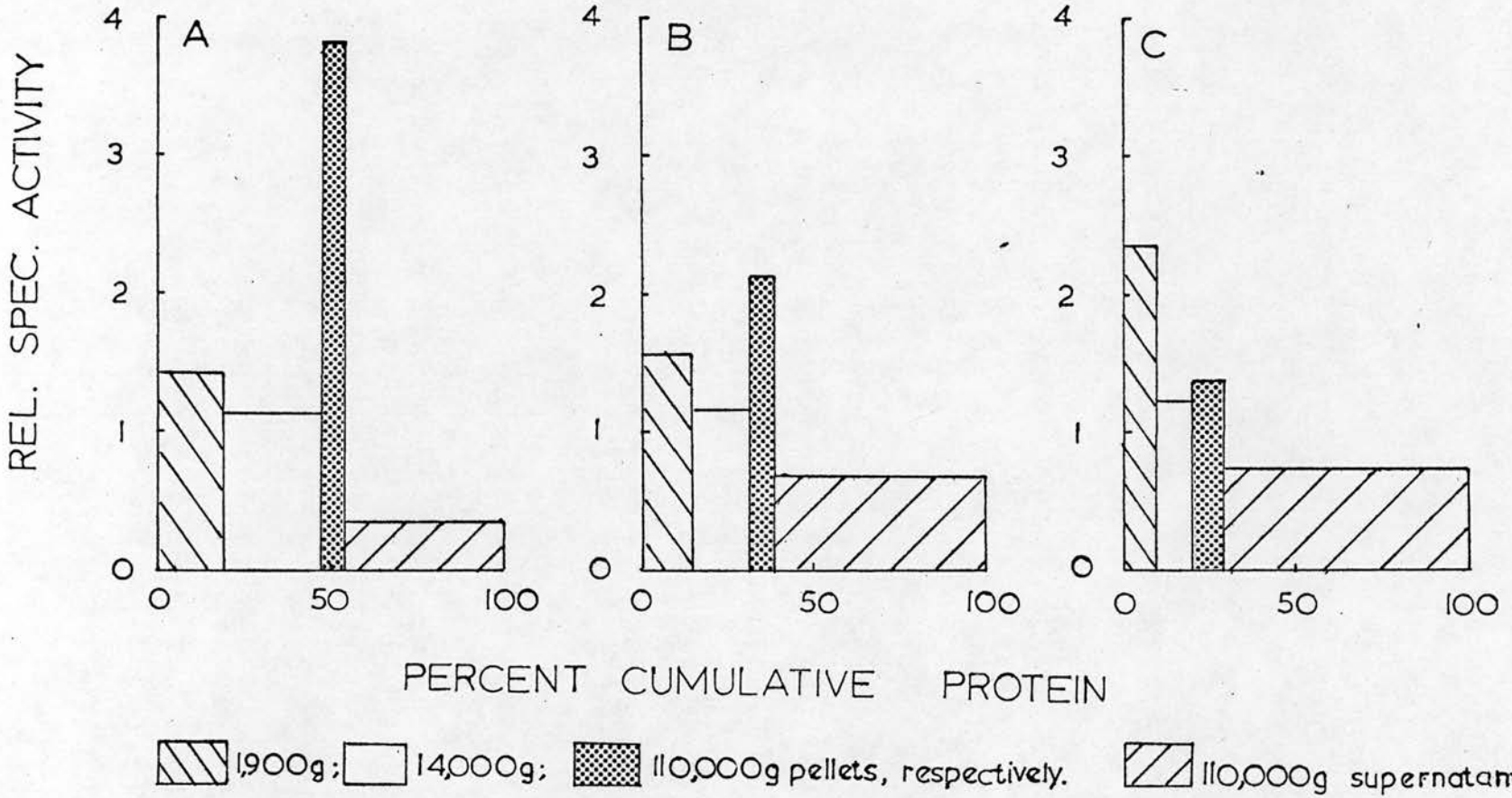


Fig. 3.3. Subcellular distribution of GP oxidase after disruption of trypanosomes by a French pressure cell. A, at 2,500 p.s.i. applied pressure; B, at 7,500 p.s.i.; C, at 15,000 p.s.i. The method is described in fig. 3.4.

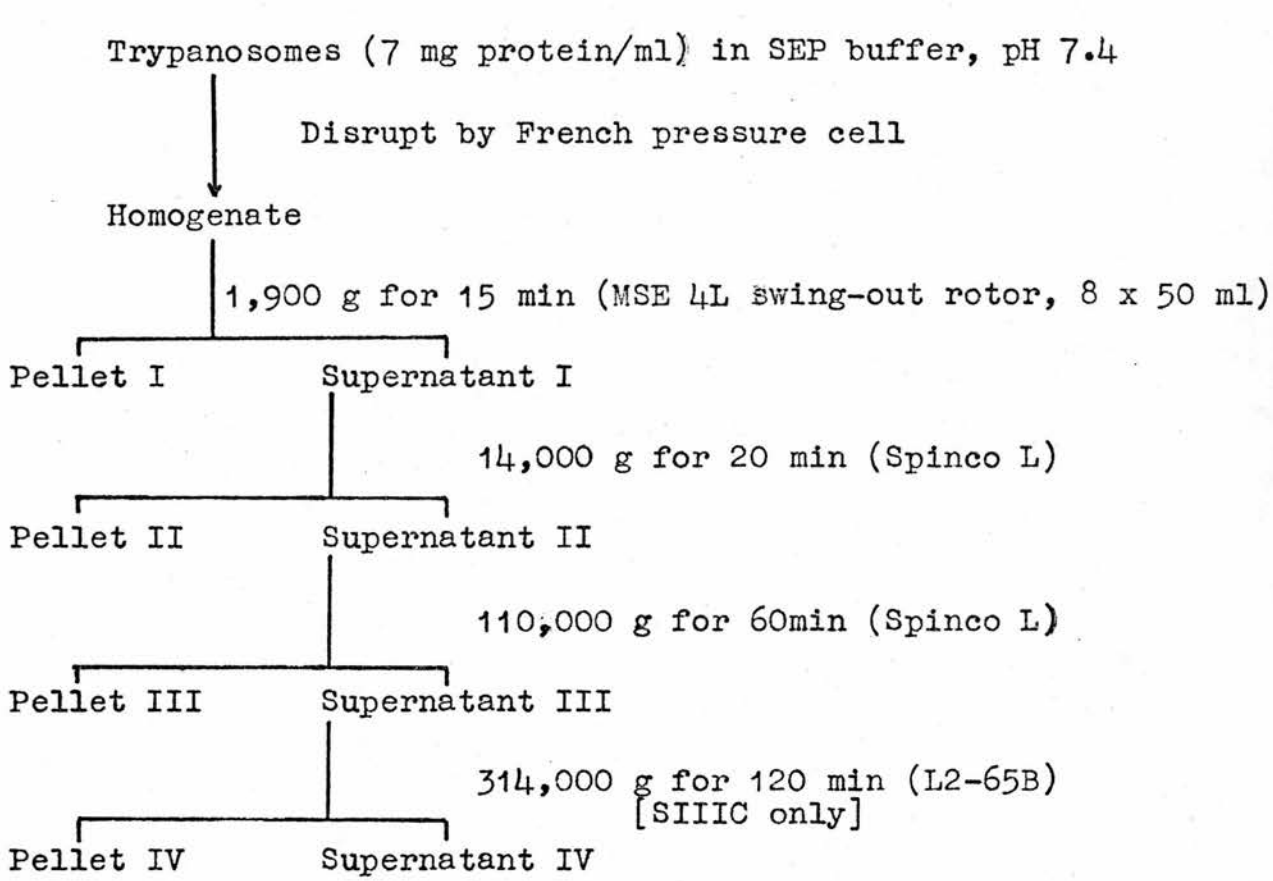


Fig. 3.4. Method used for the subcellular fractionation of GP oxidase from trypanosomes disrupted byvarying pressures in a French pressure cell.The starting specific activities in the homogenateswere 157, 124 and 100 nmol O_2/min/mg proteinfor A,B and C, respectively (fig. 3.3.). The mean percentage recovery of GP oxidase activity was 90.3 $\pm$ 1.3. Fixed angle rotors (12 x 13.5, 8 x 13.5 ml) were used in the Spinco L and L2-65B ultracentrifuges, respectively.

TABLE 3.1. Differential centrifugation of GP oxidase 'soluble' fraction from cells disrupted by a French pressure cell.

FRACTION	TOTAL GP OXIDASE ACTIVITY nmol O_2/min	TOTAL PROTEIN mg	SPECIFIC ACTIVITY nmol O_2/min/mg	RELATIVE SPECIFIC ACTIVITY	% age RECOVERED GP oxidase	% age RECOVERED Protein
110,000g supernatant(C)	1599	41.6	38.4	1	[100]	[100]
314,000g supernatant	453	25.8	17.6	0.5	28	62
314,000g pellet	882	7.6	116.1	3.0	55	18
TOTAL RECOVERED	1335	33.4	-	-	83	80

Details of the fractionation scheme as in fig. 3.4.

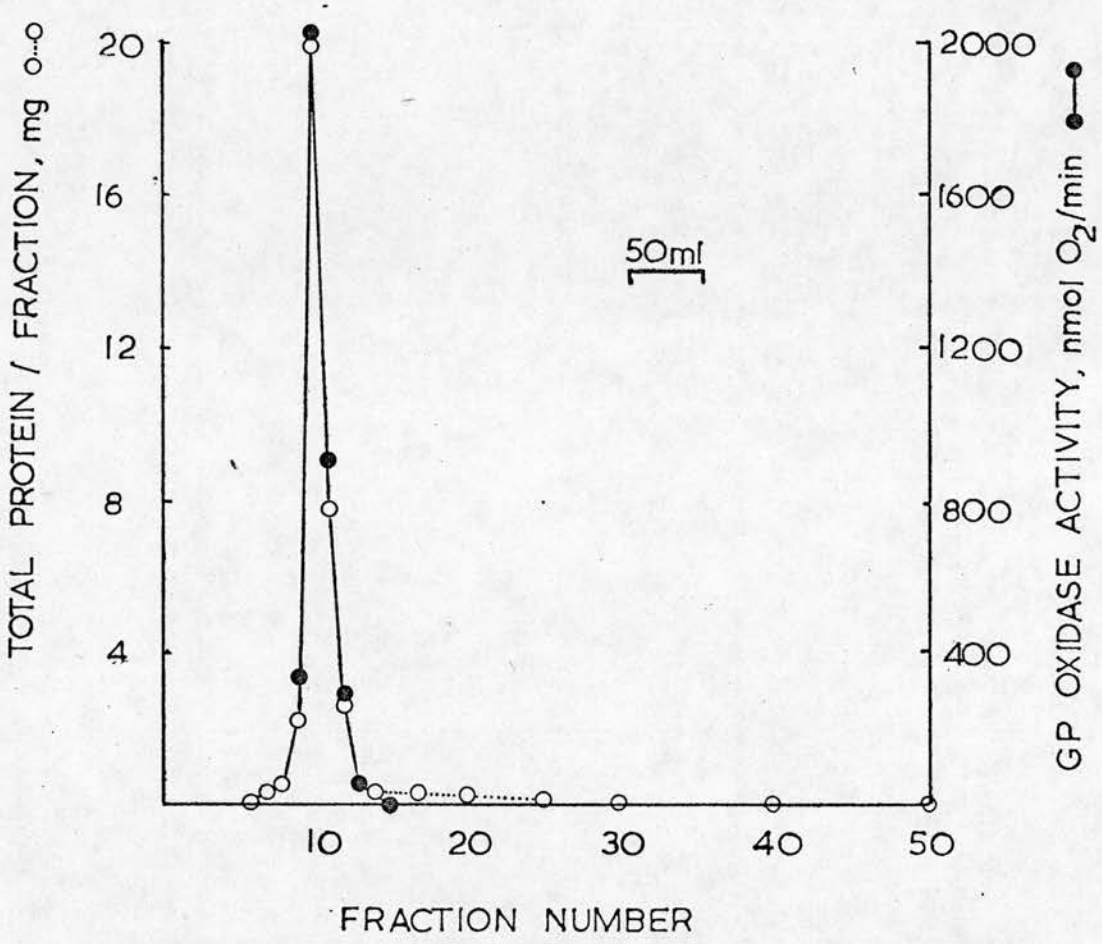


Fig. 3.5. Elution of GP oxidase from Biogel A,0.5m (50-100 mesh). The GP oxidase fraction was prepared as described in the text. Recovery of enzyme activity (corrected for loss due to inactivation over 24 h) and protein was 95% and 78%, respectively. The void volume of the column was 100 ml, using blue dextran as marker.

With greater applied pressures the specific activity of the homogenate decreases by 21 and 36% for 7,500 and 15,000 psi, respectively, indicating a corresponding inactivation of the GP oxidase. Also, there is a transfer of GP oxidase activity from the particulate fractions into the soluble 110,000g supernatant; at the greatest applied pressure of 15,000 psi some 48% of the recovered activity is found in the soluble 110,000g supernatant. When this is further centrifuged at 314,000g for 120 min at 4°C, the majority of the GP oxidase activity is found in the 314,000g pellet indicating that the enzyme is probably associated with high molecular weight membrane fragments (table 3.1). This is confirmed by gel exclusion chromatography as described below. Several GP oxidase fractions were prepared essentially as described in fig. 3.4 using 15,000 - 25,000 psi to disrupt the cells and maximise the yield of GP oxidase in the 314,000g pellet (P IV) and subsequently chromatographed on unsubstituted agarose gels. Fig. 3.5 shows the typical elution pattern of the GP oxidase on Biogel A, 0.5 m agarose. No purification is obtained as the enzyme and protein were totally excluded from the gel. Similar results to those presented in fig. 3.5 are obtained when 314,000g pellets are eluted from Biogel A,1.5 m and A,5m columns. In all experiments, the maximum purification obtained by chromatography is less than 2 fold.

Fractionation by ammonium sulphate precipitation or sonication of 314,000g pellets prepared using the French pressure cell prior to gel exclusion chromatography also gives disappointing purifications. It is concluded, therefore, that the French pressure cell, even under the most extreme conditions does not lead to

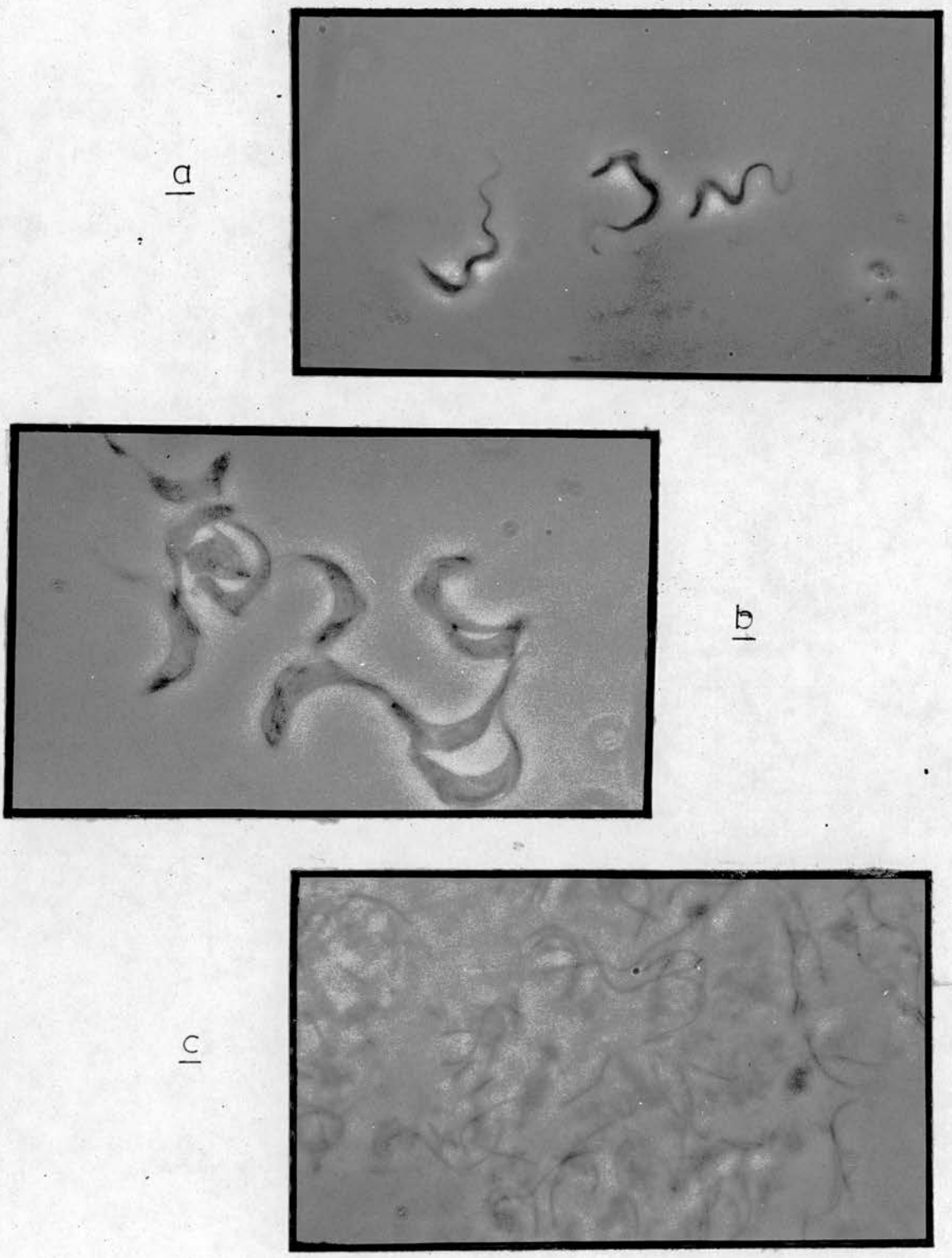


Fig. 3.6. Phase contrast photomicrographs of trypanosomes a) untreated, b) treated with 0,5% (w/v) saponin, c) after homogenising 30 times. Magnification x 1120.

TABLE 3.2. The effect of various concentrations of saponin on whole trypanosomes and their sensitivity to subsequent disruption by gentle homogenisation.

SAPONIN CONCENTRATION (% w/v)	Microscopic appearance after 15' exposure to saponin: Motility	Other observations	(a)% protein released by saponin into 2,700g supernatant	(b)Effect of homogenisation	(c)% GP oxidase released by homogenisation
0.0	++	Normal	0	100% intact	0
0.005	++	Normal	-	-	
0.025	±		-	-	
0.05	-	Cells swollen and less refractile under phase contrast microscopy	18.1 (n=1)	Majority intact	13.0(n=1)
0.25	-	Cells swollen and less refractile under phase contrast microscopy	-	-	
0.5	-	Some free vesicles visible in 0.5% saponin (i.e. slight disruption occurring)	29.4 ± 1.9 (n=7)	Complete disruption	41.5 ± 3.1(n=11)

(a) trypanosomes exposed to saponin - SEP buffer, pH 7.4, then sedimented in an 8 x 50 ml MSE 4L centrifuge (swingout bucket rotor) at 2,700g for 20 min.

(b) pelleted trypanosomes resuspended in SEP buffer, pH 7.4 and homogenised 40 strokes with a hand-operated Dounce homogeniser.

(c) homogenate centrifuged at 1,000g for 20 min (MSE 18, 8 x 50 ml fixed angle rotor) and amount of GP oxidase released into the 1,000g supernatant was determined.

solubilisation of the GP oxidase, but rather to the production of small membrane fragments with a molecular weight by gel exclusion chromatography greater than 5×10^6. This method of disruption was abandoned as unsuitable for the purification of the GP oxidase.

3. Disruption of bloodstream trypanosomes by homogenisation following pre-treatment with saponin

A number of detergents were investigated for lytic properties against trypanosomes. Except for saponin which stimulated the GP oxidase activity, all the detergents completely inhibit the GP oxidase activity when present at 1% (w/v), approximately 1 mg detergent/mg protein. The detergents tested were sodium cholate, deoxycholate and dodecylsulphate, Triton X - 100, Tween 80, Brij 35, Cemulsol, Lubrol W.X and OP 10.

When whole live trypanosomes are suspended in saponin at a concentration greater than 0.05% (w/v), the cells become swollen and immotile and appear less refractile when examined under phase contrast microscopy (fig. 3.6a, b, table 3.2). Bloodstream trypanosomes are extremely resistant to disruption by gentle homogenisation in the absence of saponin (Table 3.2). However, after prior treatment with 0.5% (w/v) saponin and its removal by centrifugation, the cells can be easily broken by gentle homogenisation. After exposure to 0.05% (w/v) saponin, although the cells are immotile and swollen, less protein is released into the saponin supernatant and the majority of the cells are intact after homogenisation. A concentration of 0.5% w/v saponin is employed in all further

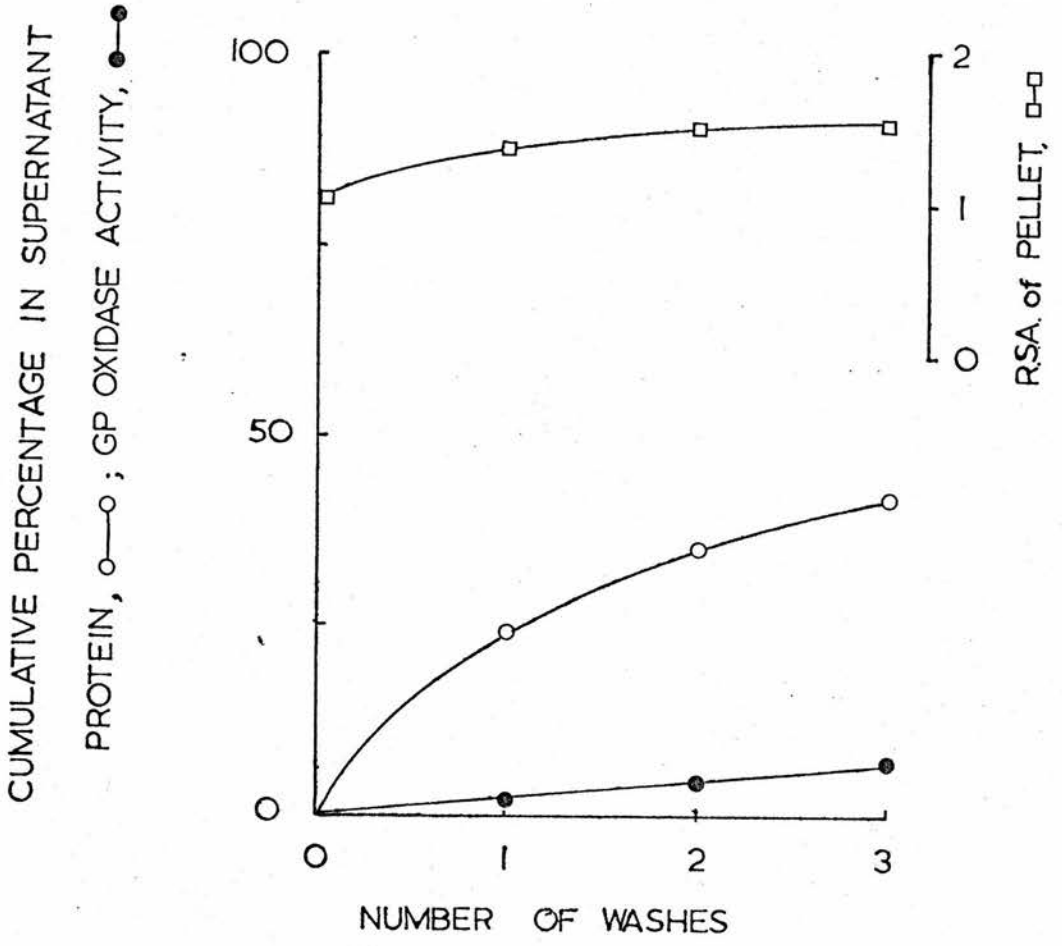


Fig. 3.7. Effect of repeated washing of whole cells treated with 0.5% (w/v) saponin. Whole trypanosomes were suspended in 0.5% (w/v) saponin in SEP buffer, pH 7.4, then sedimented at 2,700 g for 20 min in an MSE 4L centrifuge (8 x 50 ml swing-out bucket rotor). The supernatant was carefully removed and the pellet of cells gently resuspended in an equal volume of SEP buffer and centrifuged as before. Samples of pellets and supernatants were assayed for GP oxidase and protein. Mean recovery of activity and protein for each wash was 100.6% and 98.5%, respectively.

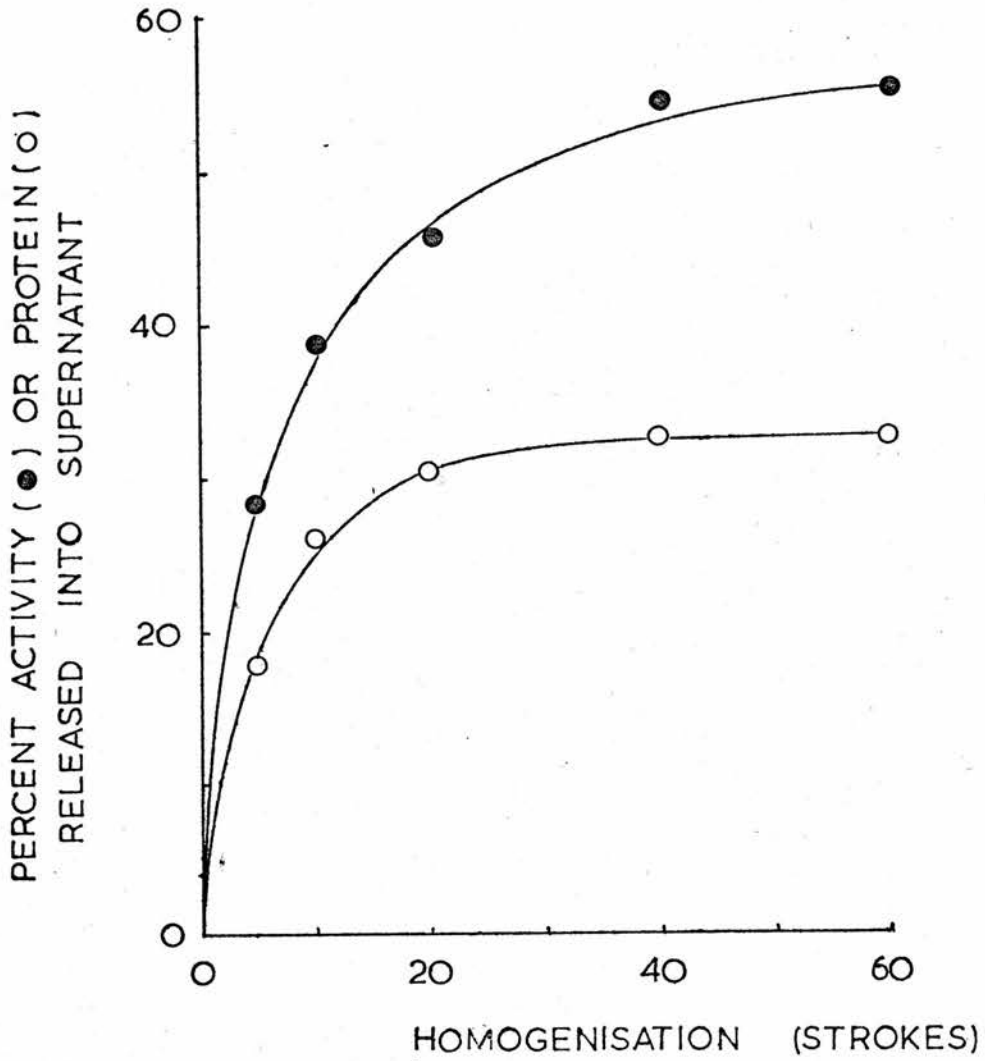
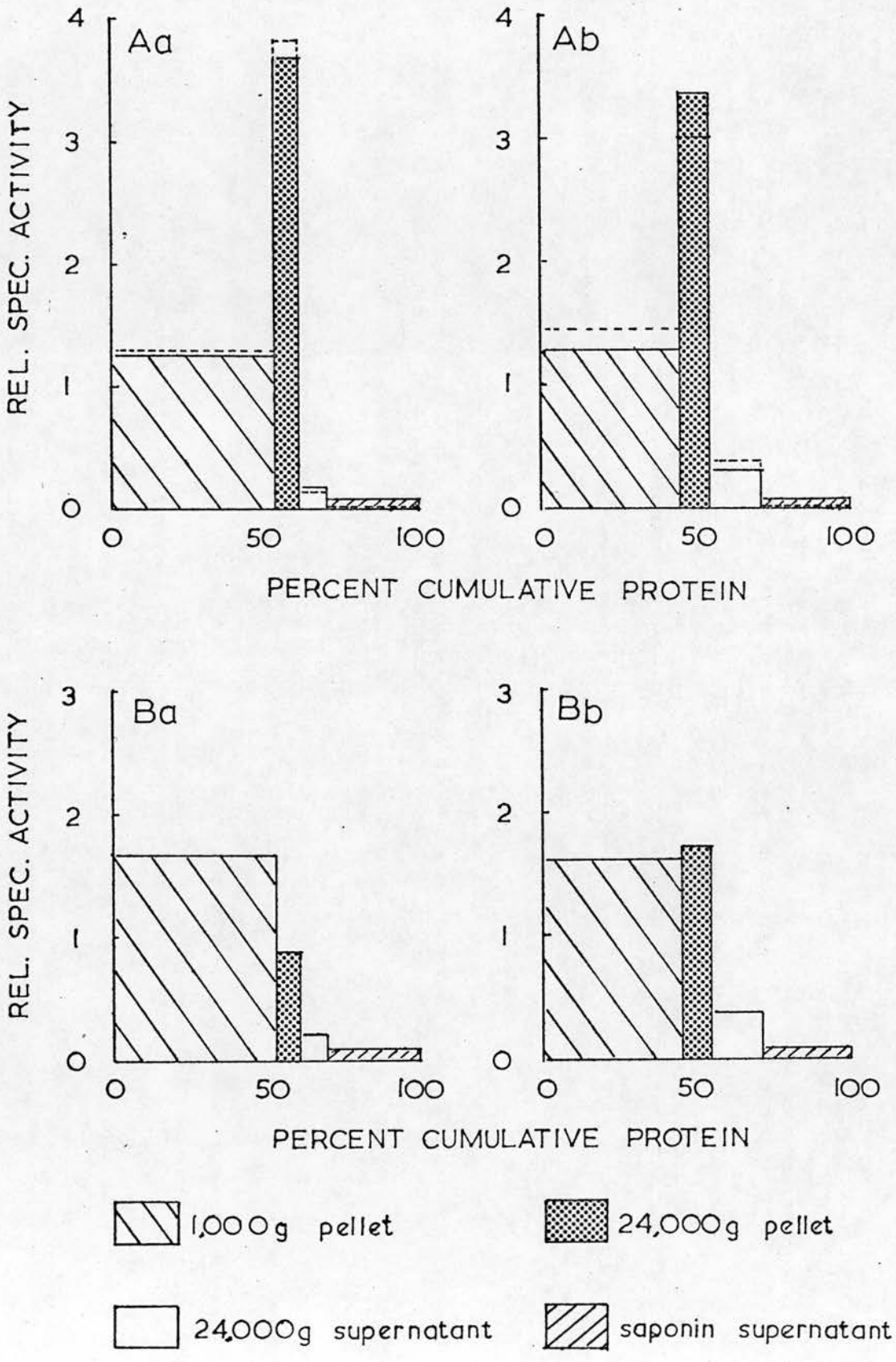


Fig. 3.8. Determination of optimal conditions for the disruption of trypanosomes pretreated with 0.5% (w/v) saponin. Whole cells were sedimented at 3,000 g for 20 min after exposure to saponin and the pellet of cells resuspended in SEP buffer, pH 7.4, then disrupted using a hand-operated Dounce homogeniser. Samples were removed after 5,10,20,40 or 60 strokes of the pestle and centrifuged at 3,000 g in an MSE 4L swing-out bucket rotor. The supernatants were removed and assayed for GP oxidase activity and protein. Percentage activity or protein released from the homogenate: GP oxidase, ● and protein, ○.

fractionation experiments. As noted in table 3.2 about one quarter of the total trypanosomal protein is removed after exposure to 0.5% (w/v) saponin. If the cells are further washed by SEP buffer, smaller amounts of protein are released from the cells and the relative specific activity of the GP oxidase in the whole cell pellet is increased to about 1.5 (fig. 3.7). After centrifuging the saponin treated cells 3 times, 7% of the total GP oxidase activity is released from the cells, indicating that the majority of the cells are still intact, but with subsequent washes the cells rapidly disintegrate. In the routine preparative scheme a single centrifugation step is used.

The amount of protein and GP oxidase activity released is also dependent on the extent of homogenisation (fig. 3.8). After 40 strokes of the homogeniser pestle the protein and GP oxidase released from the cells reaches a maximum value. 20-40 strokes of the homogeniser are required to obtain complete disruption of the cells, and, to minimise the homogenisation necessary to achieve this in each preparation, samples are routinely examined by phase contrast microscopy after 20, 30, or 40 strokes. Fig. 3.6c is a photomicrograph of the homogenate showing the extent of the disruption. Note that the majority of the flagella are intact unlike other methods of disruption such as the French pressure cell, nitrogen cavitation and sonication in which the flagella are broken into smaller fragments.


Aa
Ab
Ba
Bb
REL. SPEC. ACTIVITY
PERCENT CUMULATIVE PROTEIN
0
50
100
1
2
3
4
1,000g pellet
24,000g pellet
24,000g supernatant
saponin supernatant

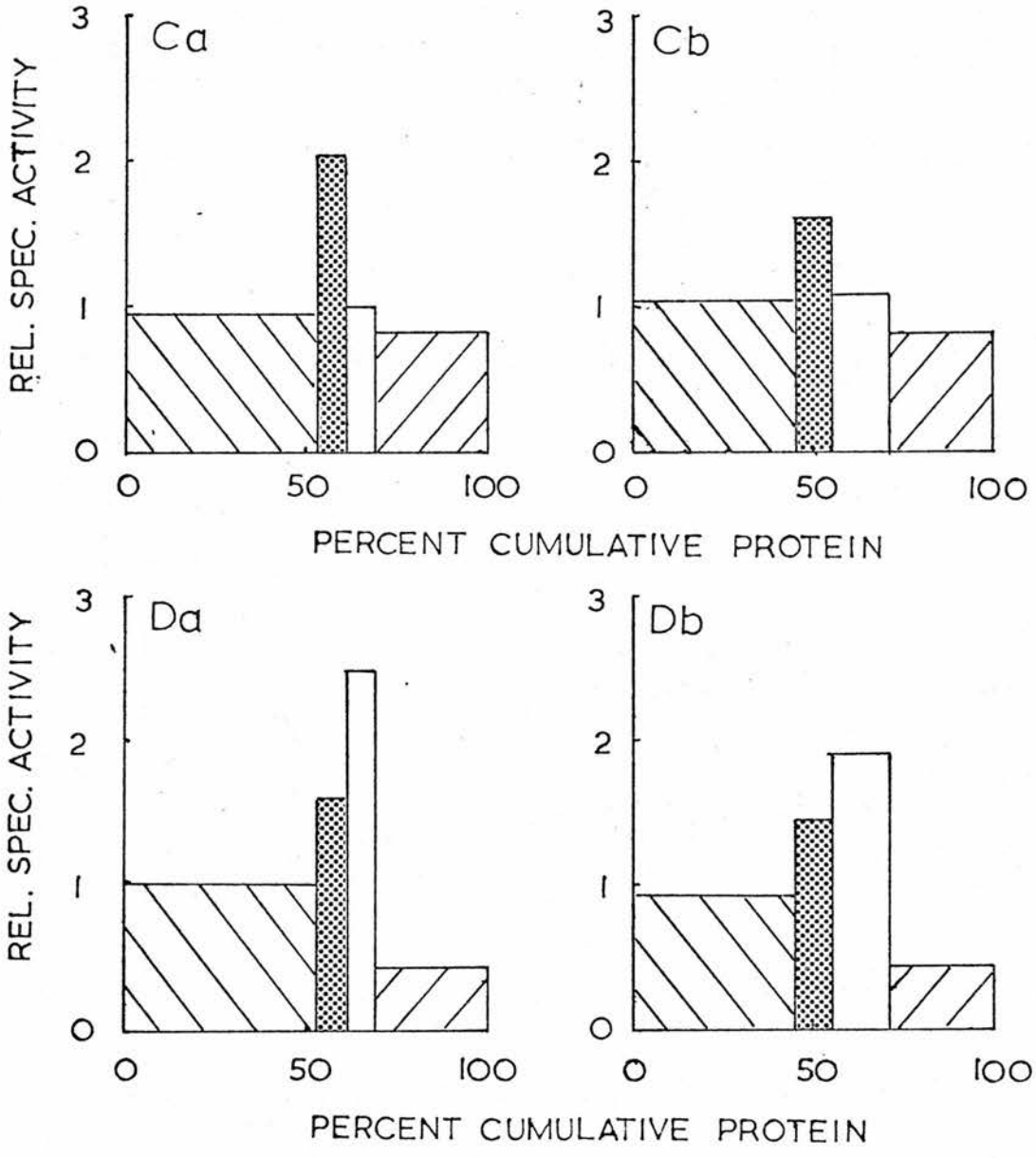


Fig. 3.9. Effect of protein concentration on the subcellular distribution of several enzymic activities prepared by the standard procedure given in fig. 2.2, omitting the 2 washes of the 1,000 g pellet and the filtration step through CM-23 cellulose. After pretreatment with saponin, the pelleted cells were resuspended in a) 1 ml PCV : 10 ml SEP buffer, or b) 1 ml PCV : 20 ml SEP buffer, pH 7.4 and homogenised with 40 strokes of a Dounce homogeniser. Enzymic activities and their recoveries (a, and b, respectively) are: GP oxidase, A, solid line (105, 108); GP tetrazolium reductase, A, dashed line (80, 99); NAD^+-dependent GPDH, B (85, 90); acid phosphatase, C (77, 83); NADH tetrazolium reductase, D (101, 102); protein (90, 94).

TABLE 3.3. Relative distribution of GP oxidase and GP dehydrogenase activities after fractionation by the standard procedure.*

FRACTION	GP OXIDASE (-PMS)		GP DEHYDROGENASE(+PMS)		PROTEIN
	-BSA	+ BSA	-BSA	+BSA	
1,000g PELLET(washed x1)	21.5	30.8	24.1	37.7	28.4
24,000g PELLET	76.5	66.0	71.8	55.6	16.5
144,000g PELLET	2.0	3.2	4.0	6.7	6.4
144,000g SUPERNATANT	0	0	0	0	24.2
SAPONIN SUPERNATANT	0	0	0	0	24.5

* as fig. 2.2, omitting the two washes of the 1,000g pellet and the filtration of the 1,000g supernatant through CM23 cellulose. Distribution of enzymic activities and protein are expressed as a percentage of the total recovered. GP oxidase was assayed in TS buffer, pH 8.0 $\pm$ bovine serum albumin (3.3 mg/ml) and the GP dehydrogenase by the addition of 1 mM PMS. Mean recovery for GP oxidase, dehydrogenase and protein was 79.5%, 75.5% and 70.0%, respectively.

4. Subcellular fractionation by differential centrifugation

The subcellular distribution of a number of enzyme activities released from cells disrupted by the saponin method is illustrated in fig. 3.9. GP oxidase and GP tetrazolium reductase show a similar pattern of distribution in the fractions with the majority associated with the 1,000g and 24,000g particulate fractions and less than 10% in the supernatant fractions. The saponin supernatant can be regarded as a soluble fraction because centrifugation at 144,700g for 60 min fails to sediment more than 5% of the protein. The more dilute homogenate (b) contains slightly more of the GP oxidase in the 24,000g pellet than homogenate (a) and therefore is routinely used in preparations of the GP oxidase. The NAD^+-dependent GPDH does not show a similar distribution in the subcellular fractions to either the GP oxidase or the GP tetrazolium reductase, but appears to be largely associated with the heavy particulate fraction. Acid phosphatase and NADH tetrazolium reductase are distributed in both soluble and particulate fractions.

Table 3.3 shows the distribution of the GP oxidase and the GP dehydrogenase (GP: PMS oxidoreductase) fractionated by the standard procedure. The distribution of both enzyme activities is essentially the same in each fraction, which is in contrast to Bide's findings (1964) in which he claimed that the distribution of these two activities was dissimilar and therefore the GP: PMS oxidoreductase was not a component of the GP oxidase. The data given in table 3.3 and the results section on inhibitors supports the view that the dehydrogenase is a component of the oxidase as inhibitors of the GP dehydrogenase are equally effective inhibitors

of the GP oxidase in crude and purified preparations (see suramin and analogues, pOMB, melarsen oxide, Tiron and o-phenanthroline).

5. Removal of contaminating flagella from glycerophosphate oxidase preparations

Examination by phase contrast microscopy shows that the 1,000 - 24,000g pellet prepared from homogenates obtained by the saponin method contains large numbers of small vesicles and flagella. Modification of the centrifugation scheme to sediment some (3,000g for 10 min) or all (7,000g for 10 min) of the contaminating flagella, thereby increasing the relative specific activity of the GP oxidase pellet (3,000 - 24,000g or 7,000 - 24,000g) results in unacceptably low yields of the enzyme. Batchwise addition of a number of materials including cellulose, carboxymethyl - (CM) and diethylaminoethyl - (DEAE) celluloses or neutral alumina (200-400 mesh) to 1,000g supernatants, followed by removal at low centrifugal forces does not markedly improve the specific activity of the final GP oxidase pellet; neither does suction through a 5 micron millipore filter, sintered glass or a double layer of fine nylon netting (MOnyl, 30 micron mesh) or suction through columns packed with glass wool, fine glass beads (no.8, Ballotini), or sodium aluminium silicate (Permutit). However rapid suction through 1 cm thick pads of cellulose powder or DEAE or CM ion exchange celluloses does lead to an improvement in purification of the GP oxidase, albeit inconsistently. When 1,000g supernatants are percolated through columns of various celluloses, the GP oxidase becomes tightly bound to the DEAE celluloses and flow

TABLE 3.4. Results of purification of GP oxidase by the standard procedure and the effect of CM 23 cellulose filtration step on the final purification and yield.

				SAPONIN TREATED CELLS				24,000g PELLET						
PREP. BUFFER	No.of expts.	No. of washes at 1,000g	CM 23 CELL.	PCV	Total Activity	Total Protein	Specific Activity	Total Activity	Total Protein	Specific Activity	RSA	Yield	Protein/ ml PCV	Activity/ ml PCV
SEP buffer pH 7.4	8	1	-	5.0	74,700	359	208 ± 34	32850	31.0	1059* ± 119	5.1* ±0.9	44.7 ± 9.3	71.7	14910
	17	2	+	4.2	62,800	359	175 ± 18	33500	18.0	1858* ± 522	10.6* ± 3.0	53.4 ± 9.4	85	14800
TS buffer pH 7.4	3	2	-	2.8	40,380	200	202 ± 6	18700	19.7	949+ ± 88	4.7+ ± 0.4	48.8 ± 13	71.4	14420
	8	2	+	6.8	10,3000	515	200 ± 24	44100	23.6	1866+ ± 437	9.3+ ± 1.9	42.8 ± 104	76	15100

All results are expressed as the mean ± standard deviation. Highly significant differences ($P<0.0005$) were found between pairs of values marked * or +, with or without CM 23 cellulose treatment. PCV: Packed cell volume; RSA: relative specific activity. Units: PCV, ml; total activity, nmol O_2/min; total protein, mg; specific activity, nmol O_2/min/mg protein; yield, %.

rates rapidly decrease in columns of the microgranular CM or DEAE celluloses. Cellulose powder and CM23 cellulose give the best results, increasing the purification by about 2 fold.

The effect of filtration through CM23 cellulose is illustrated in table 3.4. These preparations are made by the standard method outlined in fig. 2.2. Inclusion of the CM23 cellulose step in the purification scheme increases the relative specific activity of the GP oxidase in the final 24,000g preparation by a factor of 2, with a mean loss of 6% of the total starting activity (table 3.4, preparations in TS buffer). The earlier preparative method employed 0.25 M sucrose, 0.1 mM EDTA, 10 mM phosphate buffer, pH 7.4, but later work used 0.25 M sucrose, 10 mM tris buffer, pH 7.4 as phosphate ions and EDTA were found to interfere with metal analyses. The final specific activity of the GP oxidase preparations is the same, regardless of the preparative buffer used, but the mean yield of GP oxidase activity is decreased 10% when TS buffer is used. The effect of EDTA, phosphate or K^+ ions on the yield of GP oxidase prepared with sucrose - tris buffer has not been investigated.

One of the problems encountered in fractionation of the homogenates is the loosely packed nature of the 1,000g pellet after centrifugation for 10-20 min. If the rotor is decelerated with the brake applied visible mixing of the 1,000g pellet and supernatant occurs leading to lower specific activities in the 24,000g pellet, especially when the CM23 cellulose step is omitted. Therefore, for the 1,000g centrifugation steps the rotor is decelerated without applying the brake. When the 24,000g pellets prepared by the standard method are examined under phase contrast microscopy, the absence of flagellar

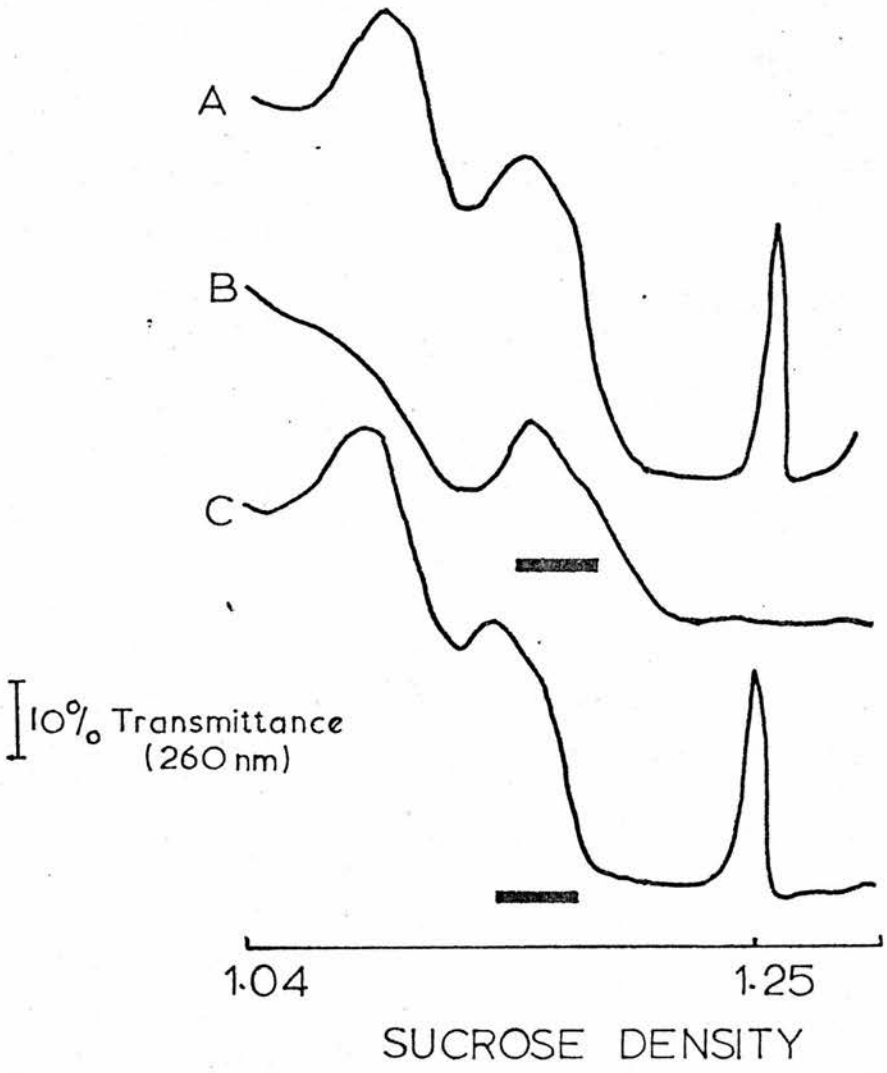


Fig. 3.10. Effect of filtration of a 1,000 g supernatant through a column of CM23 cellulose. Equal volumes (10 ml) of supernatant were loaded onto linear 0.25-2.5 M sucrose density gradients, centrifuged and fractionated as described in the methods. Tubes A and C, no treatment; tube B filtered through CM23 cellulose. The horizontal bar indicates the peak of GP oxidase activity. The peak of GP oxidase activity in tubes B and C was at d_4^{20} = 1.164 and 1.169, respectively.

TABLE 3.5. Comparison of protein estimation methods on fractions obtained from isopycnic sucrose density gradient experiments.

Experiment	Method of estimating ratio $^{B}/_{A}$	Number of individual fractions analysed	Ratio of method $^{B}/_{A}(\pm SD)$
GIIA	Linear regression analysis *	5	1.91 ± 0.08
GIII	Mean of individual fractions *	6	1.90 ± 0.19
GVII	Linear regression analysis +	20	2.00 ± 0.08
	Mean ratio		1.94 ± 0.29

Individual fractions were assayed for protein content by method A (Lowry et al. 1951) and method B (Warburg and Christian, 1942). Values marked * were determined on fractions containing the peak of the GP oxidase activity; + determined on all fractions from the gradient.

fragments is most striking compared to preparations in which the CM23 cellulose step is omitted. The mode of action of the CM23 cellulose step has not been extensively investigated, but filtration would appear to be the main factor, because cellulose powder is almost as effective as CM23 cellulose in removing flagella.

The effect of CM23 cellulose on the distribution of protein in isopycnic sucrose density gradients is illustrated in fig. 3.10. The absence of the protein peak at d = 1.25 is most striking, but there is also a slight decrease in the size of the protein peak containing the GP oxidase activity and in the load layer. The peak at d = 1.25 probably contains flagella, but unfortunately electron microscopic examination of this fraction has not been done.

6. Results of isopycnic sucrose density gradient experiments

Protein concentration measured by the method of Lowry, et al. (1951) and that of Warburg and Christian (1942) gives widely differing estimates for individual density gradient fractions even after both methods have been corrected for interference by the high concentrations of sucrose present. Table 3.5 compares the results obtained from 3 gradient experiments. It can be seen that the method of Warburg and Christian over-estimates the protein concentration by a factor of 2, whether determined on fractions containing the peak of the oxidase activity or on all the gradient fractions containing protein. Fig. 3.11 compares the fractional distribution of protein in the same gradient estimated by both methods. Compared in this way, the two methods are in good agreement throughout the gradient, except for

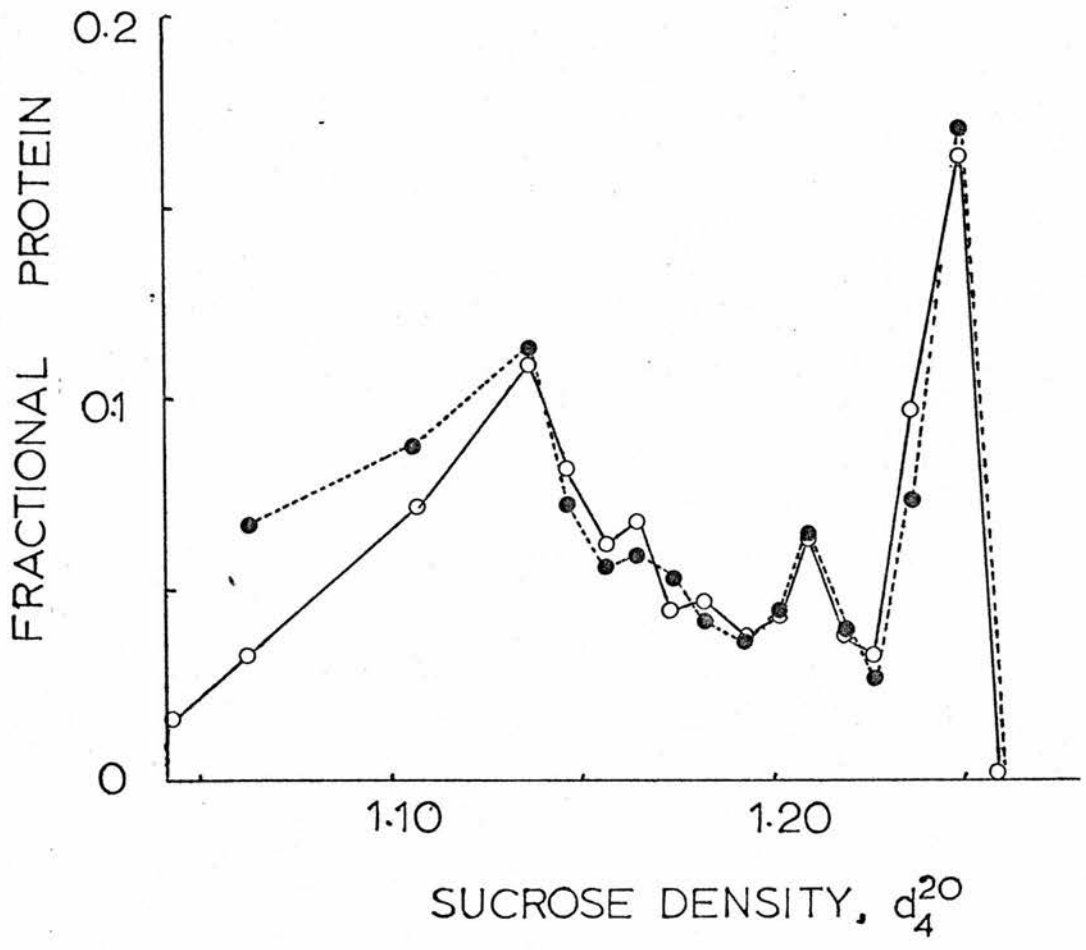


Fig. 3.11. Variation of fractional protein in a sucrose density gradient (G VII) estimated using the method of Lowry (1951), ●---●, or of Warburg and Christian (1942), o—o. Recovery of protein from the gradient using the method of Lowry was 95.4%.

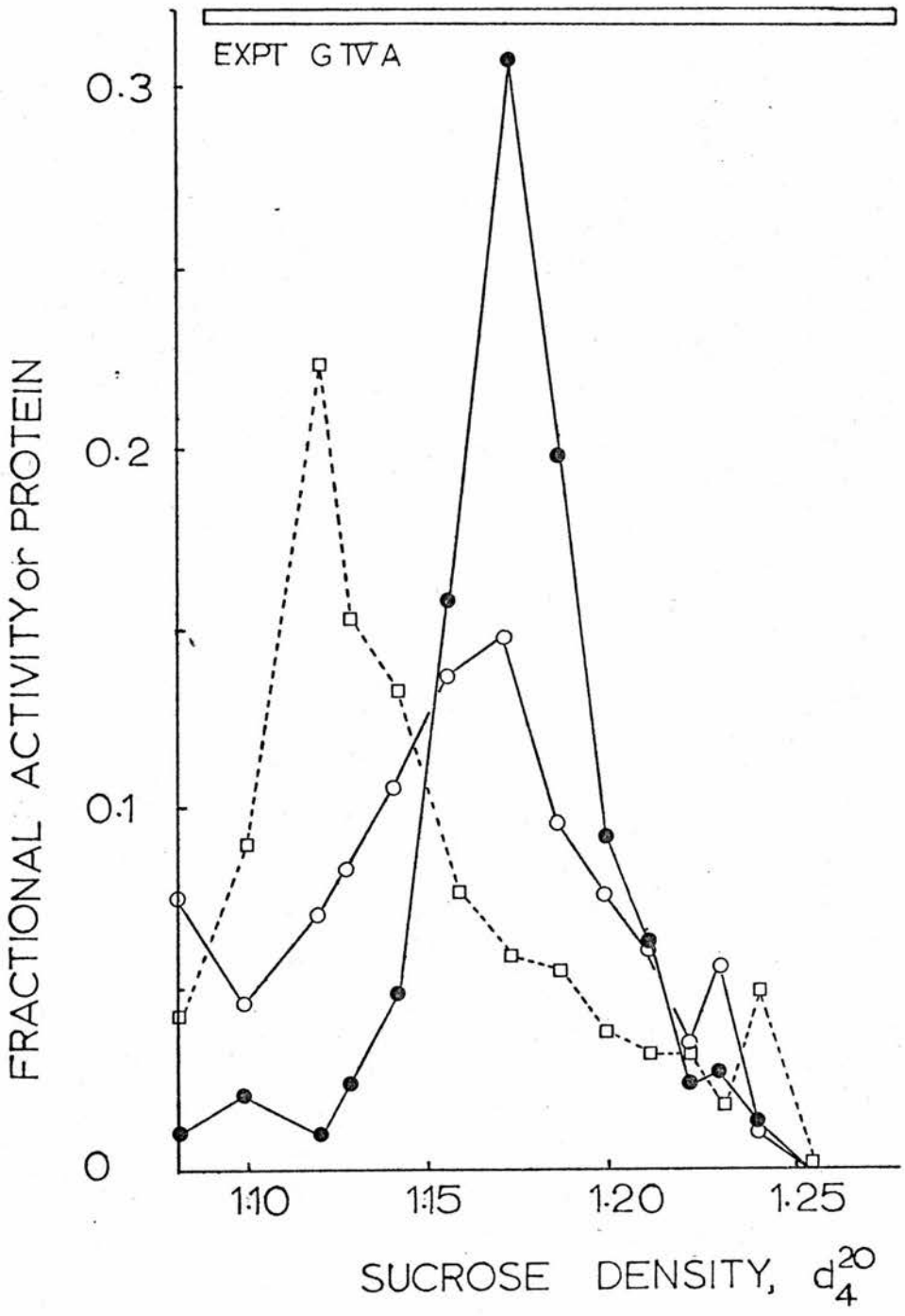


Fig. 3.12. Distribution of protein (o—o), GP oxidase (●—●) and acid phosphatase (□--□) in a sucrose density gradient after 2h. The horizontal bar indicates the linear portion of the gradient. Recovery of protein, GP oxidase and acid phosphatase was 88%, 94% and 58%, respectively. Experimental details are described in the Methods.

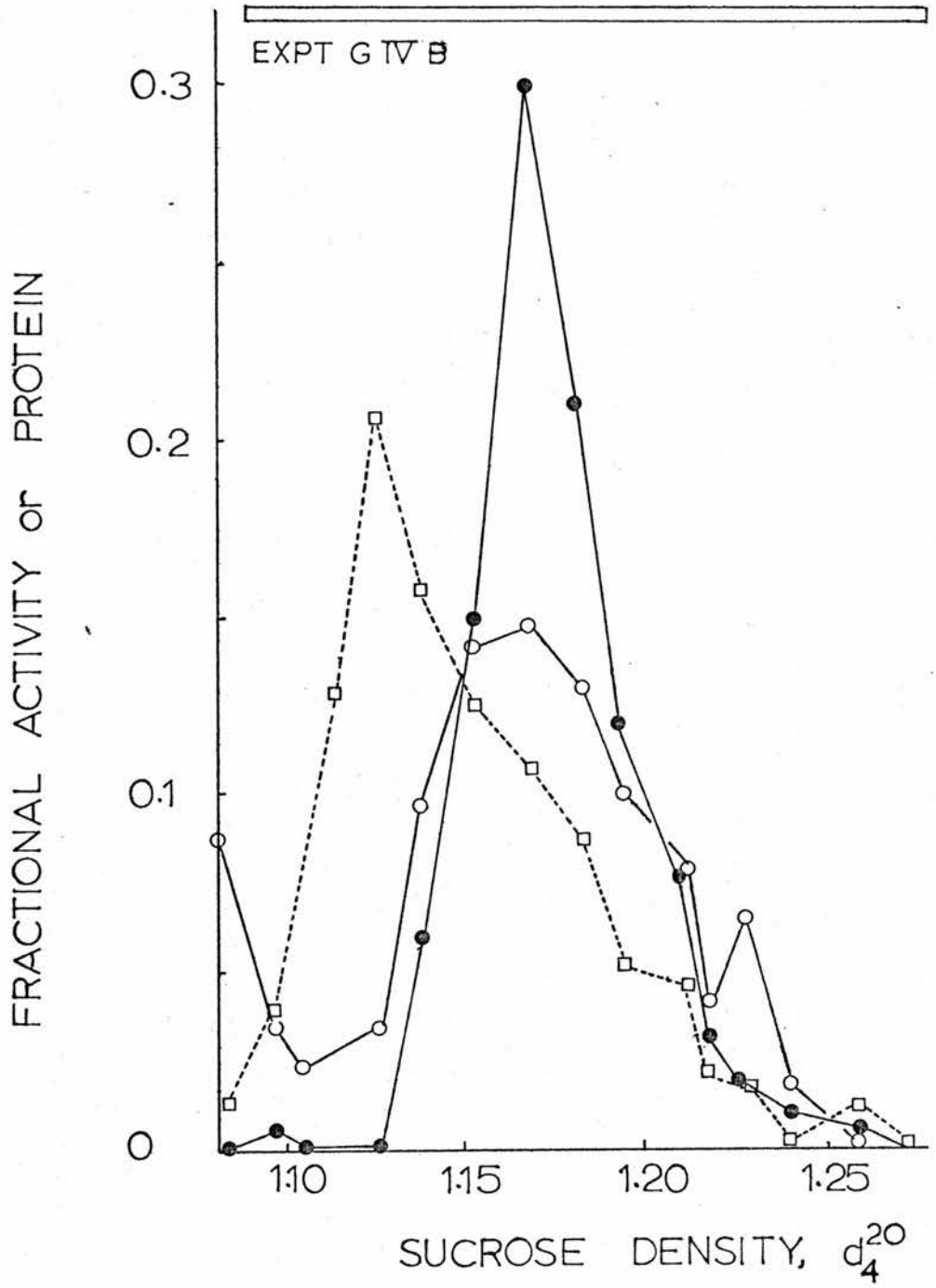
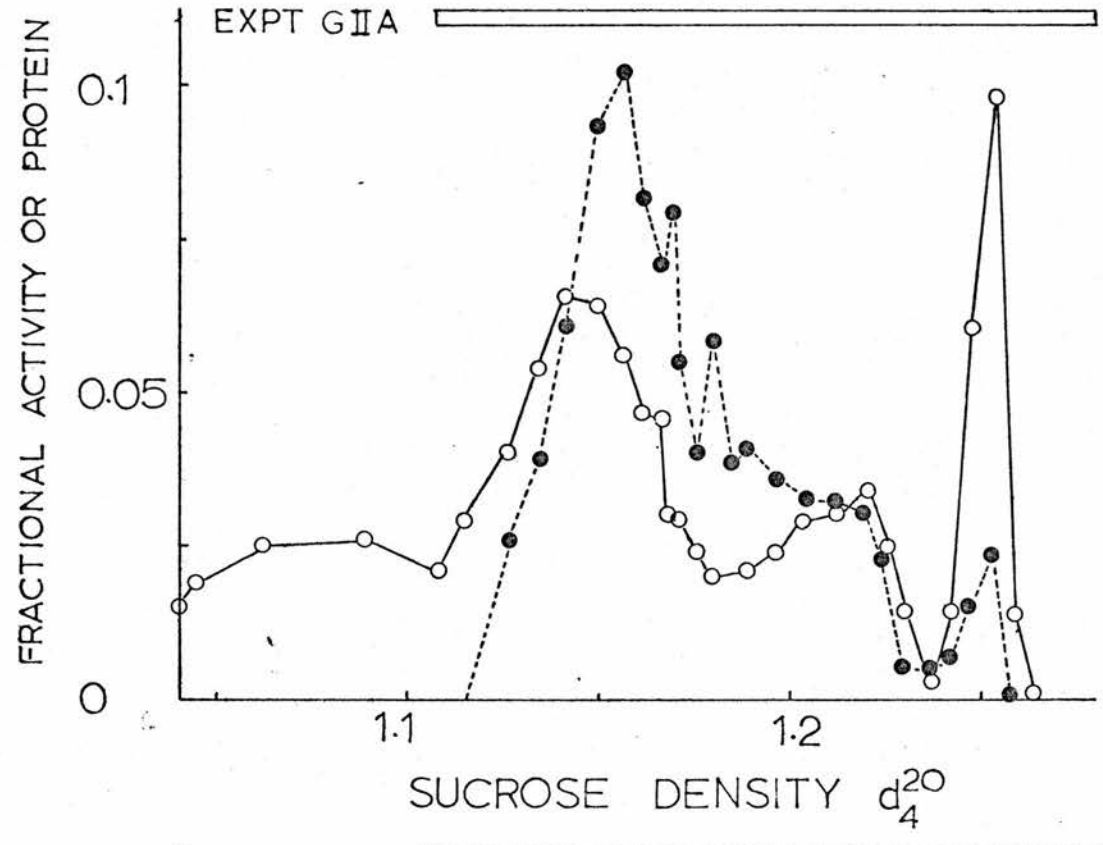


Fig. 3.13. Distribution of protein (o—o), GP oxidase (●—●) and acid phosphatase (□···□) in a sucrose density gradient after 4h. The horizontal bar represents the linear portion of the gradient. Recovery of protein, GP oxidase and acid phosphatase was 79%, 90% and 58%, respectively. Experimental details are described in the Methods.

the soluble protein in the load layer. As the method of Lowry requires such a large volume of sample from any gradient fraction (upwards of 0.5 ml/assay), the non-destructive method of Warburg and Christian is routinely employed for the assay of individual gradient fractions, and converted into equivalent Lowry protein concentrations by dividing by the factor 2.00.

The method of isopycnic sucrose density gradient centrifugation is adapted from a method developed by Borst and Opperdoes (unpublished) to whom I am most grateful for advice.

In order to establish that equilibrium of the subcellular organelles at their buoyant densities had been achieved under the conditions given in the methods, triplicate density gradients were prepared and 1 ml samples of the 24,000g pellet were layered onto two of the tubes. After centrifuging for 2 h at 23,000 rpm (38,000g at sample zone) the rotor was decelerated and one of the sample tubes removed and replaced by a blank gradient as a balancing tube and the other sample centrifuged for a further 2 h. Both gradients were fractionated into 50 drop portions and assayed for protein, acid phosphatase and GP oxidase (fig. 3.12, 3.13 respectively). After a further 2 h centrifugation the protein and acid phosphatase sedimented marginally further down the gradient, but the GP oxidase activity reaches its isopycnic density of d = 1.17 after 2 h. Note the absence of any protein peak at d = 1.25 which is removed by filtration of the 1,000g supernatant through CM23 cellulose (see fig. 3.10). Two further fractionation experiments (fig. 3.14 and 3.15) both show a protein peak in the region of d = 1.25 (d = 1.252 and d = 1.248), respectively, corresponding to the peak of NAD^+- dependent


EXPT GIIA
FRACTIONAL ACTIVITY OR PROTEIN
0.1
0.05
0
1.1
1.2
SUCROSE DENSITY d_4^{20}

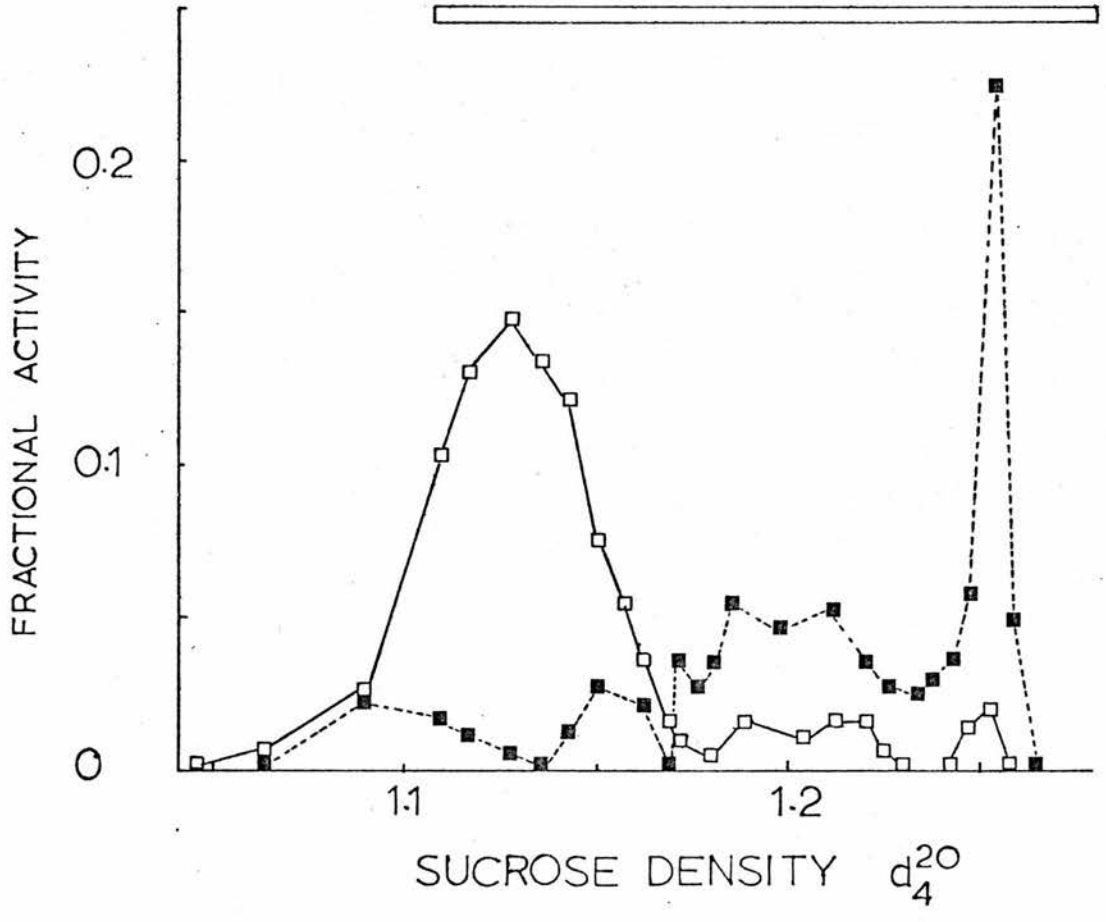

FRACTIONAL ACTIVITY
0.2
0.1
0
1.1
1.2
SUCROSE DENSITY d_4^{20}

Fig. 3.14. Distribution of glycerophosphate oxidase (●--●), protein (○—○), acid phosphatase (□—□) and NAD^+-dependent GPDH (■--■) in a sucrose density gradient after 2h. The horizontal bar represents the linear portion of the gradient. Recovery of GP oxidase and protein was 94.5% and 70%, respectively. Experimental details are described in the Methods.

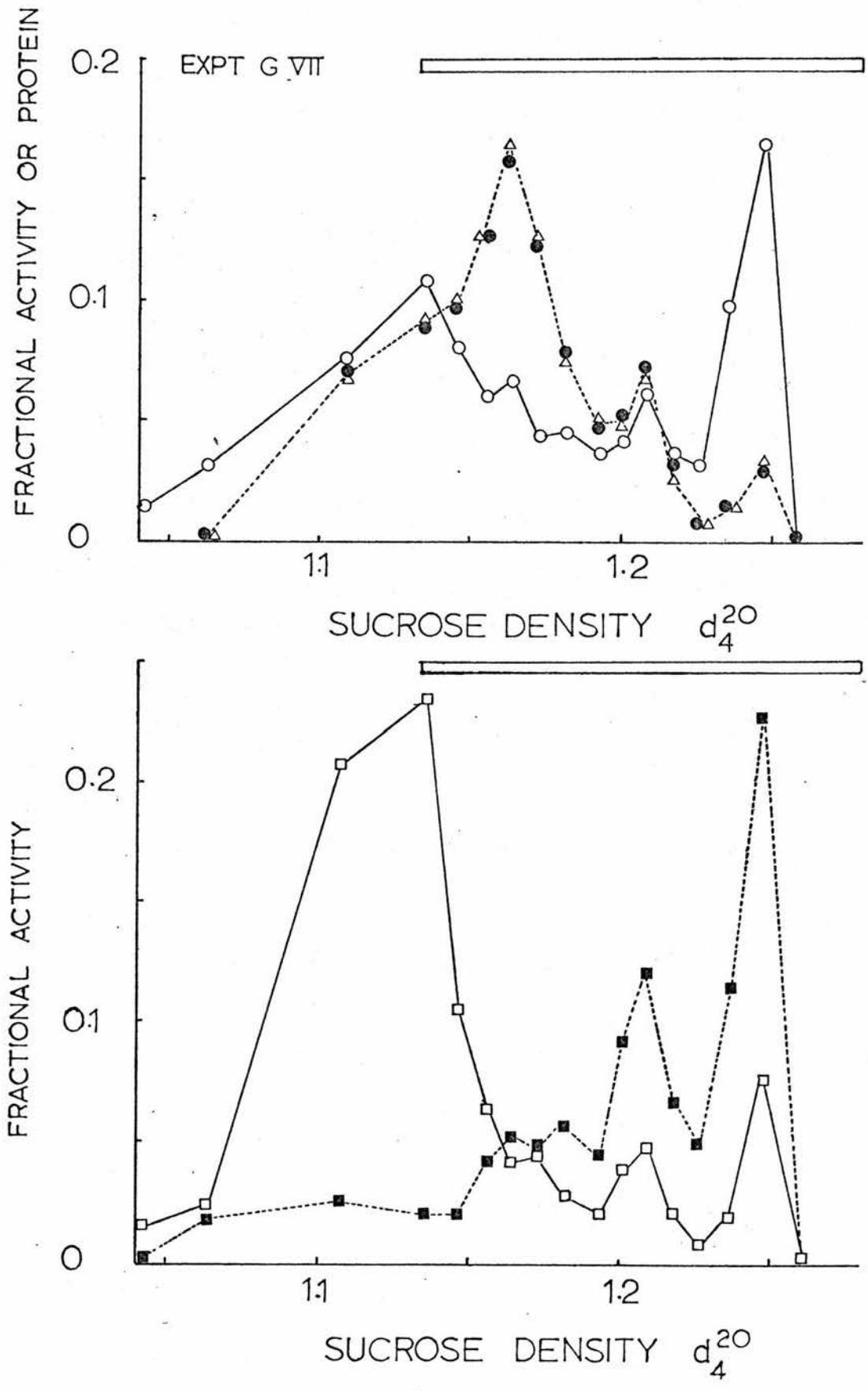

EXPT G VII
FRACTIONAL ACTIVITY OR PROTEIN
0·2
0·1
0
1·1
1·2
SUCROSE DENSITY d_4^{20}
FRACTIONAL ACTIVITY
0·2
0·1
0
1·1
1·2
SUCROSE DENSITY d_4^{20}

Fig. 3.15. Relative distribution of GP oxidase (●···●), GP tetrazolium (INT) reductase (△···△) and NAD^+-dependent glycerophosphate dehydrogenase (■···■) in a sucrose density gradient. Fractional protein (o—o) and acid phosphatase (□—□) are also indicated. The horizontal bar represents the linear portion of the gradient. The recovery of GP oxidase activity and protein was 96.3% and 95.4%, respectively.

GPDH activity. Reference to table 3.6 indicates that the specific activity of the oxidase pellet layered onto the gradient is less for experiments GIIA and GVII (1030 and 1550 nmol O_2/min/mg protein, respectively) than that of GIVA and B in which the protein peak at d = 1.25 is absent. These results suggest that partial overloading of the CM23 cellulose column is occurring in expt. GIIA and GVII. Another experiment (GIII), however, does not show a peak of protein at d = 1.25, but rather a pronounced shoulder at d = 1.22 - 1.24, which contains the peak of NAD^+- dependent GPDH activity (results not plotted).

Fig. 3.15 also compares the distribution of the GP oxidase and GP tetrazolium reductase activities in a sucrose density gradient. The fractional activity of these two enzymes is distributed identically throughout the gradient, with the NAD^+- dependent GPDH corresponding to neither.

A summary of the distribution of GP oxidase, protein, acid phosphatase and NAD^+- dependent GPDH obtained in 5 separate gradient experiments is presented in fig. 3.16. The GP oxidase activity forms a single symmetrical peak at a modal density of 1.16 - 1.18. Approximately 82% of the total protein is distributed in the linear portion of the gradients (d = 1.12 - 1.30) with a modal density of. d = 1.14 - 1.16, with 18% in the load layer and non-linear portion of the gradient (d = 1.04 - 1.12). The density cohort d = 1.24 - 1.26 has a large S.E.M. because of the marked variation in the fractional proteins at this density, which as discussed previously is probably due to breakthrough of this fraction on the CM23 cellulose column and hence variation in the purity of the oxidase containing fraction

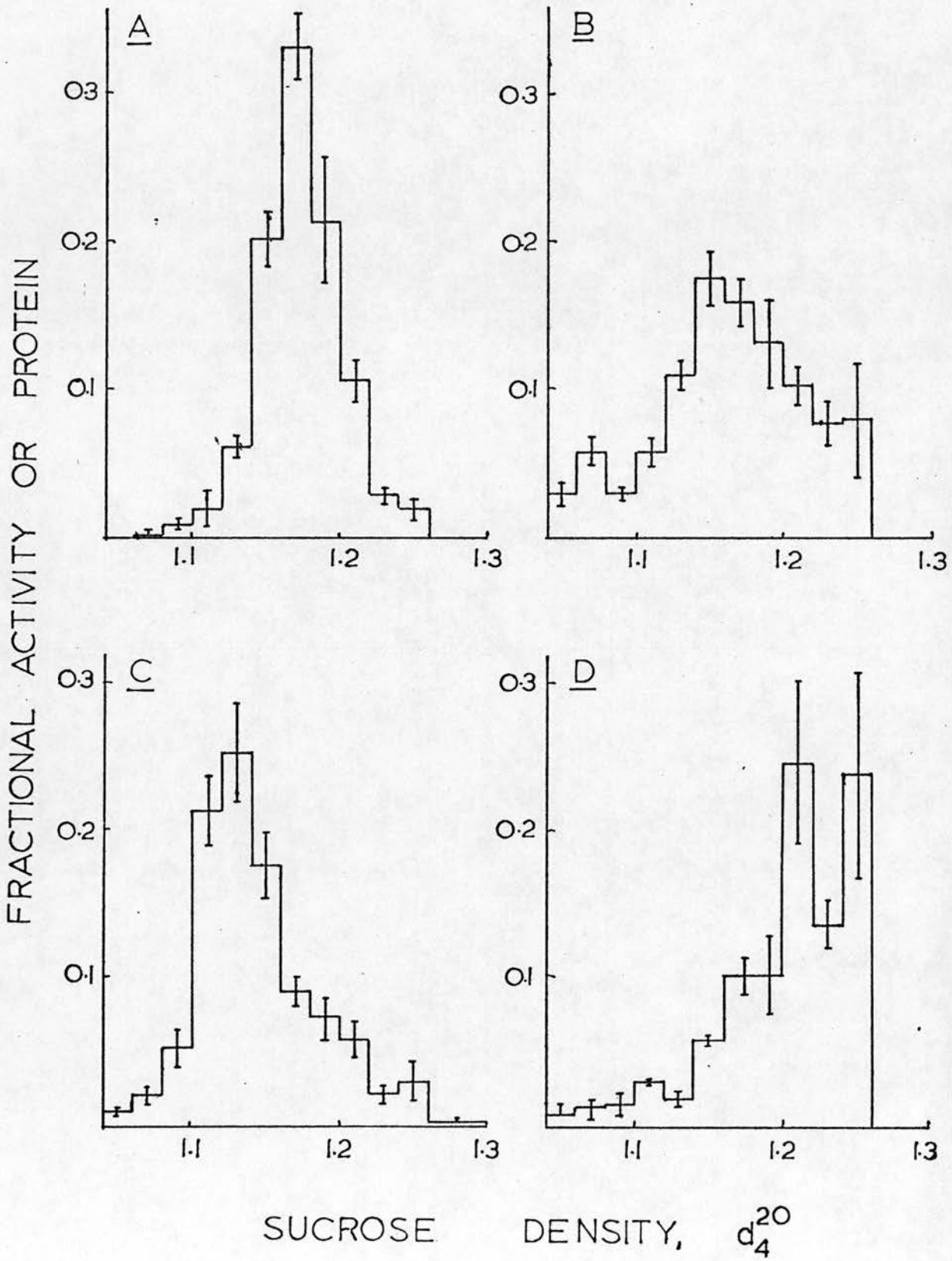

A
B
C
D
0·3
0·2
0·1
1·1
1·2
1·3
FRACTIONAL ACTIVITY OR PROTEIN
SUCROSE DENSITY, d_4^{20}

Fig. 3.16. Summary of the distribution of enzymic activities and protein in linear sucrose density gradients obtained from 5 separate experiments. Averaged density-frequency histograms were calculated for density increments of 0.02. Vertical bars represent ± standard error of the mean. Glycerophosphate oxidase, A; protein, B; acid phosphatase, C; NAD^+-dependent GPDH, D. [NAD^+-dependent GPDH was determined in 3 experiments only]. Percentage recoveries (mean ± S.E.M.) were 95 ± 2 (n=5) for GP oxidase, 58.2 ± 0.1 (n=2) for acid phosphatase and 84 ± 5 (n=5) for protein.

TABLE 3.6. Purification of GP oxidase by differential centrifugation and isopycnic sucrose density gradient centrifugation

		SAPONIN TREATED CELLS			24,000g PELLET			PEAK OXIDASE ACTIVITY FROM GRADIENT					
EXPT. NO.	T.	TOTAL ACTIVITY	TOTAL PROTEIN	SPECIFIC ACTIVITY	TOTAL ACTIVITY	TOTAL PROTEIN	SPECIFIC ACTIVITY	TOTAL ACTIVITY	TOTAL PROTEIN	SPECIFIC ACTIVITY	MODAL DENSITY	OVERALL PURIFICATION	OVERALL YIELD %
GIIA	2	15,800	103	153	6880	6.7	1030	3690	1.29	2870	1.158	18.7	23.4
GIII	4	24,370	149	164	7600	5.4	1405	4561	1.49	3067	1.176	18.7	18.7
GIVA&B	2 + 4	26,200	188	139	12400	6.2	2000	7380	2.38	3101	1.172(A) 1.169(B)	18.9	28.2
GV	2	30,560	186	164	14,820	6.9	2154	8210	1.34	6127	1.175	37.4	26.9
GVII	2	25,840	175	148	12750	8.3	1550	5960	1.58	3766	1.164	25.5	23.1
Mean				154			1628			3786	1.169	23.8	24.1
S.E.M.				± 4			± 204			± 604	± 0.003	± 3.6	± 1.7
n				5			5			5	6	5	5

The peak of the GP oxidase activity was arbitrarily selected to contain 45-60% of the total activity loaded onto the gradient. Experiment GV was used for electron microscopy (fig. 3.17). Units: total activity, nmol O_2/min; total protein (Lowry method), mg; specific activity, nmol O_2/min/mg protein. T. = time in h of gradient centrifugation procedure. Other experimental details as in the Methods.

that was loaded onto the gradient. The NAD^+- dependent GPDH forms two major peaks of modal density d = 1.20 - 1.22 and 1.24 - 1.26 clearly separate from the GP oxidase peak. The large S.E.M. for these two peaks is ascribed to the same cause as for that of the protein results. The NAD^+- linked GPDH can therefore be used as an index of purity for the GP oxidase preparations. Acid phosphatase, a lysosomal marker enzyme, equilibrates at a modal density of d = 1.14 - 1.16, which is lower than the GP oxidase, but corresponds to the modal peak of the protein. Lysosomes normally sediment in sucrose density gradients to a density of 1.22 and the reasons for this apparently anomalous behaviour is considered in the discussion section of this thesis.

In table 3.6 the purification of the GP oxidase by differential centrifugation and isopycnic sucrose density gradient centrifugation is summarised. Fractions containing the GP oxidase in highest specific activity are selected so that approximately 50% of the total activity loaded onto the gradient is included. Calculated in this manner a mean overall purification of 23.8 fold and overall yield of 24.1% is obtained relative to the specific activity of the starting material, saponin treated cells. The maximum purification is correspondingly higher and the overall yield lower if one selects a single gradient fraction. The mean purification of the GP oxidase in the 24,000g pellet loaded onto the gradient is 10.5 fold and the sucrose gradient centrifugation contributes a further 2.3 fold. The mean modal density for the GP oxidase particles is at d = 1.169.

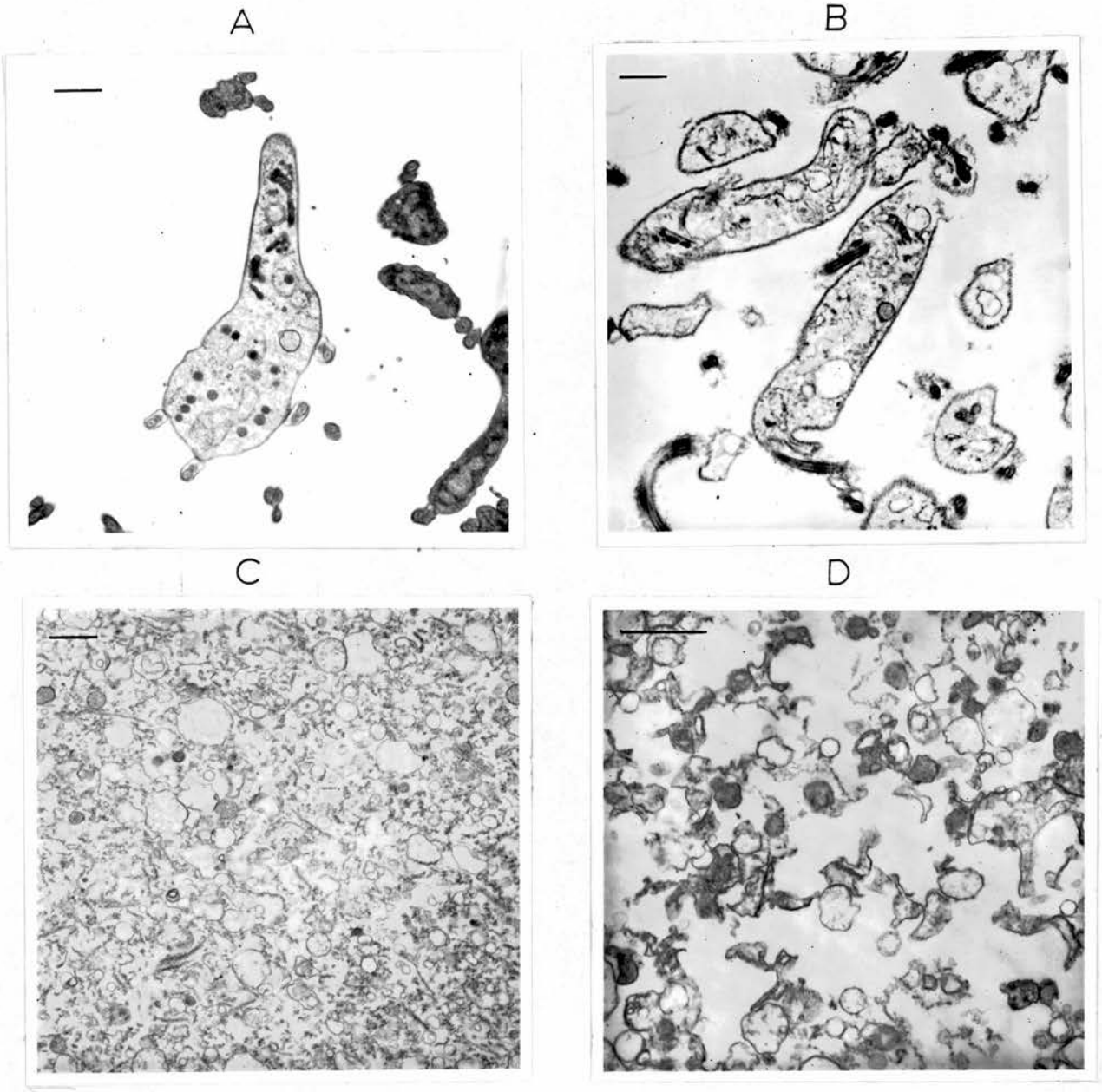


Fig. 3.17. Electron photomicrographs of various stages in the purification of the GP oxidase by the standard procedure. Samples were fixed, stained and sectioned as described in the Methods. A, an intact trypanosome; B, after treatment with and removal of 0.5% (w/v) saponin; C, 24,000 g pellet (RSA 8.0); D, sucrose density gradient fraction (experiment G V, RSA 37.0). Magnification: A,B,C x 7,200; D, x 13,200. The horizontal bar represents 1 micron.

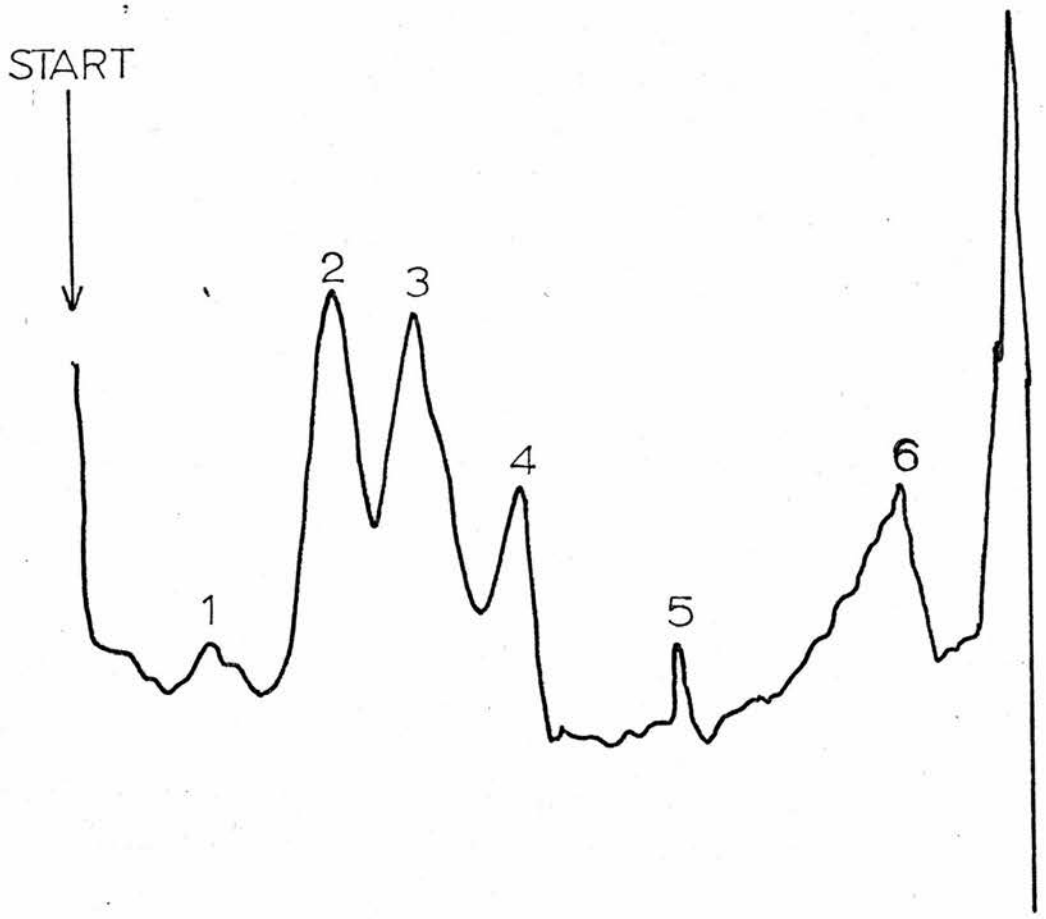


Fig. 3.18. SDS gel electrophoresis of a sucrose density gradient fraction containing GP oxidase purified 13.9 fold. Approximately 40 µg protein was applied. Other experimental details are given in the Methods.

7. Electron microscopy

Samples of various stages of the preparative method were fixed, stained and sectioned and examined by electron microscopy as described in the methods (fig. 3.17). Many of the particles in fig. 3.17d appear to have been damaged during the fixing and sectioning process, possibly due to osmotic shock on treating the gradient fractions in concentrated sucrose with the dilute sucrose-glutaraldehyde medium.

8. SDS acrylamide gel electrophoresis of a partially purified GP oxidase gradient fraction

Electrophoresis of an SDS extract of a 13.9 fold purified GP oxidase fraction was carried out as described in methods. Fig. 3.18 shows the results of gel scanning one sample containing approximately 40 µg of protein. Four main components and two minor components are visible. The slight shoulder on fraction 3 is not further resolved by increasing the amount of protein loaded onto the gel. The fraction contained 24% of the GP oxidase, 18% of the acid phosphatase and 6.5% of the total NAD^+- dependent GPDH recovered from the gradient.

CHAPTER 4

PROPERTIES OF GLYCEROPHOSPHATE OXIDASE

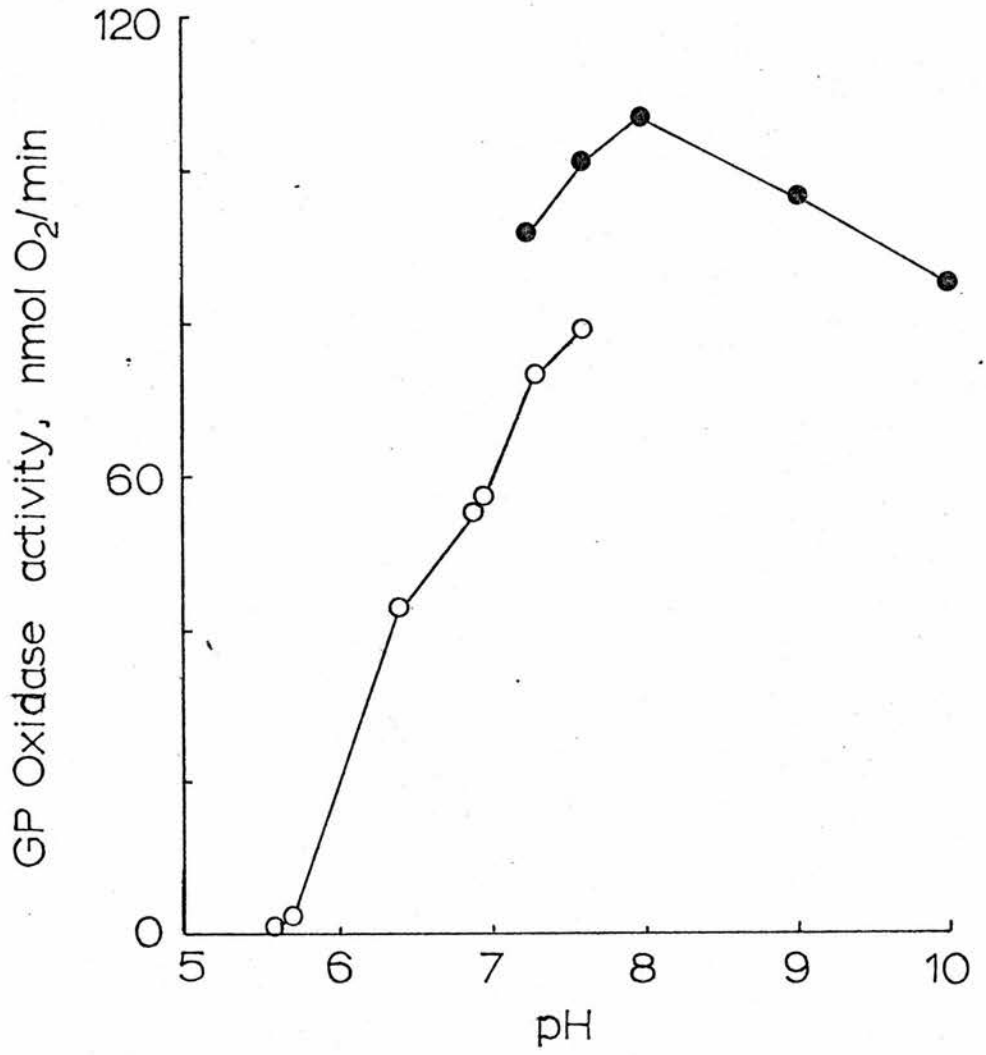


Fig. 4.1. Effect of pH on the activity of the GP oxidase. Each assay contained 2.95 ml buffer substrate (0.1 M buffer, 13.3 mM LaGP) and 0.05 ml of partially purified GP oxidase (RSA 7.1). The pH of each sample was determined using a glass electrode at about 20°C. Phosphate buffer, o ; tris buffer, ● .

1. Determination of optimal conditions for assay of glycerophosphate oxidase

The effects of pH, enzyme and substrate concentrations, metal ions, and bovine serum albumin have been investigated on the partially purified GP oxidase prepared by the saponin method.

Fig. 4.1 shows the effect of the pH of phosphate and tris buffers on the activity of the GP oxidase. To eliminate the effect of the buffering action of glycerophosphate at neutral pH values, glycerophosphate-buffer mixtures were adjusted to the desired pH and reactions initiated by the addition of the enzyme. The pH at the end of each assay was measured by a glass electrode to check that no appreciable change in pH had occurred. In tris buffer the rate of oxygen utilisation by the GP oxidase was constant throughout each assay, but in phosphate buffer at pH values less than 6.8 the GP oxidase activity decreases with time. The values plotted in fig. 4.1 are the initial rates of oxygen utilisation. It can be seen (fig. 4.1) that the activity in tris buffer is slightly higher than that in phosphate buffer at the same pH. The pH optimum in tris buffer is pH 8.0 in agreement with the value reported by Grant and Sargent (1960), but no pH optimum was obtained in phosphate buffer which is in contrast with the reported value of pH 7.4 (Grant and Sargent, 1960). Tris buffer, 0.02 M, pH 8.0 was routinely used for subsequent assays of the GP oxidase.

The effect of 0.25 M sucrose on the activity of the GP oxidase was investigated (table 4.1) and was found to have no effect, indicating that the GP oxidase and its dehydrogenase component show no latent activity and that glycerophosphate is readily available

TABLE 4.1. Effect of sucrose and bovine serum albumin (BSA) on the activity of purified GP oxidase in the presence and absence of phenazine ethosulphate (PES) phenazine methosulphate (PMS).

ASSAY CONDITIONS			FURTHER ADDITIONS			
Tris pH8.0 0.02 M	Sucrose 0.25 M	BSA 3.3mg/ml	NONE	PES 1mM	PMS 1mM	PMS/CATALASE 1mM, 183U/ml
+	-	-	102(1)	-	-	-
+	-	+	230(1)	-	-	-
+	+	-	96(3)	251(1)	250(1)	250(1)
+	+	+	227(3)	256(1)	249(1)	249(1)

Activity of the GP oxidase (nmol O_2/min) was determined using a Clark O_2 electrode. All values corrected for autoxidation rates. Figures in parenthesis refer to the number of experiments.

to the active site of the enzyme. Further supporting evidence for this is given in fig. 4.5 in that freezing and thawing GP oxidase preparations does not lead to an increase in the apparent total activity. Table 4.1 also shows that the presence of bovine serum albumin markedly activates the GP oxidase, but has no stimulatory effect on the dehydrogenase component when assayed in the presence of either phenazine ethosulphate (PES) or methosulphate (PMS). The activity of the dehydrogenase component of the oxidase is the same with either of the artificial electron acceptors PES and PMS. PES is the more stable of the two when stored in solution in ice with light excluded and has been used in preference to PMS whenever possible. Catalase does not affect the rate of oxygen consumption in the presence of PMS or PES, despite the fact that H_2O_2 is the final product of the reoxidation of the reduced electron acceptor. Catalase has been reported to be inhibited by PMS (Peel, 1972) but this would not appear to be the case when excess amounts of catalase are used. Grant and Sargent (1961) studied the stoichiometry of the GP dehydrogenase reaction in a Warburg respirometer in the presence of PMS as electron receptor, and demonstrated that the stoichiometry of 2g atom oxygen uptake/mol of substrate utilised was halved when 5 μ M catalase was present. Reduced PMS causes the univalent reduction of oxygen to form the superoxide anion (Nishikimi et al., 1972) which presumably spontaeneously dismutes to form hydrogen peroxide by the following reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Throughout the remainder of this thesis phenazine methosulphate (PMS) will be used as synonymous with phenazine ethosulphate (PES).

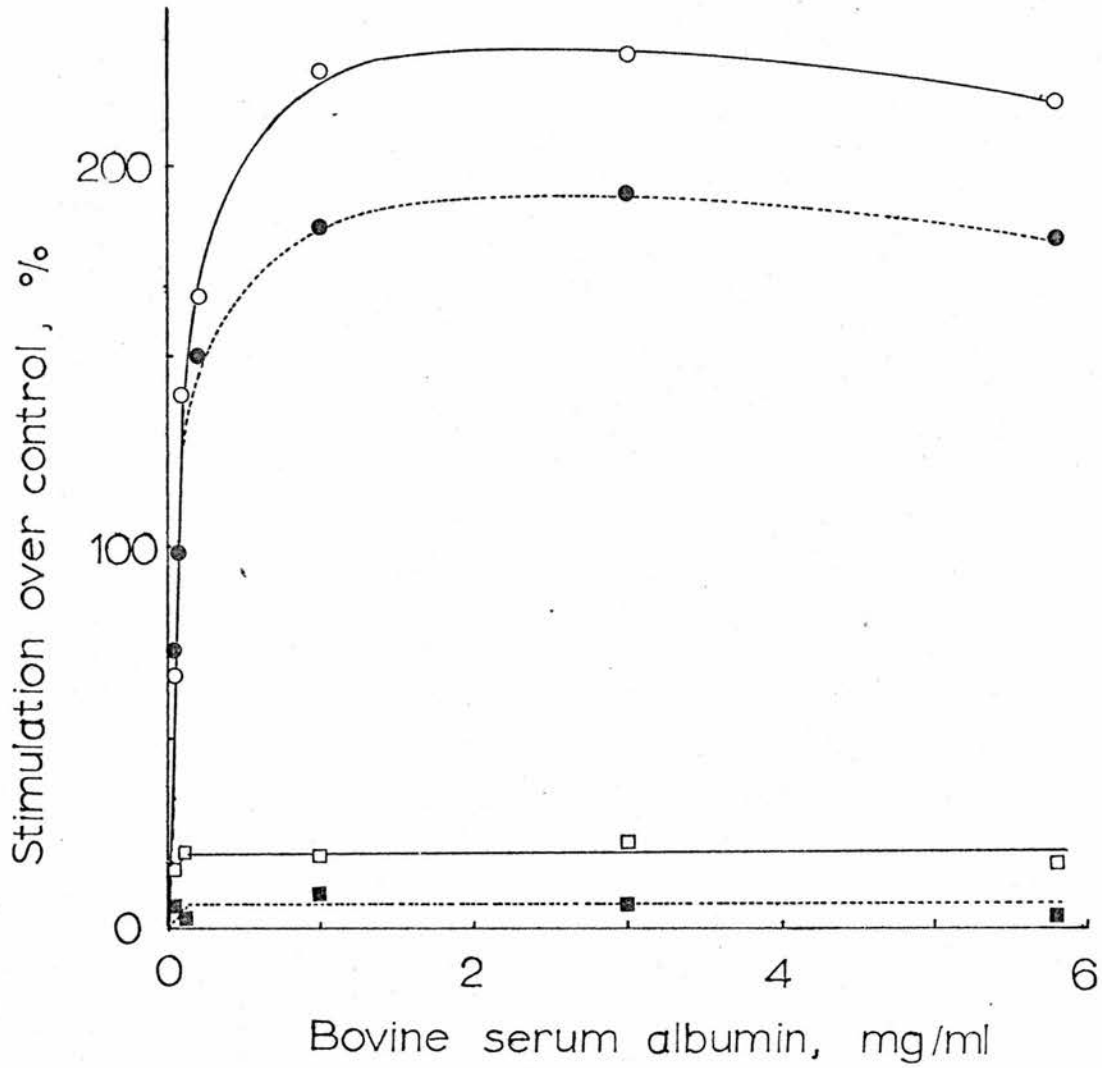


Fig. 4.2. Effect of bovine serum albumin (BSA)on the activity of purified GP oxidase and the GP dehydrogenase component. Activities were determined using a Clark O_2 electrode, containing 0.25 M sucrose, 0.02 M tris buffer, pH 8.0, BSA and 13.3 mM LαGP; in addition the GP dehydrogenase assay contained 10^{-3}M PES. BSA (fraction V, Armour Pharmaceuticals Ltd.), solid lines; BSA (fraction V, Sigma Ltd., essentially fatty acid free), dashed lines. GP oxidase, ○ , ● ; GP dehydrogenase, □ , ■ .

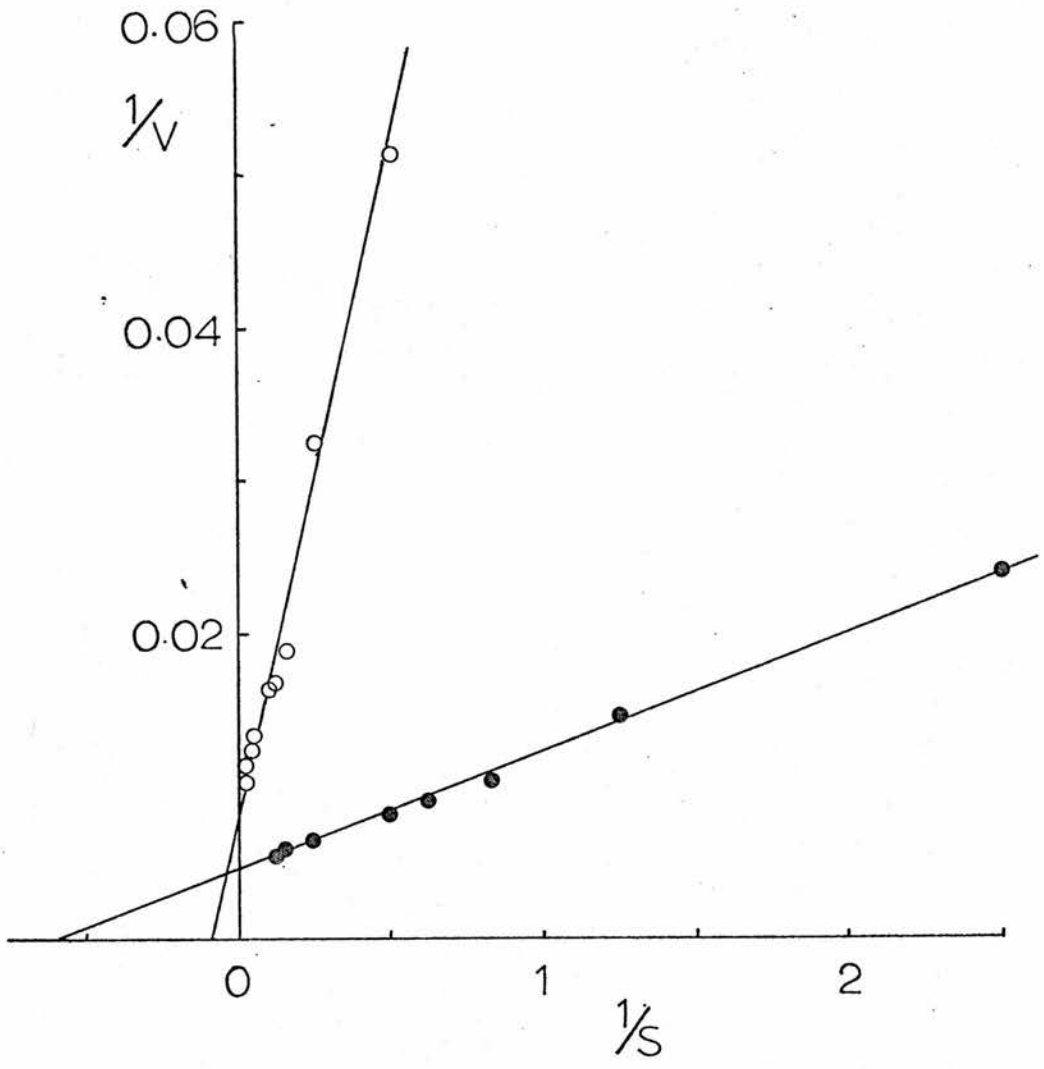


Fig. 4.3. The effect of bovine serum albumin on K_m for glycerophosphate. Double reciprocal plot of velocity (V) against substrate concentration (S) for GP oxidase in the presence, ●, and absence, ○, of BSA (3.3 mg/ml). All assays were carried out in 0.25 M sucrose, 0.02 M tris buffer, pH 8.0 using an oxygen electrode. V represents nmol O_2/min; S represents mM L-α-glycerophosphate.

Bovine serum albumin strongly activates the GP oxidase with maximum activation occurring in the presence of 2 to 3 mg BSA/ml (table 4.1, fig. 4.2). The mode of action of BSA is not known, but BSA prepared essentially free of fatty acids is slightly less effective in activating the enzyme. The GP dehydrogenase component shows a similar activity in the presence or absence of BSA indicating that a second enzyme component is being activated (fig. 4.2, table 4.1). BSA also affects the apparent K_m for glycerophosphate as well as V_{max} of the GP oxidase. In fig. (4.3) the K_m for GP in the presence of BSA is decreased from 6.5 ± 0.7 mM to 1.72 ± 0.08 mM and V_{max} increased from 100 ± 4 to 227 ± 4 nmol O_2/min.

Of the several metal ions tested on the GP oxidase and the dehydrogenase component, none are essential for optimal activity in the presence or absence of BSA. Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+} and Co^{2+} had no effect and the heavy metal ions Cu^{2+}, Zn^{2+} and Ag^{+} inhibit both the oxidase and the dehydrogenase component to the same extent (see section 4 i , thiol reagents and metal ions).

2. Stability of the glycerophosphate oxidase on storage

Fig. 4.4 shows the stability of the GP oxidase and its dehydrogenase component when stored at $0^{\circ}C$ or frozen at $-20^{\circ}C$ or $-196^{\circ}C$. At $0^{\circ}C$ both the GP oxidase and the dehydrogenase component lose activity at the same rate, 50% remaining after 3.5 days, whereas frozen at either $-20^{\circ}C$ or $-196^{\circ}C$ both activities remain essentially constant over a period of 8 months.

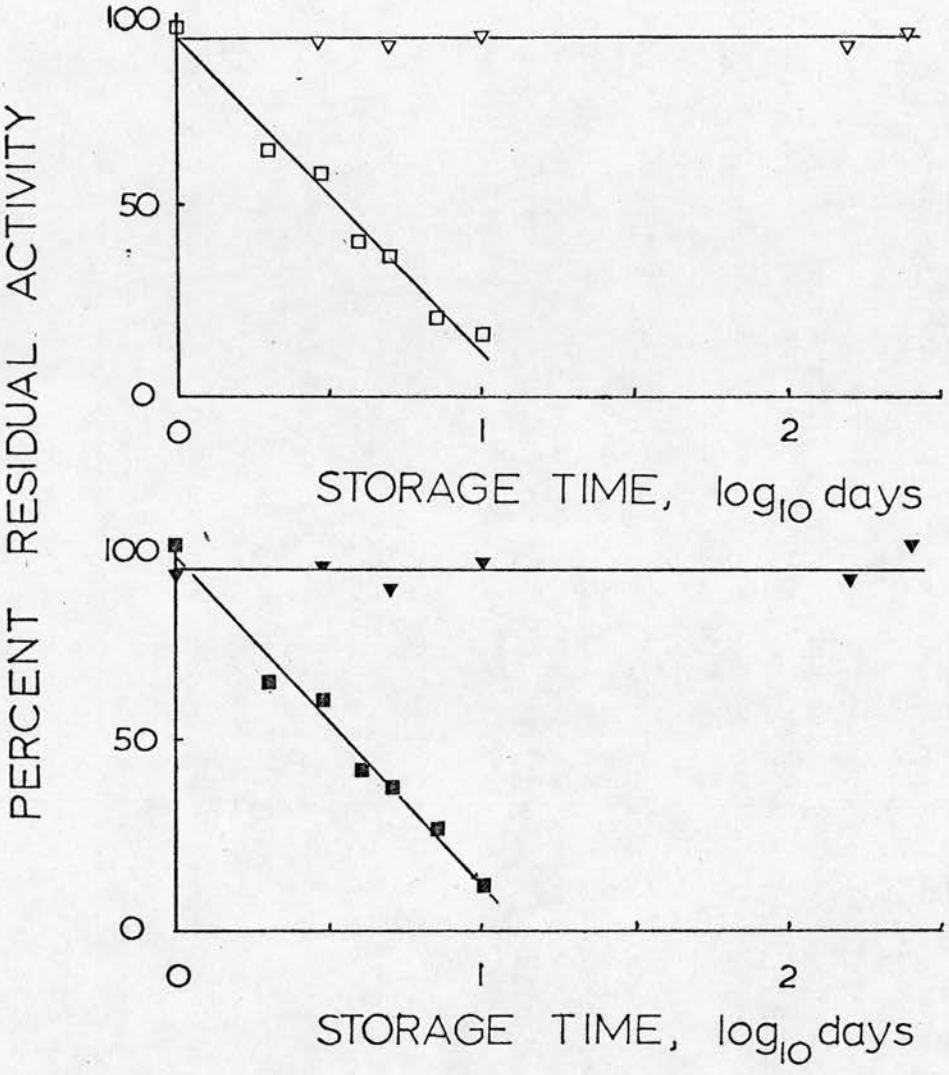


Fig. 4.4. Stability of partially purified GP oxidase in SEP buffer, pH 7.4. Samples of the GP oxidase (5 mg protein/ml, RSA, 8.0) were stored in 2 ml screw-capped vials at 0°C (in ice), or frozen at -20°C (in a deep freeze) or at -196°C (in a liquid nitrogen refrigerator). The frozen samples were rapidly thawed at 37°C and GP oxidase (open symbols) and GP dehydrogenase (closed symbols) activity determined by the standard assay method. Storage at 0°C: □,■; storage at -20°C or -196°C: ▽,▼.

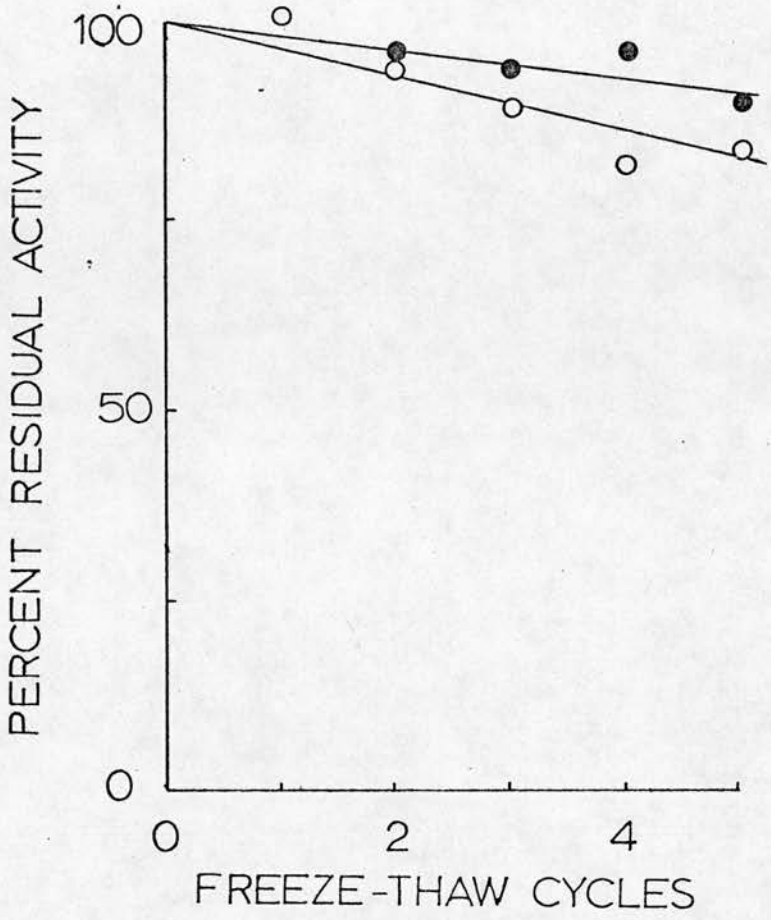


Fig. 4.5. Effect of repeated freezing and thawing on the activity of the GP oxidase (O) and its dehydrogenase component, (●). A sample containing 1 ml of partially purified GP oxidase was rapidly frozen in liquid nitrogen (-196°C) and thawed at 37°C in a water bath. Enzymic activities were assayed in a Clark O_2 electrode using the standard procedure.

TABLE 4.2. The effect of lyophilisation on GP oxidase activity in crude homogenates of trypanosomes.

Method of cell disruption/ buffer	Nitrogen cavitation in SEP buffer, pH7.4		Osmotic lysis in water	
GP Oxidase activity	nmol O_2/min/mg	%	nmol O_2/min/mg	%
Before lyophilisation	148	100	200	100
1 day after lyophilisation	-	-	45	23
39 days " "	128	86	35	18
64 " " "	127	86	37	19
239 " " "	99	67	12	6
949 " " "	79	53	10	5

After lyophilisation the samples were stored in stoppered tubes for $2\frac{1}{2}$ years at $2^{\circ}C$. Tared samples were reconstituted with water to the original volume and assayed using the standard procedure.

The effect of repeated freezing and thawing on the GP oxidase is illustrated in fig. 4.5. The GP oxidase activity is slightly less stable than the dehydrogenase component under these conditions. After freezing and thawing 5 times the concentrated enzyme samples were diluted and centrifuged at 144,700g for 1h and the supernatant assayed to estimate to what extent the enzymes had been solubilised. None of the GP oxidase activity and 4% of the dehydrogenase activity were recovered in the soluble supernatant fraction.

Lyophilisation in SEP buffer was also found to be a useful method for prolonged storage of the GP oxidase (table 4.2). Grant and Sargent (1961) reported that lyophilisation of water lysed trypanosomes resulted in complete loss of the GP oxidase activity, but retention of the dehydrogenase activity. Table 4.2 shows that lyophilisation of water lysed trypanosomes causes an immediate loss of 75% of the GP oxidase activity followed by a gradual decrease in the residual activity over a $2\frac{1}{2}$ year interval. When homogenates are lyophilised in the presence of SEP buffer, only 15% of the activity is lost immediately and half of the total activity remains after $2\frac{1}{2}$ years. Preliminary experiments indicated that all the individual components of SEP buffer, especially sucrose, had a protective effect against loss of activity on freeze drying. The mechanism of inactivation of the GP oxidase, which could occur either during freezing the enzyme and/or during lyophilisation, has not been investigated.

Acetone powders of water lysed trypanosomes contained no residual GP oxidase activity and two thirds of the dehydrogenase activity was lost. Butanol extraction of water lysed trypanosomes

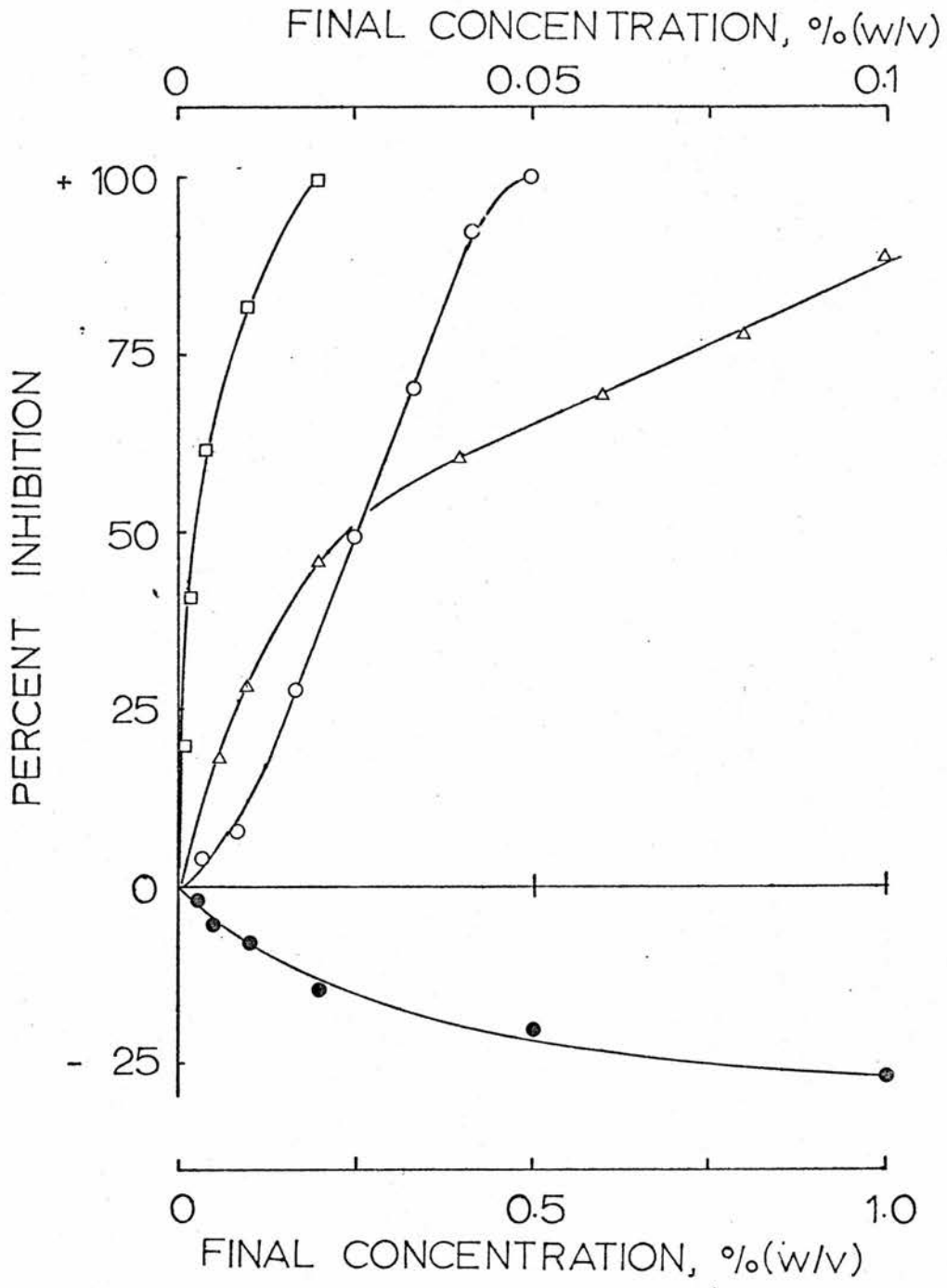


Fig. 4.6. Effect of detergents on the activity of the purified GP oxidase. The upper scale of concentration refers to the open symbols: triton X-100, □; digitonin, ○; sodium deoxycholate, △. The lower scale refers to the closed symbol: saponin, ●. Assays in TS buffer, pH 8.0, using a Clark oxygen electrode.

In this and the following Figure, the concentration of partially purified GP oxidase was 0.14 mg protein per 3ml total volume.

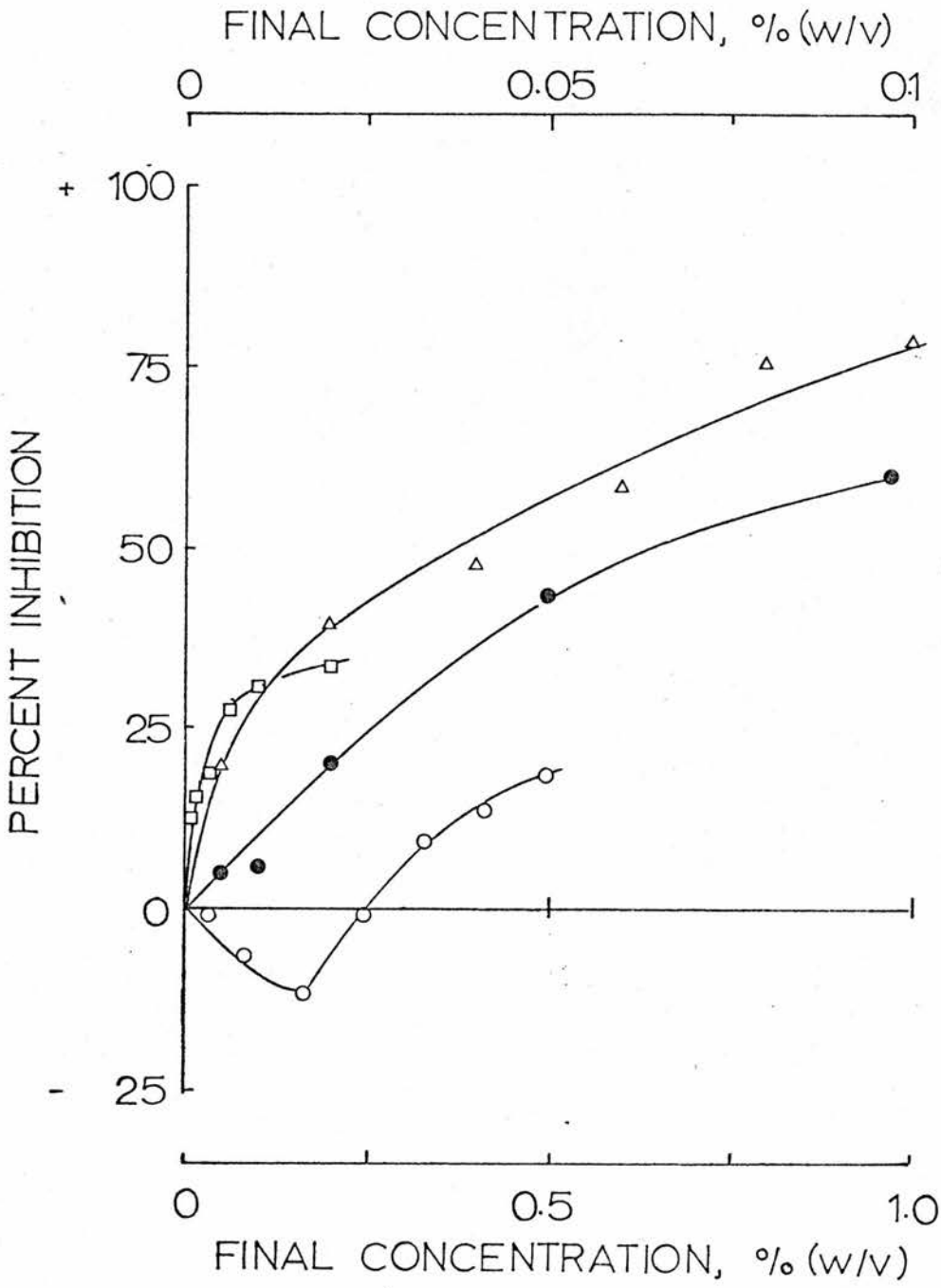


Fig. 4.7. Effect of detergents on the activity of the GP dehydrogenase component. The upper scale of concentration refers to the open symbols: triton X-100, □; digitonin, ○; sodium deoxycholate, △. The lower scale refers to the closed symbol: saponin, ●. Enzymic activity was estimated in TS buffer, pH 8.0, in the presence of 1.0 mM PMS using a Clark oxygen electrode.

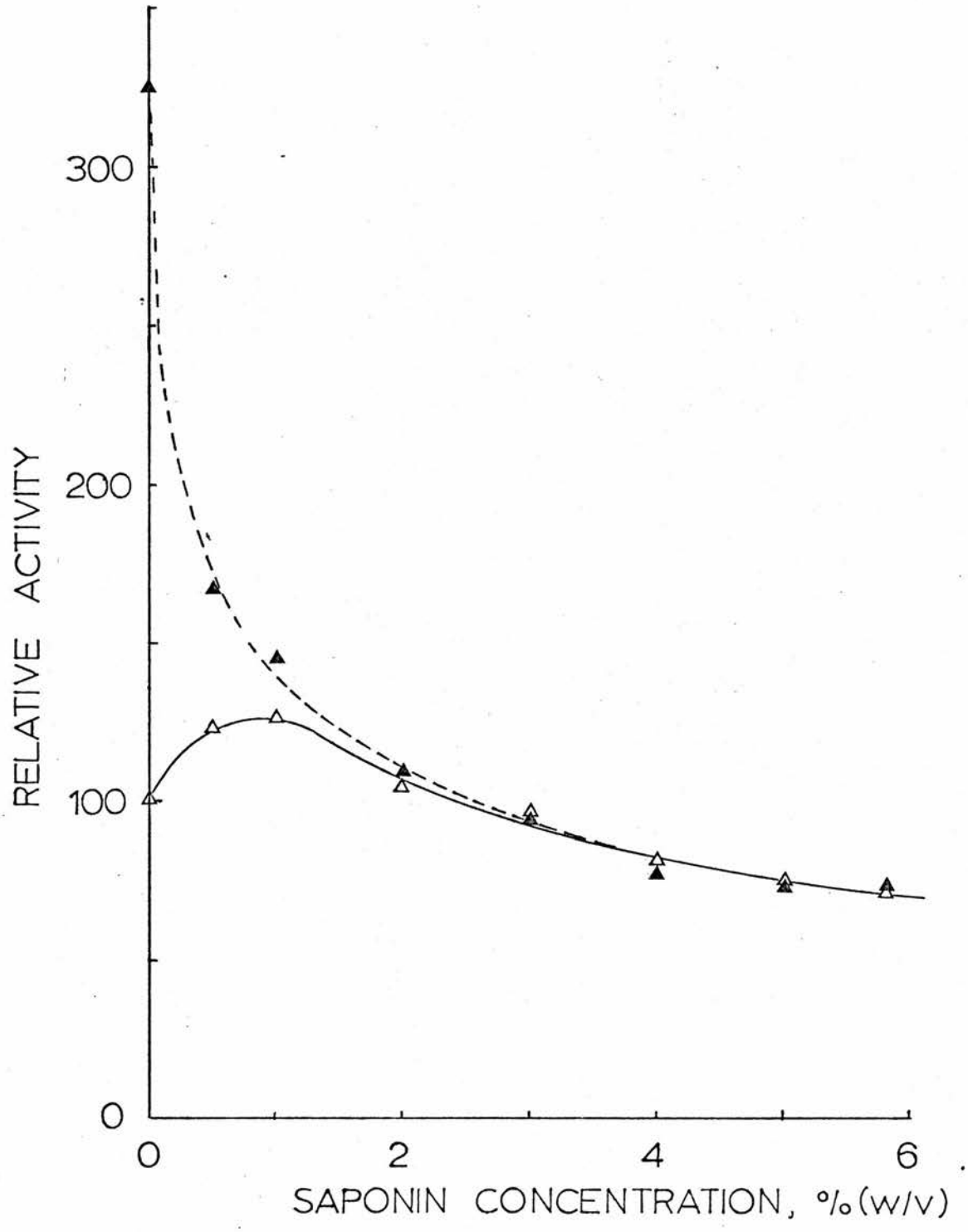


Fig. 4.8. Effect of saponin on the relative activities of the GP oxidase and its dehydrogenase component. Experimental details as in figs. 4.6, 4.7. All activities are expressed relative to the GP oxidase activity (=100) in the absence of saponin. GP oxidase, △ ; GP dehydrogenase, ▲.

by a one-phase or two-phase procedure, or butanol extraction of lyophilised water lysed trypanosomes completely inactivated both GP oxidase and the dehydrogenase component.

3. Effect of detergents on the glycerophosphate oxidase and dehydrogenase

As noted earlier the GP oxidase is inhibited by a variety of anionic and non-ionic detergents. The effect of some of these on the GP oxidase and its dehydrogenase component has been investigated (figs. 4.6, 4.7). Deoxycholate inhibits both enzyme activities to the same extent, whereas triton X-100 and digitonin preferentially inhibit the GP oxidase activity, such that when the GP oxidase activity is completely inhibited about three quarters of the dehydrogenase activity is retained.

Of the detergents tested, saponin is unique in that it activates the GP oxidase by up to 25% even at concentrations 10 fold higher than those employed with digitonin, triton X-100 or deoxycholate (fig. 4.6). The dehydrogenase component is apparently inhibited 60% at 1% (w/v) final concentration of saponin (fig. 4.7). With higher concentrations of saponin the GP oxidase activity and the GP dehydrogenase activity decrease in parallel (fig. 4.8) suggesting that the transfer of electrons to PMS has been completely inhibited by concentrations of saponin greater than 2% (w/v), but that the electron transfer to oxygen through the terminal oxidase component is relatively unimpaired.

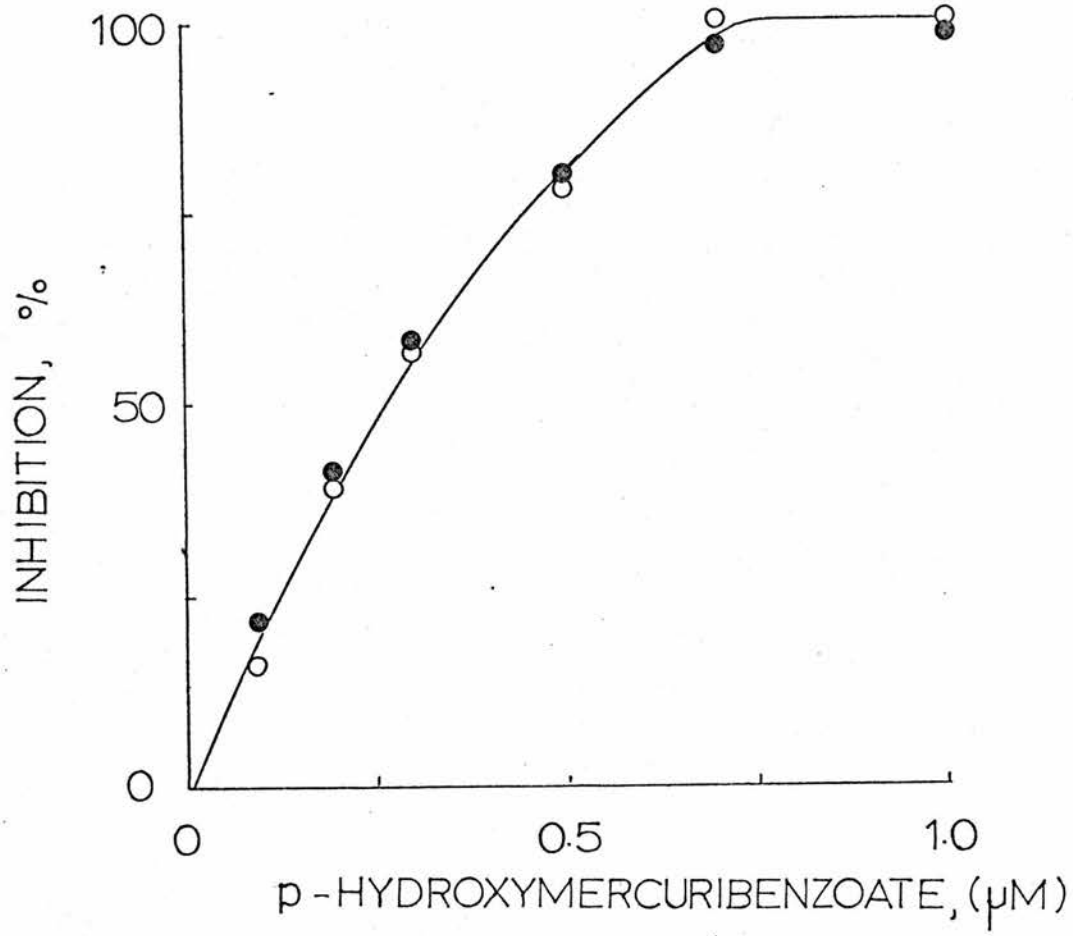


Fig. 4.9. Inhibition of GP oxidase (o) and the dehydrogenase component (●) by p-hydroxymercuribenzoate. Samples of the partially purified GP oxidase (RSA, 10.7; 0.11 mg protein) were preincubated with pOMB in TS buffer, pH 8.0, for 20 min at 37°C before the addition of 13.3 mM LαGP and then 1.0 mM PES.

TABLE 4.3. Effect of metal ions on the activity of purified GP oxidase

METAL ION	Final concentration	RELATIVE ACTIVITY	
		GP oxidase	GP dehydrogenase
CONTROL	-	100	100
Mg^{2+}	0.1 mM	94	92
Ca^{2+}	0.1 mM	90	92
Mn^{2+}	0.1 mM	94	98
Fe^{3+}	0.1 mM	96	102
Co^{2+}	0.1 mM	101	90
Cu^{2+}	0.1 mM	8	10
Zn^{2+}	0.1 mM	9	9
Ag^{+}	0.1 mM	0	0

All assays in TS buffer, pH 8.0 using a Clark O_2 electrode. GP dehydrogenase activity was measured in the presence of 10^{-3}M PMS.

4. Effects of inhibitors on the GP oxidase

i) thiol reagents and metal ions

p-Hydroxymercuribenzoate and the heavy metal ions Cu^{2+}, Zn^{2+} and Ag^{+} are effective inhibitors of the GP oxidase and dehydrogenase component (fig. 4.9, table 4.3). Inhibition by p-hydroxymercuribenzoate is initially rapid over the first minute, with maximum inhibition occurring after 20 min exposure to the reagent. 50% inhibition under these conditions occurs at 0.26 μM (0.2 μM, separate experiment). The trivalent arsenical drug, melarsen oxide, also inhibits both the GP oxidase and dehydrogenase 50% at 60 μM after 5 min preincubation at 37°C (table 4.6). Iodoacetate did not inhibit either the oxidase or the dehydrogenase component at a concentration of 1.0 mM. These findings are in general agreement with those of Grant and Sargent, (1960, 1961) and Bide (1964) for p-hydroxymercuribenzoate, heavy metal ions or iodoacetate, and Flynn and Bowman (1974) for melarsen oxide. Not surprisingly, the purified oxidase used in these studies is more sensitive to thiol inhibitors than reported by the workers mentioned above as previous studies have used crude trypanosomal homogenates. Thus p-hydroxymercuribenzoate, heavy metal ions and melarsen oxide inhibit the GP oxidase and the dehydrogenase component at a point prior to the site of reduction of the artificial electron acceptors PES or PMS. It is also possible, however that the GP oxidase is inhibited also at a point beyond the site of PMS or PES reduction, but such postulated sites must be less sensitive to inhibition by SH reagents than the dehydrogenase site as the inactivation for the dehydrogenase and the oxidase show identical sensitivities. Inhibition of oxygen

uptake in the presence or absence of PMS or PES will be subsequently referred to as type I inhibition and inhibition of oxygen uptake in the absence of PMS or PES, but not in the presence of these autoxidisable electron acceptors will be referred to as type II inhibition. Those compounds showing inhibition of both types, that is inhibiting oxygen uptake more strongly in the absence of PES, will be referred to as intermediate type inhibitors.

No alternative artificial electron donor has been found to replace glycerophosphate for the GP oxidase reaction. Ascorbate$^{+}_{-}$ TMPD, catechol, hydroquinone, juglone and duroquinone have been tested. An artificial electron donor to the terminal component of the respiratory chain could prove useful in identifying the site of action of some of the intermediate or type I inhibitors.

ii) metal ~~chelating~~ complexing compounds

A large number of compounds with metal ~~chelating~~ complexing properties have been tested as inhibitors of the GP oxidase (table 4.4). Cyanide, fluoride and azide, classical inhibitors of cytochrome oxidase, are not inhibitory even in high concentrations. Incubation with 20 mM KCN for 2h at 37°C did not inactivate the oxidase of the dehydrogenase component any faster than the control. Grant and Sargent (1961) treated the GP dehydrogenase with 10 mM KCN for 12h at 18°C or with oxidised glutathione (50 mM) for 6h at 37°C, but also failed to find any significant decrease in GP dehydrogenase activity with PMS as electron acceptor.

Two of the hydroxamic acids tested on the GP oxidase are potent

TABLE 4.4. Effect of chelating compounds on the activity of the GP oxidase and GP dehydrogenase component

CHELATING COMPOUND	SOLVENT	PREINCUBATION min	MAXIMUM CONCENTRATION, mM	INHIBITION AT MAXIMUM CONCN.		CONCN. FOR 50% INHIBITION, I_{50} mM		INHIBITION TYPE
				OXIDASE	DEHYDROGENASE	OXIDASE	DEHYDROGENASE	
o-phenanthroline	H_2O	2	15	90	90	4.4	4.4	I
dimethyl-o-phenanthroline	DMF	2	10	62	30	8.0	-	Inter.
aa'-dipyridyl	DMF	10	30	54	18	27.5	-	Inter.
Tiron	H_2O	2	20	82	91	5.5	4.5	I
TTFA	DMF	2	5	88	73	0.78	2.6	Inter.
Salicylaldoxime	DMF	2	5	75	40	1.4	-	Inter.
SHAM	DMF/H_2O	2	2	100	0	0.012	N.I.	II
*CLAM	DMF	2	2	100	0	0.015	N.I.	II
*3,4,5-trimethoxybenzhydroxamate	DMF	2	1	0	0	N.I.	N.I.	N.I.
hydroxylamine	H_2O	2	5	0	0	N.I.	N.I.	N.I.
8-hydroxyquinoline	DMF	2	5	100	0	0.165	N.I.	II
8-hydroxyquinoline-5-sulphonate	H_2O	2	5	9	0	-	N.I.	? II

TABLE 4.4. (Cont'd.)

CHELATING COMPOUND	SOLVENT	PREINCUBATION min	MAXIMUM CONCENTRATION, mM	INHIBITION AT MAXIMUM CONCN.		CONCN. FOR 50% INHIBITION, I_{50} mM		INHIBITION TYPE
				OXIDASE	DEHYDROGENASE	OXIDASE	DEHYDROGENASE	
4-hydroxyquinoline**	DMF	2	5	0	0	N.I.	N.I.	N.I.
diphenyldithiocarbazone	DMF	10	0.1	42	7	-	-	Inter.
diethyldithiocarbamate	H_2O	10	20	27	36	-	-	Inter.
Na F	H_2O	2	20	0	0	N.I.	N.I.	N.I.
NaN_3	H_2O	2	20	0	0	N.I.	N.I.	N.I.
KCN	H_2O	0-120	20	0	0	N.I.	N.I.	N.I.

All assays in TS buffer, pH 8.0, 3.0 ml total volume in a Clark oxygen electrode. GP oxidase activity was measured in the presence of 13.3 mM GP (L form) and the dehydrogenase component in the presence of 13.3 mM GP and 10^{-3}M PES or PMS.
* Chamber rinsed with 20% (v/v) DMF between assays. When DMF was used as solvent all assays, including controls, were assayed in the presence of 0.033% (v/v) DMF. N.I. signifies not inhibitory; - signifies I_{50} greater than maximum concentration of the inhibitor that was tested; Inter, intermediate type of inhibition. **Note that 4-hydroxyquinoline is not a chelating compound, but was included for comparison with 8-hydroxyquinoline.

inhibitors of type II i.e. they do not inhibit the GP dehydrogenase component. Salicylhydroxamic acid (SHAM) and 3-chlorobenzhydroxamic acid (CLAM) are equally effective inhibitors of the oxidase, but slightly stimulate oxygen uptake in the presence of PES or PMS even at concentrations greater than that required to completely inhibit the GP oxidase. Evans and Brown (1973b) found that the GP oxidase of T.brucei was completely inhibited at 0.1 mM CLAM, but did not determine the concentration required for 50% inhibition of the GP oxidase or test CLAM's effect on the dehydrogenase. For comparison, 0.1 mM CLAM in these studies inhibited the GP oxidase by 85% which is in reasonable agreement with these authors. Recently, Opperdoes et al. (personal communication) have determined the I_{50} for SHAM as 15 μM for the GP oxidase in whole cells of T.brucei, which is similar to the value determined here of 12 $\pm$ 1 μM. Trimethoxybenzhydroxamic acid (1 mM) does not inhibit the GP oxidase, yet SHAM and CLAM completely inhibit the oxidase at this concentration. Trimethoxybenzhydroxamic acid is also a poor inhibitor of the cyanide-insensitive oxidases of plants (B.T. Storey, personal communication). Hydroxylamine does not inhibit the GP oxidase at 5 mM; even 0.3 M is only slightly inhibitory. Salicylaldoxime (SAD), however, does inhibit the GP oxidase (I_{50} 1.4 mM), but, in contrast to salicylhydroxamic acid which has an extra carbonyl group and therefore a greater propensity for forming metal-chelate complexes, SAD is an inhibitor of the intermediate type. The additional carbonyl group in SHAM decreases the concentration required for 50% inhibition by about 100 fold over that for SAD (0.012 mM, 1.4 mM, respectively).

Two of the chelating agents, Tiron and o-phenanthroline, are type I inhibitors. Tiron's mode of action is liable to be complex as will be discussed later. o-Phenanthroline has been previously reported as inhibiting the GP oxidase (Bide and Grant, 1964), but no details were published for comparison with the present results.

Several of the compounds tested did not give clearly defined type I or type II inhibition; this intermediate type of inhibition could be due to a differential sensitivity of the oxidase to these inhibitors, that is to say, the second (type II) site of inhibition is more sensitive than the first (type I) site. TTFA is the most notable of these with a significantly higher I_{50} value for the dehydrogenase (table 4.4). The pattern of inhibition is complex as double reciprocal plots of percentage inhibition against inhibition concentration are non-linear. The other compounds showing intermediate type inhibition are dimethyl-o-phenanthroline, $\alpha\alpha'$-dipyridyl, salicylaldoxime, dithizone and diethyldithiocarbamate. Values for I_{50} for the dehydrogenase component were not determined for these compounds either because the maximum concentration tested was limited by solubility of the compound or concentrations in excess of 20 mM were required.

iii) miscellaneous compounds

Three other compounds giving type II inhibition are catechol, diphenylamine and hydrogen peroxide (table 4.5). In the case of hydrogen peroxide, it was not possible to determine the concentration for I_{50} because inhibition steadily increases with time and preincubation of the GP oxidase for 10 min or longer does not

TABLE 4.5. Effect of some miscellaneous inhibitors on the GP oxidase and the GP dehydrogenase component.

COMPOUND	SOLVENT	PRE-INCUBTN min	MAX CONCN mM	INHIBITION AT MAXIMUM CONCN.		CONCN. FOR 50% INHIBITION(I_{50})mM	
				OXIDASE(-PES)	DEHYDROGENASE(+PES)	OXIDASE	DEHYDROGENASE
CATECHOL	H_2O	2	2	70	0	0.6	N.I.
DIPHENYLAMINE *	DMF	2	1	93	0	0.17	N.I.
HYDROGEN PEROXIDE	H_2O +	1	2	100	0	?	N.I.
PYRROLNITRIN *	DMF	2	0.27	81	19	0.12	-
S.72 8991 *	DMF	2	0.33	96	96	0.07	0.07
SUPEROXIDE DISMUTASE	H_2O	2	100 U/ml	0	- 68	N.I.	[68% STIMULATION]

Assay method as in Table 4.4 N.I.; not inhibitory; - signifies I_{50} maximum concentration of inhibitor used.
* Electrode chamber rinsed with 20% DMF between experiments; 0.03% (v/v) DMF present in each assay. + H_2O_2 - complete inhibition after preincubation for 1 min in the presence of H_2O_2 and substrate (glycerophosphate). S.72 8991: 7-Chloro-10-hydroxy-3-(4-trifluoromethylphenyl)-3,4-dihydroacridine-1,9-(2H,10H)-dione.

inactivate the enzyme until glycerophosphate is added. Reduction of the enzyme with glycerophosphate for 3 min before the addition of peroxide does not alter the course of the inhibition. Concentrations of peroxide greater than 1 mM completely inhibit the GP oxidase within 2 minutes of addition of substrate (GP). With 0.2 mM H_2O_2 complete inhibition occurs about 10 minutes after the addition of GP. The dehydrogenase component is completely unaffected by 2 mM H_2O_2 even after 10 minutes preincubation.

Diphenylamine has been reported as inhibitory to the GP oxidase of T.brucei, but was not tested against the dehydrogenase component (Evans and Brown, 1972). The I_{50} given in table 4.5 of 0.17 mM agrees well with the published value of 0.2 mM.

Pyrrolnitrin inhibits electron transport in the flavin region of yeast and animal mitochondria (Lambowitz et al., 1972; Wong et al., 1971; Tripathi and Gottlieb, 1969; Wong and Airall, 1970), but the mode of action is uncertain and may differ between species. Pyrrolnitrin is primarily a type II inhibitor, but showing slight type I inhibition at higher concentrations (table 4.5).

iv) trypanocidal drugs

A number of chemotherapeutic agents effective against trypanosomes in vivo were tested for inhibition of the isolated GP oxidase (table 4.6). Suramin and melarsen oxide are potent type I inhibitors of the GP oxidase and dehydrogenase component, but the other drugs are not inhibitory. The trivalent arsenical drug melarsen oxide has been previously reported to inhibit the

TABLE 4.6. Effect of trypanocidal drugs on the activity of purified GP oxidase.

TRYPANOCIDAL COMPOUND	SOURCE	MAXIMUM CONCENTRATION TESTED mM	GP OXIDASE ACTIVITY
NIL	-	-	100
BERENIL	Hoechst Pharmaceuticals Ltd., Hounslow, Middlesex	0.5	142
ANTRYCIDE	I.C.I. Ltd., Macclesfield, Cheshire	0.5	127
HYDROXYSTILBAMIDINE	May and Baker Ltd., Dagenham, Essex	0.5	123
PENTAMIDINE	" " "	0.5	149
PROTHIDIUM BROMIDE	The Boots Company Ltd., Nottingham	0.5	129
ACRIFLAVINE	B.D.H. Chemicals Ltd. Poole, Dorset	0.5	95
MELARSEN OXIDE*	May and Baker Ltd., Dagenham, Essex	1.0	5
SURAMIN a) ('Antrypol')	I.C.I. Ltd., Macclesfield Cheshire	0.1	4
~~(ANTRYPOL)~~ b)	Dr. J. Williamson, N.I.M.R., Mill Hill, London.		

* Melarsen oxide was dissolved in DMF and assayed in the presence of 0.033% (v/v) DMF after 5 minutes preincubation with the enzyme. I_{50} for melarsen oxide and suramin for both GP oxidase and dehydrogenase component were 60.0 μM and 4.4 μM, respectively. The gifts of trypanocidal compounds from the companies listed above are gratefully acknowledged.

TABLE 4.7. Determination of I_{50} for suramin on crude homogenate and partially purified GP oxidase

DATE	EXPERIMENT	SAPONIN TREATED CELLS			FINAL OXIDASE PREPARATION		
		Specific Activity	Protein/3ml assay	I_{50}	Specific Activity	Protein/3ml assay	I_{50}
		nmol O_2/min/mg	mg	/uM	nmol O_2/min/mg	mg	/uM
23/9/74	1	188	0.69	4.3	2320	0.12	4.6
26/9/74	2	185	0.78	5.2	2170	0.09	5.5
23/7/74	3	-	-	-	1170	0.16	6.8
26/7/74	4	-	-	-	2160	0.07	2.0
26/6/75	5	188	0.88	5.5	1740	0.19	6.2
30/6/75	6	172	0.74	6.3	1680	0.09	3.4
7/7/75	7	-	-	-	1470	0.10	2.5
Mean $\pm$ S.E.M.			0.077 $\pm$ 0.04	5.3 $\pm$ 0.4, n=4		0.12 $\pm$ 0.02	4.4 $\pm$ 0.7, n=7

All assays in TS buffer, pH 8.0.

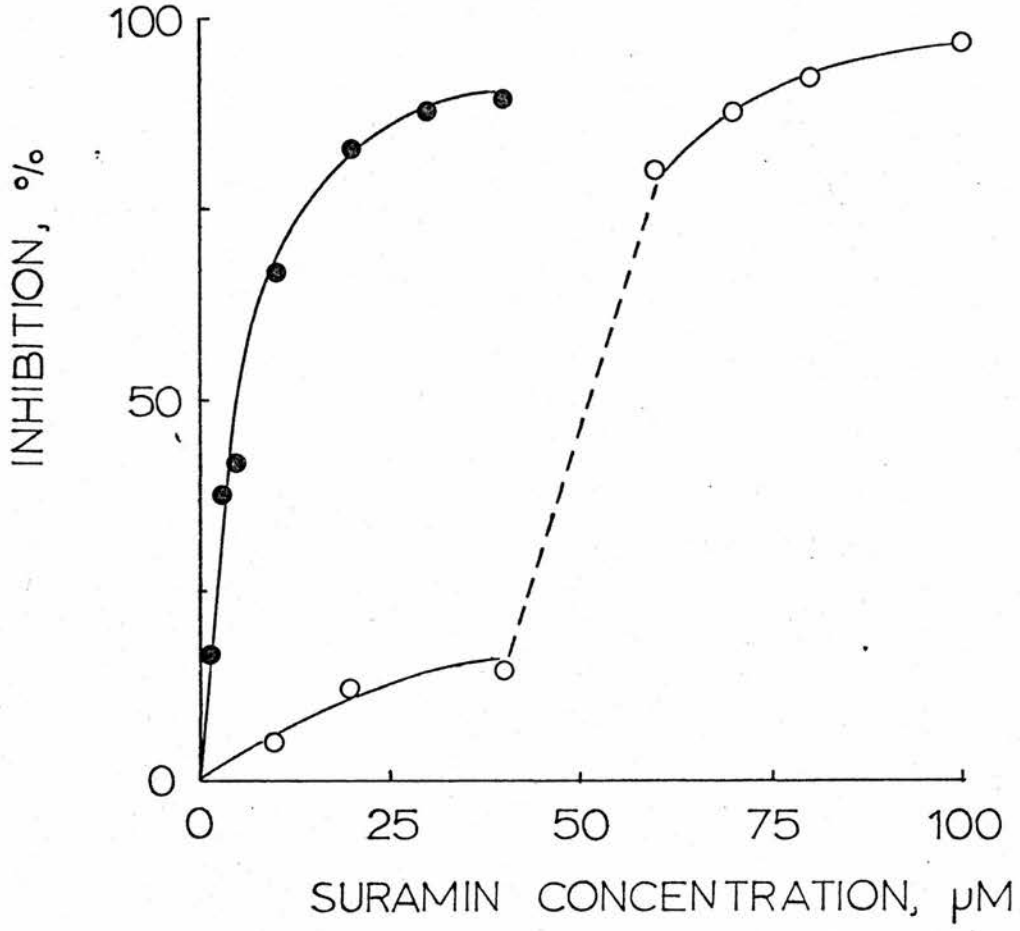


Fig. 4.10. Inhibition of partially purified GP oxidase in the presence (o) or absence (●) of bovine serum albumin (3.3 mg / ml). Assays of GP oxidase activity were done in TS buffer, pH 8.0, using a Clark oxygen electrode.

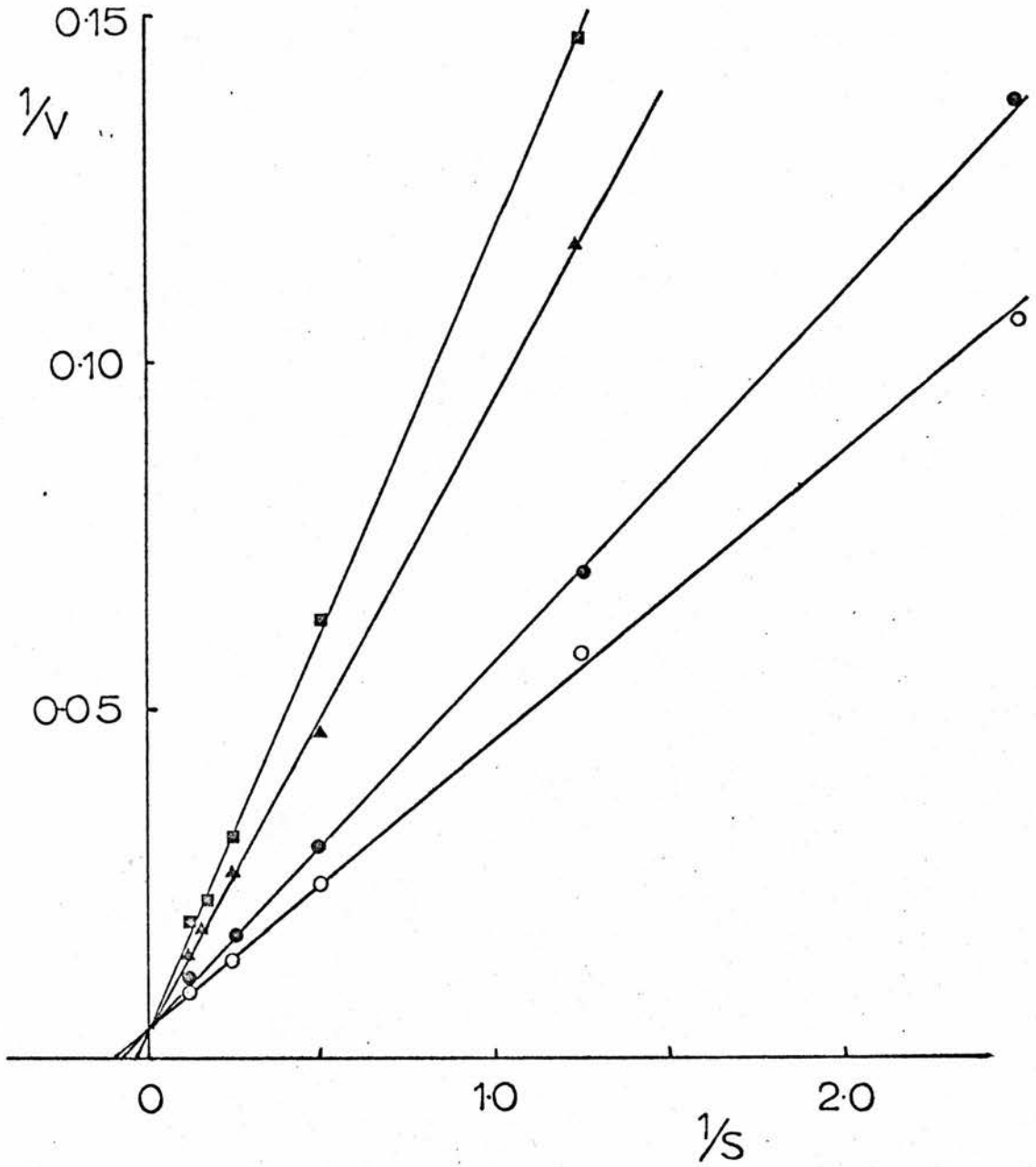


Fig. 4.11. Effect of suramin on the GP oxidase. Double reciprocal plot of velocity (V, nmol O_2/min) versus substrate concentration (S, Laglycerophosphate, mM). No inhibitor, ○; plus suramin, ●, ▲, ■, at concentrations 1,5,8 /uM, respectively. Assays in TS buffer,-pH 8.0 as described in the Methods.

GP oxidase by 15% at 20 μM, but the site of action in the GP oxidase was not determined (Flynn and Bowman, 1974). The discovery that suramin inhibits the GP oxidase has been briefly communicated (Fairlamb and Bowman, 1975), and a more extensive study is presented below.

5. Effect of suramin and its analogues as inhibitors of the glycerophosphate oxidase

Suramin is an extremely potent inhibitor of the GP oxidase and the dehydrogenase component both in crude homogenates and in purified preparations (table 4.7). The concentration required for half maximal inhibition of the oxidase is not significantly different in the crude (5.3 ± 0.4 μM) and purified samples (4.4 ± 0.7 μM) despite 85% less protein used per assay in the latter, indicating that the suramin is not preferentially bound to any significant extent to other trypanosomal protein. The inhibition pattern of the oxidase by suramin in the presence of 3.3 mg/ml BSA is biphasic with an initially slow rise in inhibition until 40 μM followed by a rapid increase to complete inhibition (fig. 4.10). Consequently the I_{50} is increased an order of magnitude (5 μM to 50 μM) by the presence of BSA. Addition of BSA reverse the inhibition of the suramin treated oxidase presumably by preferential binding of the drug to the added albumin. Competitive type of inhibition with respect to GP as substrate is obtained on kinetic analysis (fig. 4.11). Using the method of Hunter and Downs (1945) the Ki for suramin is 4.1 ± 0.4 μM.

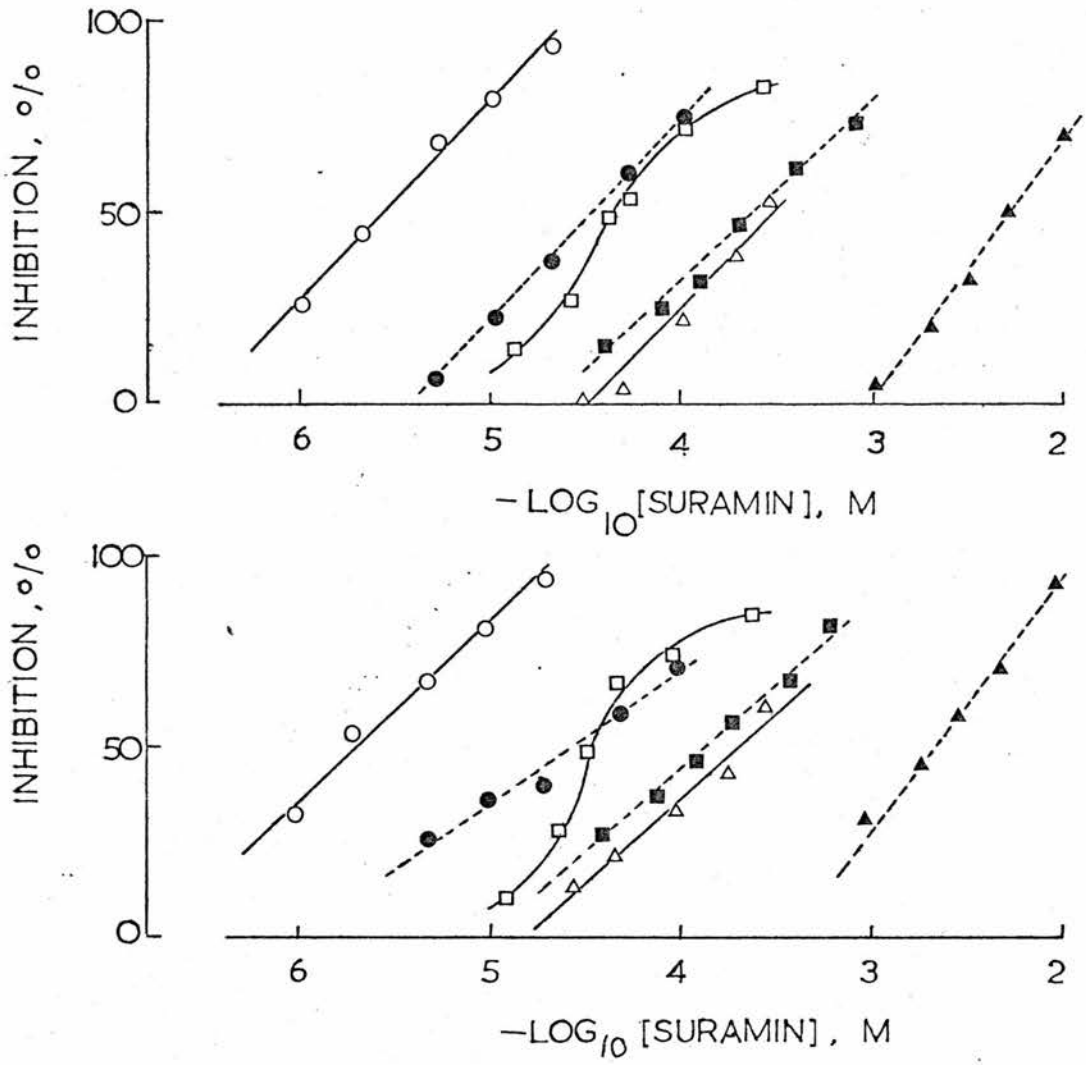


Fig. 4.12. Inhibition of the GP oxidase (upper graph) and the dehydrogenase component (lower graph) by suramin and some analogues. Methods as described in table 4.8. Suramin,(o—o); desmethyl suramin, (●···●); 'suramin nucleus', (□—□); 4-sodium monosulphonate analogue of suramin, (■···■); Antrypol amine ['half suramin'], (△—△); 1-naphthylamine-4,6,8-trisulphonic acid, (▲···▲).

TABLE 4.8. Inhibition of purified GP oxidase by suramin and related compounds

COMPOUND	CONCENTRATION FOR 50% INHIBITION x 10^{-6}M	
	GP OXIDASE	GP DEHYDROGENASE
Suramin	2.5	2.0
Desmethyl suramin	30	30
"Suramin nucleus"	40	40
Monosulphonated suramin	230	150
Antrypol amine	240	200
Naphthylamine trisulphonic acid	5000	2000

I_{50}s were determined using the standard assay procedure on a single preparation of the GP oxidase. All the compounds tested were gifts from I.C.I. Ltd., Macclesfield, Cheshire, and, with the exception of suramin, were of analytical grade only. Antrypol amine was estimated 46% pure from spectrophotometric analysis and the concentration for I_{50} has been corrected for this. "Suramin nucleus" was dissolved in DMF and all assays of this compound including controls were done in the presence of 0.033% (v/v) DMF. Naphthylamine trisulphonic acid was neutralised with KOH before use.

A number of analogues of suramin were tested for inhibition of a purified preparation of the oxidase (fig. 4.12). Plots of percentage inhibition of the oxidase or dehydrogenase component as a function of log inhibitor concentration are linear with the exception of sym-bis-(p-methyl-m-aminobenzoyl-m-aminobenzoate) carbamide [suramin nucleus"] which is sigmoid. The approximate concentrations for I_{50} are given in table 4.8. Removal of the 2 methyl groups to give desmethyl suramin results in a marked increase in I_{50} compared to suramin. Moreover, antrypol amine, equivalent to a half suramin molecule, is a much less effective inhibitor compared to suramin or desmethyl suramin. The naphthylamine trisulphonic acid end-group of suramin the least effective inhibitor tested, yet surprisingly the "suramin nucleus" is remarkably effective as an inhibitor of both oxidase and dehydrogenase component. Removal of two of the three sulphonic acid residues (monosulphonated suramin) from each naphthylamine residue also results in loss of potency of the compound as an inhibitor. Thus all modifications of the suramin molecule tested so far result in decreased potency as an inhibitor of the GP oxidase. These results will be referred to again in the discussion section as to their potential significance in the mode of action of suramin in chemotherapy.

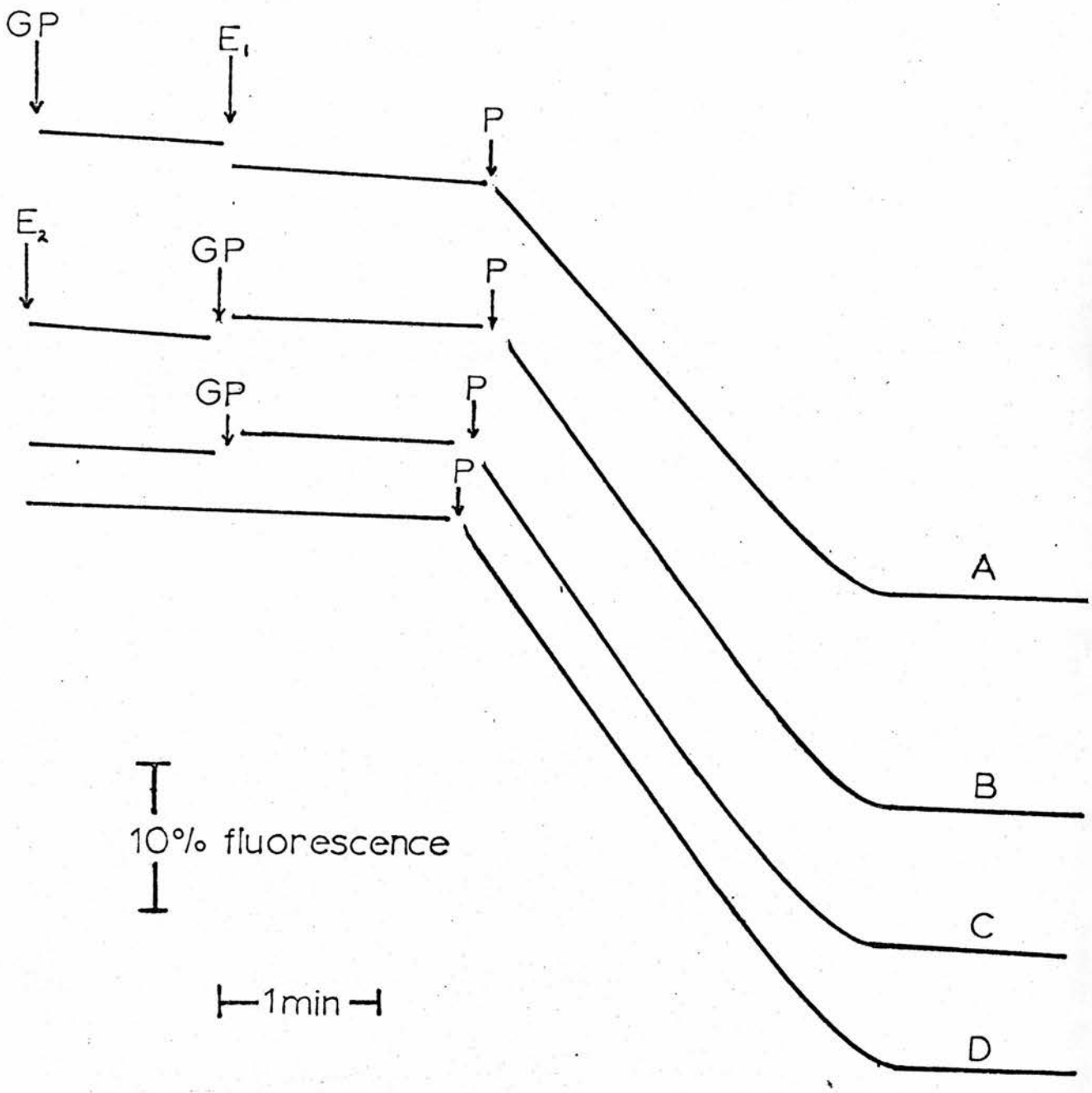


Fig. 4.13. Hydrogen peroxide production by the GP oxidase estimated using the scopoletin method. Each assay contains 0.5 /uM scopoletin, 33 /ug/ml of horse-radish peroxidase in a 3.0 ml total volume with the following additions: GP, glycerophosphate (13.3 mM, L form); P, hydrogen peroxide, 10 nmol; E_1, freshly prepared GP oxidase, 0.05 ml (66.7 nmol O_2/min); E_2, washed particulate oxidase, 0.05 ml (60 nmol O_2 /min). The decrease in fluorescence after the addition of 10 nmol H_2O_2 was: trace A, 28.5%; B, 34.0%; C, 35.8%; D, 38.5%. Traces C and D are controls for scopoletin autoxidation.

6. Tests for hydrogen peroxide as an intermediate in the reaction catalysed by glycerophosphate oxidase

i) Using scopoletin and horseradish peroxidase (HRP)

Several experiments using this method have failed to detect hydrogen peroxide as an intermediate of the reaction under any conditions. Fig. 4.13 shows a typical experiment in which purified GP oxidase is assayed directly for peroxide production in the presence of HRP and scopoletin. No peroxide production can be detected in the presence or absence of GP as substrate. The activities of the GP oxidase were assayed in an O_2 electrode to ensure that the reaction mixture did not become anaerobic during the experiment. Attempts to demonstrate peroxide production under inhibited states in the GP oxidase were also unsuccessful. Menadione, 8-hydroxyquinoline, diphenylamine and salicylhydroxamic acid all inhibit the oxidation of scopoletin by HRP. Triton X-100 does not inhibit the oxidation of scopoletin and no peroxide production can be detected even with the GP oxidase fully inhibited.

The stoichiometry of fluorescence decrease in the addition of standard amounts of hydrogen peroxide was diminished in the presence of GP oxidase (fig. 4.13). The two most likely explanations for this are that either H_2O_2 is metabolised by the GP oxidase preparations or the trypanosomal protein contains a non-fluorescent substrate that is oxidised in place of scopoletin. The cuproprotein $CP_{2.05}$ isolated free from GP oxidase activity by freezing and thawing was found to interfere with the scopoletin assay for H_2O_2, but did not appear to be enzymic because i) heating at 80°C for 5 min did not abolish this interference and ii) incubation of $CP_{2.05}$ with H_2O_2

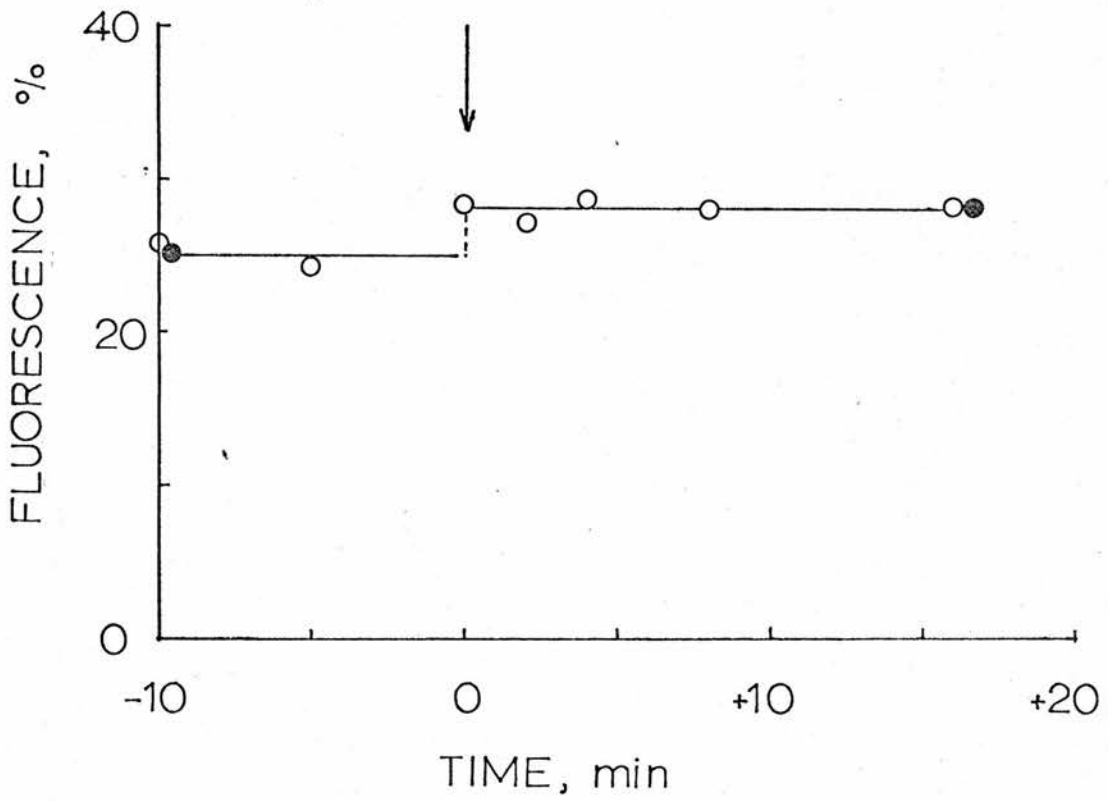


Fig. 4.14. Production of hydrogen peroxide by the glycerophosphate oxidase. Samples (0.2 ml) were removed from the incubation mixture (see text) and assayed for H_2O_2 using diacetyldichlorofluorescin and horseradish peroxidase. Glycerophosphate (13.3 mM L form) was added at the point indicated by the arrow. Open circles, experimental values; closed circles, controls. Other experimental details are given in the Methods.

for 5 min prior to estimation of the remaining H_2O_2 by scopoletin and HRP did not increase the extent to which this fraction interfered with the assay.

ii) using diacetyldichlorofluorescin (LDADCF) and horseradish peroxidase

The presence of zinc ions, necessary to minimise autoxidation of LDCF by HRP, made direct estimation of H_2O_2 production by the GP oxidase impossible as Zn^{2+} inhibits the GP oxidase reaction (table 4.3). Hence, a sampling method based on that of Hinkle et al. (1967) was used. Samples (0.2 ml) of the following incubation mixture were removed at various time intervals and assayed for H_2O_2 as described in the methods section:

2.0 ml ENK buffer, pH 7.4

0.5 ml trypanosomal homogenate (GP oxidase activity: 450 nmol O_2/min at 37°C)

3.3 ml distilled water

0.2 ml glycerophosphate (13.3 mM, L form)

The assay mixture was aerated in a 50 ml conical flask at 37°C with constant shaking and 0.2 ml samples removed at intervals before and after the addition of substrate, glycerophosphate. Fig. 4.14 shows that, after correction for fluorescence of the controls, no hydrogen peroxide production can be detected before or after the addition of GP. Thus, as quantities of H_2O_2 as low as 0.1 nmol can be reliably estimated by this method, the rate of peroxide production is less than 0.5% of the rate of oxygen consumption,

assuming a constant concentration of H_2O_2 during steady state conditions of glycerophosphate oxidation.

7. Estimation of glycerophosphate dependent peroxidase activity

Bide and Grant (1964) reported that particulate fractions containing the GP oxidase activity were able to rapidly reduce H_2O_2 in the presence of glycerophosphate. Bide (1964) established that the stoichiometry of the reaction was 1 mol H_2O_2 used/mol DHAP produced. The following direct spectrophotometric method was developed, similar to the assay for GP oxidase in which production of DHAP is coupled to the oxidation of NADH by excess NAD^+- dependent GPDH.

A "Subaseal" rubber stopper was fitted to a stoppered 3 ml quartz cuvette and 2 wide bore luer needles inserted through the rubber cap so that one needle opening was near the base of the cuvette, but not obstructing the light beam of the spectrophotometer and the second needle just pierced the cap, well above the liquid level. The following reagents were added to the cuvette and the cap replaced:

2.26 - 2.22 ml	TS buffer, pH 8.0
0.05 ml	NADH, 5 mg/ml buffer
0.10 ml	rabbit muscle NAD^+- dependent GPDH (4U)
0.01 - 0.05 ml	GP oxidase preparation

The apparatus is bubbled with oxygen-free nitrogen gas for 5 min, then the gas supply disconnected and the cuvette allowed to

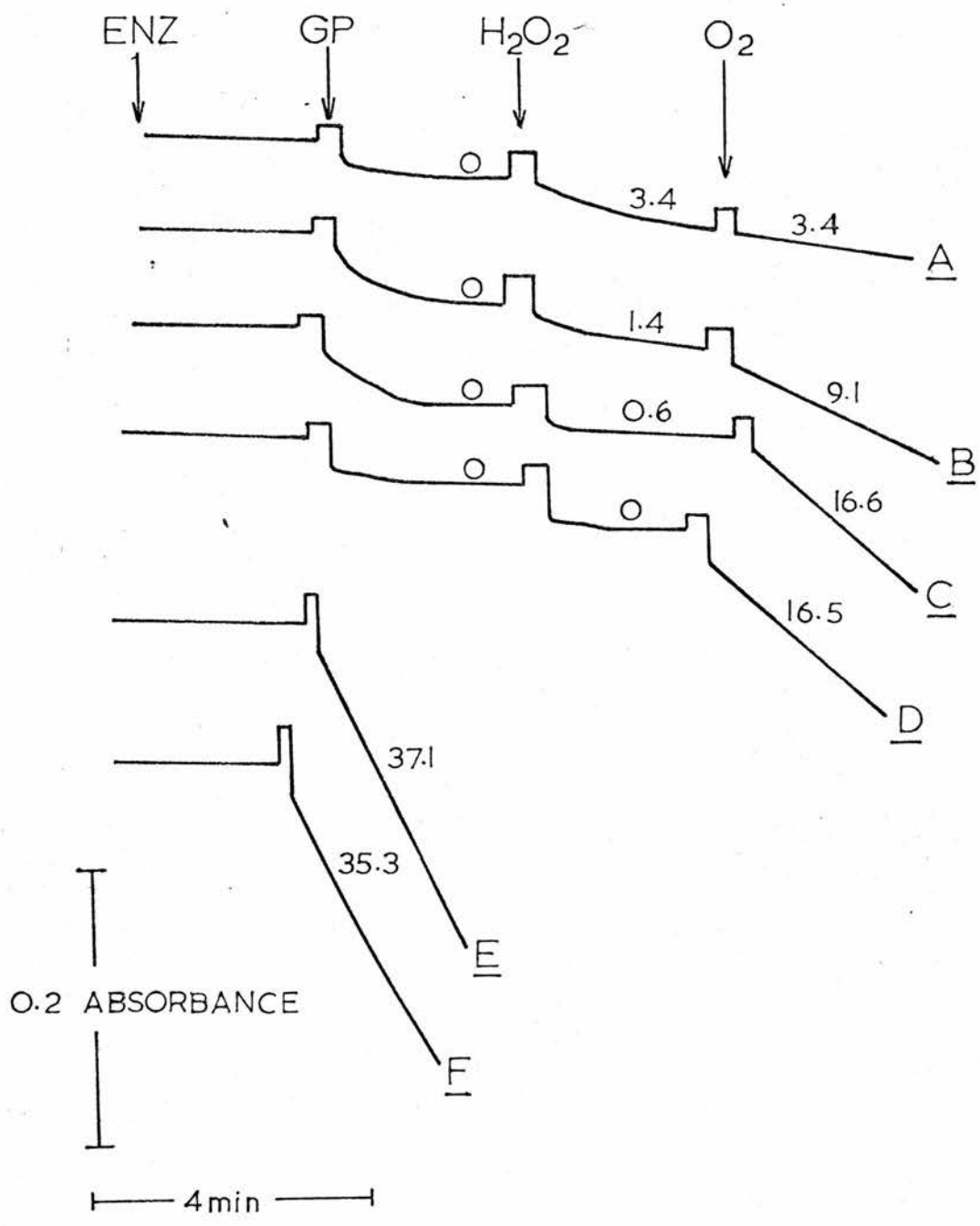


Fig. 4.15. Estimation of GP peroxidase in partially purified GP oxidase, using the coupled assay (see text). A,B,C,D were assayed anaerobically with the addition of 100, 50, 10 and 0 /uM H_2O_2 (final concentration). E and F were assayed aerobically plus 0 and 100 /uM H_2O_2, respectively. Activities in nmol NADH oxidised / min. Additions: GP oxidase, ENZ, (36.5 ng atom O_2 / min); glycerophosphate, GP (16 mM L form); O_2, bubbled 5 ml air

equilibrate to 37°C in the constant temperature housing of the spectrophotometer. Glycerophosphate (16 mM, L form) was added using a micro-syringe fitted with a fine bore needle penetrating one of the wide bore needles and the cuvette mixed. When the remaining oxygen had been scavenged, H_2O_2 (0.01 ml of varying concentrations) was added. Both GP and H_2O_2 solutions were bubbled with nitrogen to minimise the addition of oxygen to the cuvette. The decrease in extinction was recorded at 340 nm.

The results obtained by this method are presented in fig. 4.15. The maximum rate of GP peroxidase activity in the presence of 100 μM H_2O_2 was one fifth of the control (trace D). Higher concentrations of peroxide were found to be inhibitory to the GP peroxidase. Bubbling with nitrogen inactivates the GP oxidase 65% (control D versus E). Air was bubbled through the anaerobic cuvettes to estimate the residual activity of the GP oxidase after exposure to H_2O_2 in the presence of glycerophosphate. The oxidase was inhibited 50% and 100% by 50 and 100 μM H_2O_2, respectively. Yet in the presence of oxygen the oxidase was only inhibited 5% by 100 μM H_2O_2. These results confirm the finding that the GP oxidase is inhibited by peroxide only when GP is present and also may indicate that oxygen protects against inhibition of the oxidase by hydrogen peroxide.

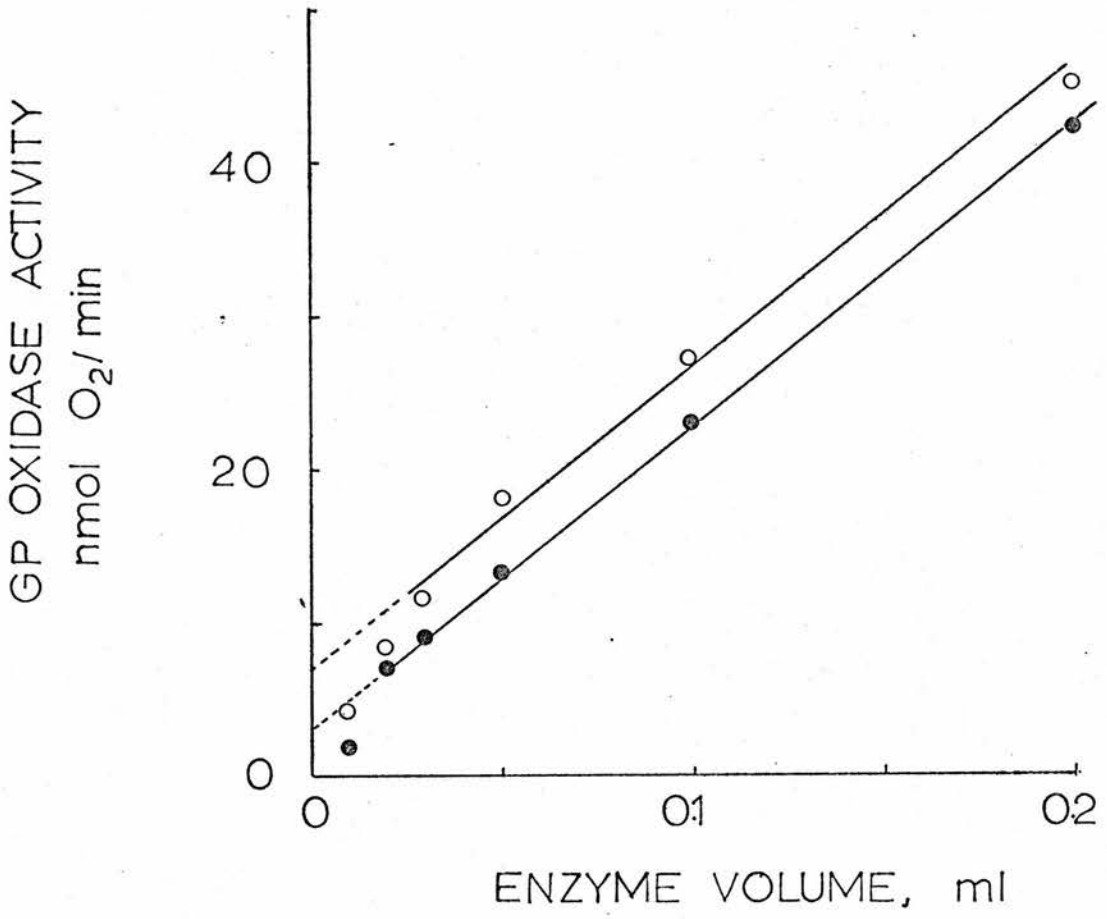


Fig. 4.16. Activity of the GP oxidase measured by a) an oxygen electrode, ●, and b) the coupled spectrophotometric assay, ○. Rates were corrected for autoxidation in a) and NADH oxidation in b). All assays in TS buffer, pH 8.0, containing 16 mM LaGP in a total volume of 2.5 ml, temperature 37°C. Other experimental details as given in the Methods. The rate of NADH oxidation has been converted into the equivalent rate of oxygen utilisation, nmol O_2/min, by dividing nmol NADH/min by the factor 2.0.

8. Stoichiometry of the glycerophosphate oxidase reaction

The oxidation of 5 µmol GP measured in the Warburg respirometer was accompanied by the utilisation of 5 µgatoms of oxygen in agreement with the findings of Grant and Sargent (1960). The addition of catalase and ethanol as a trapping mechanism for H_2O_2 (Keilin and Hartree, 1945) did not increase the oxygen utilisation above the theoretically expected oxygen uptake of 5 µgatoms.

The rate of oxygen utilisation and the rate of DHAP production were measured on the same GP oxidase preparation using the oxygen electrode and the spectrophotometric coupled assay (fig. 4.16). The rates of utilisation of oxygen (ngatom min) and the rate of production of DHAP (nmol NADH/min) are identical over the range 0.05 - 0.20 ml of enzyme after correcting for the zero activity obtained by extrapolating the linear portion of the graph to zero enzyme concentration.

9. Composition of purified GP oxidase

i) further treatment of samples before analysis

Some 15-20 preparations were required for the batch analysis of metal ions, flavin and acid-labile sulphide. 24,000g pellets prepared by the standard procedure were stockpiled and stored for up to 3 months in liquid nitrogen. Small batches of the frozen material were rapidly thawed in a 37°C water bath and combined. To concentrate the GP oxidase, the preparations were centrifuged at 30,000g for 30 min and the supernatant carefully

TABLE 4.9. Results of further fractionation of combined 24,000g pellets that had been thawed and washed with TS buffer, pH 7.4.

PREPARATION NUMBER	1				2				3			
BUFFER	SEP, pH 7.4				TS, pH 7.4				TS, pH 7.4			
FRACTIONS	TOTAL GPO ACTIVITY µmol O_2/min	%	TOTAL PROTEIN mg	%	TOTAL GPO ACTIVITY µmol O_2/min	%	TOTAL PROTEIN mg	%	TOTAL GPO ACTIVITY µmol O_2/min	%	TOTAL PROTEIN mg	%
Combined 24,000g pellets	392	(100)	239	(100)	459	(100)	317	(100)	567	(100)	497	(100)
Combined 30,000g supernatant	50.3	12.8	108	45	5.3	1.2	85	27	8.9	1.6	92	18
Debris (discoloured portion of 30,000g pellet)	3.6	0.9	2.8	1.2	6.3	1.4	4.5	1.4	-	-	-	-
30,000g pellet; COMBINED WASHED PARTICULATE OXIDASE	270	69	91.5	38	392	85	214	68	547	96	313	63
TOTAL	324	83	203	84	404	88	303	96	-	-	-	-

Specific activity of the combined 24,000g pellets were 1640, 1450 and 1140 nmol O_2/min/mg protein and of the combined washed particulate oxidase were 2950, 1830 and 1750 nmol O_2/min/mg protein for preparation 1,2 and 3, respectively.

- signifies not estimated.

removed. The pellet was resuspended in TS buffer, pH 7.4 and centrifuged as before. A small discoloured area, at the bottom of the faint yellow pellet, containing about 1% of the total protein and GP oxidase activity was left in the centrifuge tube and resuspended separately. Some losses of protein and GP oxidase activity were inevitable due to the viscous nature of the concentrated pellet. The results of this further treatment are presented in table 4.9. Freezing and thawing, especially in the presence of SEP buffer, results in the release of 25-50% of the total protein into the sùpernatant fraction. The bulk of the GP oxidase activity is recovered in the 30,000g pellet with a 30 - 80% increase in specific activity compared to the combined 24,000g pellets. The 30,000g pellet, which will be referred to subsequently as the washed, particulate oxidase, was refrozen in 2 ml aliquots until required for analysis.

ii) <u>results of analysis for flavin, metal ions, and acid-labile sulphide in washed, particulate oxidase</u>

Difference spectra were obtained on the three separate particulate oxidase preparations (fig. 4.17). The addition of glycerophosphate bleaches the GP oxidase giving troughs at 446-448 nm and ca.386 nm indicating the presence of flavin in the preparations. No other features were noted except possibly a broad and small increase in absorbance towards the red region of the visible spectrum in the GP reduced sample. The ratio of GP oxidase activity to flavin content (assuming $E_{510-465}^{nm}$ = 11 cm^{-1}, mM^{-1} and that no other interfering absorbing species were present) is the same in the

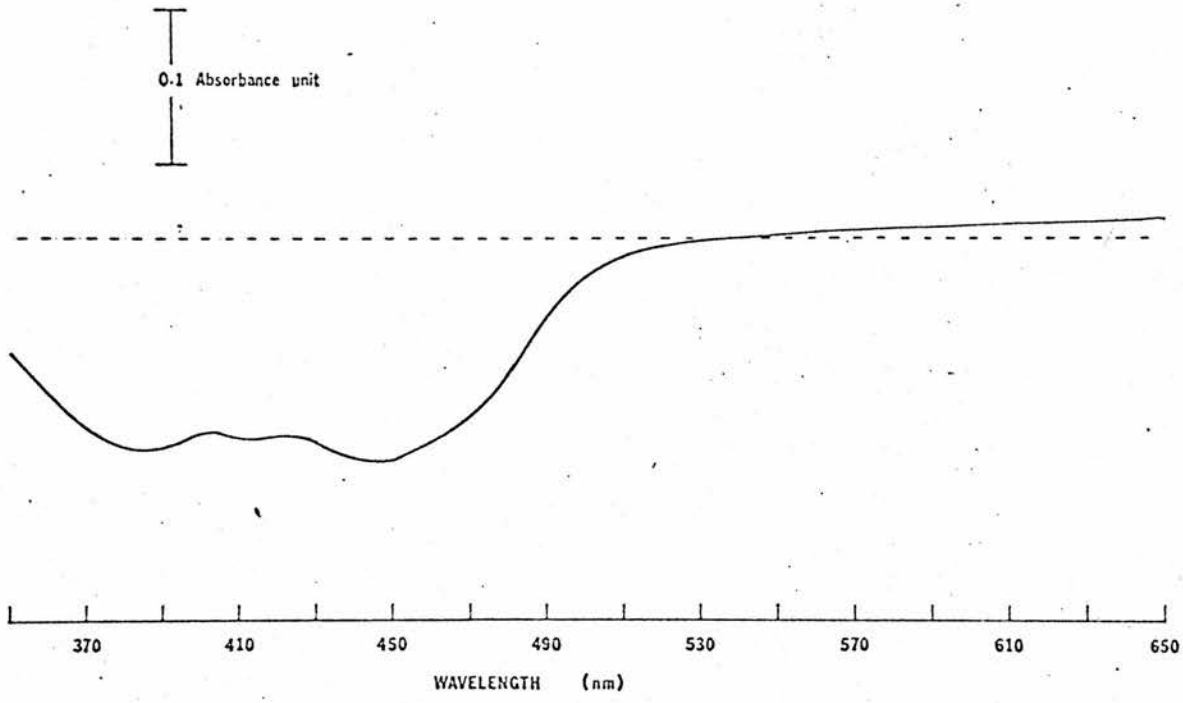


Fig. 4.17. Difference spectrum of combined washed particulate GP oxidase preparation (no. 3). Protein concentration 27.9 mg/ml; specific activity, 1750 nmol O_2/min/mg protein. Dashed line oxidised minus oxidised; solid line, reduced (13.3 mM LaGP) minus oxidised obtained one min after the addition of substrate. TS buffer, pH 7.4.

TABLE 4.10. Flavin content of bulk preparations of GP oxidase

Bulk Prepn. No.	Buffer	Absorption minima nm			$A_{510-465}^{nm}$	A Flavin nmol/ml	B GP oxidase /µmol O_2/min/ml	Ratio B/A
1	SEP pH 7.4	384-6	414	448	0.133	12.09	46.18	3.8
2	TS pH 7.4	387	-	446-448	0.053	4.82	19.33	4.0
3	TS pH 7.4	384-386	412-414	446-448	0.115	10.45	39.28	3.9

Flavin content determined by substrate reduced minus oxidised difference spectra using $E_{510-465}^{nm}$ = 11 cm^{-1} mM^{-1} (Chance, 1957). The GP oxidase activity was measured using the standard assay immediately after obtaining the difference spectrum.

TABLE 4.11. Composition of combined, washed, particulate GP oxidase preparation (no.2).

COMPONENT	METHOD	nmol/mg protein	RATIO, FAD=1	RATIO, FLAVIN=1
Acid-labile sulphide	(methylene blue formation)	~0.1	0	0
"Non-haem"Fe	(TCA extract, o-phenanthroline)	0.07	0	0
"Total" Fe	(glacial acetic acid extract thioglycolic acid bathophenanthroline)	1.31 ± 0.06	4.7	3.5
"Total" Fe	(wet ashed, bathophenanthroline)	2.8 ± 0.2	10.0	7.6
Copper	(wet ashed, dibenzyldithiocarbamate)	1.47 ± 0.02	5.3	4.0
Total flavin	(TCA extract, fluorescence at pH 6.8 after hydrolysis	0.37 ± 0.02	-	1.0
FAD	TCA " " " " " without hydrolysis)	0.278	1.0	-
Flavin	(difference spectrum, GP reduced minus oxidised)	0.282	1.0	-

Methods as described elsewhere. Linear regression of samples and standards was used to determine the standard deviation of each value. Acid-labile sulphide assay was at the limits of detection for the method.

three preparations (table 4.10). All the flavin found by the difference spectrum in preparation no.2 could be accounted for by FAD estimated by a fluorescent method (table 4.11). The total flavin content of this preparation was 33% higher than that for FAD (0.37 nmol flavin/mg protein, 0.278 nmol FAD/mg protein). As 39% of the GP oxidase activity in preparation no.2 had been lost on storage when the difference spectrum was obtained, it is possible that loss in activity of the oxidase is associated with hydrolysis of FAD to FMN and/or riboflavin. If that assumption were correct, then all the flavin in the GP oxidase could be accounted for as FAD. Further experiments are required to confirm this.

Metal analysis of the first bulk preparation gave much lower values for Cu and Fe than the second preparation, especially in those methods not requiring wet ashing of the sample, because the presence of EDTA and phosphate ions was found to interfere with the assays. The ratio of FAD (by difference spectrum, no activity loss) Cu : Fe was 1: 2.2: 3.4, but the values for Cu and Fe are probably underestimated. The results of metal analysis of the second preparation (table 4.11) gave higher values with a ratio of flavin: Cu : Fe (total, after wet ashing) of 1: 4.0: 7.6. The extraction of iron by glacial acetic acid and thioglycollic acid (Doeg and Ziegler, 1962) was not complete as some residual iron was present in the aqueous phase after extraction with bathophenanthroline in iso-amyl alcohol. Insignificant amounts of "non-haem" iron and "acid-labile" sulphide were present. Thus a ratio for flavin (FAD), copper and iron of 1: 4: 8 is tentatively proposed for the GP oxidase.

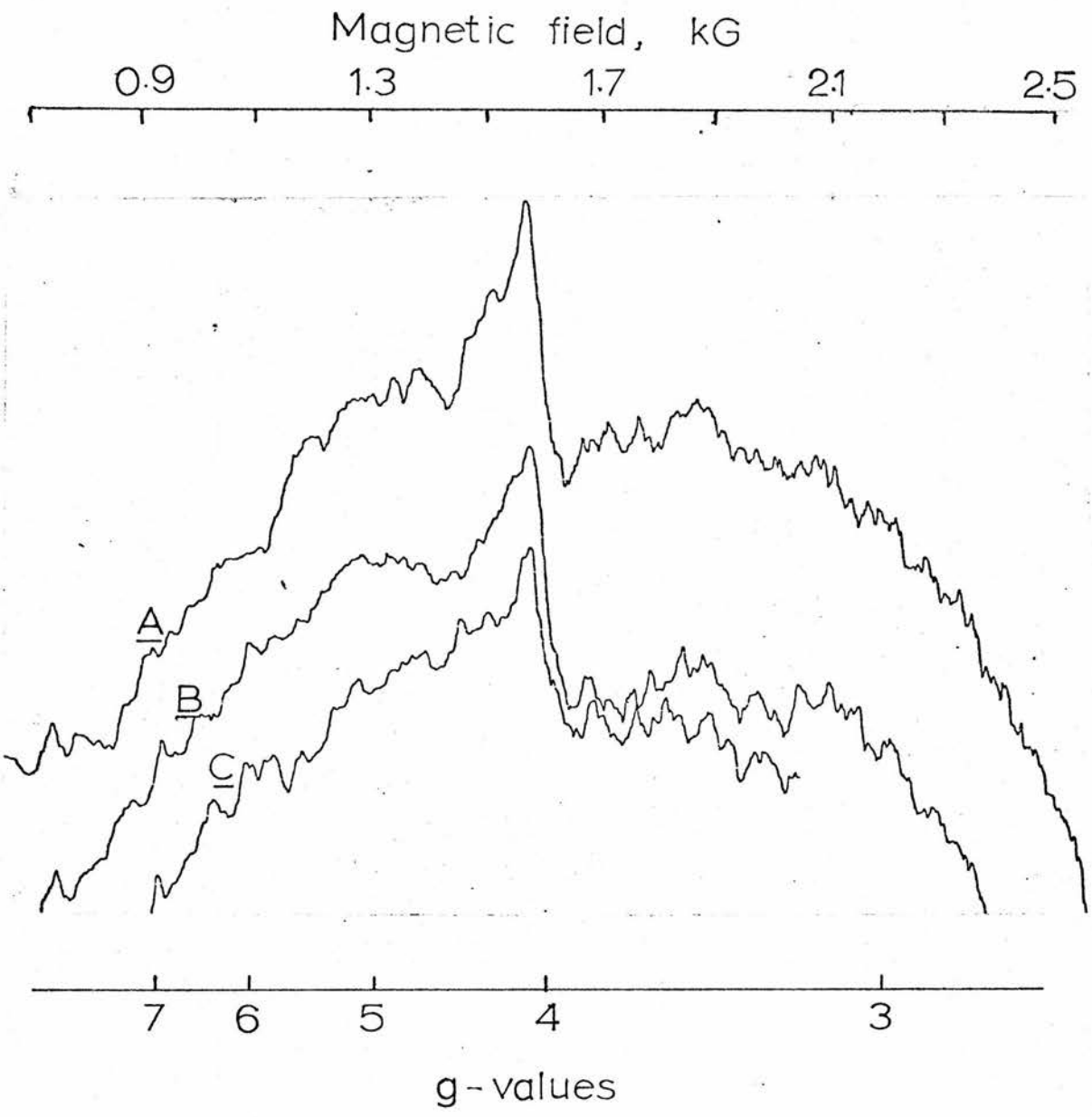


Fig. 4.18. EPR signal at g=4.1 of purified GP oxidase. Details of EPR settings are given in the Methods. A, oxidised, no additions; B, reduced with 13.3 mM LaGP; C, reduced dithionite.

[17.1 mg protein/ml]

10. Results of EPR studies

Freshly prepared purified GP oxidase contained 2, possibly 3 paramagnetic species in the oxidised and reduced forms of the enzyme. The signal at $g = 4.1$ (fig. 4.18) is typical of high-spin ferric iron in a rhombic environment. The addition of substrate (GP) does not alter the size of the signal which is found in a wide variety of organic and inorganic materials and may merely indicate contaminating iron. No signal in the region of $g = 1.94$ has been observed in substrate or dithionite reduced samples of the GP oxidase indicating the absence of iron liganded with acid-labile sulphide as is found in ferredoxins and conjugated iron-sulphur proteins. This confirms the absence of acid-labile sulphide found by analysis (table 4.11).

A second signal at $g = 2.05$ was visible in freshly prepared GP oxidase (fig. 4.19) and the height of the signal was diminished 30 - 50% by the addition of glycerophosphate or abolished by reduction with dithionite. Fig. 4.19 also shows the effect of some inhibitors on the signal at $g = 2.05$. The signal height for $g = 2.05$ in the presence of each inhibitor is approximately the same in the presence or absence of glycerophosphate (table 4.12). The type I inhibitor o-phenanthroline, increases the signal size to greater than that of the oxidised control whereas all type II inhibitors diminish the signal to about that of the reduced control. In some experiments, 8-hydroxyquinoline, but never SHAM, was found to abolish the signal at $g = 2.05$ altogether with the appearance of a signal in the region of 2.0. The signal at about $g = 2.0$ appears to be absent in the phenanthroline treated samples. The shape of the signals at $g = 2.05$ and $g = 2.0$ are slightly different in experiments

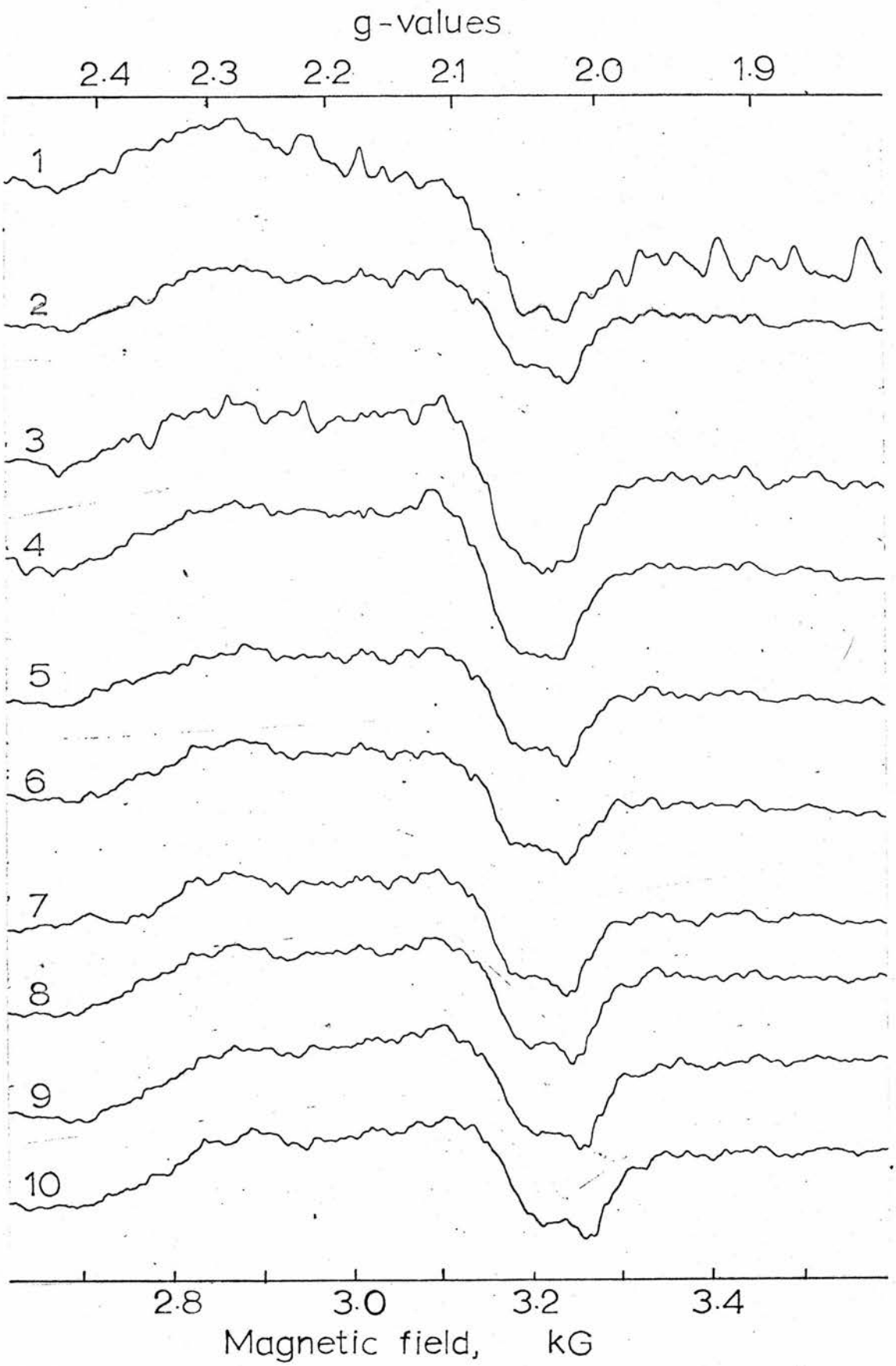


Fig. 4.19. Effect of inhibitors on the EPR signals in GP oxidase preparations. Experimental details as in table 4.12. Trace number: 1,2, controls; 3,4, + o-phenanthroline; 5,6, + SHAM; 7,8, + 8-hydroxyquinoline; 9,10, + H_2O_2; for oxidised and GP reduced samples, respectively. Assays in TS buffer, pH

TABLE 4.12. Effect of inhibitors on the EPR signals in GP oxidase preparations.

INHIBITOR	FINAL CONC. mM	PRE-INCUBATION min	INHIBITOR TYPE	SIGNAL HEIGHT			
				OXIDISED, g=2.05	-GP g~2.0	REDUCED, g=2.05	+GP g~2.0
-	-	-	-	31.0	+	22.4	+
o-phenanthroline*	30	10	I	37.1	-	36.0	-
SHAM *	3.3	2	II	22.9	+	20.9	+
8-hydroxyquinoline*	6.6	2	II	24.8	+	25.0	+
H_2O_2	3.3	5	II	25.9	+	25.1	+

0.3 ml samples of GP oxidase (1457 nmol O_2/min/mg protein, 11.2 mg protein/ml, RSA 7.7) were preincubated at room temperature with the inhibitors, then either 0.01 ml water (oxidised) or 0.01 ml GP (15 mM L form, reduced) added and vigorously aerated for 1 min before freezing.

* Signifies inhibitor added in 0.01 ml DMF. EPR settings are described in the Methods. Signal height is in arbitrary units, measured at the same signal gain throughout.

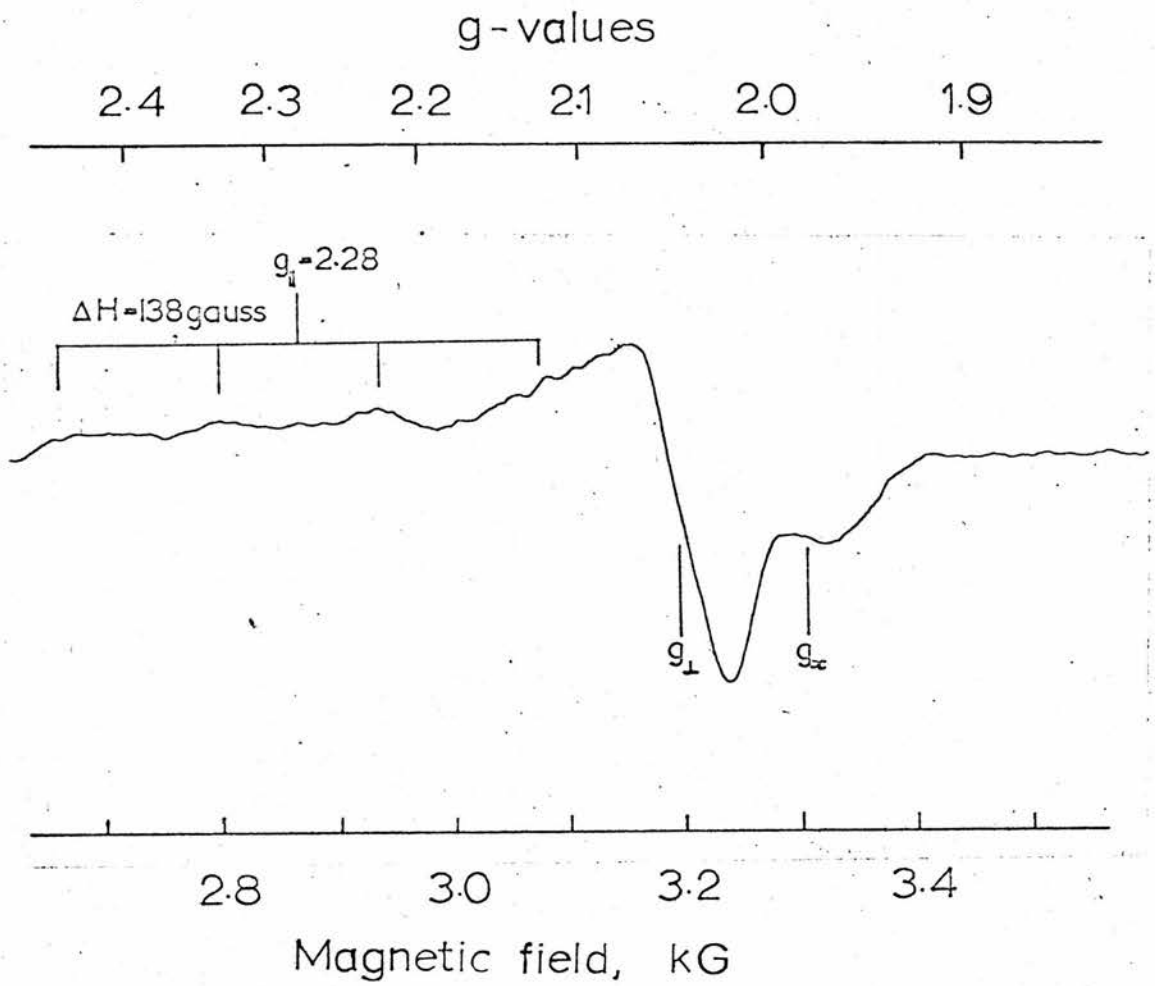


Fig. 4.20. EPR spectrum of the concentrated and dialysed soluble fraction released from the GP oxidase preparations by freezing and thawing. Protein concentration 14.7 mg/ml in 0.02 M tris buffer, pH 8.0. EPR settings as described in the Methods.

when SEP buffer, pH 7.4, was used, but the variation of the signals with the inhibitors is quantitatively similar to those presented in fig. 4.19 and table 4.12.

The main difficulty experienced in obtaining satisfactory EPR signal to noise ratios is the high concentrations of GP oxidase required involving large scale preparations. In an attempt to overcome this difficulty, the combined, washed particulate oxidase preparations were examined for EPR signals. The presence of a signal at $g = 4.1$ was confirmed, but the signal at $g = 2.05$ was completely absent. Freezing and thawing of the preparations had resulted in the release of all this component into the 30,000g supernatant. The 30,000g supernatant was centrifuged at 144,000g for 60 min and it was found that 90% of the signal at $g = 2.05$ was recovered in the soluble supernatant. This fraction was the dialysed against 0.02 M tris buffer, pH 8.0 and concentrated to a small volume with carbowax. EPR studies of this fraction show the presence of a cuproprotein with $g_x \sim 1.98$, g_y ($g_\perp$) = 2.05, g_z($g_\parallel$) = 2.28 (fig. 4.20). Four hyperfine lines are visible centred about $g_\parallel$ with a spacing (ΔH) of 138 gauss . The hyperfine splitting constant (A) for the cuproprotein was determined to be 0.015 cm^{-1} using the following equation:

$$A = \frac{(g\beta)}{(hc)} . \Delta H$$

where A = hyperfine splitting constant

g = g value for the paramagnetic species concerned

β = Bohr magneton

h = Planck's constant

c = velocity of light

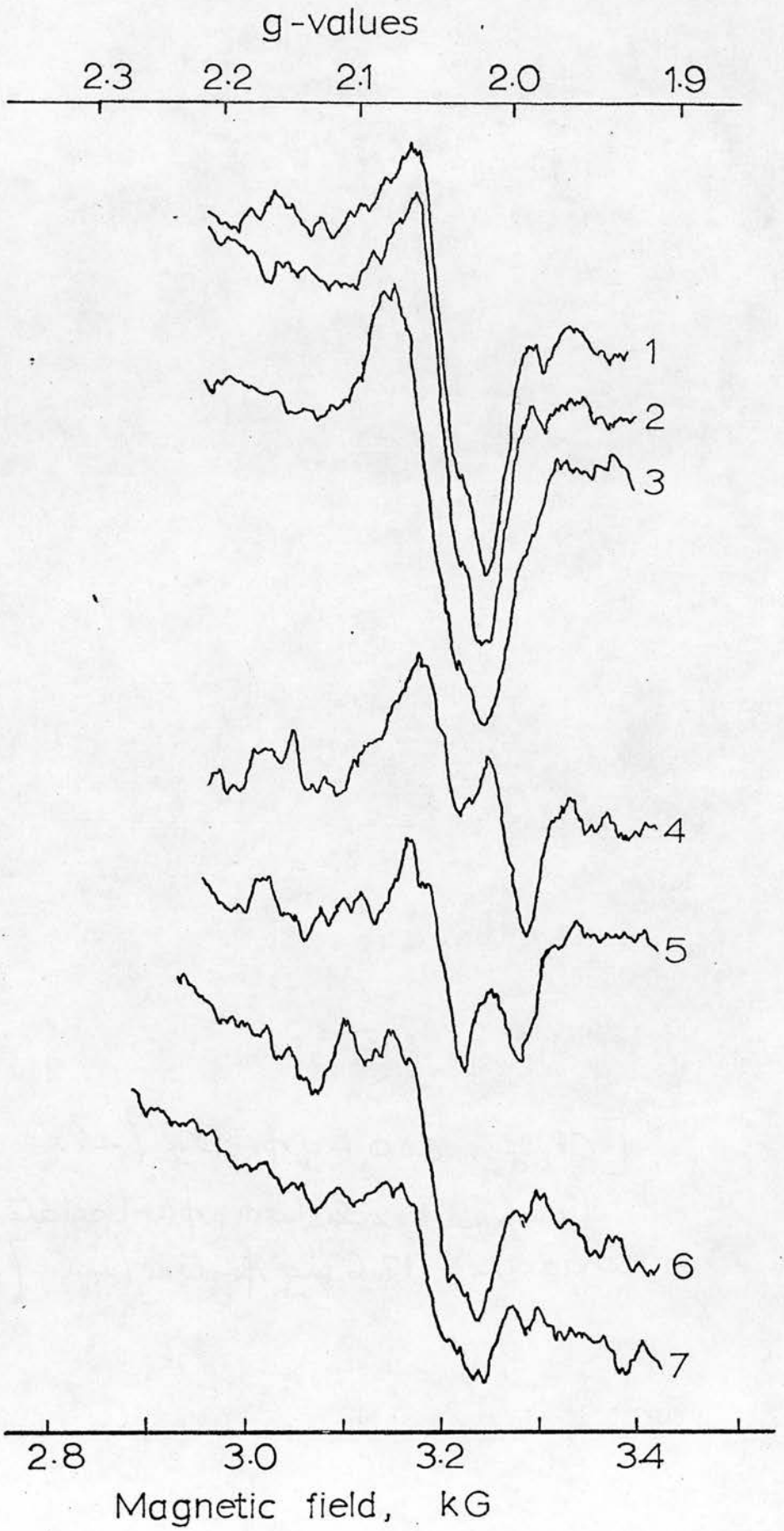
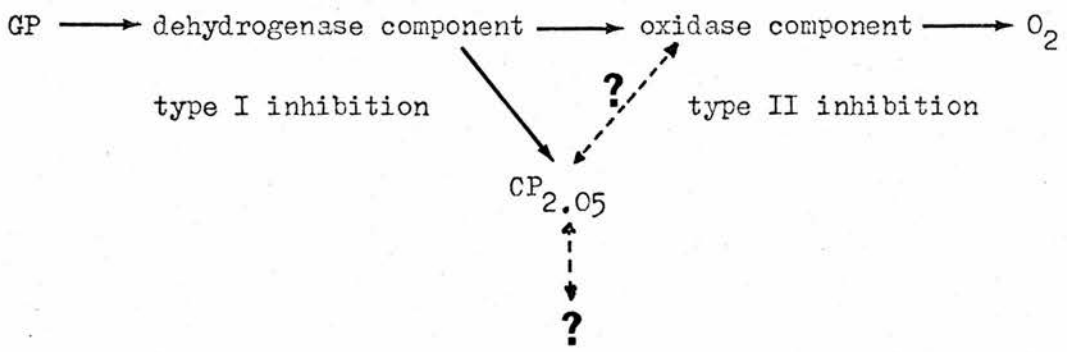

g-values
2·3
2·2
2·1
2·0
1·9
1
2
3
4
5
6
7
2·8
3·0
3·2
3·4
Magnetic field, kG

Fig. 4.21. The effect of various compounds on the soluble cuproprotein $CP_{2.05}$ isolated free of GP oxidase activity. Trace 1, no treatment; 2, $CP_{2.05}$ + glycerophosphate (13.3 mM LaGP); 3, $CP_{2.05}$ + o-phenanthroline (20 mM); 4, $CP_{2.05}$ + 8-hydroxy-quinoline (4 mM); 5, 10 /uM $CuSO_4$ + 8-hydroxy-quinoline (4 mM); 6, 0.3 ml $CP_{2.05}$ + 0.1 ml washed particulate GP oxidase (oxidised); 7, 0.3 ml $CP_{2.05}$ + 0.1 ml washed particulate oxidase (reduced, 13.3 mM LaGP). EPR settings as described in the Methods. The same signal gain setting was used throughout. Assays in SEP buffer, pH 7.4.

[$CP_{2.05}$: 4.0 mg protein/ml. Combined washed, particulate oxidase, 17.6 mg protein/ml]

The optical and near infra-red absorption spectrum showed no absorbance in the 600 - 800 nm region indicating that the copper present was of the 'non-blue' type. All 'non-blue' cuproproteins that have been studied so far by EPR techniques have hyperfine splitting constants (A) in the range 0.015 - 0.020 cm^{-1} in contrast with 'blue' type copper proteins which have a value for A in the range 0.0031 - 0.0090 cm^{-1}, i.e. have a small value for ΔH, and an extremely strong absorbance peak in the 600 - 800 nm region of the optical spectrum (Malkin and Malmstrom, 1970; Vanngard, 1972; Malmstrom, 1973). The enzymic function, if any, of this cuproprotein with a g value of 2.05 (referred to subsequently as $CP_{2.05}$) is not known. $CP_{2.05}$ does not oxidise catechol, hydroquinone, ascorbate $\pm$ TMPD, glycerophosphate, NADH or NADPH when assayed in the oxygen electrode in TS buffer, pH 8.0, and is not a GP-dependent peroxidase or catalase. A small amount of superoxide dismutase activity was associated with $CP_{2.05}$, but was too small to account for the copper estimated by analysis. Neither does $CP_{2.05}$ appear to be involved in the GP oxidase reaction as the properties of the particulate oxidase washed free from $CP_{2.05}$ appear to be the same as freshly prepared GP oxidase with regard to i) stoichiometry of the reaction ii) sensitivities to type I and II inhibitors, iii) absence of peroxide production (fig. 4.13). In the light of these negative findings the effects of inhibitors on isolated $CP_{2.05}$ was investigated (fig. 4.21). It was established that glycerophosphate alone does not reduce the copper signal, washed particulate GP oxidase alone does not reduce the cuproprotein, but in the presence of both GP and particulate GP oxidase the size of the g = 2.05 signal is diminished by 35% (fig. 4.21). The addition of o-phenanthroline

does not affect the size of the signal at g = 2.05, but 8-hydroxyquinoline partly abolishes the signal at 2.05 with the appearance of a new signal at about 2.0, similar to that found in some experiments with the GP oxidase. A mixture of 10^{-5}M Cu^{2+} and 2 mM 8-hydroxyquinoline was observed to give a similar EPR signal. These data suggest that although $CP_{2.05}$ is not an essential component of the GP oxidase it can be partially reduced by the oxidase. The inhibitor studies would suggest that the site at which $CP_{2.05}$ receives electrons is beyond the site of inhibition of o-phenanthroline, possibly as in the scheme below:



Another possibility is that $CP_{2.05}$ is an artifact of the preparative method and until some enzymic function has been identified or until the cuproprotein has been fully characterised, this possibility cannot be excluded.

CHAPTER 5

DISCUSSION

A. CELL DISRUPTION AND SUBCELLULAR FRACTIONATION

1. The effect of saponin on live bloodstream trypanosomes

Saponin has a pronounced effect on the intact trypanosome. The cells rapidly become swollen and non-motile (fig. 3.6) with the loss of both the plasma membrane and the surface coat of the pellicle (fig. 3.17). The swelling of the cells is also associated with a spreading or loosening of the subpellicular tubules and the release of soluble protein from the trypanosome. Cross (1973) has calculated from electron micrographs of T.brucei that the surface coat comprises about 10% of the total cell protein as do the soluble 4S antigens. On treatment with saponin, 29% of the total cell protein is released into a soluble fraction (table 3.2), indicating that more than just the surface coat is released. After removal of the saponin supernatant the intact swollen cells are then easily disrupted by gentle homogenisation (table 3.2 , fig. 3.8). A concentration of saponin one tenth of that required for disruption of the trypanosomes also causes cell death, but the amount of protein released by saponin into the 2,700g supernatant is significantly less at 18% and the cells are resistant to disruption by homogenisation (table 3.2). Unfortunately, the effect of saponin at these lower concentrations has not been examined by electron microscopy to show whether the surface coat was absent and whether the plasma membrane was intact or not.

Saponin has been used for the lysis of erythrocytes in the isolation of the erythrocytic stages of the malarial parasite (Christophers and Fulton, 1939) and in the preparation of surface

membranes from cercariae, schistosomula and adult worms of Schistosoma mansoni (Kusel, 1972). Saponin (0.5% w/v) alone caused considerable fragmentation of the membrane surfaces of schistosomes, but if Ca^{2+} were present during treatment fragmentation did not occur and sheets of surface membrane could be isolated (Kusel, 1972). However, Ca^{2+} has not been tested for any modifying effect on the disruption of trypanosomes pretreated with saponin. Pretreatment with digitonin has been used as a method of preparing mitochondria with respiratory control from Crithidia fasciculata (Kusel and Storey, 1972) and for subcellular fractionation studies in Crithidia fasciculata (Edwards, 1974). Digitonin has also been used for the differential fractionation of isolated rat liver mitochondria by removing the outer membrane without apparently damaging the inner membrane and matrix (Schnaitman and Greenawalt, 1968). Pretreatment of T.brucei with digitonin does facilitate the disruption of the cells by gentle homogenisation, but at the concentration of digitonin required to achieve this the GP oxidase activity is strongly inhibited. Thus, despite the structural similarities between these two detergents, saponin and digitonin would appear to act differently. Digitonin's mode of action against the GP oxidase is almost certainly as a result of its detergent properties as it inhibits the oxidase like the other anionic and non-ionic detergents tested (figs. 4.6, 4.7). Kusel and Weber (1965), however, have speculated that digitonin induced lysis of C.fasciculata was not due to its detergent action, but rather to the specific reaction of digitonin with 3-β-hydroxysteroids thought to be an integral component of the plasma membrane.

2. The effect of saponin on the internal organisation of the trypanosome

Saponin causes extensive changes in many of the subcellular organelles of the trypanosome as well as removing the surface coat and plasma membrane. Many vesicular structures have lost their homogeneous matrices or become swollen (fig. 3.17). Saponin treated cells and the 24,000g preparation of the GP oxidase have been tested for latency of acid phosphatase activity. In the presence of 0.1% (v/v) triton X-100 the acid phosphatase activity increased by 10% in the saponin treated cells, but no increase was noted in the 24,000g pellet probably indicating that the lysosomal membrane had become permeable to the substrate p-nitrophenyl phosphate. However, although some acid phosphatase activity is released into the soluble saponin supernatant (fig. 3.9) this may not be due to lysosomal disruption as large amounts of acid phosphatase are reported to be secreted into the flagellar pocket of the bloodstream, but not the culture form of the trypanosome (Jadin and Creemers, 1972; Langreth and Balber, 1975). Also, the sedimentation in sucrose density gradients of the acid phosphatase activity was entirely different from that found by Opperdoes (personal communication) when homogenates of T.brucei, obtained by grinding with silicon carbide, were centrifuged under similar conditions. Opperdoes found that acid phosphatase activity formed a single major peak equilibrating at a modal density of about 1.22, whereas in these studies the acid phosphatase activity did not enter the linear portion of the gradient, equilibrating at a density of 1.12 - 1.14 (fig. 3.1b). This sedimentation pattern is similar to that obtained when rats are administered triton WR-1339 some hours prior to isolation of

the lysosomal fraction from liver homogenates. Triton WR-1339 is taken up into the liver lysosomes and the presence of the detergent decreases the isopycnic density of the lysosomes in sucrose density gradients from 1.22 to 1.12 (Beaufay, 1969). Another possible explanation for the anomalous sedimentation of acid phosphatase in sucrose density gradients is that the enzyme is bound to, is a constituent of, small membrane fragments from broken lysosomes —— characterisation of the acid phosphatase fraction by electron microscopy could eliminate this possibility.

Not all the intracellular organelles were damaged by the presence of saponin. Particles similar to GP oxidase microbodies, nuclei and kinetoplasts appeared to be intact as judged by electron microscopy (fig. 3.17).

3. Subcellular distribution of the glycerophosphate oxidase and other enzymic activities

Several enzymic activities were assayed to establish if any of them were associated with the GP oxidase particles and to assess the purity of GP oxidase preparations. Glycerophosphate oxidase, GP tetrazolium reductase and GP: PMS oxidoreductase activities were found to be distributed identically in subcellular fractions obtained by differential centrifugation or by isopycnic sucrose density gradient centrifugation (figs. 3.9, 3.15; table 3.3). Bide (1964) had claimed that the distribution of GP: PMS oxidoreductase and the GP oxidase was dissimilar and therefore the GP: PMS oxidoreductase was not a component of the GP oxidase. But Bide's experiments are

open to criticism, because i) disruption of the cells by sonication inactivates the GP oxidase more readily than the dehydrogenase, ii) it is difficult to control for the autoxidation of PMS in Warburg assays and moreover iii) hydrogen peroxide formed from reduced PMS is reported by Grant and Sargent (1961) to inhibit the GP dehydrogenase. The fact that inhibitors of the GP:PMS oxidoreductase (suramin and analogues, p-OMB, melarsen oxide, Tiron and o-phenanthroline) are equally effective inhibitors of the GP oxidase support the view that the GP:PMS oxidoreductase is identical to the glycerophosphate dehydrogenase component of the oxidase.

Tetrazolium salts, especially INT, would also appear to be artificial electron acceptors for the GP oxidase and therefore suitable reagents for its histochemical location. Grant and Sargent (1961) and Bide (1964) have reported that the GP dehydrogenase will also reduce methylene blue, menadione, dichlorophenol-indophenol, but not cytochrome c. These findings have been confirmed, but not yet extensively studied, although they are potentially valuable probes in the further differentiation of the sites of action of the inhibitors of the GP oxidase. The distribution of NADH tetrazolium reductase activity was different from either the GP oxidase or GP tetrazolium reductase activities (fig. 3.9). Vickerman (1965) observed that formazan deposits were scattered throughout the cytoplasm of monomorphic bloodstream forms of the trypanosome after incubation with NADH and nitro-blue tetrazolium. These deposits may not in fact locate the GP oxidase as Vickerman had postulated, but some other unidentified enzyme.

NAD^+- dependent GPDH from cells disrupted after saponin pre-treatment does not sediment with the GP oxidase either in subcellular fractionation (fig. 3.9) or in sucrose gradients (figs. 3.14, 3.15, 3.16), but under these conditions appears to be wholly membrane bound and associated mainly with the largest particle fraction. The observation that other glycolytic enzymes may also be membrane bound is being investigated.

No suitable enzyme marker was identified for the promitochondrial tubule of the trypanosome which, it was anticipated, would sediment in the 24,000g pellet together with the GP oxidase microbodies. The choice of possible enzymes is limited, because in the monomorphic bloodstream form the promitochondrion is non-functional and even in the short stumpy form of the pleomorph the activities of many of the enzymes of the tricarboxylic acid are extremely low (Flynn and Bowman, 1973). Malate dehydrogenase, $NADP^+$-dependent isocitrate dehydrogenase and aspartate aminotransferase activities were identified in the monomorphic strain used in this study, but less than 10% of malate dehydrogenase and aspartate aminotransferase were found in the 24,000g pellet and the activity of the isocitrate dehydrogenase was too low for accurate analysis. Recently, an oligomycin-sensitive ATPase has been identified in the promitochondrion of the bloodstream form of T.brucei (Opperdoes and Borst, personal communication). If F_1 ATPase is present in the monomorphic strain used in these experiments, then this enzyme would be a potentially useful marker for the promitochondrion.

The subcellular distribution of the GP oxidase has been reported from bloodstream forms of T.rhodesiense (Bide, 1964; Bide and Grant,

1964) and T.equiperdum (Bayne et al., 1969a). Bide (1964) and Bide and Grant (1964) found that 80% of the GP oxidase activity from cells disrupted by ultrasonication could be recovered in two fractions (10,500g for 10 min and 41,200g for 60 min). The specific activity of the 10,500g fraction was about 2-3 times greater than the starting specific activity. Bayne et al. (1969a) disrupted T.equiperdum by repeated homogenisation in a motor-driven Potter-Elvejhem homogeniser, removing the broken cells at intervals by centrifugation at 600g for 10 min and repeating the homogenisation until a small remaining pellet was discarded. 90% of the recovered activity was found in 12,000g x 10 min pellet with a specific activity 3.4 times greater than the homogenate. In the present studies 20-30% of the GP oxidase activity was found in a twice washed 1,000g pellet and 60-70% in a 24,000g pellet. About 50-60% of the GP oxidase activity was recovered in a 14,000g pellet, i.e. about 10% less than in a 24,000g fraction. Washing the 1,000g pellet once or twice releases about 10% and 15% more of the oxidase into the supernatant. No whole cells were visible in the washed 1,000g pellet so the remaining activity may be associated with some cell organelles sedimenting faster than the GP oxidase microbodies. The final specific activity of the GP oxidase in the 24,000g pellet prepared by the standard procedure, but omitting the filtration step through CM23 cellulose, was about 1,000 nmol O_2/min/mg protein, about 5 times higher than the starting specific activity (table 3.4). Electron microscopy of the 10,500g pellet in Bide's preparation showed the presence of large numbers of flagellar fragments, as did the "light" density-gradient fraction in the preparation of Bayne et al. (1969a). Similar contamination with flagella was noted by phase contrast microscopy of the 24,000g pellet

prepared as above. However, after filtration through a column of CM23 cellulose the number of flagella in the 24,000g pellets was considerably decreased or completely absent with about 2 fold improvement in the final specific activity of 1,860 nmol O_2/min/mg protein, 9-10 fold purified overall (table 3.4). The removal of flagella and pellicular microtubules was confirmed by electron microscopy (fig. 3.17) and by the absence of a peak at d = 1.25 on the sucrose density gradient (fig. 3.10). However, it was noted that in some gradient experiments a peak at d = 1.25 was present and that this was related to a lower specific activity of the 24,000g pellet presumably indicating that some breakthrough of flagella and pellicle had occurred at the CM23 cellulose stage of the preparation. The optimal conditions for the amount of CM23 cellulose/ml packed cells was not determined, but with more than 5 ml packed trypanosomes per 10 gm wet CM23 cellulose the specific activity of the GP oxidase preparation was decreased. For quantities greater than 5 ml, 2 columns were used, as simply increasing the column length of CM23 cellulose markedly decreased the flow rate. The protein peak at d = 1.25 has not been examined by electron microscopy to confirm the presence of flagella and pellicular microtubules. The location of the NAD^+-dependent GPDH in this density fraction has already been noted and as it is almost absent from the GP oxidase peak, this enzyme could be used to assess the purity of GP oxidase preparations.

The GP oxidase on isopycnic sucrose density gradient centrifugation has a modal equilibrium density of 1.169 (table 3.6) which is in agreement with the value of d ~ 1.18 found by Opperdoes (personal communication) using a similar method with the important difference

that cells were disrupted by grinding with silicon carbide. Thus saponin treatment does not appear to affect the equilibrium density of the oxidase particles. In two gradient experiments small peaks of GP oxidase containing about 15% of the activity loaded onto the gradient were present at densities of d = 1.21 and 1.25 (fig. 3.14, 3.15 experiments GIIA, GVII, respectively). As noted above the specific activity of the GP oxidase loaded onto these gradients was lower than expt. GIVA and B (fig. 3.12, 3.13) and also both gradients show a protein peak at d = 1.25. Thus the GP oxidase associated with these minor peaks could be associated with flagella and pellicle. A minor peak of GP oxidase activity at d ~ 1.22 was noted in experiments done by Opperdoes (personal communication) which would tend to confirm this observation. In contrast, Bayne et al. (1969a) found that the GP oxidase formed a main peak at a density of d = 1.10. However, the isopycnic density for the oxidase particles was unlikely to be achieved after centrifuging at 16,000g for 20 min, and the 3 to 4 fold increase in specific activity at the peak of the GP oxidase activity has been achieved by the rapid sedimentation of other contaminating protein. The overall maximum purification achieved by these authors was 14 fold. In the experiments presented here the purification achieved by density gradient centrifugation was 2-3 fold or an overall mean purification of 23 times the starting specific activity and an overall yield of 24%. The maximum purification in a single fraction could be somewhat higher than this, but the yield of GP oxidase activity was considerably lower.

Attempts to solubilise the glycerophosphate oxidase were unsuccessful. The non-sedimenting oxidase fraction prepared using

the French pressure cell (144,000g supernatant) did not contain GP oxidase in a soluble form, but, rather, bound to small membrane fragments, as this fraction had a molecular weight greater than 5×10^6 by agarose gel exclusion chromatography and could be sedimented at 314,000g for 60 min.

4. Localisation of the glycerophosphate oxidase and purity of the final preparations

To sum up the previously mentioned findings, then, and to relate this to the ultrastructural appearance of the fractions it can be said that the mean purification achieved by differential centrifugation in the 24,000g pellet was about 9-10 fold higher (1860 nmol O_2/min/mg protein) than the starting specific activity (200 nmol O_2/min/mg protein). Electron microscopic examination of an 8 fold purified preparation shows the pellet to consist of vesicular structures of varying sizes, endoplasmic reticulum and lesser amounts of pellicular microtubules and the occasional flagellar fragment. No promitochondrial fragments were identified, but are presumed to be present in the 24,000g pellet. The vesicular structures are bounded by a single membrane and appear to be swollen or to have lost their contents. Vesicles like the microbodies claimed by Bayne et al. (1969a) to contain the GP oxidase activity were present in small numbers. Their diameters range from 0.3 to 0.5 μ in agreement with Bayne et al. (1969a). Considerable amounts of acid phosphatase, NAD^+-dependent GPDH and NADH tetrazolium reductase activities are found in these preparations.

After centrifugation of the 24,000g pellet on linear sucrose density gradients the mean purification of the GP oxidase increases to 24 fold overall, and the maximum purification achieved was 37 fold overall, with specific activity 6,100 nmol O_2/min/mg protein (table 3.6). Electron microscopic examination of this 37 fold purified oxidase fraction shows many distorted vesicles and a complete absence of pellicles, flagella and endoplasmic reticulum present in the 24,000g pellet (fig. 3.17). Many of the vesicles are empty or distorted and a considerable amount of amorphous material is lying outside the vesicles presumably as a result of damage sustained during or after fixation. Some of the particles appear to have been fixed in the process of discharging their amorphous matrix. Promitochondria cannot be identified in these sections. It is not known whether saponin selectively damages the promitochondrion or alters its equilibrium density in sucrose gradients or whether filtration through CM23 cellulose removes these particles. From fig. 3.16 it can be seen that for the density cohort d = 1.16 to 1.18 the percentage of total activity recovered ($\pm$SEM) in this fraction for GP oxidase, acid phosphatase, NAD^+-dependent GPDH and protein are 33 ± 5, 9 ± 2, 10 ± 2 and 16 ± 4, respectively. Unfortunately, no data are available on the relative abundance of these enzymes compared to the starting homogenate in any gradient experiment.

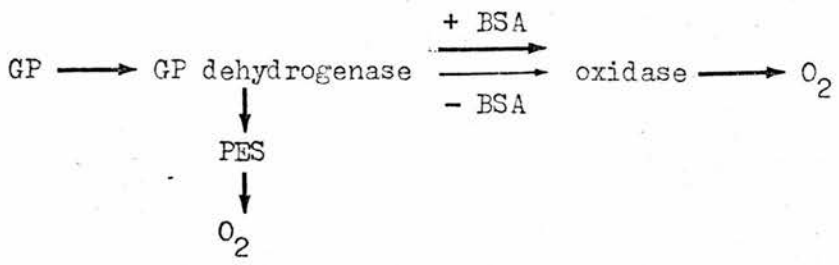
Electrophoresis of SDS extracts of a 14 fold purified GP oxidase preparation shows the presence of 4 main protein bands and 2 minor components (fig. 3.18). Increasing the protein concentration 5 fold revealed a third minor component. Gel electrophoresis of more highly purified GP oxidase fractions has not been carried out to establish which of these components are an integral part of the GP oxidase microbodies or merely protein contaminants.

Thus, although the GP oxidase mircobodies obtained by a combination of differential and density gradient centrifugation still show evidence of impurities, as judged by enzymic analysis and electron microscopy, this preparative method is suitable for the isolation of purified GP oxidase in satisfactory yields.

B. PROPERTIES OF THE GLYCEROPHOSPHATE OXIDASE

1. Conditions for optimal assay

The pH for optimum activity for the GP oxidase was determined to be $pH_{\frac{1}{2}}$ = 8.0 in agreement with Grant and Sargent (1960). The activity at any given pH was slightly higher in tris buffer than in phosphate buffer (fig. 4.1). The pH optimum for the GP dehydrogenase component using phenazine ethosulphate or methosulphate as electron acceptor has not been determined. Metal ions were not found to be essential for optimal activity and Cu^{2+}, Zn^{2+} and Ag^{+} were inhibitory (table 4.3). The addition of ADP and Mg^{2+} had no effect on the activity of the GP oxidase suggesting that the GP oxidase is not coupled to the phosphorylation of ADP in agreement with Grant and Sargent (1960) and Bide (1964). The detergent saponin activates the GP oxidase in the absence of BSA, but apparently inhibits the transfer of electrons to PMS and PES. Bovine serum albumin (BSA) activates the GP oxidase by up to 3 fold, but has little effect on the GP dehydrogenase component (table 4.1, fig. 4.2). Both the apparent K_m and V_{max} for the GP oxidase were altered by the addition of BSA when assayed in TS buffer, pH 8.0. The presence of BSA decreases the K_m for GP from 6.5 $\pm$ 0.7 to 1.72 $\pm$ 0.08 mM and increases the V_{max} from 100 $\pm$ 4 to 227 $\pm$ 4 nmol O_2/min (fig. 4.3). BSA prepared free of fatty acids was almost as effective in activating the GP oxidase as BSA that had not been purified. The activation curve was similar for both types of BSA and the concentration required for maximum activity was 3 mg/ml. In the presence of BSA the activity of the GP dehydrogenase was slightly greater than that of the GP oxidase indicating that the second component of the oxidase is rate limiting. These findings can be summarised by the following scheme:



2. Stability

The GP oxidase and its dehydrogenase component are extremely unstable when stored in solution. Grant and Sargent (1960) found that water-lysed preparations lost 70% of the original oxidase activity after 24h at 2°C when exposed to air. Bide (1964) reported that the stability of the oxidase was improved by storing the enzyme at 4°C in 0.25 M sucrose adjusted to pH 7.4 in the presence of 0.5 mM GP and under nitrogen. Under these conditions 90% of the activity remained after 6 days. From fig. 4.4, it can be seen that, when stored in SEP buffer, pH 7.4 at 0° and exposed to air, GP oxidase and GP dehydrogenase activities are lost at the same rate, with 50% remaining after 3.5 days. In samples frozen at -20°C or -196°C both activities remain essentially constant over a period of 8 months. Freezing and thawing the enzyme samples 5 times resulted in the loss of 16% of the GP oxidase and 10% of the dehydrogenase activity (fig. 4.5). Both activities are labile at 37°C, with 50% loss in activity after $1\frac{1}{2}$ h, so exposure to this temperature during the thawing of frozen samples must be minimised. Freezing and thawing the partially purified samples prepared for metal and flavin analysis releases 20-45% of the total protein into a 30,000g supernatant fraction without apparently affecting the particulate GP oxidase and dehydrogenase activity (table 4.9). A copper

protein giving an EPR signal at $g = 2.05$ was also completely dissociated from the GP oxidase activity and does not appear to be a component of the GP oxidase.

3. Specificity of the GP oxidase reaction

Grant and Sargent (1960) and Bide (1964) found that the stoichiometry of the GP oxidase reaction was 1 mol L α GP utilised/g atom O_2 and that dihydroxyacetone phosphate and H_2O were the products according to the equation:

$$GP + \tfrac{1}{2} O_2 \longrightarrow DHAP + H_2O$$

It was confirmed that the oxidation of 5 μmol GP by the GP oxidase measured in a Warburg respirometer was accompanied by the utilisation of 5 μg atoms of oxygen. The addition of catalase and ethanol as a trapping agent for H_2O_2 (Keilin and Hartree, 1945) did not increase the oxygen utilisation above the theoretical limit of 5 μg atoms.

The rate of oxygen utilisation and the rate of production of DHAP were found to be identical (1 ng atom O_2/min per 1 nmol DHAP/min) when assayed on the same sample of GP oxidase (fig. 4.16). Catalase did not affect the rate of oxygen utilisation (measured in the oxygen electrode) or the rate of production of DHAP (measured spectrophotometrically by the coupled assay). These experiments indicate that H_2O_2 is not an end product of the GP oxidase reaction in agreement with the findings of Grant and Sargent (1960), Bide (1964) and Bide and Grant (1964).

Attempts to measure the affinity for oxygen of the GP oxidase using the equipment available was unsatisfactory because of the low apparent K_m, estimated to be less than 2 μM. Ideally, a closed system with a rotating or vibrating electrode for measuring low oxygen concentrations should be used for the determination of oxygen affinity.

4. Tests for hydrogen peroxide as an intermediate of the GP oxidase reaction

Grant and Bowman (1963), Bide and Grant (1964) and Grant (1966) have proposed that the GP oxidase may consist of an L-glycerol-3-phosphate: oxygen oxidoreductase and a substrate specific peroxidase (L-glycerol-3-phosphate: hydrogen peroxide oxidoreductase) catalysing the reactions:

$$GP + O_2 \longrightarrow DHAP + H_2O_2 \qquad (1)$$

$$GP + H_2O_2 \longrightarrow DHAP + 2H_2O \qquad (2)$$

Sum:
$$2GP + O_2 \longrightarrow 2DHAP + 2H_2O \qquad (3)$$

If this were so one might expect trace amounts of H_2O_2 from reaction (1) to accumulate in the reaction medium if reaction (2) were rate limiting. If reaction (1) were rate limiting then under steady state conditions a constant small amount of H_2O_2 might still be detectable.

Two sensitive fluorescent methods were used for the estimation of trace amounts of H_2O_2. The sensitivity of the scopoletin method was limited because stoichiometric (1 : 1) oxidation of

scopoletin by H_2O_2 was not found (fig. 2.6) and small decreases in fluorescence may not be detectable against the high background fluorescence of the scopoletin substrate. The stoichiometry of the fluorescence decrease with the addition of standard amounts of peroxide was further diminished when GP oxidase was present (fig. 4.13). Nonetheless, no hydrogen peroxide was detected in freshly prepared GP oxidase or in the washed particulate GP oxidase preparations in the presence or absence of the substrate GP. Neither was H_2O_2 detectable when the GP oxidase was inhibited by triton X - 100. Loschen et al. (1971) demonstrated the production of H_2O_2 in pigeon heart mitochondria in the presence of succinate and antimycin A, but not with malate or glutamate in the presence of rotenone. It is probably the absence of catalase and GSH-peroxidase which enables a direct determination of H_2O_2 in pigeon heart mitochondria, but not in rat liver where these two enzymes are present. It was suggested by these workers that the H_2O_2 probably originates from the completely reduced flavin component of succinate dehydrogenase.

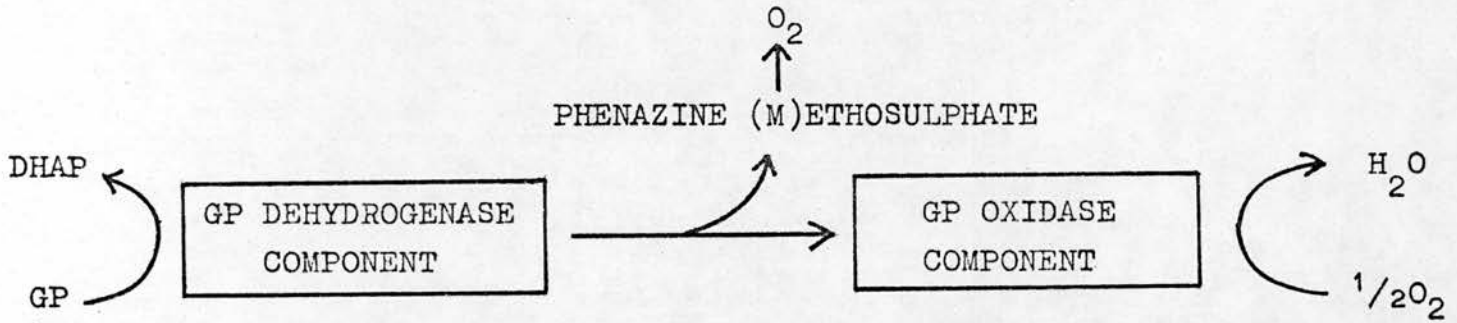
The oxidation of dichlorofluorescin (LDCF) to its fluorescent product dichlorofluorescein (DCF) by HRP was more sensitive in detecting hydrogen peroxide than the scopoletin method. Again no accumulation of hydrogen peroxide was detected in the GP oxidase reaction (fig. 4.14) and the rate of production of H_2O_2, if any, is less than 0.5% of the rate of oxygen consumption.

Thus, failure to detect H_2O_2 by these two fluorescent methods or to alter the stoichiometry of the GP oxidase reaction by means of a catalase and ethanol trap indicates that peroxide is not released from the particulate oxidase into the reaction medium.

The possibility that peroxide exists as a transient enzyme-bound intermediate cannot be excluded.

5. Estimation of glycerophosphate peroxidase activity

Bide and Grant (1964) reported that particulate fractions containing GP oxidase activity would rapidly reduce hydrogen peroxide in the presence of glycerophosphate. However, Bide (1964) found that the activity of the "GP peroxidase" never exceeded 1/3 that of the GP oxidase, suggesting that this enzyme is not a functional part of the GP oxidase reaction. One puzzling feature noted by Bide (1964) was that the activity of the GP peroxidase was not proportional to enzyme concentration, but was found to increase exponentially with increasing protein concentration. The maximum "GP peroxidase" activity found in this study never exceeded 20% of the GP oxidase activity in fresh preparations (fig. 4.15). Concentrations of H_2O_2 greater than 10^{-4}M inhibited the "GP peroxidase" reaction in a manner similar to that of the GP oxidase. Acetone powders or lyophilised preparations in which the GP oxidase was inactive, did not contain "GP peroxidase" activity. These findings suggest that "GP peroxidase" activity is dependent on a functional GP oxidase. Thus one possibility is that non-enzymic decomposition of H_2O_2 is occurring with the evolution of oxygen, that is, in turn, utilised by the GP oxidase. However, at the concentrations of protein and peroxide used, no O_2 evolution was detected in the oxygen electrode. On the other hand, peroxide could be used as an alternative substrate to oxygen by the GP oxidase with inactivation of the enzyme. In any case, regardless of whether the "GP peroxidase" is a true



TYPE I INHIBITORS

pOMB, Melarsen oxide, Cu^{2+}, Zn^{2+}, Ag^{+}
o-Phenanthroline, Tiron
Suramin and its analogues
S.72 8991

TYPE II INHIBITORS

SHAM, CLAM, 8-Hydroxyquinoline
Diphenylamine, Pyrrolnitrin
Catechol
H_2O_2

INTERMEDIATE TYPE INHIBITORS

Bathophenanthroline, Thenoyltrifluoroacetone (TTFA)
αα' Dipyridyl, Salicylaldoxime
Dithizone, Diethyldithiocarbamate

Fig. 5.1. Summary of the effects of various inhibitors of the GP oxidase.

enzymic activity or not, it is concluded that the GP oxidase: GP peroxidase model proposed by Grant and Bowman (1963), Bide and Grant (1964) and Grant (1966) is not substantiated by these experiments.

6. Inhibitors of the glycerophosphate oxidase and its dehydrogenase component

A summary of the effects of inhibitors of the GP oxidase is given in fig. 5.1. Before considering possible models to fit this data some further discussion of the results is necessary.

i) hydrogen peroxide

~~The GP oxidase is rapidly inhibited by high concentrations of H_2O_2 in the presence of substrate.~~ As discussed earlier lower concentrations of peroxide are inhibitory only when the oxidase, H_2O_2 and substrate GP were present (fig. 4.15). Oxygen would appear to protect against inhibition of the GP oxidase by hydrogen peroxide (fig. 4.15). However, at 2 mM H_2O_2 although the GP oxidase is completely inhibited within 1 minute, the dehydrogenase activity is not affected indicating that peroxide is a type II inhibitor (table 4.5). This finding does not agree with that of Grant and Sargent (1961) who reported that the dehydrogenase component of the oxidase in lyophilised material was inhibited by H_2O_2 in the presence of metal ions or enzyme ash and that the addition of catalase, EDTA, KCN or especially 8-hydroxyquinoline protected against inhibition. Cyanide or EDTA are reported to inhibit Cu^{2+} or Fe^{2+} catalysed oxidation of thiol groups (Harrison, 1924) while 8-hydroxyquinoline

is reported to have no effect (Bernheim and Bernheim, 1939) which is contrary to the findings of Grant and Sargent (1961) where 8-hydroxyquinoline was a more effective protecting agent than cyanide or EDTA. However, the discrepancy with the present findings may be due to changes in the GP dehydrogenase component induced by the lyophilisation and sonication procedures used by Grant and Sargent or due to the longer time of exposure of the enzyme to peroxide necessary in the manometric method of assay.

ii) chelating compounds

Inhibition of the GP oxidase by chelating compounds can be classified into type I, II or intermediate type inhibition (table 4.4). The majority of chelating compounds tested on the GP oxidase show an intermediate type inhibition (fig. 5.1). The exceptions to this are the type II chelating agents, SHAM, CLAM and 8-hydroxyquinoline and the type I chelating agents o-phenanthroline and Tiron. The type II chelating agents are heterocyclic compounds where oxygen and nitrogen serve as donor atoms to the metal and are the most effective inhibitors of all the chelating agents tested against the GP oxidase, having a value for I_{50} of less than 0.2 mM. In the case of 8-hydroxyquinoline (oxine), the addition of a sulphonic-acid-group at position 5 virtually abolishes oxine's inhibitory effect despite the fact that 8-hydroxyquinoline-5-sulphonic acid and oxine have the same stability constants for metals (Albert, 1953). However, unlike oxine, it has no antibacterial properties whatsoever and has a low oil/water partition coefficient (Albert, 1953). In the case of substituted aza-oxines, it has been shown that increasing antibacterial action can be correlated with increasing partition

coefficient (Albert et al., 1954), but studies on the inhibition of the GP oxidase by a large number of substituted 8-hydroxyquinolines or benzhydroxamic acids would be necessary to establish whether this mechanism operates here. The identification of iron and copper in the partially purified GP oxidase preparations could explain the three patterns of inhibition by chelating compounds, assuming that both metals are constituents of the enzyme. Thus type II compounds could be chelating only with copper, whilst type I compounds chelate most strongly with iron and intermediate compounds could be regarded as binding both metals, but preferentially copper. Iron has been identified as being a component of the GP dehydrogenase component (Grant and Sargent, 1961) and copper would appear to be located beyond the site of PMS reduction, that is to say, in the second enzymic component. Most chelating agents show approximately the same order of preference for metals which in general follows the following sequence in order of increasing avidity (stability constant): K^{+}, Na^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{3+} (Albert, 1973; Vallee and Wacker, 1970). However, the stability constants of coordination complexes determined for free metal ions in an aqueous environment may not necessarily apply to the interaction of chelators with the metals of a metalloprotein because the geometry of the metalloprotein and the steric properties of the chelating agents, for example, may importantly affect the interaction and modify binding. Moreover, the number of metal-protein bonds and the stability constants of the metalloprotein complexes are not known. Finally, as discussed above, the partition coefficient of the particular chelating agent can also modify its inhibitory properties. For these reasons, it is impossible to theorise why

any of chelating agents in table 4.4 are type I, II or intermediate inhibitors.

Rigorous proof that the GP oxidase is a metalloenzyme requiring both copper and iron for activity is at present lacking. The following are valuable criteria according to Vallee and Wacker (1970):

i) with progressive purification of the enzyme the ratio of metal: protein should approach a fixed limit;

ii) the removal of the functional metal by chelating agents or by lowering the pH to provide a metal-free apoprotein should show concurrent loss of activity, and, following its readdition, restoration of enzymic activity;

iii) the native metal ion may be exchanged either for its own radioactive isotope or for the stable or radioactive isotope of other metals can be a useful method for the characterisation of binding sites and catalytically active centres of metalloenzymes.

Until studies such as these have been done on the GP oxidase the role of copper and iron in the function of this enzyme must remain open to doubt.

In the case of Tiron, a number of chemical features of the molecule could be responsible for its inhibitory action on the GP oxidase. Tiron is a chemical trapping agent for superoxide anion (O_2^-) produced by reduced flavin or metalloflavoproteins in the presence of oxygen (Fridovich and Handler, 1962; Miller and Massey, 1965; Miller, 1970b; Miller and Rapp, 1973). Tiron is also a catechol and a reducing agent capable of forming a stable o-semiquinone in the physiological pH range (Miller and Rapp, 1973). Although

catechol itself is an inhibitor of the GP oxidase, it is a type II inhibitor, I_{50} 0.6 mM (table 4.5) as opposed to Tiron, which is type I, I_{50} 5.5 mM (table 4.4) and therefore the mode of action of Tiron at site I is unlikely to be due to its properties as a catechol. Tiron is also a polysulphonated compound as are the analogues of suramin in fig. 5.1, table 4.8, all of which are type I inhibitors. Finally, Tiron also has metal ion chelating properties, but which of these properties are involved in its action as a type I inhibitor is not clear.

It is worth noting at this point that superoxide anion does not appear to be a free intermediate in the GP oxidase reaction as superoxide dismutase does not affect the rate of oxygen utilisation by the enzyme (table 4.5). Neither does the GP oxidase reduce ferricytochrome c, in contrast to substrate-reduced dihydroorotate dehydrogenase (Miller and Massey, 1965) and xanthine oxidase (McCord and Fridovich, 1968) which are known to form superoxide anion, considered to be the reductant for ferricytochrome c (Massey et al., 1969). These findings seem to rule out Tiron acting as an O_2^- trap at site I. The possibility remains that superoxide anion could be a tightly enzyme-bound intermediate in the GP oxidase reaction so that superoxide dismutase and ferricytochrome c would be unable to scavenge O_2^-. Superoxide dismutase apparently stimulates the oxygen utilisation of the dehydrogenase component by 68% when assayed with PMS. Reduced PMS catalyses the univalent reduction of oxygen to superoxide anion (Nishikimi et al., 1972), but the underlying mechanism of how superoxide dismutase increases the rate of oxygen utilisation by reduced PMS is not clear. If the rate of

reduction of PMS by the GP dehydrogenase is unaffected by superoxide dismutase, then this enzyme must in some way accelerate the transfer of electrons from the reduced electron acceptor to oxygen.

iii) thiol inhibitors

p-Hydroxymercuribenzoate (pOMB), the heavy metal ions Cu^{2+}, Zn^{2+} and Ag^{+} and melarsen oxide are all type I inhibitors of the GP oxidase (fig. 4.9, table 4.3, 4.6). All the above compounds show a marked initial inhibition followed by a gradual increase in inhibition over 5-20 min to a constant value. pOMB is particularly effective with an I_{50} of 0.2 - 0.3 μM. Grant and Sargent (1961) found that this inhibition could be completely reversed by incubating the treated GP dehydrogenase with 1 mM glutathione at 37°C for 10 min, but this effect has not been investigated in this study. The simplest explanation of the inhibition of the GP oxidase by heavy metal ions would be the formation of metal mercaptides, however, Grant and Sargent (1961) suggest that metal ions are enhancing the inhibition of the GP dehydrogenase by hydrogen peroxide produced by the oxidation of reduced PMS (see hydrogen peroxide section). Thus thiol groups are essential for catalytic activity of the GP oxidase. This does not necessarily imply that they are involved in the transfer of electrons from GP to O_2, nor that they are present at the active site for GP, as conformational changes in the protein due to the reaction of thiol reagents at sites remote from the catalytic centre could equally inactivate the enzyme.

iv) Miscellaneous inhibitors

As discussed earlier diphenylamine and pyrrolnitrin are both inhibitors of mitochondrial respiration in the region of electron transfer from flavin to cytochrome b (Baker, 1963; Sharpless and Butow, 1970; Lambowitz et al., 1972; Wong et al., 1971; Tripathi and Gottlieb, 1969; Wong and Airall, 1970). Diphenylamine is a type II inhibitor of the GP oxidase and pyrrolnitrin is a type II inhibitor at low concentrations, but showing intermediate type inhibition at high concentrations (table 4.5). Thus it is proposed that both these inhibitors act at the same site which lies beyond the site of PMS reduction and, by analogy with the results of other workers, acts between flavin and the terminal part of the respiratory chain. The mode of action of these inhibitors is unknown.

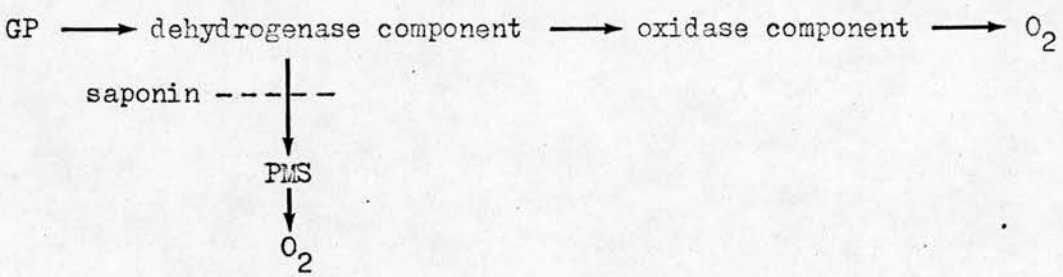
Compound S.72 8991 which is an experimental antimalarial compound derived from acridine is a potent type I inhibitor of the GP oxidase inhibiting 50% at 70 μM (table 4.5). Acriflavine, on the other hand, does not inhibit the GP oxidase. S.72 8991 has not been tested against intact trypanosomes in vivo or in vitro, but these preliminary results should be further investigated.

v) detergents

Deoxycholate inhibits both the GP oxidase and GP dehydrogenase activities to the same extent, whereas triton X-100 and digitonin preferentially inhibit the GP oxidase, such that when the oxidase is completely inhibited about 75% of the dehydrogenase activity is retained. Preliminary experiments have indicated that triton X-100 partially solubilises the GP dehydrogenase component, so that

inhibition could be due to a dissociation of this component from the terminal part of the respiratory chain. Another possibility is that triton X-100 or digitonin remove some lipid soluble cofactor mediating electron transfer from the dehydrogenase to the other components of the oxidase. These two possibilities can not be differentiated at present. The loss of the GP oxidase activity by lyophilisation in water or by acetone extraction could also be due to either of these possibilities.

The effect of saponin on the GP dehydrogenase activity is unique amongst all the inhibitors tested in that reduction of PMS appears to be completely inhibited at concentrations greater than 2% (w/v), yet the electron transfer through the terminal oxidase component appears to be relatively unimpaired (fig. 4.8). Saponin presumably prevents PMS reaching its site of reduction in some way, yet without interfering with the normal route of transfer of electrons to oxygen. These results are consistent with the following scheme:



7. Components of the glycerophosphate oxidase

The results of analysis so far have identified flavin (FAD), copper and iron in two bulk particulate oxidase preparations. The ratio of flavin (FAD) to copper to iron in one preparation was found

to be 1: 4: 8 (table 4.11). The other preparation gave lower values for total copper and iron because of interference by EDTA and phosphate ions. The flavoprotein difference spectra obtained with these preparations are proportional to the GP oxidase activity present (table 4.10) and in one preparation all the flavin found by spectral analysis could account for all the FAD found by chemical analysis using a fluorescent method (table 4.11). Thus FAD is strongly implicated in electron transfer in the GP oxidase reaction. No other notable features were present in the 350 - 650 nm range of the spectrum (fig. 4.17) indicating that iron and copper chromophores, if undergoing a valence change on reduction by substrate, do not show characteristic absorbance bands of the iron-sulphur-flavoproteins, such as in xanthine oxidase and aldehyde oxidase (Rajagopalan and Handler, 1964), or of the 'blue' copper proteins found at about 600 - 610 nm (Malkin and Malmstrom, 1970).

The nature of the protein ligands binding the iron in the GP oxidase preparations is not known, but certain ligands can be eliminated. First, the lack of acid-labile sulphide and the lack of a characteristic EPR signal at $g = 1.94$ confirms the absence of iron-sulphur-protein chromophores in the difference spectrum. Secondly, as haem is not detectable in bloodstream trypanosomes (Flynn and Bowman, 1973) and is not found in absolute or difference spectra of the oxidase, porphyrin-bound iron is also excluded. As the estimation of total iron by extraction with glacial acetic and thioglycollic acids gave values about half of that found after wet-ashing of the samples, the possibility of an contaminating chelating compound interfering with those methods not employing wet-ashing cannot be eliminated. The difficulties encountered in obtaining

sufficient material for these analyses have hampered this line of research.

Xray fluorescence has confirmed the presence of copper, nickel and iron in these preparations. Molybdenum was not detected by this method.

Insufficient material has prevented analysis for free thiol content, lipid content and the detection of lipid soluble coenzymes that may be implicated in the GP oxidase. Further analysis in these areas is required.

8. EPR studies

In freshly prepared GP oxidase, 3 paramagnetic species were observed at - 172°C giving signals at g = 4.1, 2.05 and ~2.0. The signal at g = 4.1 (fig. 4.18) is typical of high-spin ferric iron in a rhombic environment and the addition of substrate GP does not alter the size of this signal. Similar signals at g = 4.2 are found in a wide variety of organic and inorganic materials and may merely indicate contaminating iron. In NADH dehydrogenases of low molecular weight this signal may be derived from non-haem iron originally associated with these proteins, which has undergone a change in environment during enzyme preparation (Beinert, 1972). No signal in the region of g = 1.94 has been observed under any conditions confirming the absence of iron liganded with acid-labile sulphide. In particulate preparations washed free of cuproprotein a signal at g = 4.1 is still present, but no other signals due to iron have been identified. EPR spectral analysis at liquid helium

temperatures should be carried out to eliminate the possibility that iron or copper signals, too broad to be detected at -172°C, may be present.

The other two signals at g = 2.05 and ~2.0 are released from the particulate oxidase by freezing and thawing and it has been established that i) the cuproprotein released does not appear to be essential for the activity of the GP oxidase ii) it is a "non-blue" type copper protein of unknown function and iii) it can be used as a redox probe for the GP oxidase at a site that cannot be differentiated from that of PMS reduction. The significance of the partial reduction of the copper in this protein is unknown, but failure to reduce all the Cu^{2+} present suggests two functionally different types of copper exist, or even different cuproproteins. Partial denaturation of the GP oxidase with release of copper and subsequent binding to protein could also explain these observations, for similar reasons to the signal at g = 4.2.

The remaining copper in the particulate oxidase is not detectable by EPR, but this does not necessarily imply that it is diagmagnetic (Cu^{+}) since extensive line broadening of the EPR signal in a binuclear Cu^{2+} complex can occur. The structure of this unit is not known, but it has been suggested that EPR-nondetectable copper might exist as a strongly coupled Cu^{2+} - Cu^{2+} pair (Fee et al., 1969). If they were diamagnetic due to spin pairing, they would give no EPR absorption, or, even when showing some paramagnetism, would yield only very broad signals owing to dipolar interaction (see Malkin and Malmstrom, 1970 and Vanngard, 1972 for further discussion). The only method by which the diamagnetic state of "EPR-nondetectable copper"

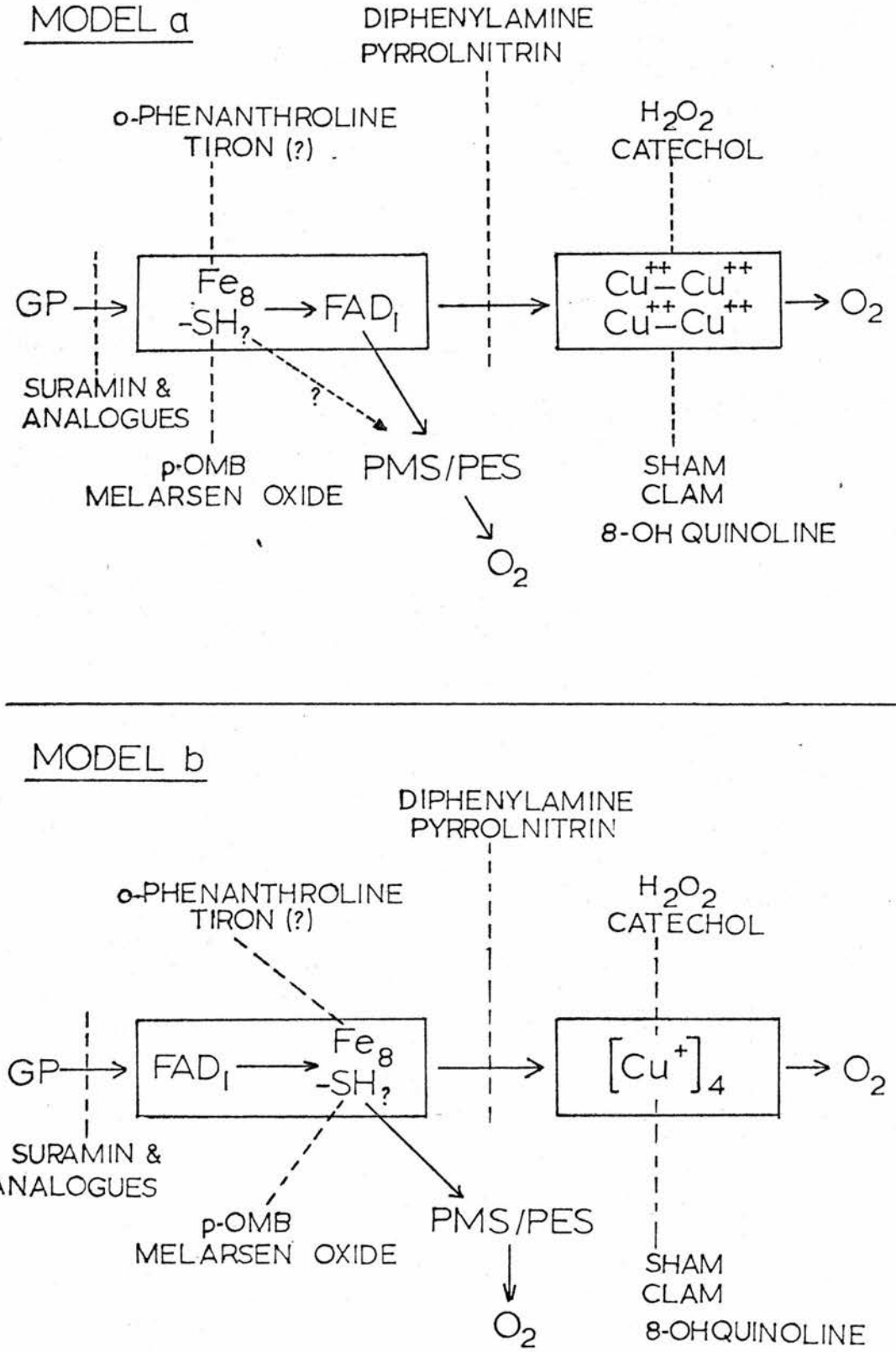


Fig. 5.2. Tentative models of the GP oxidase. GP dehydrogenase component: Fe/SH, FAD; terminal oxidase component: Cu.

can be determined is by measurements of the magnetic susceptibility of the sample, which have not yet been carried out on the GP oxidase.

9. Proposed models for the glycerophosphate oxidase

From the results obtained to date, the glycerophosphate oxidase appears to be a membrane bound enzyme complex consisting of at least two components: an L-glycerol-3-phosphate dehydrogenase and a second component catalysing the terminal electron transfer from the GP dehydrogenase to molecular oxygen.

A tentative electron transfer scheme is given (fig. 5.2) in which it is proposed that:

i) the iron and copper identified in GP oxidase preparations are involved in electron transfer.

ii) copper is involved in the terminal oxidase component catalysing a 4 electron transfer to oxygen.

iii) FAD is situated in the GP dehydrogenase component.

Although other more complex models could be proposed, the schemes given in fig. 5.2 would be consistent with all the evidence available so far and should form a suitable basis for the design and interpretation of further experiments.

i) GP dehydrogenase component

Bide and Grant (1964) suggested that iron and thiol groups were closely related within the dehydrogenase component and thus have been allocated a single site before, or after, the FAD (fig. 5.2a,b,

respectively). The fact that thiol inhibitors and the chelators, o-phenanthroline and Tiron, act as type I inhibitors would support the location of thiols and iron at a closely related site in the dehydrogenase. The site of PMS reduction is not known, but PMS could accept electrons from either FAD or Fe/SH in model a, but in model b reduction of PMS by FAD is not allowed, as it would not then fit the experimental data that Fe or SH reagents inhibit the reduction of PMS. The precise mode of action of diphenylamine and pyrrolnitrin is not known, but they are reported to inhibit mitochondrial electron transport subsequent to the flavoprotein component of the respiratory chain. As diphenylamine and pyrrolnitrin have no chelating properties, it is proposed that they act at a site different from the other type II inhibitors SHAM, CLAM, catechol and 8-hydroxyquinoline (see terminal oxidase component). To conform to the experimental data, these two inhibitors must act beyond the site of PMS reduction in either model. In the case of model b, this would be between Fe/SH and copper, thereby making this scheme less likely, as this would not conform to the site of action proposed for mitochondrial electron transport.

Suramin is a competitive inhibitor of the GP oxidase with respect to substrate, glycerophosphate, and is therefore consistent with the "bridge hypothesis" of Wills and Wormall (1950a). Thus it is proposed that suramin and its analogues prevent the binding of GP at the active site of the dehydrogenase (see section C).

ii) terminal oxidase component

The allocation of copper at this site can be justified for a number of reasons. The absence of detectable hydrogen peroxide and

the low apparent K_m for O_2 for the GP oxidase is presumptive evidence that the enzyme catalyses a four electron transfer to oxygen, forming water. Cytochrome c oxidase and the 'blue' copper-containing oxidases (ascorbic acid oxidase, laccase, caeruloplasmin) constitute the only enzymes which reduce both atoms of molecular oxygen to water (Malkin and Malmstrom, 1970). Each molecule of these oxidases contains three distinct forms of copper: Type 1 Cu^{2+} ('blue'), Type 2 Cu^{2+} ('non-blue') and diamagnetic copper (Cu^{2+} - Cu^{2+}), (Malkin and Malmstrom, 1970) although Vanngard (1972) considers that cytochrome oxidase 'blue' copper has a combination of $g_{||}$ and A values such that it falls distinctly outside the range of Type 1 copper ions found in the "blue" cuproproteins. Proteins with only Type 1 copper, for example, stellacyanin, cannot be readily reoxidised by oxygen and substrate oxidation in laccase is thought to occur primarily at this Cu^{2+} ion. Oxidases with only Type 2 Cu^{2+}, for example, galactose oxidase, work on two-electron substrates reducing oxygen only to the peroxide level (Vanngard, 1972) and it has also been suggested that this 'non-blue' Cu^{2+} may stabilise an intermediate such as peroxide, in the reduction of oxygen in the laccase reaction (Malkin and Malmstrom, 1970). Type 2 Cu^{2+} shows an unusual affinity for the anions CN^-, F^- and N_3^- and as Type 2 Cu^{2+} is absent from the GP oxidase, this may explain its lack of inhibition by these reagents. It has also been suggested that Type 2 Cu^{2+} represents a form of Cu^{2+} found in denatured enzymes, giving an artifactual signal $g = 2.05$ analagous to the ubiquitous EPR signal at $g = 4.2$ of high-spin ferric iron. Neither type 1 or type 2 copper are found in the GP oxidase prepared free of $CP_{2.05}$, which would appear to place this enzyme in a category separate from

cytochrome c oxidase and the "blue" copper oxidases. As the copper in the GP oxidase is diamagnetic, it could exist either as Cu^{+} or in diamagnetic Cu^{2+} - Cu^{2+} pairs. It is difficult to conceive how Cu^{+} could be involved in electron transfer to oxygen without change in valence, but in model b (fig. 5.2) Cu^{+} could possibly act by destabilising the comparatively unreactive oxygen molecule. Alternatively, in the case of a diamagnetic Cu^{2+} - Cu^{2+} pair, which has been proposed as a two-electron-accepting unit for laccase (Malkin and Malmstrom, 1970), valency change to Cu^{+} - Cu^{+} would not be detectable by EPR. It is tempting to suggest that, as 4 copper atoms per mol FAD are present in the GP oxidase, these could be arranged in two Cu^{2+} - Cu^{2+} pairs, capable of a simultaneous four electron transfer to O_2.

Attempts to oxidise artificial electron donors by the copper in GP oxidase have been unsuccessful. Hydroquinone, catechol, and ascorbate $\pm$ TMPD are inhibitors of the enzyme, as is the case of other copper-containing oxidases which can be markedly inactivated during the catalytic reaction (Malmstrom and Ryden, 1968). The inhibition of the GP oxidase at site II by nitrogen-oxygen ligands (SHAM, CLAM and 8-hydroxyquinoline) or oxygen-oxygen ligands (H_2O_2 and catechol) is also suggestive evidence for copper at this site. The possibility that other cyanide-insensitive plant oxidases may contain copper does not appear to have been investigated, yet as all the cyanide-insensitive oxidases tested so far have proved to be sensitive to inhibition by substituted benzhydroxamic acids, it is suggested that the possibility of copper as a functional part of these enzymes has been overlooked.

C. INHIBITION OF THE GLYCEROPHOSPHATE OXIDASE BY SURAMIN AND ITS ANALOGUES

Suramin is as potent an inhibitor of the GP oxidase in crude homogenates as in partially purified preparations (table 4.7). Since there is 85% less protein in assays of the partially purified preparations, suramin is not bound preferentially to any significant extent to other trypanosomal protein. Suramin is a competitive inhibitor with respect to glycerophosphate as substrate with a Ki of 4.1 ± 0.4 µM (fig. 4.11) and inhibition of the GP oxidase by suramin can be reversed by the addition of BSA, presumably by preferential binding of suramin to the added BSA (fig. 4.10). Yet suramin does not affect motility, glycolysis or respiration of intact bloodstream trypanosomes even after a few hours exposure (Williamson, 1970). However, with longer exposures (6-12h) to the drug administered to the host animal, some workers have reported a depression of glycolysis and respiration of isolated intact trypanosomes (see Introduction). This delay in action could be attributed to the probable mode of uptake of the drug, namely the ingestion of suramin bound to plasma protein by pinocytosis from the flagellar pocket of the trypanosome. Then, after fusion of the phagosomes with lysosomes, forming secondary lysosomes the suramin would be released by proteolysis of the carrier plasma protein. Two factors may delay the release of suramin from the phagosome. First, protein digestion is reported to be retarded in hepatic parenchymal cells and in the Kupffer cells of the liver, presumably by inhibition of the proteases (Davies *et al.*, 1971; Buys *et al.*, 1973). Second, it has

recently been reported that suramin interferes with phagosome-lysosome fusion in isolated mouse macrophages grown in culture (D'Arcy Hart and Young, 1975). Both of these effects may delay the release of suramin in the trypanosome.

If the observations by Opperdoes and Borst (personal communication) are correct, that trypanosomes can survive after respiration is completely abolished by SHAM, as a result of salvaging the "high energy" phosphate of glycerophosphate by transphosphorylation to glucose or hexose monophosphate, then glycolysis would procede with a net yield of 1 mol ATP per mol glucose metabolised to pyruvate and glycerol. Similarly, the observation that trypanosomes can survive at least some hours anaerobically could also be explained by the "phosphate-salvage" hypothesis (Fulton and Spooner, 1956; Ryley, 1956, 1962). However, it has not been shown that the blood-stream trypanosome can survive indefinitely under conditions where the GP oxidase is inoperative. Assuming glycolysis in these cells is already proceding at near its maximum rate, then halving the ATP concentration within the cell could be progressively deleterious to the cell. Moreover, if the observation that glycolysis in trypanosomes is depressed after several hours exposure to suramin is correct then an additional site of inhibition is required to explain suramin's depression of glycolysis, as it has been shown (Ryley, 1956) that under anaerobic conditions glycolysis procedes at the same rate as that aerobically. These experiments should be repeated with the improved isolation techniques now available, to exclude the possibility that the effects of suramin on glycolysis and respiration were due to the contaminating white blood cells and platelets

present in the preparation and not the trypanosomes.

The investigation of the effect of several analogues of suramin has shown that suramin is the most effective of all the compounds available (tables 1.1, 1.2, 1.3). Changing the structure by the removal of the methyl groups increases the concentration required for 50% inhibition by about 10 fold, which is in good agreement with that of Fourneau et al. (1924) who reported an approximate increase in the minimum curative dose of 15 fold (table 1.1). Of the other compounds tested, as far as is known, none has any trypanocidal activity. The finding that the 'suramin nucleus' (table 4.8) was almost as effective as desmethyl suramin in inhibiting the GP oxidase and much more effective than either Antrypol amine or monosulphonated suramin would indicate that some of the inhibitory properties of suramin may not reside simply in the spacing between the terminal naphthylamine trisulphonic acid residues as had been previously supposed. Naphthylamine trisulphonic acid itself is an extremely weak inhibition of the GP oxidase, yet the removal of these two groups from each end of the suramin molecule (leading to the "suramin nucleus") or of two of the three sulphonic acid groups from both naphthylamine trisulphonic acid groups of suramin (leading to monosulphonated suramin) also decreases the inhibition by these compounds compared to suramin. It was not possible to obtain further analogues of suramin such as those given in tables 1.1, 1.2, 1.3. Therefore, as proposed in the Introduction, if an enzyme is to be the principal site of action for suramin, then it should be possible by the use of analogues of this drug to demonstrate parallel effects on the trypanocidal activity in vivo and on the

inhibition of the isolated enzyme *in vitro*. This hypothesis would appear to hold true in the case of the GP oxidase, because analogues of suramin with little or no chemotherapeutic action are not effective inhibitors of the enzyme and a close correlation was found between therapeutic activity and inhibitor action against the oxidase. Taken together with the fact that the GP oxidase is highly sensitive to suramin, these findings suggest that this enzyme is at least one of the principal sites of action of suramin. This is the first report of any trypanosomal enzyme significantly inhibited by suramin at concentrations less than 10^{-5} M.

LIST OF REFERENCES

Articles denoted ** were not consulted in the original.

ADAMS, A., ASHLEY, J.N. and BADER, H. (1956) J. Chem. Soc. 3739-3744.

ALBERT, A. (1953) Biochem. J. 54, 646-654.

ALBERT, A. (1973) Selective Toxicity, 5th edition, Chapman and Hall, London.

ALBERT, A., HAMPTON, A., SELBIE, F.R. and SIMON, R.D. (1954) Brit. J. Exp. Path. 35, 75-84.

ALLSOPP, B.A. and NJOGU, A.R. (1974) Parasitol. 69, 271-281.

ALTMAN, F.P. (1972) An Introduction to the Use of Tetrazolium Salts in Quantitative Enzyme Cyto-chemistry, Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

ANALYTICAL METHODS COMMITTEE (1967) Analyst 92, 403-407.

ANDREAE, W.A. (1955) Nature (London) 175, 859-860.

ANGELOPOULOS, E. (1970) J. Protozool. 17, 39-51.

APTED, F.I.C. (1970) in The African Trypanosomiases (Mulligan, H.W., ed.), pp.684-710, Allen and Unwin Ltd., London.

BACCHI, C.J., HUTNER, S.H., CIACCIO, E.I. and MARCUS, S.M. (1968) J. Protozool. 15, 576-584.

BAERNSTEIN, H.D. (1963) J. Parasitol. 49, 12-21.

BAKER, J.E. (1963) Arch. Biochem. Biophys. 103, 148-155.

BAUER, H. and BECKER, J. (1928) Arbeiten aus dem Staatsinstitut fur Experimentelle Therapie und dem Georg Speyer-Hause zu Frankfurt A.M. 21, 10-25.

BAYNE, R.A., MUSE, K.E. and ROBERTS, J.R. (1969a) Comp. Biochem. Physiol. 30, 1049-1054.

BAYNE, R.A., MUSE, K.E. and ROBERTS, J.F. (1969b) Comp. Biochem. Physiol. 30, 61-72.

BEAUFAY, H. (1969) in Lysosomes in Biology and Pathology (Dingle, J.T. and Fell, H.B., eds.), Vol.2, chapter 18, pp.516-546, North-Holland Publishing Co., Amsterdam.

BEAUFAY, H., BENDALL, D.S., BAUDHUID, P., WATTIAUX, R. and de DUVE, C. (1959) Biochem. J. 73, 628-637.

BEERS, R.F. and SIZER, I.W. (1952) J. Biol. Chem. 195, 133-140.

BEILER, J.M. and MARTIN, G.J. (1948) J. Biol. Chem. 174, 31-35.

BEINERT, H. (1972) in Biological Applications of Electron Spin Resonance, (Swartz, H.M., Bolton, J.R. and Borg, D.C., eds.) pp.352-410, Wiley-Interscience, New York.

BERNHEIM, F. and BERNHEIM, M.L.C. (1939) Cold Spring Harbour Symp. Quant. Biol. 7, 174-182.

BIDE, R.W. (1964) A Study of Glycerophosphate oxidase of Trypanosoma rhodesiense, Ph.D. thesis, Aberdeen University.

BIDE, R.W. and GRANT, P.T. (1964) Fedn. Eur. Biochem. Soc. 1st meeting, abstract A90.

BOURSWELL, J.C. and WORMALL, A. (1939) Biochem. J. 33, 1191-1200.

BOWMAN, I.B.R. (1974) in Trypanosomiasis and Leishmaniasis with special reference to Chagas' Disease, CIBA Foundation Symp. 20, pp.255-271, Elsevier, Amsterdam.

BOWMAN, I.B.R. and FLYNN, I.W. (1975) in Biology of the Kinetoplastida (Lumsden, W.H.R., ed.), Academic Press, New York, in press.

BOWMAN, I.B.R., SRIVASTAVA, H.K. and FLYNN, I.W. (1972) in Comparative Biochemistry of Parasites (Van den Bossche, H., ed.) pp.329-342, Academic Press, New York.

BRAND, T. von (1951) in Biochemistry and Physiology of Protozoa (Lwoff, A., ed.), vol. 1, pp.177-234, Academic Press, New York.

BRAND, T. von (1966) Biochemistry of Parasites, Academic Press, New York.

BRANDT, R. and KESTON, A.S. (1965) Anal. Biochem. 11, 6-9.

BROWN, R.C., EVANS, D.A. and VICKERMAN, K. (1973) Int. J. Parasitol 3, 691-704.

BRUMBY, P.E. and MASSEY, V. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), vol. 10, pp.463-474, Academic Press, New York.

BUYS, C.H.C.M., ELFERINK, M.G.L., BOUMA, J.M.W., GRUBER, M., and NIEWENHUIS, P. (1973) RES: J. Reticuloendothel. Soc. 14, 209-223.

CHANCE, B. (1957) in Methods in Enzymology (Colowick, S.P. and KAPLAN, N.O.) vol. 4, pp.273-329, Academic Press, New York.

CHRISTOPHERS, S.R. and FULTON, J.D. (1939) Ann. Trop. Med. Parasit. 33, 161-170.

CROSS, G.A.M. (1973) Trans. Roy. Soc. Trop. Med. Hyg. 67, p.261.

CHELLO, P.L. and JAFFE, J.J. (1972) Comp. Biochem. Physiol. 43B, 543-62.

DANGERFIELD, W.G., GAUNT, W.E., and WORMALL, A. (1938) Biochem. J. 32, 65-70.

D'ARCY HART, P. and YOUNG, M.R. (1975) Nature (London) 256, 47-49.

DAVIES, M., LLOYD, J.B. and BECK, F. (1971) Biochem. J. 121, 21-6.

DEWEY, H.M. and WORMALL, A. (1946) Biochem. J. 40, 119-124.

DOEG, K.A. and ZIEGLER, D.M. (1962) Arch. Biochem. Biophys. 97, 37-40.

EDWARDS, C. (1974) Studies of Structure and Function in Crithidia fasciculata, Ph.D. thesis, Cardiff, University of Wales.

ELSEN, V. and LOVEDAY, C. (1973) Br. J. Pharm. 49, 678-87.

EVANS, D.A. and BROWN, R.C. (1972) J. Protozool. 19, 365-369.

EVANS, D.A. and BROWN, R.C. (1973a) Trans. Roy. Soc. Trop. Med. Hyg. 67, 258.

EVANS, D.A. and BROWN, R.C. (1973b) J. Protozool. 20, 157-160.

FAIRLAMB, A.H. and BOWMAN, I.B.R. (1975) Trans. Roy. Soc. Trop. Med. Hyg. 69, (2), p.268.

FAUST, E.C., RUSSELL, P.F. and JUNG, R.C. (1970) Craig and Fausts' Clinical Parasitology, 8th edn., Lea and Febiger, Philadelphia.

FEE, J., MALKIN, R., MALMSTROM, B. and VANNGARD, T. (1969) J. Biol. Chem. 244, 4200-4207.

**FENYVESSY, B. von and REINER, L. (1928) Biochem. Z. 202, 75.

FINELLE, P. (1974) Joint WHO/FAO African Trypanosomiasis Information Service, Tryp/Inf/74.55.

FLYNN, I.W. and BOWMAN, I.B.R. (1973) Comp. Biochem. Physiol. 45B, 25-42.

FLYNN, I.W. and BOWMAN, I.B.R. (1974) Comp. Biochem. Physiol. 48B, 261-273.

FOGO, J.K. and POPOWSKY, M. (1949) Anal. Chem. 21, 732-734.

FORTES, P.A.G., ELLORY, J.C. and LEW, V.L. (1973) Biochem. Biophys. Acta. 318, 262-272.

FOUREAU, M., TREFOUEL, J. (M.et Mme.) and VALLEE, J. (1924) Annal. Inst. Pasteur. 38, 81-114.

FRIDOVICH, I. and HANDLER, P. (1962) J. Biol. Chem. 237, 916-921.

FULTON, J.D. and SPOONER, D.F. (1956) Biochem. J. 63, 475-481.

FULTON, J.D. and SPOONER, D.F. (1959) Exp. Parasitol. 8, 137-162.

GERZELI, G. (1955) Riv. Parastiol. Roma. 16, 209-215.

GOTTLIEB, M., ZAHALSKY, M. and ZAHALSKY, A.C. (1972) J. Parasitol. 58, 1008-1009.

GRANT, P.T. (1966) Symp. Soc. Gen. Microbiol. 16, 281-293.

GRANT, P.T. and BOWMAN, I.B.R. (1963) Biochem. J. 89, 80-90 (PROCEEDINGS).

GRANT, P.T. and FULTON, J.D. (1957) Biochem. J. 66, 242-250.

GRANT, P.T. and SARGENT, J.R. (1960) Biochem. J. 76, 229-237.

GRANT, P.T. and SARGENT, J.R. (1961) Biochem. J. 81, 206-213.

GRANT, P.T., SARGENT, J.R. and RYLEY, J.F. (1961) Biochem. J. 81, 200-206.

HARRISON, D.C. (1924) Biochem. J. 18, 1009-1022.

HARVEY, S.C. (1949) J. Biol. Chem. 179, 435-453.

HAWKING, F. (1937) J. Pharmacol. 59, 123-156.

HAWKING, F. (1938) Ann. Trop. Med. Parasitol, 32, 313-331.

HAWKING, F. (1939) Ann. Trop. Med. parasitol. 33, 13-19.

HAWKING, F. (1963) Ann. Trop. Med. Parasitol. 57, 255-261.

HILL, G.C. and ANDERSON, W.A. (1970) Exp. Parasitol. 28, 356-380.

HILL, G.C. and BONILLA, C.A. (1974) J. Protozool. 21 (5), 632-8.

HILL, G.C. and HUTNER, S.H. (1968) Exp. Parasitol. 22, 207-212.

HINKLE, P.C., BUTOW, R.A., RACKER, E., and CHANCE, B. (1967) J. Biol. Chem. 242, 5169-5173.

HINTON, R.H., BORGE, M.L.E., and HARTMAN, G.C. (1969) Anal. Biochem. 29, 248-256.

HENRY, M.F. and NYNS, E.D. (1975) Sub- Cell. Biochem. 4, 1-65.

HOFFMAN-BERLING, H. (1955) Biochim. Biophys. Acta. 16, 146-154.

HUNTER, A. and DOWNS, C.E. (1945) J. Biol. Chem. 157, 427-446.

ISSEKUTZ, B. (1933a) Arch. Exp. Path. Pharmak. 173, 479-498.

ISSEKUTZ, B. (1933b) Arch. Exp. Path. Pharmak. 173, 499-507.

JACQUES, P.J., HUYBRECHTS-GODIN, G. and SMEESTERS, C. (1975) Biochem. Soc. Trans. 3, (1) 155-157.

JADIN, J.M. and CREEMERS, J. (1972) Trans. Roy. Soc. Trop. Med. Hyg. 66, 8-9.

JAFFE, J.J., McCORMACK, J.J. and MEYMARIAM, E. (1972) Biochem. Pharmacol. 21, 719-731.

JANCSO, N. and JANCSO, H. (1934) Zbl. Bakt., Abt. I, Orig., 132, 257-292.

JANCSO, N. and JANCSO, H. (1935) Z. Immun Forsch. 84, 471-504.

KAHAN, D., ZAHALSKY, A.C. and HUTNER, S.H. (1968) J. Protozool. 15, 385-390.

KEILIN, D. and HARTREE, E.F. (1945) Biochem. J. 39, 293-301.

KESTON, A.S. and BRANDT, R. (1965) Anal. Biochem. 11, 1-5.

KESTON, A.S., BRANDT, R. and KAHAN, E. (1965) Anal. Biochem. 11, 23-29.

KIELLEY, W.W. (1963) in Methods in Enzymology (S.P. Colowick and N.O. Kaplan, eds.) Vol.6, p.276, Academic Press, New York.

KING, T.E. and MORRIS, R.O. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.) vol. 10, pp.634-637, Academic Press, New York.

KUSEL, J.P. and STOREY, B.T. (1972) Biochem. Biophys. Res. Comm. 46, 501-507.

KUSEL, J.P. and WEBER, M.M. (1965) Biochim. Biophys. Acta. 98, 632-639.

KUSEL, J.R. (1972) Parasitol. 65, 55-69.

LAMBOWITZ, A.M., SMITH, E.W. and SLAYMAN, C.W. (1972) J. Biol. Chem. 247, 4850-4858.

LANGRETH, S.G. and BALBER, A.E. (1975) J. Protozool. 22, 40-53.

LAYTON, D. and AZZI, A. (1974) Biochem. Biophys. Res. Commun. 59, 322-5.

LEHMANN, D.L. (1964) Ann. Trop. Med. Parasitol. 58, 189-191.

LINHARDT, K. and WALTER, K. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H.U., editor) pp.783-785, Academic Press, London and New York.

LOSCHEN, G., FLOHE, L. and CHANCE, B. (1971) FEBS lett. 18, 261-264.

LOVENBERGE, W., BUCHANAN, B.B. and RABINOWITZ, J.C. (1963) J. Biol. Chem. 238, 3899-3913.

LOWRY, O.H., ROSEBROUGH, N.H., FARR, A.L. and RANDALL, R.J. (1951) J. Biol. Chem. 193, 265-275.

MAIZEL, J.V., Jr. (1969) in Fundamental Techniques in Virology (Habel, K. and Salzman, N.P., eds.), pp.334-362, Academic Press, London and New York.

MALKIN, R. and MALMSTROM, B.G. (1970) Adv. Enzymol. 33, 177-244.

* MALMSTROM, B.G. and RYDEN, L. (1968) in Biological Oxidations (Singer, T.P., ed.), pp.415-438, Interscience Publishers.

MARTENS, R.I. and GITHENS, R.E., Sr. (1952) Anal. Chem. 24, 991-993.

MASSEY, V., STRICKLAND, S., MAYHEW, S.G. HOWELL, L.G., ENGEL, P.C. MATTHEWS, R.G., SCHUMAN, M. and SULLIVAN, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 891-897.

McCORD, J.M. and FRIDOVICH, I. (1968) J. Biol. Chem. 243, 5753-5760.

McCORD, J.M. and FRIDOVICH, I. (1969) J. Biol. Chem. 244, 6049-6055.

* MALMSTROM, B.G. (1973) Biochem. Soc. Trans. 1, 47-48.

MERCIER-PAROT, L. and TUCHMANN-DUPLESSIS, H. (1973) Cr. Soc. Biol. 167, 1518-22.

MILLER, R.W. (1970a) Anal. Biochem. 35, 181-192.

MILLER, R.W. (1970b) Can. J. Biochem. 48, 935-939.

MILLER, R.W. and MASSEY, V. (1965) J. Biol. Chem. 240, 1466-1472.

MILLER, R.W. and RAPP, U. (1973) J. Biol. Chem. 248, 6084-6090.

NEWTON, B.A. (1962) in Drugs, Parasites and Hosts (Goodwin, L.G. and Nimmo-Smith, R.H., eds.), p.142, J.P.A. Churchill Ltd., London.

NEWTON, B.A. (1968) Ann. Rev. Microbiol. 22, 109-130.

NEWTON, B.A., CROSS, G.A.M. and BAKER, J.R. (1973) Symp. Soc. Gen. Microbiol. 23, 389-373.

**NICHOL, M. and MESNIL, F. (1906) Ann. Inst. Pasteur. 20, 417.

NISHIKIMI, M., RAO, N.A. and YAGI, K. (1972) Biochem. Biophys. Res. Commun. 46, 849-854.

ORMEROD, W.E. (1951) Brit. J. Pharmacol. Chemother. 6, 334-341.

ORMEROD, W.E. and SHAW, J.J. (1963) Brit. J. Pharmacol. 21, 259-72.

PEEL, J.L. (1972) in Methods in Microbiology (Norris, J.R. and Ribbons, D.W., eds.), vol. 6B, pp.1-24, Academic Press, New York.

PERSCHKE, H. and BRODA, E. (1961) Nature (London) 190, 257-258.

QUASTEL, J.H. (1931) Biochem. J. 25, 1121-27.

RAJAGOPALAN, K.V. and HANDLER, P. (1964) J. Biol. Chem. 239, 1509-1514.

RINGLER, R.L. and SINGER, T.P. (1959) J. Biol. Chem. 234, 2211-2217.

ROEHL, W. (1926) Arch. Schiffs-u. Tropenhyg. 30, 103-111.

RYLEY, J.F. (1956) Biochem. J. 62, 215-222.

RYLEY, J.F. (1962) Biochem. J. 85, 211-223.

RYLEY, J.F. (1966) Proc. Int. Cong. Parasitol. pp.41-42.

SACKTOR, B. (1961) Ann. Rev. Entomology. 6, 103-130.

SACKTOR, B., PACKER, L. and ESTABROOK, R.W. (1959) Arch. Biochem. Biophys. 80, 68-71.

SCHNAITMAN, C. and GREENAWALT, J.W. (1968) J. Cell Biol. 38, 158-175.

SHARPLESS, T.K. and BUTOW, R.A. (1970) J. Biol. Chem. 245, 58-70.

SIMPSON, A.M. and SIMPSON, L. (1974) J. Protozool. 21, 774-781.

SIMPSON, L. (1972) Int. Rev. Cytol. 32, 139-207.

SMITH, P.A.S. (1965) The Chemistry of Open Chain Organic Nitrogen Compounds, Vol. I, W.A. Benjamin Inc., New York.

SPINKS, A. (1948) Biochem. J. 42, 109-116.

SRIVASTAVA, H.K. and BOWMAN, I.B.R. (1971) Comp. Biochem. Physiol. 40B, 973-981.

SRIVASTAVA, H.K. and BOWMAN, I.B.R. (1972) Nature, New Biol. (London) 235, 152-153.

STECK, E.A. (1971) The Chemotherapy of Protozoon Diseases, Vol. III, section 3, pp.11.31-11.40, Division of Medical Chemistry, Walter Reid Army Institute of Research, Washington D.C. 20012.

TAYLOR, A.E.R., LANHAM, S.M. and WILLIAMS, J.E. (1974) Exp. Parasitol. 35, 196-208.

TOWN, B.W., WILLS, E.D. and WORMALL, A. (1949) Nature. 164, p.233.

TOWN, B.W., WILLS, E.D., WILSON, E.J. AND WORMALL, A. (1950) Biochem. J. 47, 149-158.

TRIPATHI, R.K. and GOTTLIEB, D. (1969) J. Bacteriol. 100, 310-318.

UDENFRIEND, S. (1962a) Fluorescence Assay in Biology and Medicine, vol. 1, p.241, Academic Press, New York.

UDENFRIEND, S. (1962b) Ibid. pp.96-124.

VALLEE, B.L. and WACKER, W.E.C. (1970) in The Proteins (Neurath, H., ed.), vol. 5, Metalloproteins, 2nd. edn., Academic Press, New York.

VAN DE BOGART, M. and BEINERT, H. (1967) Anal. Biochem. 20, 325-334.

VANNGARD, T. (1972) in Biological Applications of Electron Spin Resonance, (Swartz, H.M., Bolton, J.R. and Borg, D.C., eds.), pp.411-447, Wiley-Interscience, New York).

VICKERMAN, K. (1965) Nature (London) 208, 762-766.

VICKERMAN, K. (1969) J. Cell Sci. 5, 163-193.

VICKERMAN, K. (1972) Symp. Brit. Soc. Parasitol. 10, 71-91.

VICKERMAN, K., and LUCKINS, A.G. (1969) Nature (London) 224, 1125-1126.

VIERTHALER, R.W. and BOSELLI, A. (1939) Arch. Schiffs-u. Tropenhyg. 43, 149-160.

VOGEL, A.I. (1961) A Textbook of Quantitative Inorgance Analysis, 3rd edn., pp.370-371, Longmans, London.

WADDY, B.B. (1970) in The African Trypanosomiases, (Mulligan, H.W., ed.), pp.711-725, Allen and Unwin Ltd., London.

WARBURG, O., and CHRISTIAN, W. (1942) Biochem. Z. 310, 384-421.

WARING, M.J. (1965) Molec. Pharmacol. 1, 1-13.

WILLIAMSON, J. (1970) in The African Trypanosomiases (Mulligan, H.W., ed.), pp.125-221, Allen and Unwin Ltd., London.

WILLIAMSON, J. and BROWN, K.N. (1964) Exp. Parasitol. 15, 44-68.

WILLS, E.D. (1952) Biochem. J. 50, 421-425.

WILLS, E.D. (1954) Biochem. J. 57, 109-120.

WILLS, E.D. and WORMALL, A. (1949) Biochem. J. 44, xxix P.

WILLS, E.D. and WORMALL, A. (1950a) Biochem. J. 47, 158-170.

WILLS, E.D. and WORMALL, A. (1950b) Nature (London) 165, 813-814.

WONG, D.T., HORNG, J.S. and GORDEE, R.S. (1971) J. Bacteriol. 106, 168-173.

WONG, D.T. and AIRALL, J.M. (1970) J. Antibiot. (Tokyo) 23, 55-62.

YORKE, W., MURGATROYD, F. and HAWKING, F. (1931) Ann. Trop. Med. Parasitol. 25, 313-349.

ZEISS, H. and UTKINA-LJUBOVTZOVA, X. (1930) Z. Immun-Forsch. 68, 170-181.