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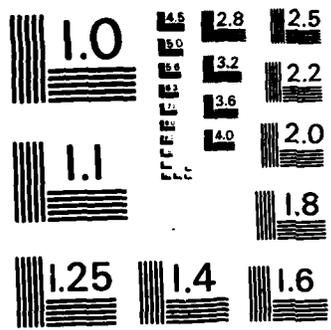
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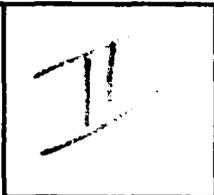
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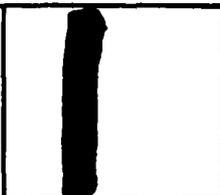


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DISTRIBUTION AND CHARACTERIZATION OF ANTIGENS FOUND
IN SUBCELLULAR FRACTIONS OF AFRICAN TRYPANOSOMES

John McLaughlin

For the Period ~~March 1, 1979~~ ^{August 1979} to August 20, 1979

(ANNUAL REPORT)

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FOREWARD

Experiments involving laboratory animals have been in accordance with all relevant guidelines regarding humane treatment.

ABSTRACT

Work has just commenced on a project aimed at characterizing the subcellular distribution and nature of antigens found in the African trypanosome, Trypanosoma rhodesiense, with an eventual aim of assessing such antigens for their potential in immune prophylaxis. To date attempts have been made to identify enzymes that could be used as markers to characterize the various subcellular fractions obtained after isopycnic gradient centrifugation. Differential centrifugation has established the gross distribution of various enzyme activities between particle fractions and a soluble (cell sap) fraction. A surface membrane ATPase has been tentatively identified which shows no Na^+/K^+ stimulation or inhibition in the presence of ouabain. Results using several phosphatase substrates suggest the presence of at least two distinct latent phosphatases, differing in subcellular distribution. One is an acid phosphatase, principally active against β -glycerophosphate and localized in the smaller subcellular particles. The other having a slightly higher pH optimum is more evenly distributed between small and large particles and principally active against β -glycerophosphate. An authentic glucose-6-phosphatase may be present but it is not associated with microsomal elements as in other cells. A neutral sulfhydryl proteinase showed the greatest enrichment of all enzymes in the large particle fraction. An oligomycin sensitive ATPase was detected and an α -glycerophosphate dehydrogenase, both presumably mitochondrial in association. Two glycolytic enzymes investigated, hexokinase and phosphofructokinase, were highly latent and associated with large subcellular particles.

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SUMMARY

This report describes progress for the period March 1, 1979 through August 20, 1979 for the current project concerning the subcellular distribution and characterization of antigens in the African trypanosome, Trypanosoma rhodesiense. During this period the major effort has focussed on attempts to recognize enzyme activities that will aid in the identification of subcellular fractions obtained after isopycnic centrifugation of T. rhodesiense. In addition the distribution of enzyme activities after differential centrifugation of total cell homogenated has been determined. This has aided in the characterization of individual enzymes and will provide a means for the preliminary separation of cell components prior to isopycnic gradient centrifugation.

The following enzyme activities were investigated, with their presumed localization given in parentheses: proteinase; acid phosphatase and glycosidases (lysosomes), glucose-6-phosphatase, NADPH-cytochrome c oxidoreductase, diaphorase and nucleoside diphosphatase (endoplasmic reticulum); hexokinase, phosphofructokinase and NAD linked α -glycerophosphate dehydrogenase (glycosomes); 5'-mononucleotidase; alkaline p-nitrophenylphosphatase and $\text{Na}^+ \text{K}^+$ stimulated Mg-ATPase (surface membrane); oligomycin sensitive Mg-ATPase and DCPIP linked α -glycerophosphate dehydrogenase (mitochondria) and alkaline inorganic pyrophosphatase (cell sap).

From the results obtained it appears that the major proteolytic activity in this organism is due to a sulfhydryl proteinase possibly analogous to cathepsin B. No glycosidases could be demonstrated using various

4-methylumbelliferyl glycosides as substrates. Phosphatase activity was demonstrable toward a range of substrates but distribution data, particularly with β -glycerophosphate as substrate indicates a quite different localization to proteinase. Both enzymes are lysosomal in distribution in most eukaryotic cells. From the inhibitor studies, pH optima and distribution data with various substrates, the existence of a least two distinct phosphatases (or isoenzymes) is suggested. In this connection results obtained tentatively indicates an authentic glucose-6-phosphatase, though distribution data is not compatible with localization within endoplasmic reticulum. Clearly further work is required to confirm the existence of a substrate specific phosphatases. Activity to all phosphatase substrates exhibited at least 50-60% structure linked latency. While nucleoside diphosphatase was also present, most of the activity was associated with the cell sap not with structural elements of the cell.

Present results confirmed the high degree of latency and sedimentability of those enzymes previously associated with a novel trypanosomal organelle, the glycosome.

ATPase activity showed some sensitivity toward oligomycin, but most of the activity was tentatively identified as being associated with surface membrane. There was no indications of either alkaline p-nitrophenolphosphatase or 5-mononucleotidase being present as surface membrane markers.

A) INTRODUCTION

This report details work performed during the period March 1 through August 20, 1979, being the first six months of the first year of the current project. The aim of this project, which was fully described in the original proposal, is to characterize the nature and subcellular distribution of antigens in the African trypanosome, Trypanosoma rhodesiense, with an eventual aim of assessing their potential in immune prophylaxis. As was also pointed out in the original proposal a comprehensive analysis of the organellar association and nature of antigens in the African trypanosomes has not been undertaken previously. An essential aspect of such an investigation is the ability to identify the nature (organellar derivation) of cell fractions based upon chemical but more importantly reliable enzyme markers. The initial stages of this project, reported here within, have been concerned with a survey of potential marker enzymes and development of suitable assay procedures. In addition we report the development of a successful homogenization procedure and the results of cell fractionation using differential centrifugation.

B) METHODS

1) Maintenance of Parasites

The Wellcome CT strain of T. rhodesiense is maintained in ICR/HA Swiss mice at the Rane Laboratory (University of Miami). For collection of trypanosomes a minimum of 30 - 40 retired breeder mice (\approx 40g) are infected with 0.1 ml diluted whole blood (0.1 ml in 50 ml 0.85% NaCl) and bled by cardiac puncture 72h later. The separation of trypanosomes from blood utilized a DEAE cellulose procedure after that described by Lanham and Godfrey (1970).

2) Preparation of DEAE cellulose

Approximately 100g lots of Sigma DEAE cellulose (coarse mesh) are prepared as follows:

Firstly fines are removed by repeated washings with distilled water after which time the cellulose is cycled from base (1.0N NaOH - 0.75M NaCl) to acid (IN HCl) to base in two 11 x 5 cm Buchner funnels. After the final exposure to base the cellulose is extensively washed with de-ionized water to give a final pH of 6.5-7.0. In order to achieve satisfactory flow rates it is imperative to ensure as complete removal of fines as possible during the initial washing of the cellulose. A 300 ml volume of buffered glucose (0.36% NaCl, 1.25% glucose 0.1M Tris pH 7.8) is then passed through the DEAE cellulose, packed in a Buchner funnel, followed by 200 ml of the same buffer containing 10 I.U. heparin/ml.

3) Collection of Parasites

Approximately 50 ml of infected mouse blood, collected on ice, is layered on top of the packed bed of DEAE cellulose under gentle vacuum. A 300 ml volume of heparinized buffered glucose is then passed through the DEAE cellulose and discarded, after which time trypanosomes are found in the effluent and collected. The parasites are sedimented by centrifugation (1500 rpm Sorvall SS-34 rotor with 50 ml polycarbonate tubes) and twice washed with 250 mM sucrose; 10 mM HEPES; 2.5mM EDTA; 5mM KCl pH 7.2 (buffered sucrose). For enzyme assays performed directly on this suspension, cells are disrupted using 2% Triton X-100 added to a final concentration of 0.2% and the suspension gently mixed with a Pasteur pipette. In certain instances such as the cell fraction experiments, cells were disrupted by homogenization with glass beads (see below).

4) Assay Procedures for Potential Markers Enzymes

Enzyme activities to be used as subcellular markers were chosen based upon their subcellular localization in other cells or in a few instances based upon previous work with African trypanosomes. There is need for great caution in assuming the applicability of marker enzymes established for cells other than those under investigation, particularly when the cells are widely separated phylogenetically. Also, in order to distinguish overlapping enzyme activities i.e. the phosphates, it is necessary to obtain a certain amount of kinetic data to establish the degree of specificity of individual enzymes. In addition kinetic data is required to optimize assay conditions and permit good recovery in cell fractionation experiments.

The enzyme assays described are grouped according to their established organellar association in other cell types, with the exception of certain glycolytic enzymes associated with the glycosome, a recently described organelle unique to certain trypanosomes (Oppendoes and Borst 1977). The method currently in use is first described followed by any comments regarding the properties of that particular enzyme in T. rhodesiense. Throughout, the term enzyme in assay procedures refers to the total cell homogenate or individual cell fraction for which enzyme activity is being assayed.

a) Lysosomes

A characteristic property of lysosomes is the association of a range of acid hydrolases such as the various proteinases, phosphatases and glycosidases.

1) Proteinase. The procedure currently employed follows that described by McLaughlin and Muller (1979). To 250 λ 4% urea denatured hemoglobin (adjusted to pH 6.5) are added 125 λ 250mM Tris-cacodylate pH 6.5; 25 λ 2% Triton X-100; 50 λ 10mM dithiothreitol and 50 λ enzyme. After a 30 minute incubation at 37°C, the reaction is stopped using 1.5 ml 0.5 M trichloroacetic

acid, and following centrifugation at 3000 rpm (Sorvall HS4 rotor with 00482 multiplace carriers) for 10 minutes an aliquot (25 - 50 λ) of the supernatant added to 2.0 ml 0.1 M 0.12M phosphate pH 7.2. To this sample is then added 1.0 ml Fluram solution (10mg/100ml anhydrous acetone) and after mixing, the tubes are read fluorometrically at $Ex=390nm$ and $Em=475nm$. Enzyme activity is calculated using a calibration curve obtained with 1-20 n mole L-leucyl-L-leucine. 1 Unit of enzyme activity is that required to liberate 1.0 μ mole L-leucyl-L-leucine equivalents per minute at 37°C.

Proteolytic activity is also assayed using azocasein as substrate, as described by McLaughlin and Muller (1979). To 150 λ 1.25% azocasein in 250mM phosphate pH 6.0 are added 10 λ 2% Triton X-100, 25 λ 10mM dithiothreitol, 50 λ enzyme to a final volume of 250 λ . After a 1.0h incubation at 37°C, 500 λ 0.5M trichloroacetic acid is added, the precipitated protein removed by centrifugation as described above and the supernatant fraction added to 2.0ml of 0.5N NaOH. The absorbance is then read at 420nm. Activity is expressed in arbitrary units in which 1 Unit \equiv an absorbance change of 0.1 per hour, at 420 nm. after incubation at 37°C.

The limited amount of data available so far suggests that a major portion of the proteolytic activity of T. rhodesiense is due to a sulfhydryl proteinase, possibly of the cathepsin B type (Barrett 1977). With both substrates activity is 50 - 60% higher at pH 6.0 as compared to pH 7.5. At the lower pH 1.0mM $CuSO_4$ and 2.0mM iodoacetamide give inhibitions of 63 and 40% (hemaglobin) and 89 and 45% (azocasein) respectively. Addition of either 1.0mM dithiothreitol or mercaptoethanol has produced up to 200% activation, though results are variable. Such variable results have been previously reported for crude preparations of thiol proteinases such as calhepsin B (Otto 1971). Neither EDTA nor $MgCl_2$

(up to 5mM) have any effect upon activity with either substrate at both pH 6.0 and 7.5, suggesting the absence of neutral EDTA sensitive proteinases in this trypanosome. Using acid denatured hemoglobin at pH 3.5 we have also been unable so far to detect the presence of acid proteinase activity analogous with calhepsin D in higher eukaryotic cells.

It is of some interest that thiol proteinases, rather than other types of proteolytic enzymes have been described in a number of other protozoa (McLaughlin and Muller 1979; McLaughlin and Faubert 1977; Levy et al 1976).

ii) Phosphatase. One of the more reliable marker enzymes for lysosomes in general is acid phosphatase assayed using β -glycerophosphate as substrate. Activity is now routinely assayed using 1.0ml of 15.0mM β -glycerophosphate in 50mM acetate pH 4.0 containing 0.1% Triton X-100 to which is added 10-30 λ enzyme. After incubation at 30°C for 20 minutes the reaction is terminated by adding 500 λ 8.0% sulfosalicylic acid and the precipitated protein removed by centrifugation as described above. To the withdrawn supernatant fraction is added 1.5 ml of the Triton-molybdate reagent (McLaughlin and Meerovitch 1976), and after incubating the whole at 30°C for 10 minutes the tubes read spectrophotometrically at 790 nm. The concentration of released orthophosphate is then determined using a calibration curve for 10-15 n moles PO_4 .

1 Unit of enzyme activity \equiv 1 mole of PO_4 released per minute at 30°C.

As can be seen from the accompanying pH activity profile (Fig 1) pH 4.0 gives optimum activity, with no indication of any alkaline activity, even in the presence of added Mg^{2+} . In keeping with "typical" non-specific acid phosphatase from various other sources (Hollander 1973) the trypanosomal enzyme is inhibited by both F- (100% using 1mM NaF) and tartrate (80% using 10mM Na K tartrate).

Other substrates have also been investigated: p-nitrophenyl-phosphate; α -glycerophosphate and glucose-6-phosphate. Hydrolytic activity toward the first of these substrates is routinely assayed using 1.0ml, 10mM p-nitrophenylphosphate in 50mM acetate pH 5.5 containing 0.1% Triton X-100. After a 10 minute incubation at 30°C, 3.0ml glycine stopping solution is added (Muller 1973) and the absorbance of the released p-nitrophenol read at 410nm. Reaction conditions for α -glycerophosphate were the same as for the β -isomer except for the use of an acetate buffer at pH 5.5.

The use of p-nitrophenylphosphate as a substrate for lysosomal acid phosphatase is widespread though it is not as specific as β -glycerophosphate since it can be hydrolysed by both microsomal acid phosphatase (Lin and Fishman 1972) and glucose-6-phosphatase if present (Nordlie 1971). In contrast to α -glycerophosphate, p-nitrophenylphosphate hydrolysis is optimal at a higher pH (pH 5.5-6.0) as shown in Fig 2. Noticeable also is the lack of any alkaline activity, again even with the addition of Mg^{2+} . Alkaline phosphatase, using p-nitrophenylphosphate as substrate has been employed as a marker enzyme for plasma membranes (see below).

Fig 3 shows the pH profile for activity toward α -glycerophosphate which also shows an optimum activity at pH 5.5-6.0. In this instance we also compared the effect of 10mM tartrate on the pH activity profile. It is apparent that tartrate exerts a much more pronounced inhibitory effect at lower pH values, 78% at pH 4.0 as compared to 37% at pH 5.5. Possibly this indicates the presence of two distinct phosphatase (or iso-enzymes) having different pH optima and sensitivity to tartrate inhibition. As will become apparent below the cell fractionation experiments lend additional support to this proposal.

Certainly further experiments are required to differentiate the specificity and localization of phosphohydrolases in T. rhodesiense.

Glucose-6-phosphate as a substrate is discussed below in connection with the potential use of glucose-6-phosphatase as an endoplasmic reticulum marker.

iii) Glycosidases Attempts to detect the presence of individual glycosidases against a range of 4-methylumbelliferyl glycosides (N-acetylglucosamine; N-acetylgalactosamine, galactose, xylose and fucose) using fluorometric procedures described by Barrett and Heath (1977) have so far revealed a complete absence or activities too low to be of any value in cell fractionation work.

b) Endoplasmic Reticulum

To date a number of the more easily assayed presumptive microsomal enzymes have been investigated: glucose-6-phosphatase (Nordlie and Arion 1966) NADPH-cytochrome c oxidoreductase (Omura et al 1967), diaphorase-NAD(P)H -ferricyanide oxidoreductase, (Wallach and Kamat 1966) and nucleoside diphosphatase (Amar Costesecet al 1974). The latter activity is also associated with elements of the Golgi apparatus.

i) Glucose-6-phosphatase - Inorganic Pyrophosphatase Activity against glucose-6-phosphate has been demonstrated using the following assay system: 1.0ml 15mM glucose-6-phosphate in 60mM cacodylate pH 6.0 containing 10mM Na K tartrate and 0.1% Triton X-100. After incubating with enzyme for 10 minutes at 30°C, the reaction is stopped with 500 λ 8% sulfosalicylic acid and precipitated protein removed by centrifugation and released orthophosphate

measured as for β -glycerophosphate. The potentially related inorganic pyrophosphatase was assayed as follows: to 375 λ 6.0mM sodium pyrophosphate; 50mM acetate pH 5.5, were added 25 λ 2% Triton X-100 and 20 λ enzyme to a final volume of 450 λ . After incubating for 20 minutes at 30°C, 50 λ 8% sulfosalicylic acid containing 10mM CuSO_4 (to prevent non-specific PPI hydrolysis by complex formation with Cu^{2+} , Woltgens and Ashman 1970) are added and precipitated protein removed by centrifugation as above. The supernatant fraction is then removed to 1.0ml ice cold de-ionized water at which time 1.5ml of the Triton-molybdate reagent is added, and the concentration of orthophosphate determined as referenced above.

Incontrast to the non-specific acid phosphatase activity toward β -glycerophosphate, hydrolysis of glucose-6-phosphate was inhibited only 16% in the presence of 10mM tartrate, and displayed optimum activity at pH 6.0 (see Fig 4). This preliminary data suggests the existence of an authentic glucose-6-phosphatase and that this same enzyme maybe in part responsible for the splitting of p-nitrophenyl-phosphate. Mammalian liver glucose-6-phosphatase is known to be a multifunctional enzyme (Nordlie 1971) being capable for instance of also functioning as an inorganic pyrophosphatase. Acid pyrophosphatase activity is readily demonstrable, and as will be discussed more fully exhibits a similar subcellular distribution after differential centrifugation, to glucose-6-phosphatase.

ii) Cytochrome C-NADP oxido-reductase and Diaphorase

Using procedures described by Omura et al (1967) for cytochrome c-NADP reductase, and Wallach and Kamat (1966) for diaphorase, neither enzyme was present above 1mU/mg protein. The absence of the former enzyme was not suprising in view of the lack of cytochromes in the blood stages of African trypanosomes. Neither enzyme has been investigated further.

iii) Nucleoside Diphosphatase. Activity is presently assayed using 1.0ml 2mM ADP; 5mM MgCl₂ in 30 mM glycyl-glycine pH 8.5, both with and without 0.1% Triton X-100. The pH activity profile shown in Fig 5 exhibits as expected an alkaline pH optimum (see Yamazaki and Hayaisha 1968). Preliminary results on cation requirements also agree with previous work (loc. cit.) with Mg²⁺ being twice as effective in stimulating activity (5 fold increase as compared to Ca²⁺).

As will be discussed below the distribution data casts doubt on the presumed association of this enzyme with endoplasmic reticulum or golgi apparatus.

c) Glycosomes

These organelles unique to certain trypanosomes are synonymous with the microbodies referred to in the earlier literature (i.e. Bayne et al 1969) being termed glycosomes by Opperdoes and Borst 1977, who convincingly demonstrated them to contain most of the enzymes involved in glycolysis in Trypanosoma brucei. For the current project two of these enzymes are being assayed, hexokinase and phosphorfructokinase.

i) Hexokinase To 900λ 3.7mM glucose; 7.5mM MgCl₂; 45mM HEPES pH 7.5 are added 40λ 2% Triton X-100; 10-20λ enzyme; 100λ NADP solution (25mg/ml), 100λ 4mM dithiothreitol and 10λ glucose-6-phosphate dehydrogenase (Sigma Type IX, 100 U/ml in 0.025% bovine serum albumin, 20mM Tris pH 7.5). After an equilibration period of 3.0 minutes the reaction was started by adding 30λ 220mM ATP and the change in absorbance at 340nm recorded at 25°C for 1-2 minutes, using a Hitachi-Coleman 124 double beam spectrophotometer, equipped with a cuvette positioner and linear pen recorder.

ii) Phosphofructokinase: To 2.5 ml 30 mM glycyl-glycine, 30mM β -Glycerophosphate; 1.2 mM EDTA; 7.2 mM $MgCl_2$; 3.6mM $(NH_4)_2SO_4$ pH 8.2 were added 100 λ 30mM fructose-6-phosphate; 100 λ 30mM ATP; 100 λ 3mM dithiothreitol; 50 λ NADH (10mg/ml); 150 λ 2% Triton X-100; 50 λ aldolase (Sigma Grade I, 36 U/ml) and 10 λ triose-phosphate isomerase (Sigma Type I, 8 U/ml) both enzymes contained in 25mM glycylglycine pH 8.0. After a 3.0 minute equilibration, 20 λ T. rhodesiense enzyme was added and the change in absorbance at 340nm recorded as above for 2-3 minutes.

In both instances enzyme activities are calculated using $E_{mM} = 6.2$ for NAD(P)H, 1 Unit \equiv the oxidation/reduction of 1.0 μ mole NADH/NADP per minute at 25°C.

d) Surface Membrane

The potential importance of surface membrane components apart from glyco-protein coat material as antigens was of special interest. Thus a number of enzyme markers specific for surface (plasma) membrane preparations in other cells have been investigated.

i) 5-mononucleotidase The assay of activity using 5-AMP as substrate was exactly as described by Michell and Hawthorn (1965), with released orthophosphate being measured as for α -glycerophosphate. Specific activity of less than 5mU per mg protein have been found indicating the lack of a specific 5-mononucleotidase. At pH 5.0 in the presence of only 50mM acetate, activity of 15-20mU is observed, presumably due to non-specific acid phosphatase activity. Although considered one of the most authentic surface membrane enzymes, in mammalian cells at least, results with various other protozoa

(i.e., Trypanosoma cruzi, Pereira et al 1978; Entamoeba histolytica, McLaughlin and Muller in preparation, and T. brucei Voorhies et al 1979), with the possible exception of Acanthamoeba custellani (Schultz and Thompson 1969), all demonstrate low activities and a complete absence of any specific 5-mononucleotidase.

ii) Na⁺ K⁺ Stimulated Mg-ATPase. Using the assay procedure of Wallach and Kamat (1966), with released orthophosphate determined as above, no activity of this type can be demonstrated. Whilst a Mg-ATPase can be measured, 30-40mU per mg protein, no further stimulation is observed in the presence of Na⁺ and/or K⁺. In addition in the presence of up to 0.5mM ouabain, a relatively specific inhibitor of Na⁺ K⁺ stimulated ATPases, no inhibition has been observed. These findings are in contrast with those of Voorheis et al (1979) who found evidence of a putative surface membrane ouabain sensitive Na⁺ K⁺-mg ATPase in T. brucei. Clearly further work is required to determine what portion of the ATPase activity occurring in T. rhodesiense might be surface membrane associated.

iii) Alkaline p-nitrophenylphosphatase. This enzyme activity especially with p-nitrophenylphosphate as substrate in the presence of Mg²⁺ has been utilized as a marker for plasma membrane fractions in several studies, though it is not entirely specific (Lauter et al 1971). As mentioned in the previous section on phosphatases (Sect. 4a(ii)) none of the substrates tested displayed any alkaline pH optima, even with added Mg²⁺.

e) Mitochondria

Mitochondrial function in the blood stages of African trypanosomes is highly repressed, however Opperdoes et al 1977 a claimed the presence of an oligomycin sensitive Mg-ATPase which they used as a mitochondrial marker.

Since we have been unable to demonstrate the presence of an oligomycin sensitive

ATPase by measuring orthophosphate release, an ATP regenerating assay system was used in order to overcome the likely potent product inhibition, due to ADP, observed for various other ATPases. The assay is based upon that described by Pullman *et al* 1960. To 500 λ NADH solution (containing 15.0ml 70mM tris-acetate pH 7.4; 1.25ml 45mM MgCl₂ and 0.5ml 22mM NADH) were added 200 λ 2.75mM p-enolpyruvate; 200 λ 10mM ATP; 15 λ lactate dehydrogenase (Sigma Type II 500u/ml) and 20 λ pyruvate kinase (Sigma Type II). ATPase activity was measured after addition of 10-50 λ *T. rhodesiense* enzyme source, by recording the change in absorbance at 340nm at 25°C for 3.0 minutes after ignoring the first 0.5 minutes. Since we are still attempting to obtain reproducible results with this assay, we did not utilize it in the cell fractionation experiments reported below. However in the presence of 2.25 μ g/ml of oligomycin up to 50% of the ATPase activity measured is inhibited.

f) Miscellaneous Enzyme Activities.

i) Mg²⁺ dependent alkaline inorganic PPIase. In order to assay this PPIase 1.0ml of 1.0mM sodium pyrophosphate; 20 mM MgCl₂ 30mM tris-acetate pH 7.5 is incubated with 15 λ enzyme at 30°C for 20 minutes. After adding 500 λ 8% sulfosalicylic acid 10mM CuSO₄, precipitated protein is removed by centrifugation and orthophosphate formation measured as described above for β -glycerophosphate.

This enzyme activity is of ubiquitous occurrence in most cell types, being exclusively associated with cell sap. For this latter reason it is a good index of the degree of cell disruption achieved during homogenization, its presence in fractions other than the final supernatant and pellet being due to contamination with whole cells (see below).

ii) α -glycerophosphate dehydrogenase

Two different assay systems are routinely followed: one measuring NAD

linked activity using dihydroxyacetone as substrate; the other using dichlorophenol-indophenol (DCPIP) as electron acceptor and α -glycerophosphate as substrate.

For NAD linked activity 900 λ 55mM HEPES 1.5mM EDTA pH 7.5 were incubated with 25 λ 20mM dihydroxyacetone (lithium salt); 20 λ NADH soln. (7.4 mg/ml) and 50 λ 2% Triton X-100, 20-50 λ enzyme being added to start the reaction. The change in absorbance at 340nm was then recorded for 2-3 minutes at 25°C.

With DCPIP as electron acceptor 2.7 ml α -glycerophosphate solution (519mg α -glycerophosphate in 22.5 ml K-phosphate pH 7.6 plus 2.25ml 2% Triton X-100 and 15.7ml H₂O) were added to 0.45ml 475mM DCPIP and the change in absorbance at 600nm recorded for 1-2 minutes at 25°C; activity was calculated using E_{mM} of 21.0 for DCPIP at pH 7.6.

Opperdoes and Borst (1977) have produced evidence showing the NAD linked α -glycerophosphate dehydrogenase to be associated with microbodies later identified as glycosomes (see section 4c). The reverse reaction, assayed using DCPIP and α -glycerophosphate was found previously (Opperdoes *et al* 1977b) to be associated with mitochondria, being synonymous with the activity described as α -glycerophosphate oxidase.

c) CELL FRACTIONATION

The differential centrifugation of a cell homogenate is an useful preliminary step prior to more analytical density gradient isopycnic centrifugation. Moreover, although further kinetic data and inhibitor studies remain to be performed on the enzymes investigated so far, fractionation by differential centrifugation has in itself proved useful in enzyme characterization.

For both experiments described below purified and washed suspensions of T. rhodesiense were obtained from approximately 80-100 ml infected mouse blood, processed as described previously (section B2). A cell homogenate was then prepared by grinding a heavy suspension of cells (2.0ml) in 250mM buffered sucrose with 15.0g acid washed glass beads (Sigma Type 1-W) using about 15 firm strokes in an all glass pestle and mortar. The homogenate was then diluted to about 5.0ml and glass beads removed by sedimentation using a 30 sec. centrifugation at 500g. The beads were washed with 3.0ml buffered sucrose and the pooled homogenate processed as below.

In all instances centrifugations were performed using a Sorval RC-5B centrifuge with an SS-34 rotor equipped with 425 adapters and 16 x 88 mm polycarbonate screw cap centrifuge tubes.

1) Differential Centrifugation - Experiment A

The T. rhodesiense cell homogenate was first centrifuged at 2,500 rpm for 3.0min to sediment nuclei, flagellar, intact and partially intact cells. The resulting supernatant was then centrifuged at 5,500 rpm for 10 minutes, the resulting pellet resuspended in buffered sucrose, centrifuged once more under identical conditions and the supernatant fractions combined. The washed pellet contained presumptive lysosomes and the pro-mitochondria. The combined supernatant fraction was finally centrifuged at 19,000 rpm for 1.0h to sediment a microsomal fraction with a final supernatant cell sap. Cell fractions were stored continuously on ice, all assays being completed within 2-3 days, those for both α -glycerophosphate dehydrogenases and both glycolytic enzymes being performed within 24h.

The above fractionation scheme is similar to that used by Muller 1973 for another flagellate, Tritrichomonas foetus. The specific activities for enzymes in the original homogenate, cumulative percentage distributions in the various fractions and final recovery are shown in Table 1. For protein determinations aliquots of homogenate and fractions were added to 200 μ l 1N NaOH and protein determined using the procedure of Lowry et al (1951). The data obtained is also presented as a series of relative specific activity plots (Fig, 6) showing the relative specific activity for each fraction in relation to the percentage protein content of that fraction. The original cell homogenate of course would be equivalent to a relative specific activity of 1.0.

2) Differential Centrifugation - Experiment B

A cell homogenate prepared as for "Experiment B" was first centrifuged for 4.0 minutes at 2,500 rpm, the supernatant fraction removed and the sediment resuspended in buffered sucrose and centrifugation repeated. The combined supernatant fractions were then centrifuged for 10.0 min. at 8,000 rpm, the resulting pellet being washed once with buffered sucrose. These combined supernatant fractions were finally centrifuged for 1.0h at 19,000 rpm, the resulting supernatant removed and the pellet resuspended in buffered sucrose. The data obtained, presented in the same format as above, are shown in Table 2 and Fig. 7.

The results from both experiments indicate more than 85% of the cells in both experiments to have been disrupted by the homogenization procedure used based on the localization of Mg^{2+} dependent alkaline PP_i ase in the final supernatant (S_2) fraction. In addition, the high recovery in the particulate fractions of all those enzymes considered to be associated with structural elements of the cell indicates no extensive damage to organelles with subsequent release of enzymes.

The one exception, however, was the distribution of nucleoside diphosphatase, used as a microsomal-golgi body marker for mammalian cells, but more than 60% in the soluble fraction for the present investigation. In mammalian cells this has been reported to be a latent membrane-lipid associated enzyme (O'Toole 1975), however, a soluble nucleoside diphosphatase has been reported from plant cells (Hunt et al 1977). Possibly this enzyme in T. rhodesiense is much less tightly membrane bound, being released during homogenization of the cells. Before eliminating this enzyme as a subcellular marker, we will compare the subcellular distribution of activity using two other substrates, inosine diphosphate and thiamine pyrophosphate, also susceptible to hydrolysis by nucleoside diphosphatase (Yamazaki and Hayaishi 1968).

Another indication of structural integrity of organelles after homogenization is the retention of a high degree of latency by certain particle associated (sedimentable) enzyme activities. In this context, latency refers to enzymes associated with subcellular particles where free access of substrate to enzyme is prevented by a limiting membrane impermeable to that substrate. As can be seen from Table 3, at least 50% of all

phosphatase activities were still latent after cell homogenization, with more than 60% for both p-nitrophenylphosphate and glucose-6-phosphate. In most studies of this type, the aim would be to maintain a 70% latency, i.e., only 30% of the activity being released by the homogenization procedure employed. However, in order to successfully disrupt the trypanosome cell, and thus avoid the contamination of the initial fractions with a high percentage of whole cells, quite drastic homogenization is required. At present it is considered preferable to obtain as full cell breakage as possible and accept latency values for the various phosphatases slightly lower than optimum.

The enzymes associated with the glycosome all display very high degrees of latency, at least 90%, and confirm the findings of Opperdoes and Borst (1977) for T. brucei.

Total Mg-ATPase is non-latent; the presence of Triton X-100 causes a 25% decrease in activity. Such sensitivity to added Triton X-100 has been noted for other ATPases, including that associated with another protozoan T. foetus (Lloyd, Lindmark and Muller in press). The lack of latency for this trypanosomal ATPase indicates the enzyme to occupy extrinsic membrane sites. In addition, two experiments were performed in which the ATPase activity, measured according to orthophosphate liberation, of whole cells osmotically protected with 250mM sucrose, was compared with that for cells disrupted by homogenization with glass beads. The whole cells had activity levels 76% and 83% respectively of those for the cell homogenate, indicating the ATPase to be localized on the external side of the surface membrane (an ecto-enzyme). Since after homogenization the surface membrane is probably forming open membrane sheets, known from other cells to be sedimentable at low centrifugal forces, then the present results showing

most of the ATPase in the N and P₁ fractions would be compatible with a surface membrane localization for this ATPase.

One of the interesting points of comparison, evident from both experiments A and B was the marked association of proteinase activity with fraction P₁, containing presumptive lysosomes, and the association of acid phosphatase (β -glycerophosphate) with fraction P₂, presumed to be analogous to microsomes. Both of these hydrolases are unequivocally lysosomal in association in higher eukaryotic cells. Activity toward the other phosphatase substrates showed less enrichment in fraction P₂, particularly glucose-6-phosphate and inorganic pyrophosphate. This is unexpected in view of the microsomal association of glucose-6-phosphatase in other cells. Further studies, particularly the ability to act as a phosphotransferase, remain that will hopefully provide more information as to whether this enzyme is synonymous with authentic glucose-6-phosphatase. The distribution of phosphatase activity toward α -glycerophosphate was dramatically altered in the presence of 10 mM tartrate, as shown in Figure 7. The activity recovered in fraction P₂ was reduced by about 50% whilst both fraction N and P₁ showed increases of 37% and 21% respectively. This suggests as mentioned previously [Methods Sect. 4a (ii)] that distinct phosphohydrolase activities toward α -glycerophosphate are present in T. rhodesiense and associated with more than one population of subcellular particles. As with other phosphatase activities the nature of the organelles with which these hydrolases are associated will have to await further studies, though it is clear even now that these are major differences when compared with other cells.

The two glycolytic enzymes hexokinase and phosphofructokinase were both almost totally sedimentable, their distribution between fractions P₁ and

P_2 depending on centrifugation conditions. With the use of a lower centrifugal force for the sedimentation of fraction P_1 (Experiment A), both enzymes were preferentially concentrated in fraction P_2 . In Experiment B, where a higher centrifugal force was applied, both enzymes now showed highest relative specific activities in fraction P_1 . This latter distribution for these enzymes is similar to that obtained after differential centrifugation of T. brucei by Opperdoes and Borst 1977, who subsequently employed gradient centrifugation to demonstrate the existence of a discrete organelle, the glycosome, referred to earlier. Our studies show NAD-linked α -glycerophosphate dehydrogenase, which these same authors also located in the glycosome, to have a distribution similar to hexokinase and phosphofructokinase. However, it will only be possible to confirm such a localization in T. rhodesiense after isopycnic gradient centrifugation of fractions P_1 and P_2 . The DCPIP linked α -glycerophosphate dehydrogenase also showed a similar distribution after differential centrifugation but is supposedly mitochondrial in localization according to Opperdoes et al 1977^b. Mitochondria would be expected to sediment predominantly with fraction P_1 , and once more isopycnic centrifugation will be required to ascertain the exact localization.

The present procedure for the homogenization of cells appears by all the criteria discussed below to be satisfactory, apart from possible excessive damage to the phosphatase containing structures. It has been possible to achieve a high percentage of disrupted cells, more so than were apparently obtained by Opperdoes et al 1977^a and Opperdoes and Borst 1977, based upon the comparatively high recovery of a presumptive cell sap enzyme, enolase, in fraction N. Moreover the extended centrifugation used by these authors to sediment fraction N appears to have resulted in a loss of P_1 enzymes to fraction N.

The scheme of centrifugation used in Experiment A did not give as good enrichment of enzyme in fractions P_1 and P_2 due to the failure to sediment sufficient non-specific structural protein into fraction N. It is of interest that Experiment B in which conditions were closer to those of Opperdoes and Borst 1977, gave maximum enrichment of hexokinase and phosphofructokinase in fraction P_1 . This in contrast to the maximum enrichment of these enzymes in fraction P_2 in experiment A where lower centrifugation speeds were used. None of the other enzymes studied displayed such a marked shift in distribution.

In future work the intention is to utilize the differential centrifugation scheme described in Experiment B followed by isopycnic gradient centrifugation of each of the fractions N, P_1 and P_2 . Sufficient number of marker enzymes are or will be established to enable the characterization of the fractions obtained after isopycnic centrifugation. Special attention however must be given to establishing a reliable surface membrane marker, the Mg-ATPase being a potential lead. Because of the numerous drawbacks inherent in the use of external covalent markers (Lin and Wallach 1973), it would be preferable to utilize some naturally occurring surface membrane component. One potentially useful marker associated with the Golgi bodies of other eukaryotic cells might be galactosyl transferase, particularly in view of the presence of a surface membrane glycoprotein coat. However, the assay is expensive in terms of substrate and will probably have to await the renewal of this proposal for a second year.

TABLES AND FIGURES

Table 1: Differential fractionation of a T. rhodesiense cell homogenate according to Experiment A.

Table 2: Differential fraction of a T. rhodesiense cell homogenate according to Experiment B.

Table 3: Latency of various sedimentable enzymes in T. rhodesiense.

Figure 1: pH activity plot for phosphatase activity using β -glycerophosphate as substrate.

Figure 2: pH activity plot for phosphatase using p-nitrophenylphosphate as substrate.

Figure 3: pH activity plot for phosphatase using α -glycerophosphate as substrate \pm tartrate.

Figure 4: pH activity plot for hydrolysis of glucose-6-phosphate in the presence of tartrate.

Figure 5: pH activity plot for nucleoside diphosphatase activity using ADP as substrate.

Figure 6: Relative specific activity plots for various enzymes after differential centrifugation according to Experiment A.

Figure 7: Relative specific activity plots using the protocol of Experiment B.

Total Activity Level
In Original Cell Homogenate
(m U*/mg protein)

Enzyme	Total Activity Level In Original Cell Homogenate (m U*/mg protein)	Percentage Distribution In Cell Fractions			Recovery (%)	
		N	P ₁	P ₂ S ₂		
Protein	72.6 mg	6.7	28.5	18.4	46.4	140
α-glycerophosphate dehydrogenase	70	6.9	49.6	32.3	11	125
Hexokinase	378	5	44	44	7	108
Phosphofructokinase	429	5.2	33.4	57.4	4	140
Nucleoside Diphosphatase	24	9.2	17.1	9	64.6	145
Mg ²⁺ dependent PPIase	42	3.6	4.1	3.6	88.7	150
Acid PPIase	45	8	49	30	13	95
Proteinase	26	3.9	57.2	24.6	14.3	130
Mg ²⁺ ATPase	40	12	47	19.7	21.3	125
Phosphatase						140
α-glycerophosphate	15	10.9	37.2	39	12.8	123
β-glycerophosphate	20	9.1	23.2	61.5	6.1	135
β-nitrophenylphosphate	31	6.5	37.1	40.6	15.8	
glucose-6-phosphatase	26	9.0	46	32	13	

TABLE 1 Differential Fractionation of T. rhodesiense showing percentage distribution and recovery for a cell homogenate fractionated according to the protocol in the text for "Experiment A".

* Units of enzyme activity are defined in the text.

Enzyme	Total Activity Level In Original Cell Homogenate (m U*/mg protein)	Percentage Distribution in Cell Fractions			Recovery (%)	
		N	P1	P2		
Protein	87.8 mg	19.2	21.6	9.4	49.8	102
α-glycerophosphate dehydrogenase	93	18.1	51.3	18.3	12.2	102
α-glycerophosphate dehydrogenase (NAD ⁺ linked)	183	9.0	64.7	25.4	0.9	102
Hexokinase	301	15.2	57.5	20	7.0	98.3
Phosphofructokinase	403	11.7	67.6	16.1	4.6	76
Nucleoside Diphosphatase	26	11.8	7.0	11.4	69.8	172
Mg ²⁺ dependent PPIase	54	7.4	6.7	3.6	85.8	165
Acid PPIase	35	30.5	33.0	17.5	19.0	114
Proteinase	19	11.4	69.6	10.1	8.8	99.8
Mg ²⁺ -ATPase	33	31	32.8	11.1	25.1	103
Phosphatase						
α-glycerophosphate ^a	19	28.7	32.9	34	4.4	99
β-glycerophosphate	25	24.8	30.2	39.7	5.3	105
α-glycerophosphate ^b	12	39.5	40	19.6	0.9	80
ρ-nitrophenylphosphate	41	24.3	27.6	27.2	20.9	101
glucose-6-phosphate	23	33.7	34.5	22.8	8.8	92.6

TABLE 2 Differential fractionation of T. rhodesiense showing percentage distribution and recovery for a cell homogenate fractionated according to the protocol in the text for "Experiment B".

* Units of enzyme activity are defined in the text.

a = no addition; b = assayed in the presence of 10mM NaK tartrate

<u>ENZYME</u>	<u>FREE ACTIVITY</u>	<u>TOTAL ACTIVITY</u>	<u>LATENCY</u>
Phosphatase			
α-glycerophosphate	10.0	21.5	50
β-glycerophosphate	12.0	28.0	57
p-nitrophenylphosphate	13.7	43.0	68
glucose-6-phosphate	12.9	28.0	64
Acid Inorganic Pyrophosphatase	17.6	43.0	59
Mg-ATPase (pH 8.6)	45	34	0
Hexokinase	8	402	98
Phosphofructokinase	22	441	95

TABLE 3: Latency of Various Sedimentable Enzymes in *T. rhodesiense*

For free activity, assays were performed in the presence of 250 mM sucrose. Total activity was measured by incorporation of Triton X-100 to a final concentration of 0.1% in the same assays.

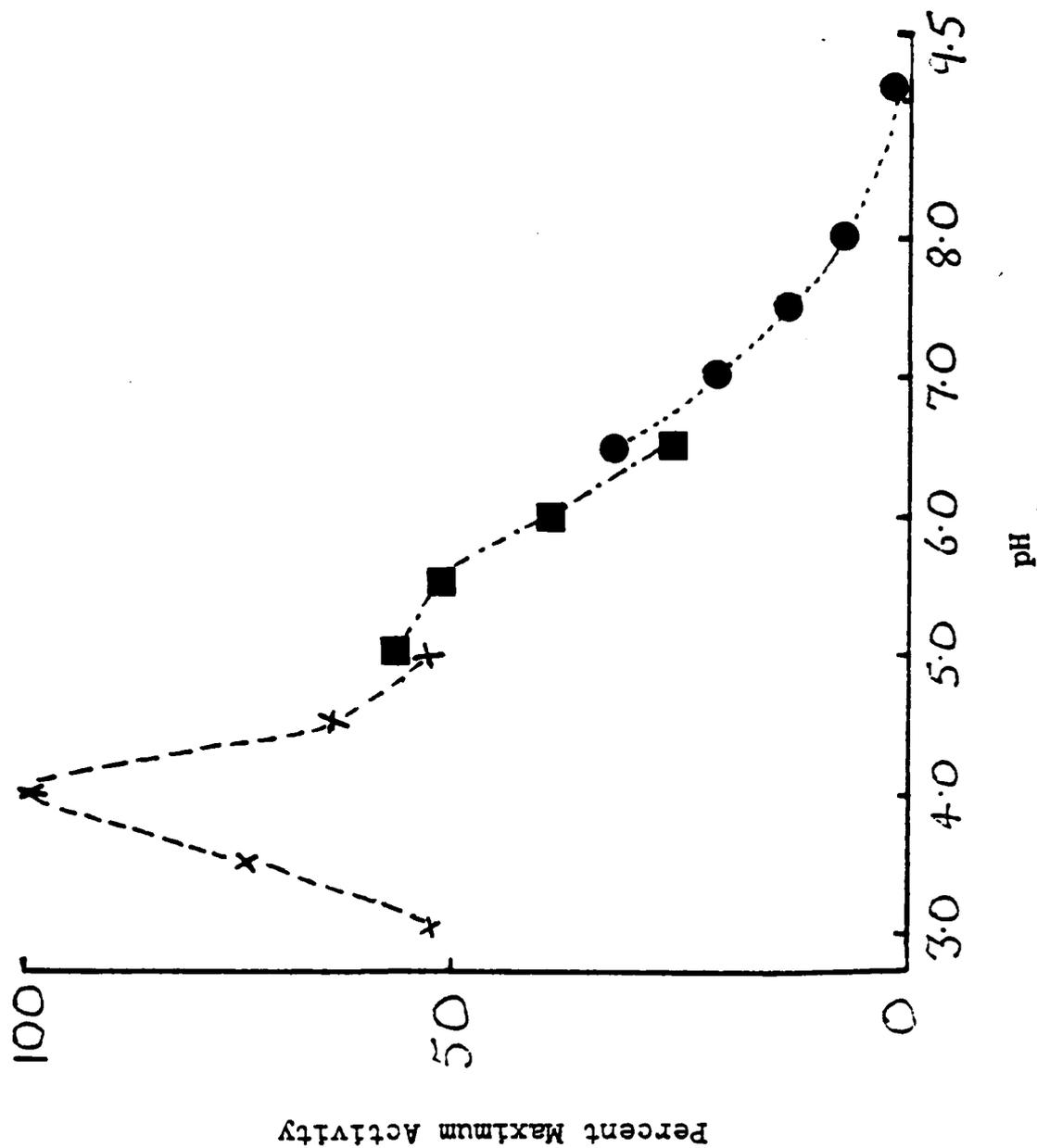
$$\text{Latency} = \frac{\text{total activity} - \text{free activity}}{\text{total activity}} \%$$

FIGURE 1

x pH related activity plot for phosphate activity toward β -glycero-phosphate.

- x x 75mM formate buffer
- ■ 75mM acetate buffer
- ● 75mM tris-acetate

Assay conditions as in text.



Percent Maximum Activity

pH

FIGURE 2

pH related activity plot for phosphatase activity toward
p-nitrophenylphosphate. Symbols as for Figure 1.

Percent Maximum Activity

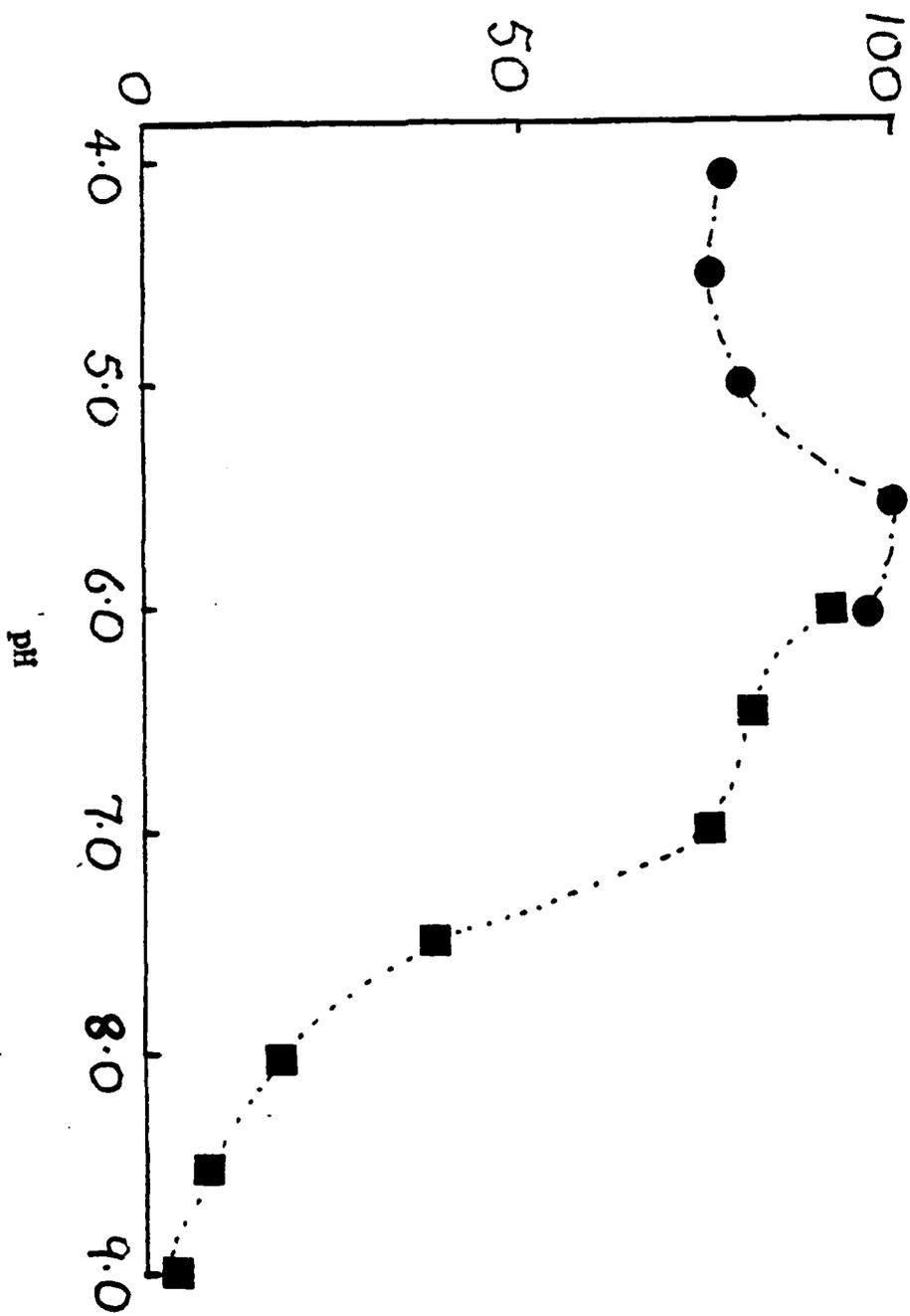


FIGURE 3

pH related plot for phosphatase activity toward α -glycerophosphate
in the presence and absence of 10mM tartrate.

- ■ 75mM acetate buffer
- ● 75mM tris-acetate
- ● 75mM acetate buffer and 10mM NaK tartrate
- ● 75mM tris-acetate and 10mM NaK tartrate

Percent Maximum Activity

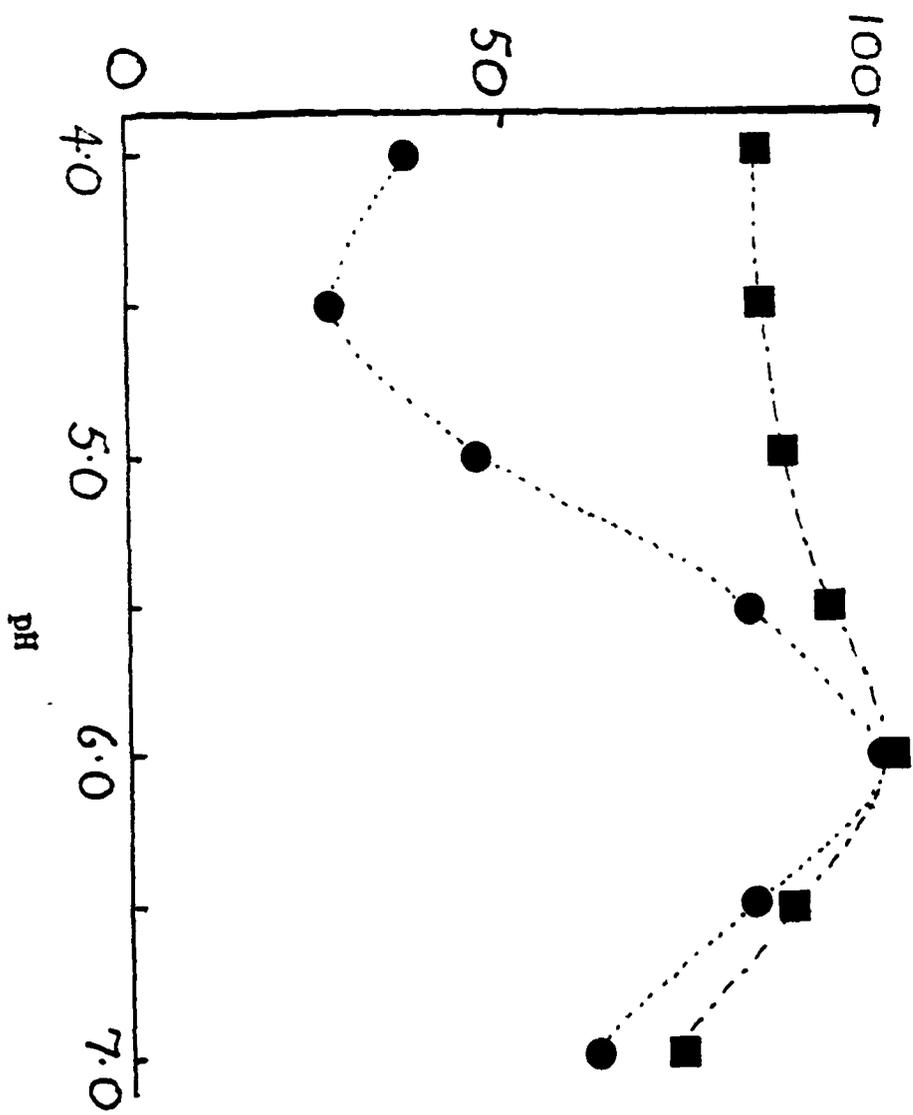


FIGURE 4
pH activity profile for the hydrolysis of glucose-6-phosphate
in the presence of 10mM tartrate (glucose-6-phosphatase) Buffer:
60mM tris-cacodylic acid.

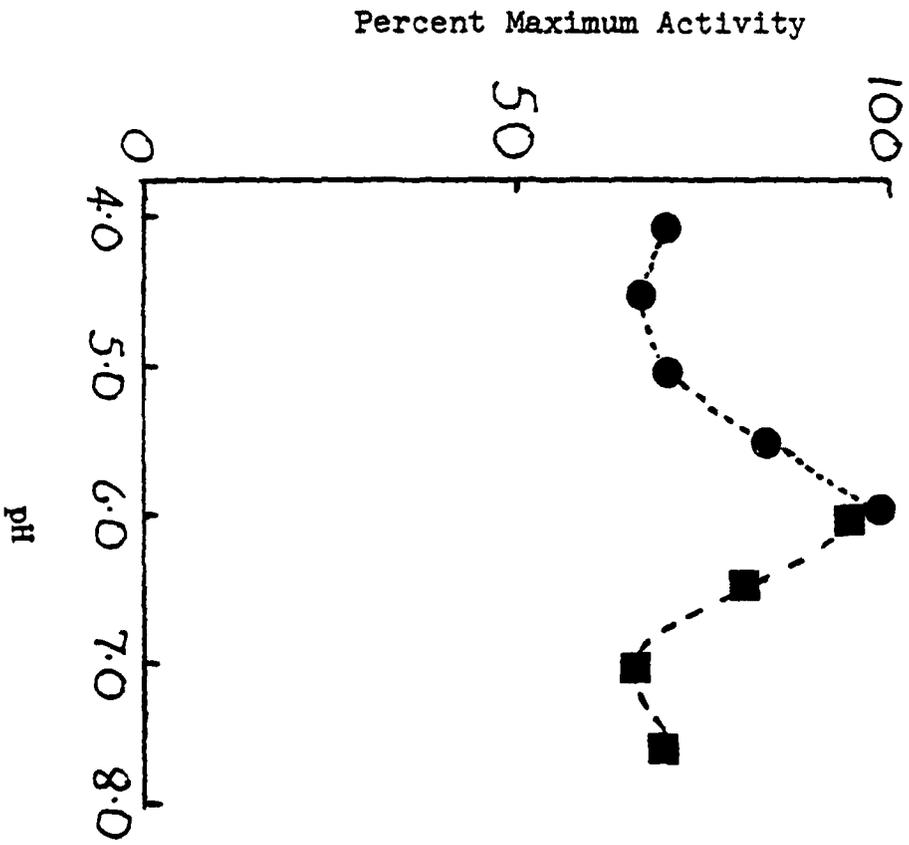


FIGURE 5

pH related activity plot for nucleoside diphosphatase using
ADP as substrate. Assay as in text. Symbols as for Figure 1.

Percent Maximum Activity

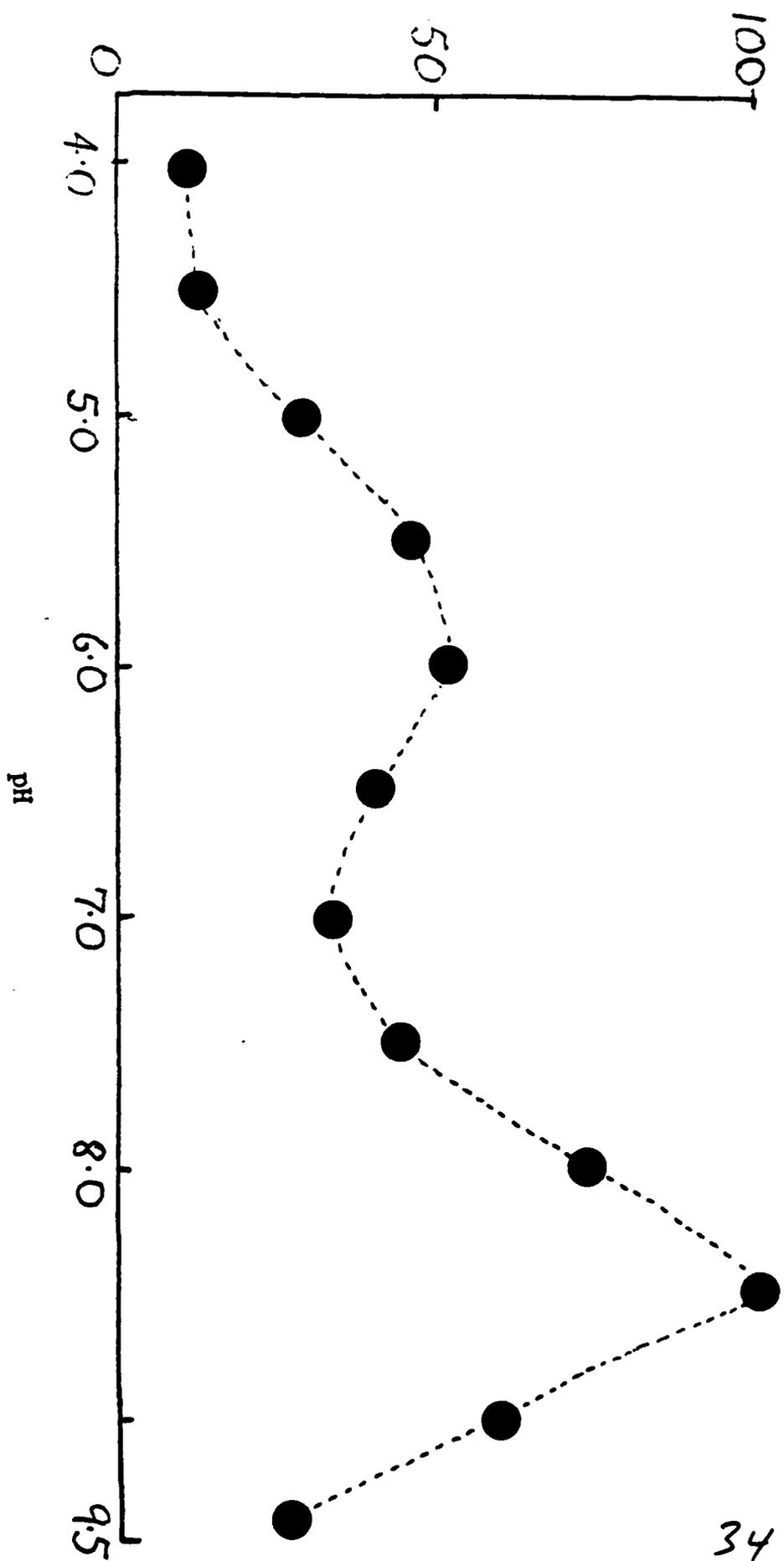
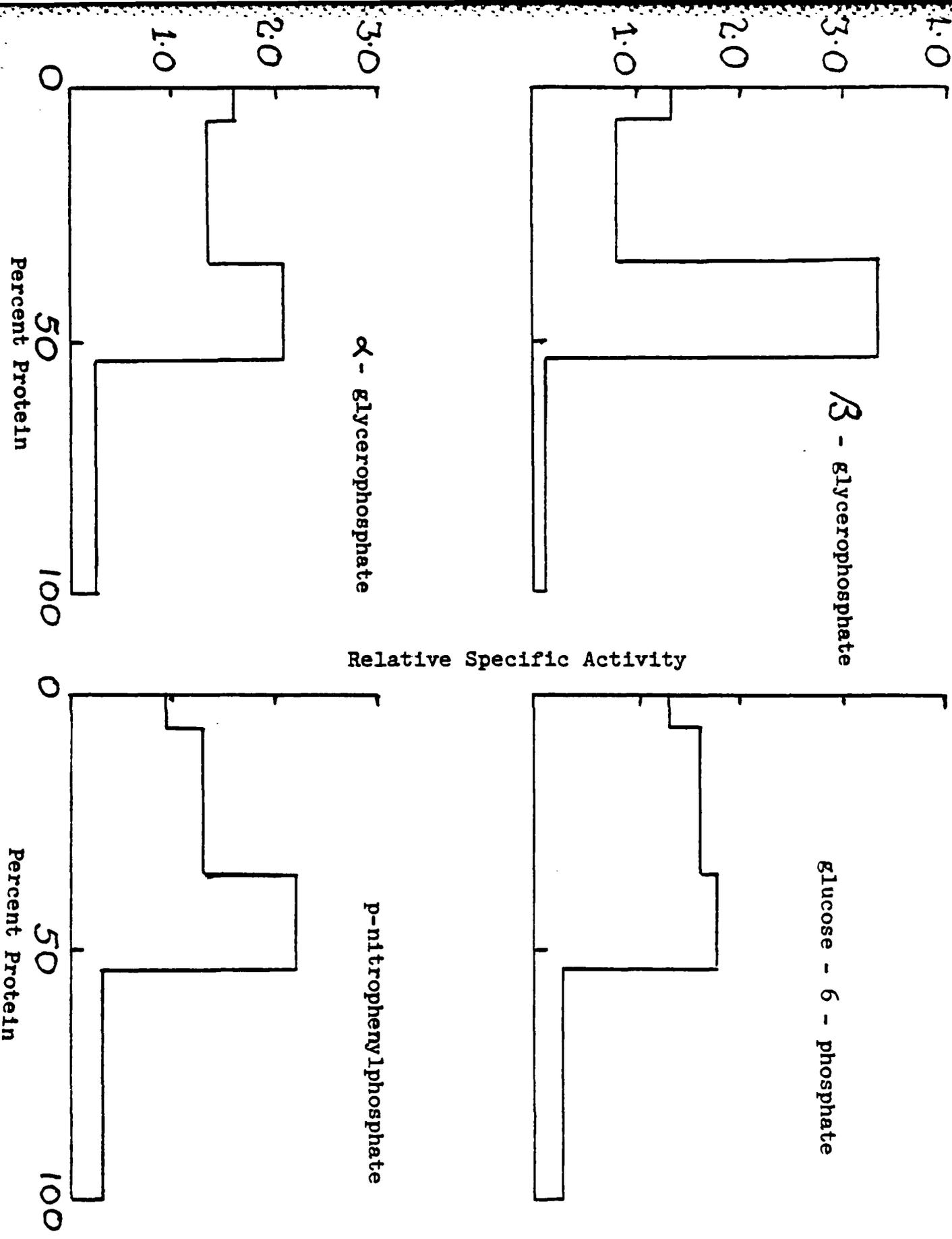
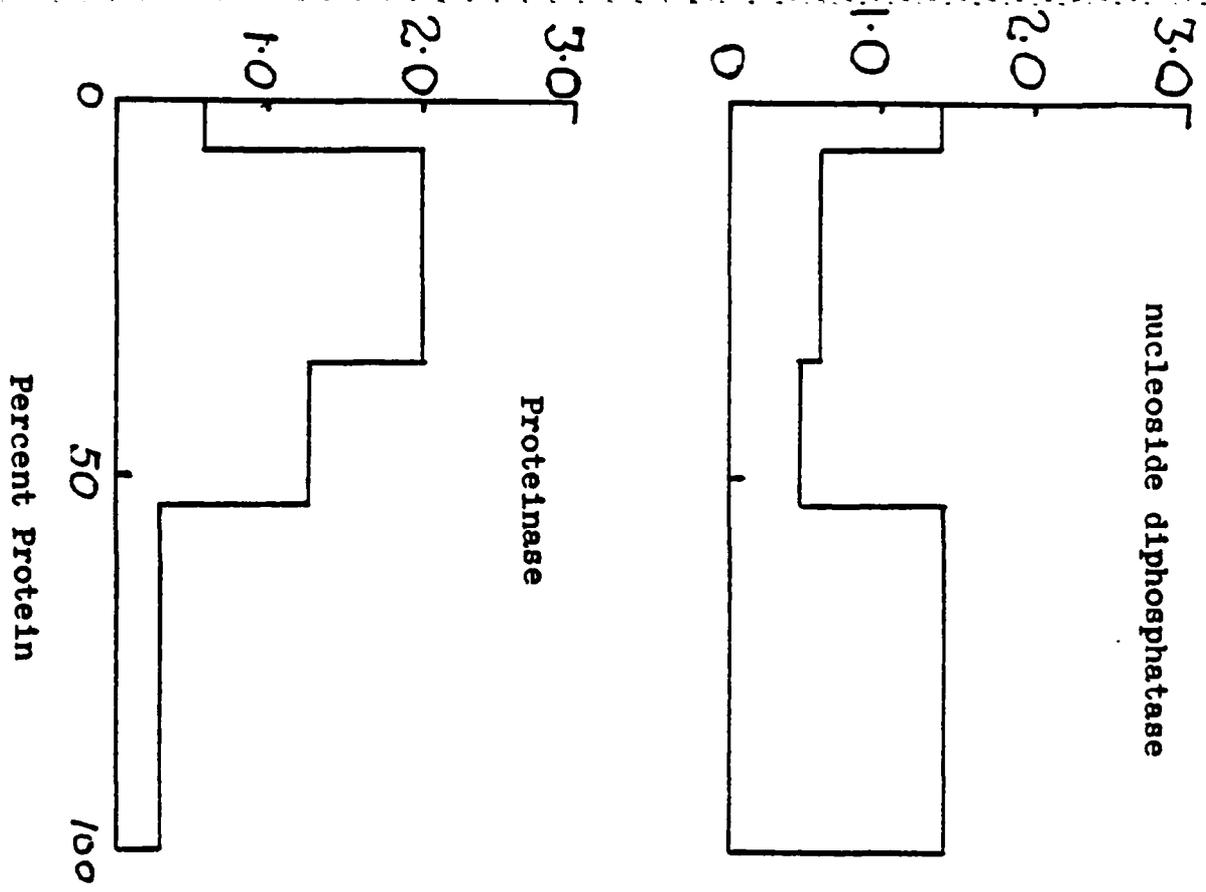


FIGURE 6

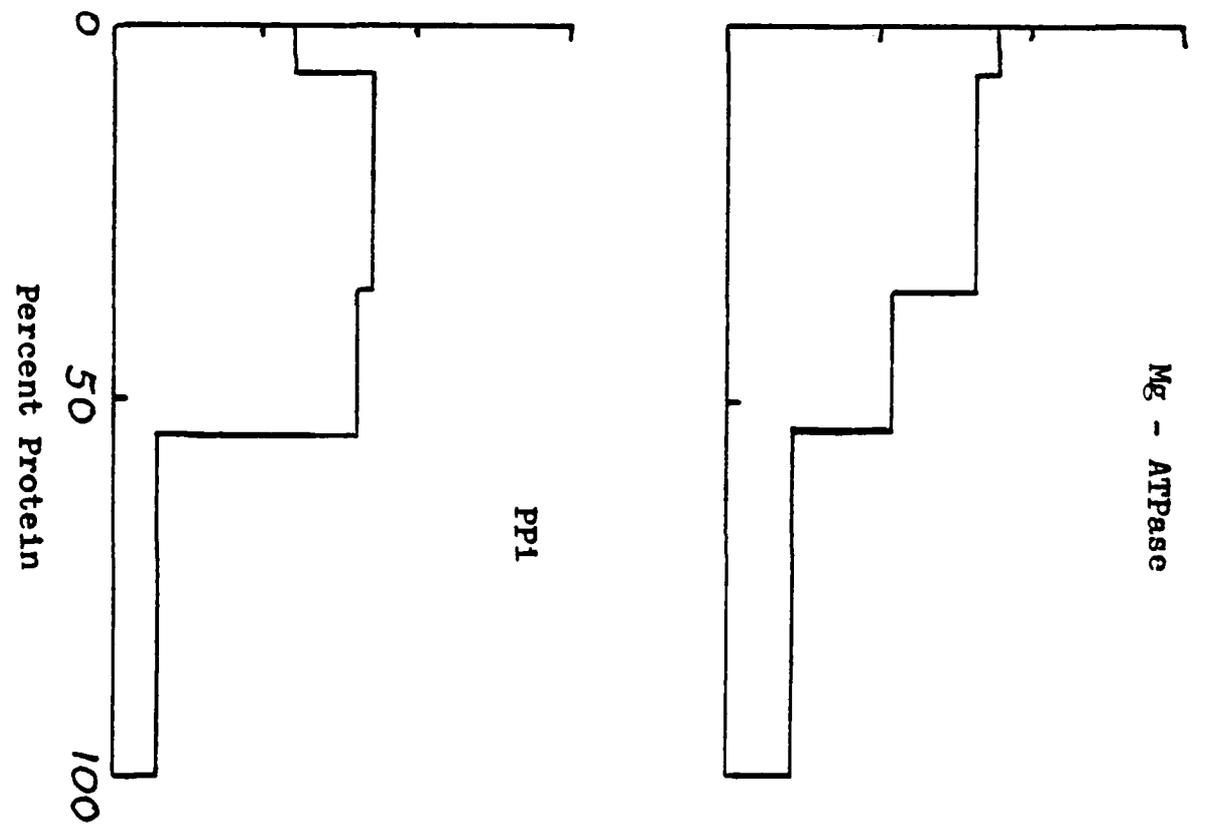
Differential centrifugation of a total cell homogenate of T. rhodesiense showing relative specific activities for selected enzymes as a function of percentage protein for each fraction. Blocks in histograms are arranged from left to right in order of increasing centrifugal force and represent fraction N, P₁, P₂, and S₂ respectively.

Phosphatase Activities





Relative Specific Activity



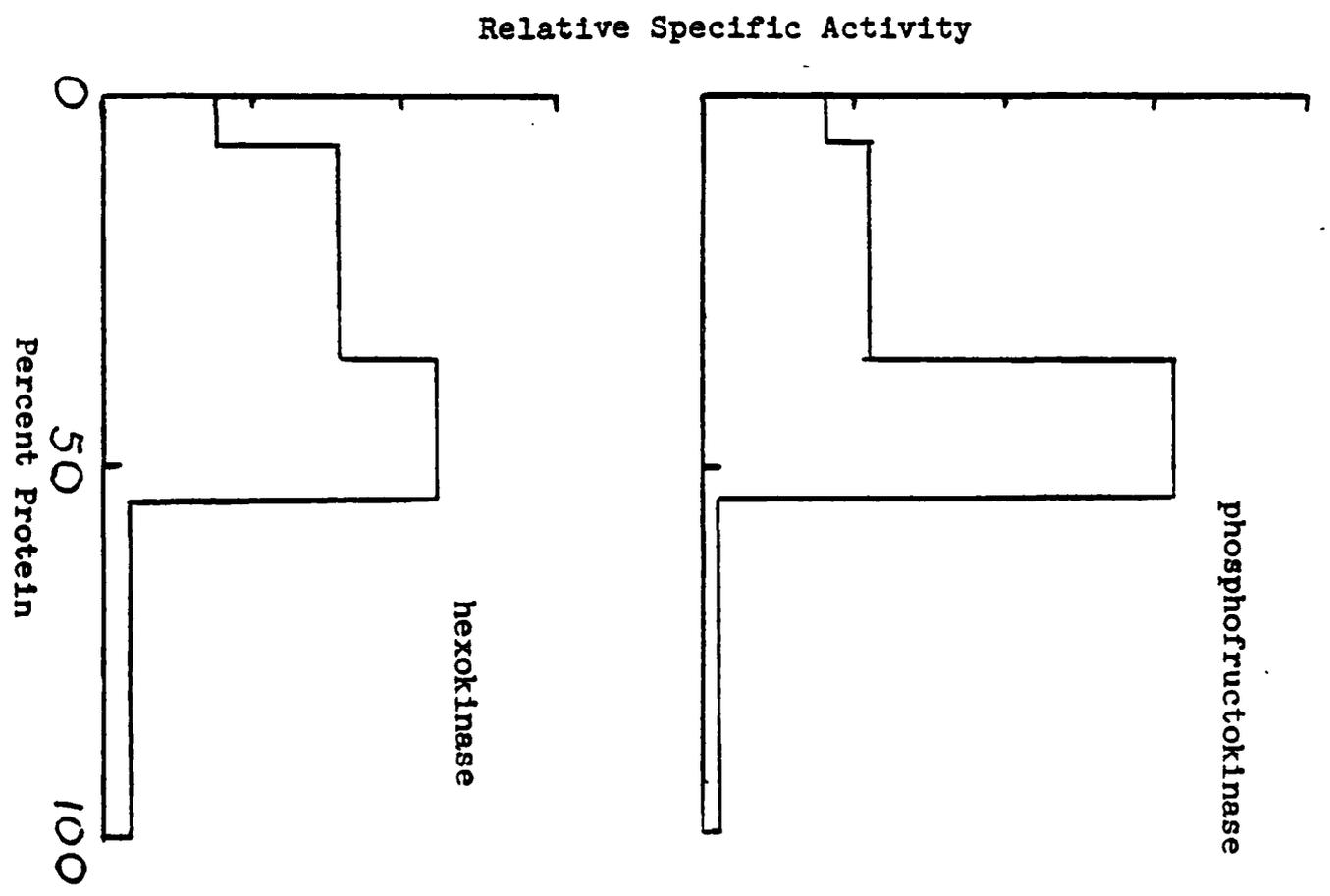
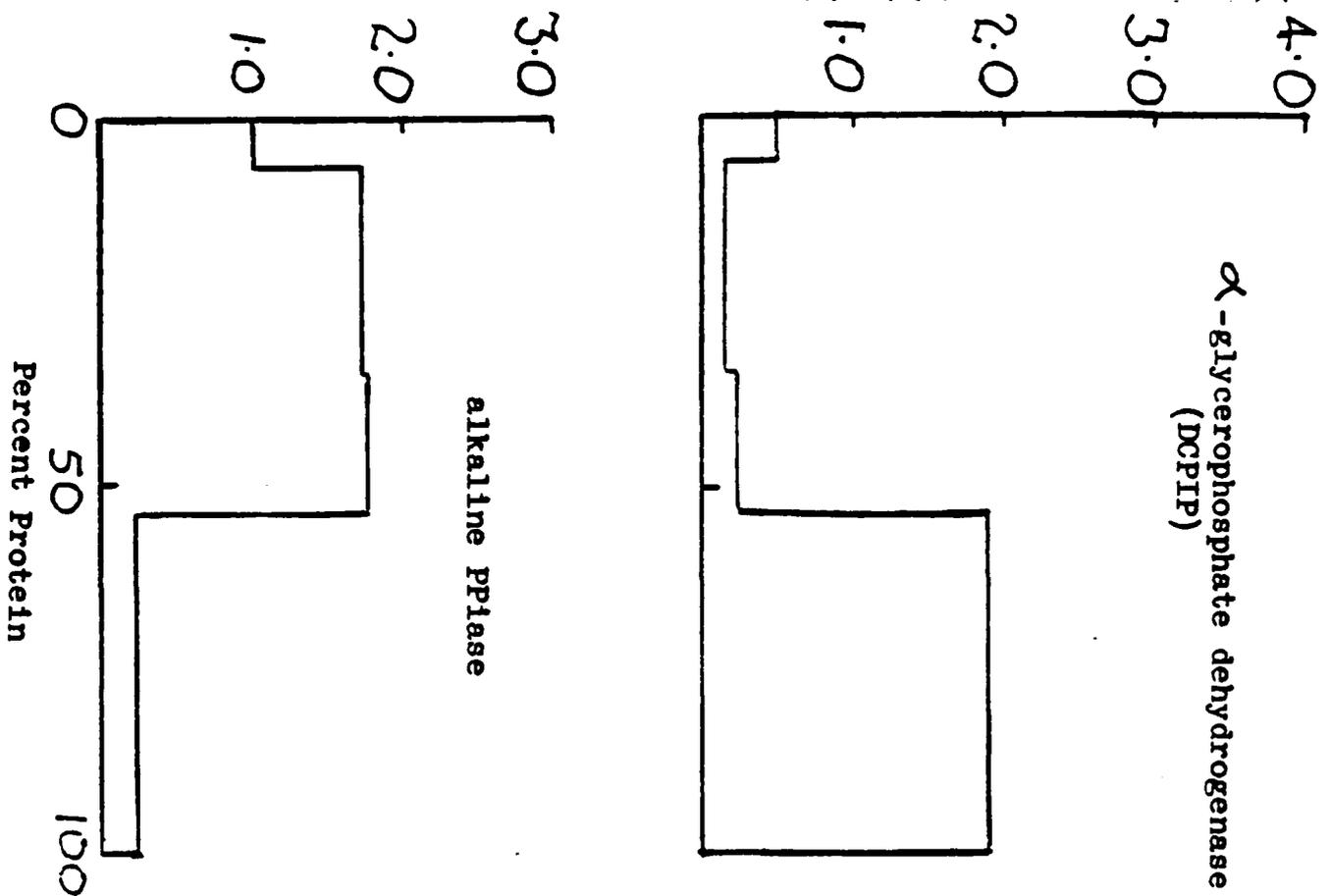
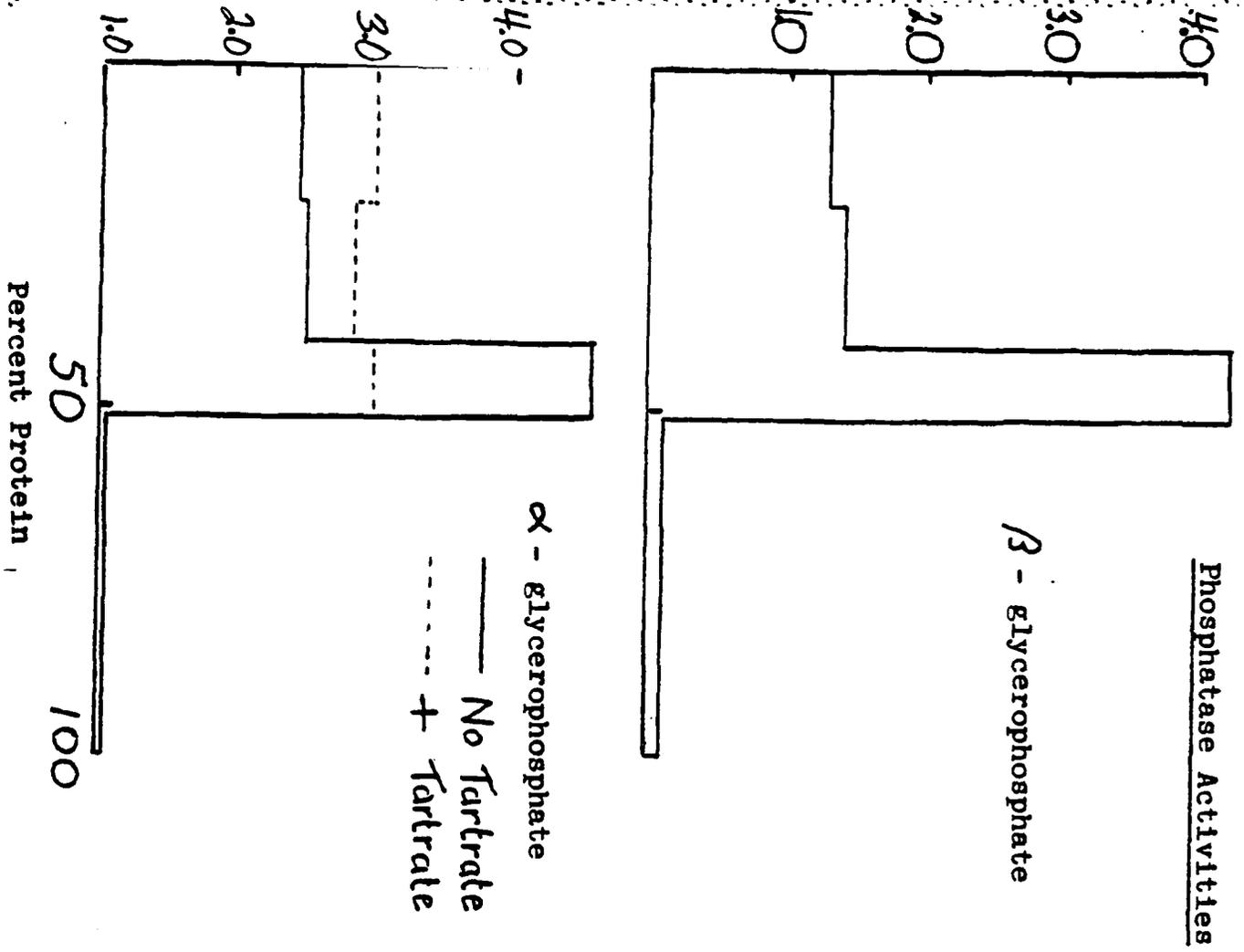
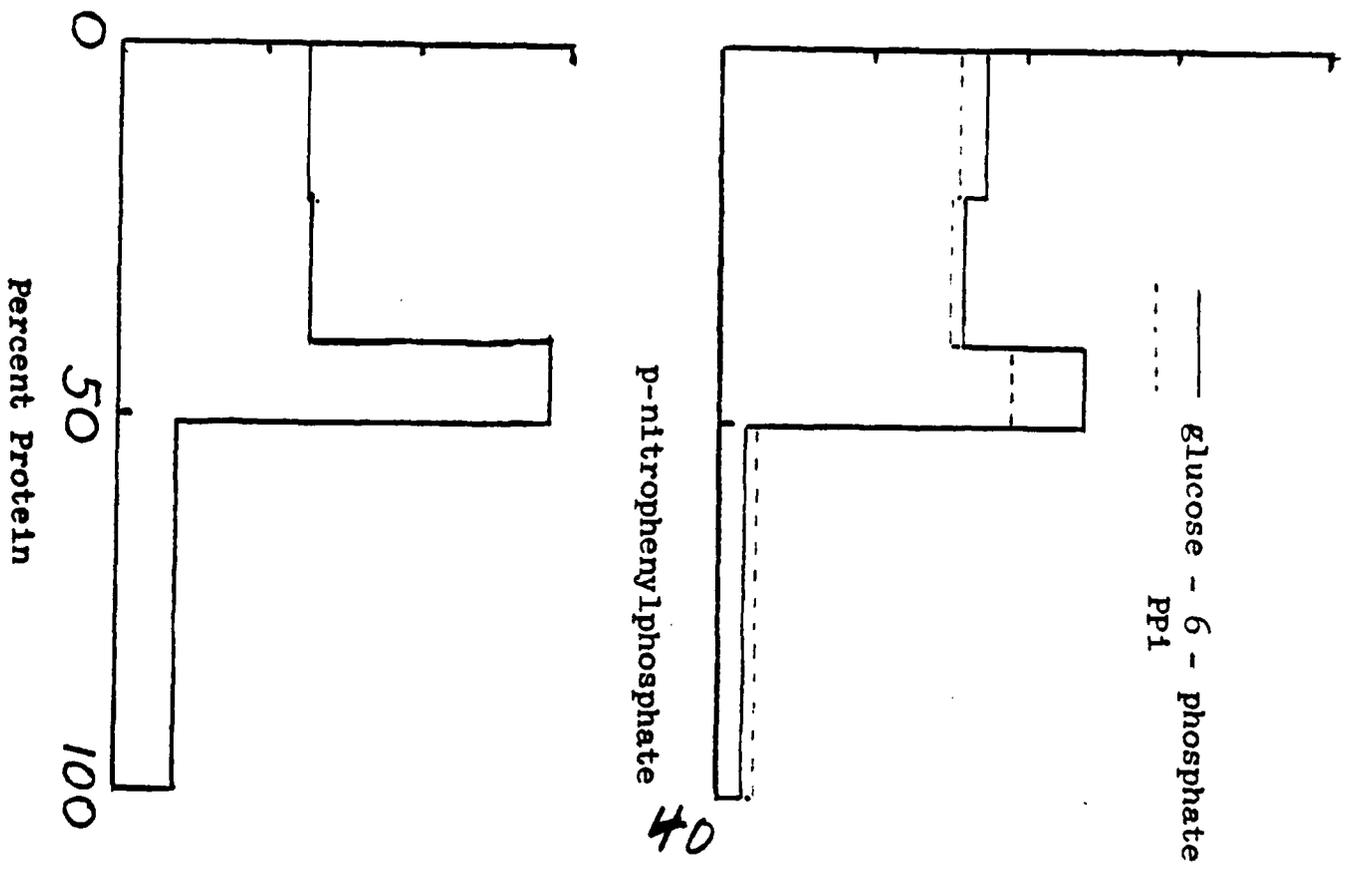


FIGURE 7

Differential centrifugation of a total cell homogenate of T. rhodesiense as a series of relative specific activity plots. Protocol according to Experiment B. See Figure 6 for further explanation.

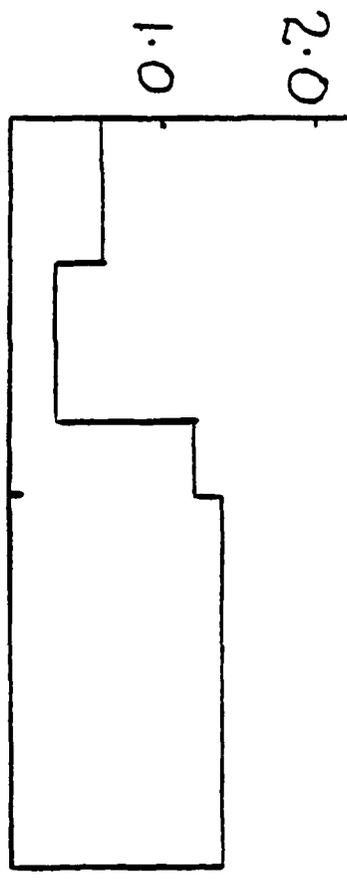


Relative Specific Activity

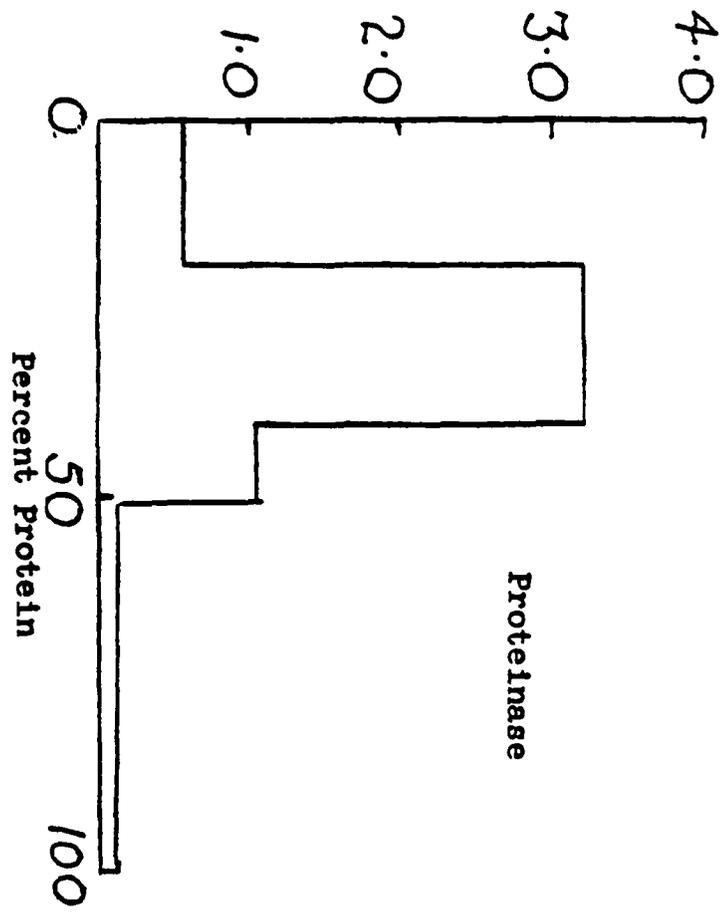


40

nucleoside diphosphatase

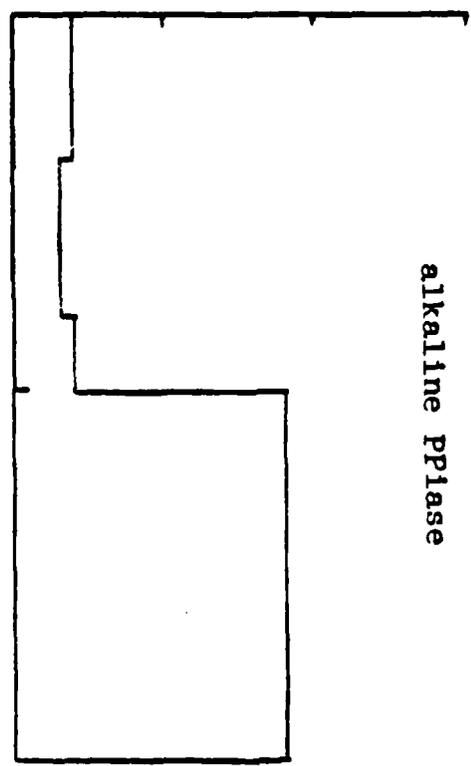


Protelnase

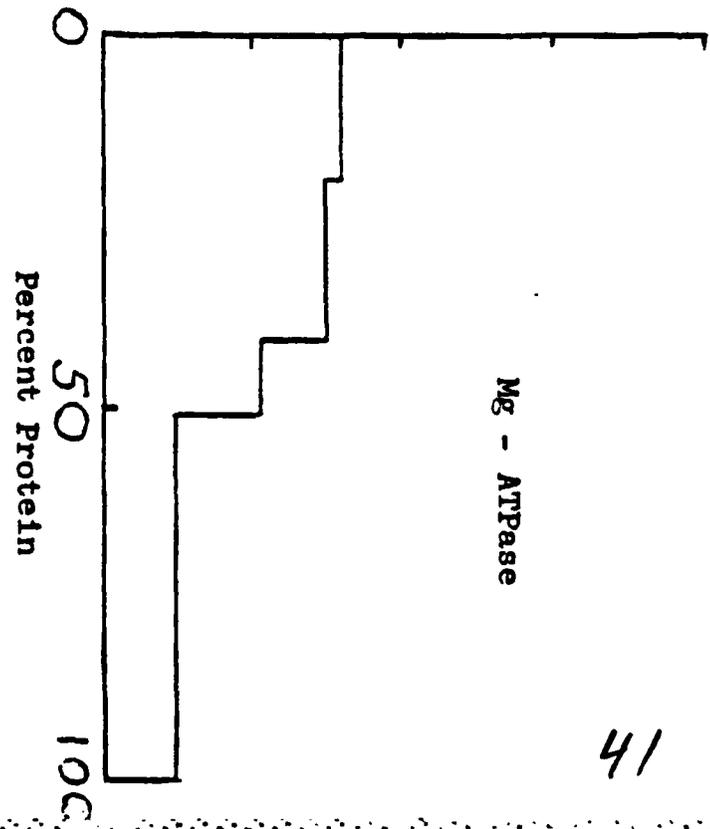


Relative Specific Activity

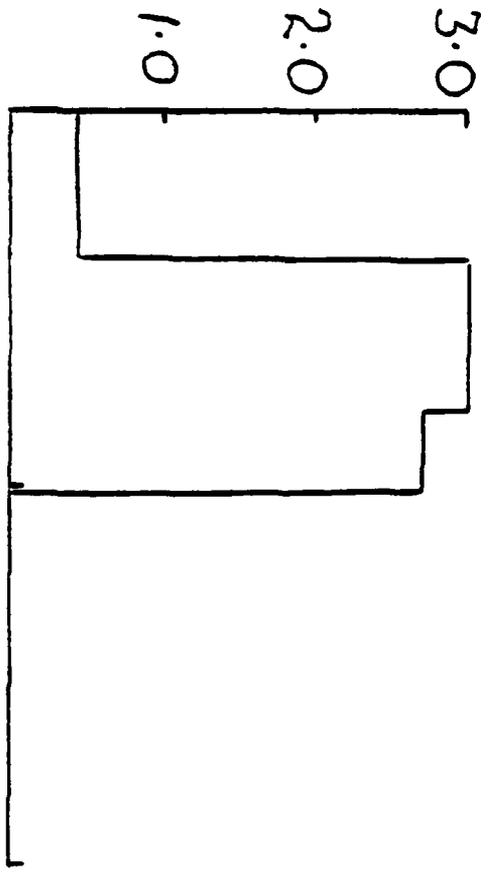
alkaline PPlase



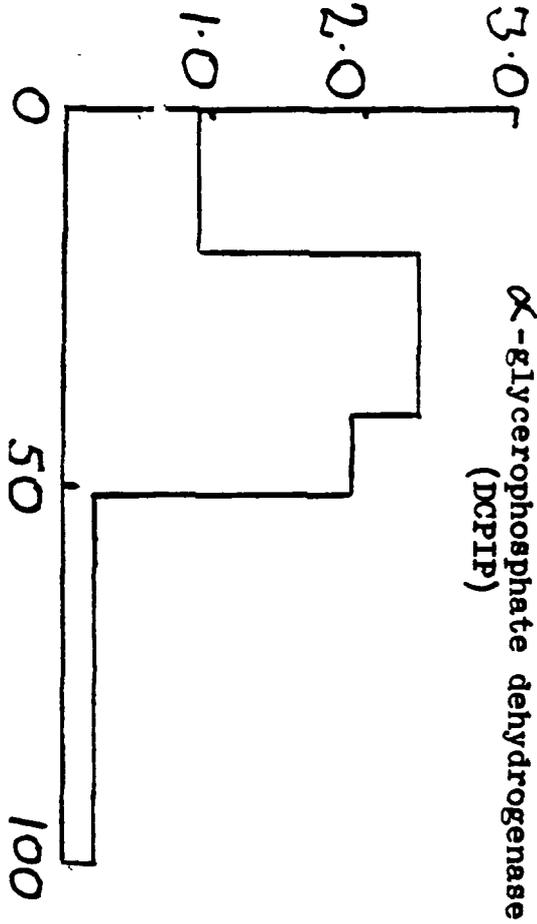
Mg - ATPase



NAD linked α -glycerophosphate dehydrogenase

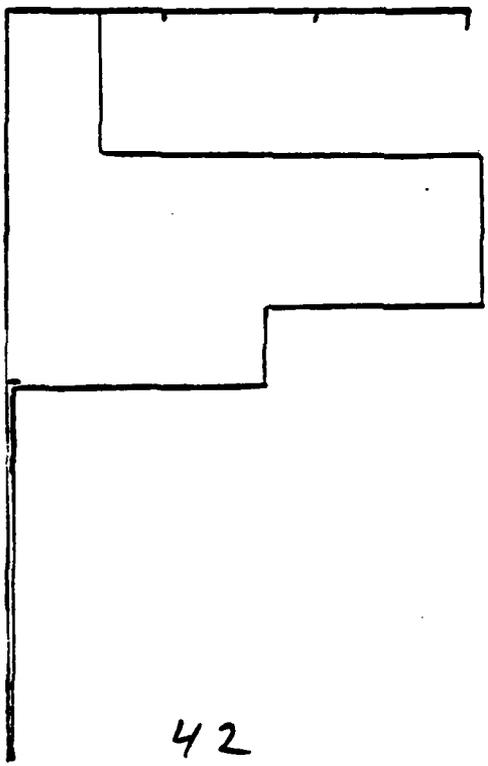


α -glycerophosphate dehydrogenase (DCPIP)

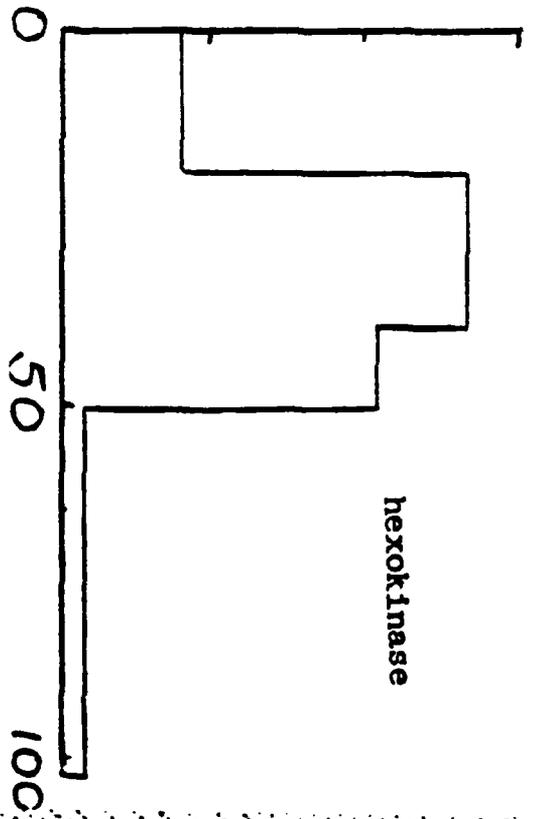


Relative Specific Activity

phosphofructokinase



hexokinase



Percent Protein

ACKNOWLEDGEMENTS

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