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Changes In Sulfhydryl Groups Of Honeybee Glyceraldehyde
Phosphate Dehydrogenase Associated With Generation Of The
Intermediate Plateau In Its Saturation Kinetics†

†Department of Biochemistry
University of Massachusetts
Amherst, Massachusetts 01002

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W. G. Gelb[‡], J. F. Brandts[§] and J. H. Nordin^{*}

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ABSTRACT

Experiments with honeybee (Apis mellifera) and rabbit muscle GPDH were conducted to obtain information at the chemical level regarding anomalous saturation kinetics of the honeybee enzyme. Results demonstrate that the enzyme's sulfhydryl groups are implicated in the process. Measured by DTNB titration, native honeybee GPDH has one less active SH than the native rabbit muscle enzyme and displays changes in overall sulfhydryl reactivity after preincubation with G-3-P or G-3-P plus NAD^+ . The total DTNB reactive sulfhydryls of rabbit muscle GPDH are not changed by preincubation with NAD^+ or G-3-P; honeybee GPDH, under certain conditions of preincubation with these ligands, shows a decrease of two total DTNB reactive SH groups.

This difference has been confirmed by an independent experiment in which the two enzymes were carboxymethylated with ^{14}C -bromoacetic acid. The loss of SH groups in honeybee GPDH is not a result of its acylation by G-3-P. After generation of the stable form of the honeybee enzyme with G-3-P plus NAD^+ it is possible to regenerate the anomalous kinetic curve by treatment of the enzyme with dithiothreitol. It is proposed that in honeybee GPDH, an intrachain disulfide bond forms in conjunction with the conversion of the enzyme from the "metastable" to stable state.

FOOTNOTES

[‡]Present address: Pharmacia Fine Chemicals Inc., Piscataway, New Jersey.

[§]Department of Chemistry, University of Massachusetts.

*To whom all inquiries should be directed.

[¶]Abbreviations used: GPDH, Glyceraldehyde phosphate dehydrogenase; G-3-P, Glyceraldehyde-3-phosphate; NAD^+ , NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide respectively; IAA, iodoacetic acid; DTNB 5,5'-dithiobis (2 nitro benzoic acid); Tris, tris-hydroxy methylamino methane; DTT; dithiothreitol; NEM, N-ethyl maleimide, SDS; sodium dodecyl sulfate.

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INTRODUCTION

Intermediate plateau and transition regions in ligand saturation curves have been reported for a number of enzymes. These include glutamate dehydrogenase (Le John and Jackson, 1968), phosphoenol pyruvate carboxylase (Corwin and Fanning, 1968), adenosine diphosphoglucose pyrophosphorylase (Gentner and Preiss 1968), cytidine triphosphate synthetase (Levitski and Koshland 1969), pyruvate kinase (Somero, 1969) lactate dehydrogenase (Somero and Hochatchka, 1969), acetylcholine esterase (Kato et al. 1972) and L-threonine dehydratase (Kagan and Dorozhko 1973). However, except for glutamate dehydrogenase no satisfactory explanation for these anomolous kinetics is presently available (Teipel and Koshland, 1969). A similar transition in substrate saturation curves of glyceraldehyde phosphate dehydrogenase (GPDH)ⁿ (E.C. 1.2.1.12) obtained from the honeybee Apis mellifera (Figure 1) and carpenter ant Camponotus pennyslvanicus have also been reported by us. (Gelb and Nordin 1969 a, b; Gelb et al.

Inspection of the transition regions of many of these curves (for example, Figure 1) shows that simple binding phenomena or "isozymes" cannot explain such cooperativity (Hill coefficient of the inset transition is approximately 40.) Honeybee GPDH has been found by direct experiment to have a maximum of 4 NAD^+ binding sites (Gelb et al. 1970). This

value equals the number reported for rabbit muscle GPDH (Conway and Koshland 1968).

The anomolous kinetics of trout lactate dehydrogenase (Somero and Hochatchka 1969) and Alaskan king crab pyruvate kinase (Somero 1969) as well as carpenter ant and honeybee GPDHs occur in organisms known to adapt to a wide environmental temperature range. It is therefore of interest to examine the chemical nature of these transitions to attempt any assessment of their possible significance in thermal compensatory mechanisms.

Based on the results of earlier studies (Gelb et al. 1970) it was proposed that honeybee GPDH exists in two forms; one, obtained when the enzyme is isolated, displays the transition and a second stable form, that does not display this phenomenon, obtained by a preincubation of the first ("metastable") form with NAD^+ plus D,L-G-3-P.

In the case of honeybee GPDH, abolition of the transition by preincubation of the "metastable" form of the enzyme with NEM (Gelb et al. 1970) suggested the possibility that the enzyme's sulfhydryl groups are somehow associated with the interconversion between the two forms of the enzyme.

This communication details the results of experiments with honeybee GPDH which provide additional evidence for the participation of the enzyme's sulfhydryl groups in the transition from the "metastable" to the stable form. A preliminary

report of this work has been presented (Gelb and Nordin 1971).

MATERIALS AND METHODS

D,L-G-3-P diethylacetal, monobarium salt; β NAD⁺, IAA, DTNB, 2-mercaptoethanol and Tris were purchased from Sigma Chemical Co. Urea was a product of Mann Laboratories. Uniformly labelled ¹⁴C-fructose 1,6-diphosphate (69 mCi/mM) was obtained from Amersham-Searle, and C¹⁴-bromoacetic acid (methyl labelled, 5 mCi/mM) from International Chemical and Nuclear Corporation. DTT was purchased from Calbiochem. Honeybees were either purchased from commercial suppliers or were a gift from the Entomology Department, University of Massachusetts. Rabbit muscle GPDH and fructose diphosphate aldolase and bovine liver catalase were obtained from Sigma, trypsin from Nutritional Biochemicals and bovine serum albumin from Pentex Inc. All were crystalline preparations.

PREPARATION OF GLYCERALDEHYDEPHOSPHATE. D,L-G-3-P diethylacetal monobarium salt was converted to the free acid according to suppliers instructions. ¹⁴C-D-G-P was prepared by a modification of the method of Hall (1960), using 50 microcuries of U-¹⁴C-fructose 1,6diphosphate and crystalline rabbit muscle fructose diphosphate aldolase. The specific radioactivity of the isolated ¹⁴C-D-G-3-P was estimated by determining the chemical concentration of D-G-3-P enzymatically, using rabbit

muscle GPDH and excess NAD^+ followed by a radioactivity determination in a Packard Tri-Carb liquid scintillation spectrometer. The product had a specific activity of 1.76×10^9 cpm/millimole.

GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE ASSAY. Measurements of the specific activities of honeybee and rabbit muscle GPDH were performed at 25° and pH 8.5 by the method of Velick (1955) as described elsewhere (Gelb et al., 1970). The standard assay buffer contained 5 mM sodium pyrophosphate, 5 mM sodium arsenate and 1 mM EDTA. For certain experiments the pH of this buffer was 9.0. Total protein concentrations were obtained from absorbance measurements at 280 nm assuring an extinction coefficient of $1.06 \text{ cm}^2 \text{ mg}^{-1}$ Murdock and Koeppel, 1964; Marquardt et al. 1968. In some experiments protein was determined by the method of Lowry et al. (1951).

PREPARATION OF HONEYBEE GPDH. Crystalline honeybee GPDH was prepared by a modification of the procedure of Marquardt et al. (1968). The details of the method employed and the specific activities of preparations have been published earlier (Gelb et al., (1970), Nordin et al., (1970)).

POLYACRALYMIDE GEL ELECTROPHORESIS. Electrophoresis was conducted as described by Davis (1964). A modification of the procedure described by Weber and Osborn (1969) was employed for electrophoresis in the presence of S.D.S. using a 10% gel. The

stable form of honeybee GPDH was prepared by incubating 0.5 ml of "metastable" enzyme (5 mg per ml) in assay buffer pH 9.0 which contained 2 mM NAD^+ and 3.6 mM D,L-G-3-P. Enzyme in each state and standards of bovine serum albumin, catalase, aldolase and trypsin also 5 mg per ml in the above buffer, were diluted tenfold in 0.01 M sodium phosphate, 1% SDS, 0.01 M iodoacetic acid at pH 7.0 and allowed to react for two hours at 37°. Approximately 12 micrograms of each protein were subjected to electrophoresis for 4 hours at a constant current of 8 milliamperes per tube. Following electrophoresis the gels were stained and destained according to the procedure of Chrambach et al. (1967). .05% bromphenol blue was used as tracking dye. While the method of Weber and Osborn (1969) employs 2-mercaptoethanol, it was omitted in these experiments since it would lead to reduction of any disulfide bonds present in the GPDH.

DTNB STUDIES. For the titration of native rabbit muscle and honeybee GPDHs with DTNB, enzyme crystals were harvested by centrifugation and dissolved in one ml of assay buffer to give protein concentrations between 1.0 and 1.4 mg per ml. To these solutions, appropriate additions of D,L-G-3-P (50 mg per ml) and/or 0.1 M NAD^+ were made and they were then incubated 2 minutes at 20°. Each mixture was then applied to a Sephadex G-25 (coarse) column (1.0 x 45 cm) which had been previously equilibrated with 0.1 M imidazole, 1 mM EDTA, pH 7.0. The fractions containing GPDH activity were pooled and

additional imidazole buffer added to bring the final enzyme concentration to 0.2-0.3 mg per ml. One ml of the enzyme solution was placed in a cuvette and the reaction initiated by the addition of 5 microliters of 0.01 M DTNB. The reaction was monitored at 412 nm with a Gilford model 240 spectrophotometer equipped with a Honeywell Electronic 19 recorder.

To determine the total DTNB-reactive sulfhydryl content of the denatured enzyme, 0.46 gms of solid urea was added per ml, to another aliquot of the pooled diluted enzyme solution eluted from the Sephadex G-25 column, described above. This solution was then incubated for 15 minutes at 20°C and divided into 4, one ml samples. Ten microliters of 0.01 M DTNB was added to each sample and the optical density changes at 412 nm measured periodically for one hour, at which time the reactions had reached completion. After subtraction of the appropriate blank readings at 412 nm the average concentration of reacted DTNB in the four cuvettes determined using an extinction coefficient for the thionitrobenzoate ion of 13,600 liter mole⁻¹ cm⁻¹ at 412 nm (Ellman, 1959).

SPECIFIC ACTIVITY OF ¹⁴C BROMOACETIC ACID. ¹⁴C Bromoacetic acid was assayed for specific activity by its reactivity with urea denatured rabbit muscle GPDH. (Independent titration of the urea denatured enzyme with DTNB indicated it to contain 13.0 sulfhydryl groups per mole). Two milligrams of crystalline rabbit muscle GPDH were dissolved in one ml of assay buffer,

pH 9.0, followed by the addition of 0.46 g of solid urea. After 15 minutes at 20°, 20 microliters of ^{14}C bromoacetic acid was added. The mixture was incubated one hour and then applied to a Sephadex G-25 column (1.0 x 45 cm) which was preequilibrated with 0.1 M imidazole, 6 M urea pH 7.0. One ml fractions were collected and the eluate assayed for radioactivity by liquid scintillation spectrometry and for protein by the method of Lowry et al. (1951). The standard curve of protein versus absorbance at 500 nm was linear in all regions examined for the carboxymethylated GPDH in urea. Based on protein concentration and radioactivity, a specific activity of 6.23×10^9 cpm/millmole of ^{14}C bromoacetic acid was obtained for the ^{14}C carboxymethylated enzyme.

STUDIES WITH ^{14}C BROMOACETIC ACID. The procedure followed is a modification of the method of Zahler and Cleland (1968) for the specific determination of disulfide bonds. Crystalline honeybee or rabbit muscle GPDH's were harvested by centrifugation at 12,000 x g for 10 minutes and the pelleted crystals dissolved in one ml of assay buffer pH 9.0 (final protein concentrations 1.3-1.8 mg per ml). Next, the appropriate additions, if any, of D,L-G-3-P (50 mg per ml) and/or of 0.1 M NAD^+ were made to obtain the desired state of honeybee GPDH, and the mixture incubated for 2 minutes at 20°C. Then, 0.46 gms of solid urea was added followed by a 5 minute incubation at 20°C. Next 13 microliters of 0.1 M iodoacetic acid were added to carboxymethylate all free sulfhydryl groups, and

the mixture was allowed to incubate an additional one hour at 20°C. The solution was then dialyzed against two changes (250 ml each) of 0.05 M Tris, pH 9.0, for 2 hours to remove excess IAA, and unreacted substrates and products of the enzymic reaction. The enzyme solution was concentrated to approximately 0.5 ml by rolling the dialysis membrane in dry Sephadex G-200. 0.18 g of urea was added to 0.3 ml of concentrated enzyme solution followed by 0.1 ml of 3mM DTT and the mixture incubated 40 minutes at 20°C to allow reduction of any disulfide bonds in the enzyme. Next, 0.2 ml of 1.0 M Tris pH 8.1, 0.5 ml of 5 mM sodium arsenite (in 1.0 M Tris pH 8.1) and 0.32 gm of urea were added to the enzyme solution followed by a 15 minute incubation at 20°C. The sodium arsenite added in this step serves to complex reduced DTT rendering it less accessible to reaction with the bromoacetic acid (Zahler and Cleland 1968). Twenty microliters of ^{14}C bromoacetic acid solution was then added and the mixture allowed to incubate 15 minutes at 20°C and then an aliquot was applied to a Sephadex G-25 (fine) column (1.0 x 50 cm) preequilibrated with 0.1 M Tris, 6 M urea at pH 8.1. One ml fractions were collected and the eluate assayed for protein (Lowry et al., 1951) and radioactivity.

STUDIES WITH ^{14}C D-G 3-P. 0.7 ml of a suspension of honeybee GPDH crystals (5 mg per ml) was harvested by centrifugation at 12,000 x g for 10 minutes and the pellet dissolved in one ml

of assay buffer, pH 9.0. 25 μ l of ^{14}C D-G-3-P (4 mg/ml) or 25 μ l of ^{14}C D-G-3-P plus 10 μ l of 0.1 M NAD^+ was made followed by an incubation for 2 minutes at 20°C. 0.46 gms of solid urea was then added and the mixture incubated an additional 5 minutes. Next, 13 microliters of 0.1 M IAA were added and the enzyme solution allowed to incubate for one hour at 20°C. The protein solution was applied to a Sephadex G-25 (fine) column (1.0 x 50 cm), previously equilibrated with 0.1 M Tris, 6 M urea at pH 8.1. One ml fractions were collected and each fraction assayed for protein concentration (Lowry et al., 1951) and radioactivity.

RESULTS

A comparison of the effects of DTNB on native rabbit muscle and honeybee GPDHs indicates fundamental differences exist between the two enzymes with regard to their relative sulfhydryl reactivities.

Figure 2 compares the number of fast reacting sulfhydryls in the two enzymes as well as the result of thionitrobenzoate addition to the proteins on their catalytic activities. Rabbit muscle GPDH contains 3.5 to 4 sulfhydryl groups capable of reacting within 30 seconds with DTNB and all enzymatic activity is destroyed within this same period. However, honeybee GPDH displays only 2.5 to 3 fast reacting sulfhydryls. There was a concomitant loss of 75% of the enzymes catalytic activity but 25% remained even after titration of several additional SH groups during the 10 minute experimental period.

Additional differences in sulfhydryl reactivities of the native enzymes were noted when comparing DTNB titrations after exposure to various combinations of D,L-G-3-P and NAD^+ followed by gel filtration of Sephadex G-25 to remove the ligands. Figures 3 and 4 illustrate the results of these experiments. Curve 2 in Figure 3 shows the DTNB titration of "metastable" honeybee GPDH preincubated alone and after preincubation in 2 mM NAD^+ . Experimentally, the two curves were indistinguishable and are shown as one. Identical results

were also obtained with rabbit muscle GPDH as shown in Curve 1 of Figure 4. Similarly, when honeybee or rabbit muscle GPDHs were preincubated with 2 mM NAD^+ plus 3.6 mM D,L-G-3-P prior to titration with DTNB, a decrease in sulfhydryl reactivity, relative to incubation without ligands was observed for both enzymes (Figure 3, Curves 3 and 4 and Figure 4, Curve 3). Incubation of honeybee GPDH with NAD^+ plus G-3-P converts it from the "metastable" to the stable state (Gelb et al., 1970). The conversion occurs regardless of which ligand, NAD^+ or G-3-P, is in excess. Since the rabbit muscle enzyme does not show any change in kinetic properties with this treatment (Gelb et al., 1970) it served as a control for the honeybee enzyme. Preincubation of "metastable" honeybee enzyme with 3.6 mM D,L-G-3-P alone (Figure 3, Curve 1) caused a marked increase in the sulfhydryl reactivity over that obtained with "metastable" honeybee enzyme (incubated alone) or with NAD^+ (Figure 3, Curve 2). However, the rabbit muscle enzyme exhibited no corresponding increase under the same conditions, (Figure 4, Curve 1).

Table 1 lists the total DTNB reactive sulfhydryl groups, obtained with denatured rabbit muscle and honeybee GPDHs subjected first to various preincubation conditions as described in Materials and Methods. The total DTNB reactive sulfhydryl titer of rabbit muscle GPDH was unchanged by the various incubations. In contrast, the total DTNB reactive sulfhydryl titer of honeybee GPDH was changed under certain conditions. When the "metastable" enzyme is preincubated alone its total DTNB reactive

sulfhydryl content was 16 per 140,000 grams of enzyme. When it was pretreated with NAD^+ plus G-3-P; G-3-P alone; or with G-3-P and then after Sephadex G-25 chromatography with NAD^+ , its sulfhydryl content decreased to approximately 14. Upon incubation of the "metastable" enzyme with either NAD^+ or NADH prior to reaction with DTNB, no decrease in sulfhydryl titer was observed as compared to control values. Although the data in Table 1 suggests that with honeybee GPDH certain preincubation conditions may result in disulfide bond formation, a change in sulfhydryl titer from 16 to 14 represents a small absolute difference. Therefore, another experiment using an independent method was conducted to confirm these results. A modification of the method of Zahler and Cleland (1968) was employed to quantitate changes in disulfide bonds in the two GPDHs. With this method, the protein is completely carboxymethylated with IAA to block any SH groups present. After removal of unreacted IAA, any disulfide bonds are reduced with DTT. The protein is then subjected to a second carboxymethylation using ^{14}C -bromoacetic acid. The absolute number of sulfhydryl groups determined using this technique is much smaller and larger percentage changes can be seen. The choice of ^{14}C -bromoacetic acid rather than DTNB, as described by Zahler and Cleland (1968), was made to alleviate interference by the simultaneous reaction of DTNB with the reducing agent DTT. Figure 5 shows the elution profiles of urea denatured honeybee GPDH after carboxymethylation with IAA and ^{14}C -bromoacetic acid. Approximately 5 times more

^{14}C acetate is incorporated into protein with both the stable enzyme and "metastable" enzyme that had been pre-incubated with G-3-P than with "metastable" enzyme which had been preincubated alone. Table 2 gives the quantitative data obtained from these profiles in terms of the number of moles of ^{14}C -carboxymethyl groups incorporated into each 140,000 grams of honeybee and rabbit muscle (profiles not shown) GPDH under various conditions. This data is in excellent agreement with the evidence presented in Table 1 and suggests that a disulfide bond is formed when honeybee GPDH is converted from the "metastable" to the stable form; (change from 0.4 to 2.2 SH groups per 140,000 g GPDH) or incubated with G-3-P (change from 0.4 to 2.0 SH groups per 140,000 g GPDH), and 2). The above treatments have no significant effect on rabbit muscle GPDH. A control experiment was also conducted to test the possibility that the incorporation of ^{14}C -carboxymethyl groups into honeybee GPDH as shown in Figure 3, was a result of prior removal of acyl groups (of G-3-P) from GPDH by transesterification to DTT prior to carboxymethylation with ^{14}C -bromoacetic acid. Reaction of GPDH with certain acyl compounds has been shown to occur with via certain of its sulfhydryl and lysine residues (Matthew et al., 1967). Figure 6 shows the elution profiles obtained when honeybee GPDH is incubated with either 0.59 mM ^{14}C -D-G-3-P alone (top frame) or with 1 mM NAD^+ plus 0.59 mM ^{14}C -D-G-3-P. Neither

sample was subjected to incubation with DTT as was done in the experiment illustrated in Figure 3. Therefore, any covalent bonds formed between G-3-P and the enzyme could not be destroyed by this reagent. Calculations using the data from Figure 6 shows that only 0.2 mole of $^{14}\text{C}-\underline{\text{D}}-\text{G}-3-\text{P}$ was introduced per mole of enzyme when it was incubated with radioactive substrate alone and no incorporation was observed when $^{14}\text{C}-\underline{\text{D}}-\text{G}-3-\text{P}$ plus NAD^+ were incubated with the enzyme.

If, in fact, the conversion from the "metastable" to stable form of GPDH occurs in conjunction with disulfide bond formation it should be possible to regenerate the transition in the kinetic saturation curve by subjecting the stable form to reduction. The upper frame of Figure 7 shows the saturation kinetics obtained with "metastable" honeybee GPDH. The middle frame shows kinetic data obtained with stable enzyme prepared by incubation of an aliquot of metastable enzyme (assay in upper frame) with 3.6 mM $\underline{\text{D}},\underline{\text{L}}-\text{G}-3-\text{P}$ and 2 mM NAD^+ . As was reported previously (Gelb and Nordin, 1969; Gelb, *et al.*, 1970), the transition is abolished under these conditions. When stable enzyme is incubated with 2.4 mM DTT for one hour and the kinetic saturation curve again analyzed, the results shown in the lower frame of Figure 7 are obtained. Although the magnitude of the observed transition (ΔV_{max}) varied somewhat from experiment.

to experiment, (top and bottom frames; see also Gelb et al. 1970) the regeneration of the transition always occurred when the stable form of the enzyme was incubated with DTT. These results indicate that the conversion from metastable to the stable state can be caused by incubation with the ligands G-3-P + NAD⁺ and that the conversion back to the "metastable" state occurs when the stable form is treated with DTT, a reagent capable of reducing disulfide bonds.

Polyacrylamide gel electrophoresis of "metastable" and stable honeybee GPDHs at pH 8.3 (Davis 1964) failed to reveal any difference in their mobilities. It has been shown previously that the two forms were also inseparable by gel filtration (Gelb et al. 1970). Attempts by Marquardt et al. (1968) to distinguish between holo and apo forms of honeybee GPDH by electrophoretic technique were also unsuccessful. Figure 8 represents the relative mobilities of the subunits of the two states of honeybee GPDH subjected to polyacrylamide gel electrophoresis in SDS (Weber and Osborn, 1969) but in the absence of thiols. The marker proteins employed and their minimum molecular weights were: bovine serum albumin, 68,000 (Tanford, et al., 1967); catalase, 60,000 (Sund et al., 1967); aldolase, 40,000 (Kawahara and Tanford, 1966); and trypsin, 23,000 (Dayhoff and Eck, 1967). From the data molecular weight of the subunits from both states was estimated

to be between 35,000 and 36,000, a value in agreement with the literature for GPDH protomers (Harrington and Karr, 1965; Harris and Perham, 1965). Disulfide bond formation is not occurring between subunits of honeybee GPDH since higher molecular weight bonds could not be detected by this technique. This suggests that the disulfide bond is intra subunit in nature.

DISCUSSION

The present study extends the view that oxidation of sulfhydryl groups occur in conjunction with the kinetic transition in honeybee GPDH and provides some insight into this difference in behavior between the two GPDH's. It remains to be determined whether similar transitions in other enzymes also occur with changes in their SH group chemistry. Several experiments show conclusively that the sulfhydryl reactivities of honeybee and rabbit muscle GPDH's differ. Results in Figure 2 clearly indicate both a difference in the number of "fast" sulfhydryl groups between the two native enzymes and a striking difference in the effect DTNB has in destroying their catalytic activities. The relative reactivities of the sulfhydryls of honeybee GPDH have not been investigated previously, but results obtained regarding the number of "fast" sulfhydryls in rabbit muscle GPDH are in excellent agreement with the observations of others. Studies with o-iodosobenzoate (Olson and Park, 1964); flourodinitrobenzene (Shaltiel & Soria, 1969) and p-mercuribenzoate (Boross et al., 1969) show that there are four fast reacting sulfhydryl groups in pig and rabbit muscle GPDH's. It is assumed that four such groups exist in honeybee GPDH also. Because DTNB

reacts with only 2.5 to 3 sulfhydryls per mole of honeybee GPDH within 30 seconds, it seems one "reactive" SH is protected from reaction with this reagent. That this result is real is reinforced by the fact that only 75% of the enzyme's catalytic activity is destroyed in the same time period (as opposed to complete loss of activity in the rabbit muscle enzyme). The slow inactivation of honeybee GPDH during the following 10 minutes could be explained either by slow reactivity of one additional "active" sulfhydryl group with DTNB (after 3 moles of thiol reagent are bound) or slow denaturation of the enzyme under the experimental conditions employed. The structural environment allowing for DTNB reactivity of the "fast" sulfhydryl group is clearly different in the two enzymes, however. Kinetic experiments by Boross et al. (1969) with pig muscle GPDH and by Kemp and Forest (1968) with phosphofructokinase show that sulfhydryl groups in the native enzymes vary in their DTNB reactivities, presumably because of activation or conformational restraints imposed by neighboring amino acids.

Further evidence for differences in sulfhydryl reactivity between the two native GPDH's is seen in Figures 3 and 4. Rabbit muscle enzyme shows no significant changes either in the number of "fast" sulfhydryls titrated with DTNB or in sulfhydryl reactivity with DTNB under the conditions employed. Furthermore, preincubation of either enzyme with NAD^+ alone resulted

in no change while preexposure to NAD^+ plus G-3-P caused a small decrease in sulfhydryl reactivity. However, while rabbit muscle GPDH was not effected by preincubation with G-3-P, this treatment resulted in a marked increase in the DTNB reactivity of honeybee GPDH compared to preincubation with NAD^+ . It is possible that exposure to G-3-P causes a partial unfolding of honeybee GPDH, allowing greater reactivity of its SH groups. This property is not seen with the rabbit muscle enzyme.

The total number of DTNB-reactive sulfhydryls in the denatured enzymes is also affected differently by preincubation of the native GPDHs with ligands. The data in Table 1 show that rabbit muscle GPDH does not undergo significant change in this parameter as a result of various preincubation conditions. However, preincubation with certain combinations of ligands results in a decrease of two total DTNB reactive sulfhydryls in the honeybee enzyme. This suggests the possibility of a disulfide bond being formed in honeybee GPDH. The results with ^{14}C bromoacetic acid (Table 2) indicate that, per mole of enzyme, 1.6 to 1.8 sulfhydryls are unavailable for S-carboxymethylation by iododacetate after honeybee GPDH is preincubated with either G-3-P or G-3-P plus NAD^+ . This value is in excellent agreement with the change in total DTNB reactive SH groups (Table 1). In agreement with the data in Table 1, the sulfhydryl titer of rabbit muscle GPDH was not changed by

preincubation as measured by S-carboxymethylation. The base value of 0.6 to 0.8 mole of ^{14}C carboxymethyl group incorporated per mole of rabbit muscle enzyme may represent an endogenous amount of mixed disulfides present after its isolation (Parker and Allison, 1969).

Conversion of honeybee GPDH from the "metastable" to the stable form causes a disappearance of the transition region in the saturation curve (Figure 7). Regeneration of the anomolous kinetic curve can be accomplished by incubation of the newly formed stable enzyme with dithiothreitol. Coupled with evidence presented in Tables 1 and 2 this experiment strongly suggests preincubation of "metastable" GPDH causes the formation of a disulfide bond. Which sulfhydryls are involved is not known but curves 2 and 3 of Figure 3 tend to rule out the "fast" titratable SH groups since essentially the same numbers are present in both forms of the enzyme. That variability exists in both presence and position of sulfhydryl groups of representative mammalian (pig muscle) and arthropod (lobster) GPDH's has been conclusively shown by sequencing studies (Davidson et al., 1967; Harris and Perham, 1968). Therefore, it is possible that structural differences in the locations of sulfhydryl groups permit disulfide bond formation in honeybee GPDH but prohibit it in rabbit muscle GPDH under identical conditions of exposure to G-3-P and G-3-P plus NAD^+ . Intramolecular disulfide bond

formation has been demonstrated after chemical treatment of enzymes. Guinea pig liver transglutaminase undergoes a dithiothreitol reversible inactivation when preincubated with DTNB in the absence of calcium ion. Loss of activity in the enzyme coincides with the loss of two detectable sulfhydryl groups (Connellan and Folk, 1969). On the basis of mapping experiments the SH groups involved were concluded to be those that bind glutamine while the single SH groups required for all catalytic activities of the enzyme was not part of the disulfide bond. This is similar to the observations noted in the present study. Harris and Perham (1964) reported the formation of intrachain disulfide bonds in mammalian GPDH upon treatment of the native enzyme with o-iodosobenzoate. This resulted in complete inactivation of the enzyme. Parker and Allison (1969) showed that the immediate product of the reaction between o-iodosobenzoate and the active site sulfhydryls of pig muscle GPDH is not a disulfide but probably a sulfenyl derivative. This decomposes when the enzyme's conformation is changed to permit formation of a disulfide bond. Interestingly, the present studies show that the loss of 2 sulfhydryls occurs with only slight changes in enzymatic activity and the loss in SH groups is induced by the natural substrates of the enzyme.

Honeybee and carpenter ant GPDH's display another striking difference with respect to rabbit muscle GPDH. The two former enzymes are not inactivated or dissociated by physiological levels of ATP at 0° as is rabbit muscle enzyme (Gelb and Nordin, 1970; Oliver et al., 1971). Low temperature stability

of their GPDH's probably plays a role in overwintering (Gelb et al., 1971; Oliver et al., 1971). Greene and Feeney (1970) have reported similar stability of the GPDH from the cold adapted Antarctic fish Dissostichus mawsonii.

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TABLE 1

Effect of Added Ligands on the Total DTNB-Reactive Sulfhydryls
of Urea Denatured Rabbit Muscle and Honeybee GPDHs

Addition*	SH GROUPS / 140,000 gms GPDH	
	Rabbit Muscle	Honeybee
None	12.9 ± 0.2 (2)	16.0 ± 0.1 (2)
NAD ⁺ (2 mM) plus G-3-P (3.6 mM)	12.7 ± 0.2 (4)	14.1 ± 0.3 (3)
NAD ⁺ (3 mM) plus G-3-P (1.8 mM)	13.1 ± 0.2 (4)	14.5 ± 0.1 (3)
G-3-P (3.6 mM)	13.6 ± 0.3 (2)	14.0 ± 0.1 (2)
G-3-P (3.6 mM); then after Sephadex G-25, NAD ⁺ (0.02 mM)	13.2 ± 0.2 (2)	13.7 ± 0.1 (2)
NAD ⁺ (2 mM)	13.2 ± 0.3 (4)	15.9 ± 0.1 (4)
NADH (2 mM)	13.1 ± 0.2 (4)	15.8 ± 0.3 (4)

*All concentrations expressed as D, L G-3-P

Numbers in parentheses refer to the number of determinations

TABLE 2

C¹⁴-carboxymethyl groups
mole of GPDH

Incubation	Honeybee	Rabbit Muscle
None	0.4	0.8
3.6 mM <u>D,L</u> G-3-P.	2.0	0.6
2 mM NAD ⁺ plus 3.6 mM <u>D,L</u> G-3-P	2.2	0.8

LEGENDS TO FIGURES

Figure 1: Comparison of the Saturation Kinetics of Rabbit Muscle and Honeybee GPDHs (Figure 2 of Gelb *et al.* 1970). All reactions were carried out at 30° and pH 9.0. Assay cuvettes contained enzyme and D,L-G-3-P in standard assay buffer. Reactions were initiated by the addition of increments of NAD⁺. The final volume was 1.0 ml. 0—0, rabbit muscle enzyme; 0—0, honeybee enzyme. The error bars show the range of values for 4 individual assays. The absolute maximum velocities for both enzymes were identical. The inset shows a more detailed representation of the transition area for honeybee GPDH obtained in a separate experiment in which smaller increments of NAD⁺ were used. $\frac{V_0}{V_{max}}$ = the initial velocity of the reaction at the various NAD⁺ concentrations, V_{max} = the initial velocity of the reaction at $28 \times 10^{-5} M$ NAD⁺.

Figure 2: Effect of DTNB on Enzymatic Activities of GPDHs. Each reaction mixture contained enzyme 0.3 mg per ml in 0.1 M imidazole, 1 mM EDTA at pH 7.0 and 20° in a final volume of 1.0 ml. The reaction was initiated by the addition of 5 microliters of .01 M DTNB. GPDH activity measurements were then

made on suitable aliquots either before addition of DTNB or after adding it to the reaction mixture. Upper frame, "metastable" honeybee GPDH; lower frame, rabbit muscle GPDH. The continuous curve represents the DTNB titration and the curve with the closed circles, enzymatic activity.

Figure 3: Titration of Native Honeybee GPDH with DTNB
 "Metastable" honeybee GPDH 1.0-1.4 gm per ml was preincubated at 20° in 1 ml of assay buffer pH 9.0 for 2 minutes with or without ligands at the final concentrations shown below. The entire reaction mixture was then applied to a Sephadex G-25 column equilibrated with 0.1 M imidazole, 1 mM EDTA pH 7.0. The pooled enzyme fractions were adjusted to 0.3 mg protein per ml with the above imidazole buffer and a 1.0 ml aliquot reacted with 5 microliters of .01 M DTNB. The curves obtained were from enzyme samples subjected to the following preincubations:

- 1) Enzyme and 3.6 mM D,L-G-3-P;
- 2) Enzyme alone or with 2.0 mM NAD⁺;
- 3) Enzyme and 2.0 mM NAD⁺ plus 3.6 mM D,L-G-3-P;
- 4) Enzyme and 1.8 mM D,L-G-3-P plus 3.0 mM NAD⁺.

Figure 4: Titration of Native Rabbit Muscle GPDH with DTNB.
 This experiment was performed under conditions identical to those used in Figure 3. 1) Three

superimposable curves which represent rabbit muscle GPDH alone or with NAD^+ , or with G-3-P; 2) Enzyme and NAD^+ plus G-3-P with either ligand in excess with respect to the other (two superimposable curves).

Figure 5: Reactivity of ^{14}C Bromoacetic Acid With the Two States Of Honeybee GPDH.

See Materials and Methods Section for experimental details.

Upper frame, "metastable" enzyme; middle frame, stable enzyme (prepared by preincubating "metastable" GPDH with 2 mM NAD^+ plus 3.6 mM $\underline{\underline{\text{D}}}, \underline{\underline{\text{L}}}$ -G-3-P); lower frame metastable enzyme preincubated with 3.6 mM $\underline{\underline{\text{D}}}, \underline{\underline{\text{L}}}$ -G-3-P only. 0—0, protein; O — O ; radioactivity.

Figure 6: Reactivity of ^{14}C $\underline{\underline{\text{D}}}$ -G-3-P With Honeybee GPDH.

See Materials and Methods Section for experimental details. Upper frame; enzyme preincubated in 0.59 mM ^{14}C $\underline{\underline{\text{D}}}$ -G-3-P; lower frame, enzyme preincubated in 1 mM NAD^+ plus 0.59 mM ^{14}C $\underline{\underline{\text{D}}}$ -G-3-P. 0—0; protein; O — O ; radioactivity.

Figure 7: Regeneration of The Transition in The Kinetic Saturation Curve of Honeybee GPDH.

Honeybee GPDH 0.25 mg/ml in assay buffer pH 9.0

was analyzed for the kinetic transition by diluting a suitable aliquot 200 fold in the assay cuvette. To a 1.0 ml aliquot of this solution was added D,L-G-3-P (3,6 mM final concentration) and NAD^+ (2.0 mM final concentration). After incubating 5 minutes at 20° a 5 microliter aliquot was transferred to an assay cuvette for analysis of enzymatic activity at this point. The remaining reaction mixture was applied to a 1.4 x 25 cm column of Sephadex G-25.

To a 0.3 ml fraction of the column eluate was added 0.2 ml of 6 mM DTT. After 60 minutes at 0° , an assay was performed on an aliquot of this mixture.

Each data point in the three frames represents the average of 2 to 4 separate titration experiments. The dotted lines represent the results obtained in a repeat experiment in which another sample of "metastable" enzyme was preincubated with G-3-P and NAD^+ as described above.

Figure 8: SDS Electrophoresis of Subunits Obtained From The Two States of Honeybee GPDH.

For experimental details see Materials and Methods Section. 1) "metastable" GPDG; 2) stable GPDH. Mobility = mobility of each protein relative to Bromphenol blue marker dye.

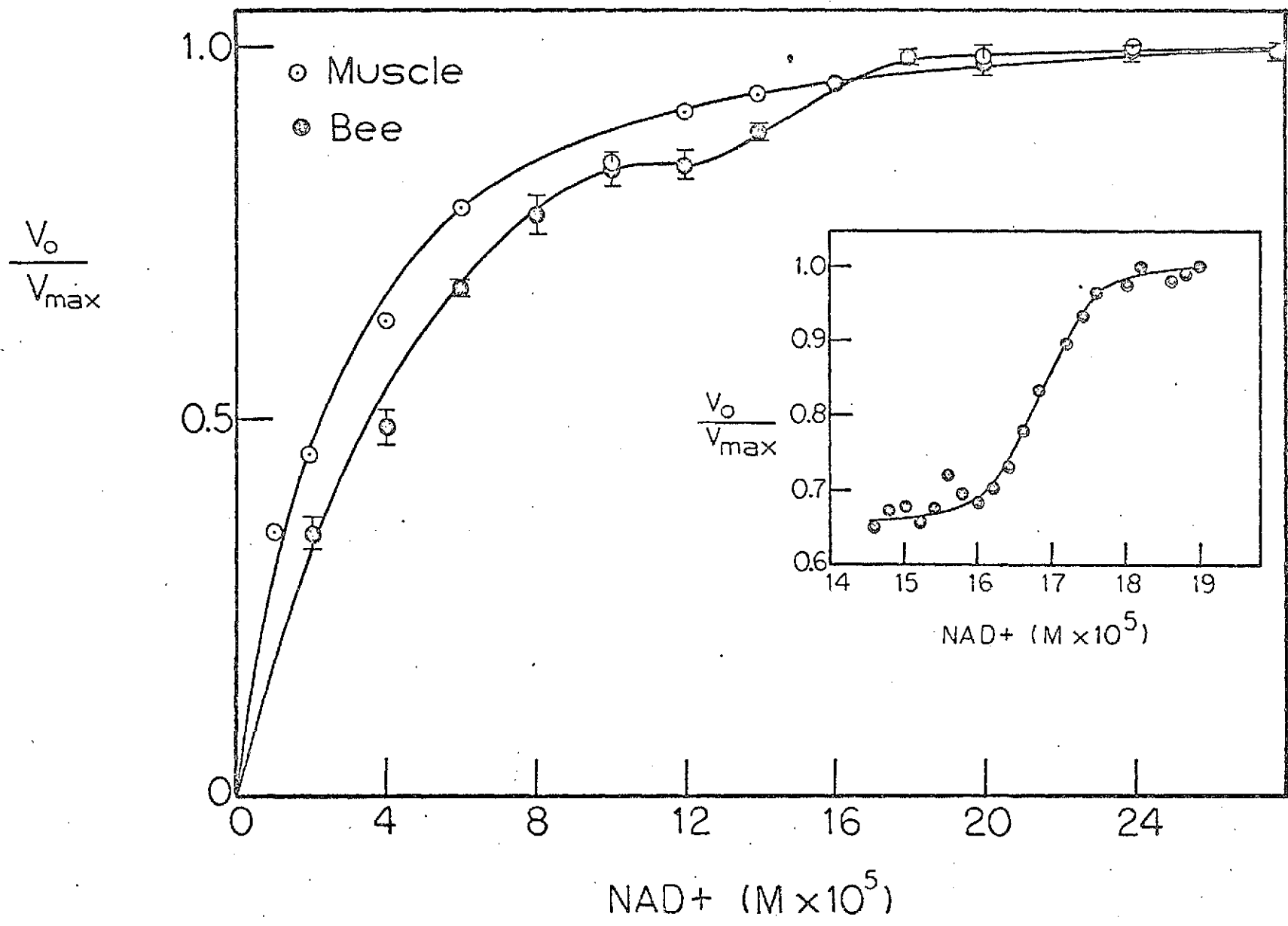


Figure 1

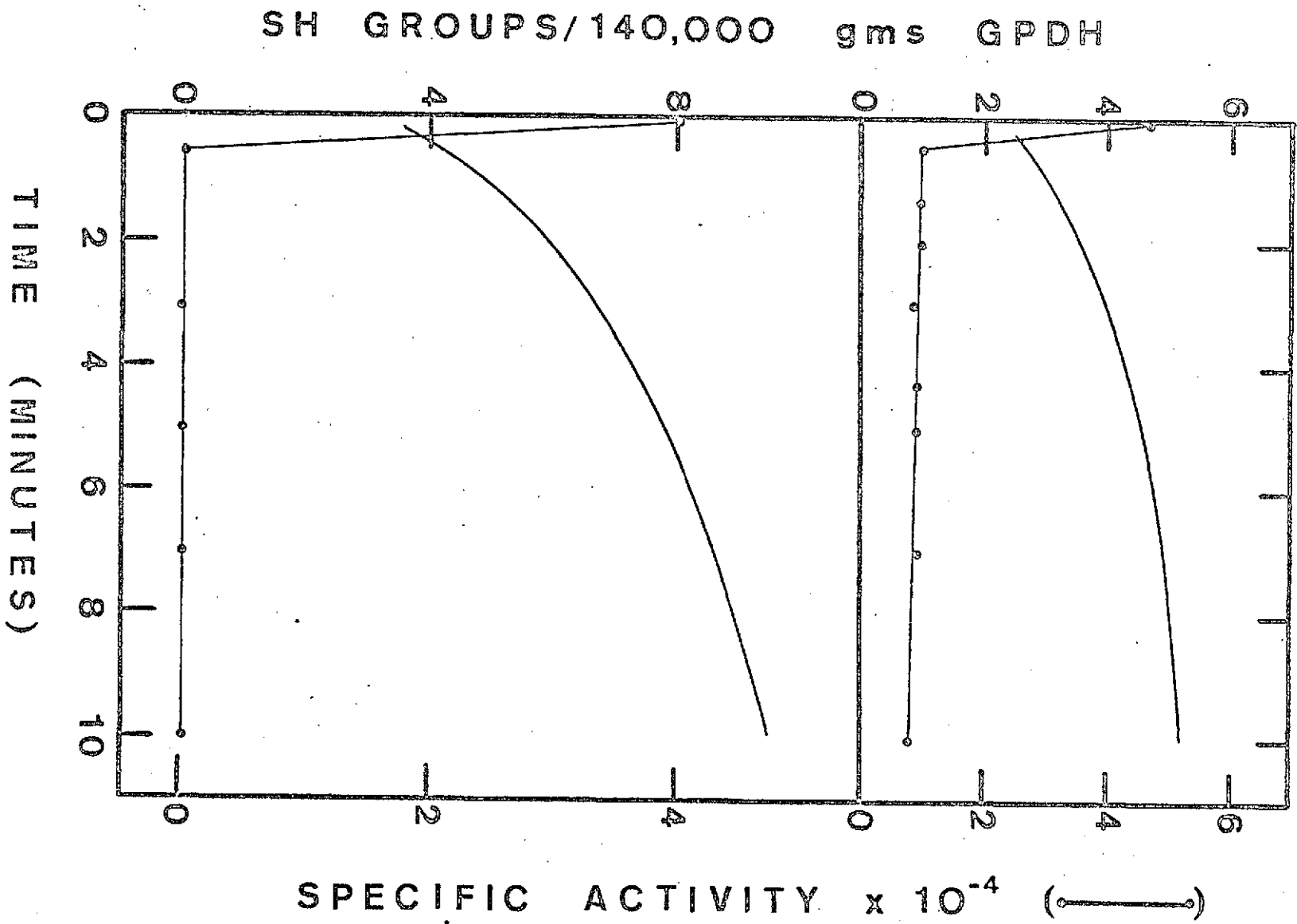


Figure 2

Figure 3

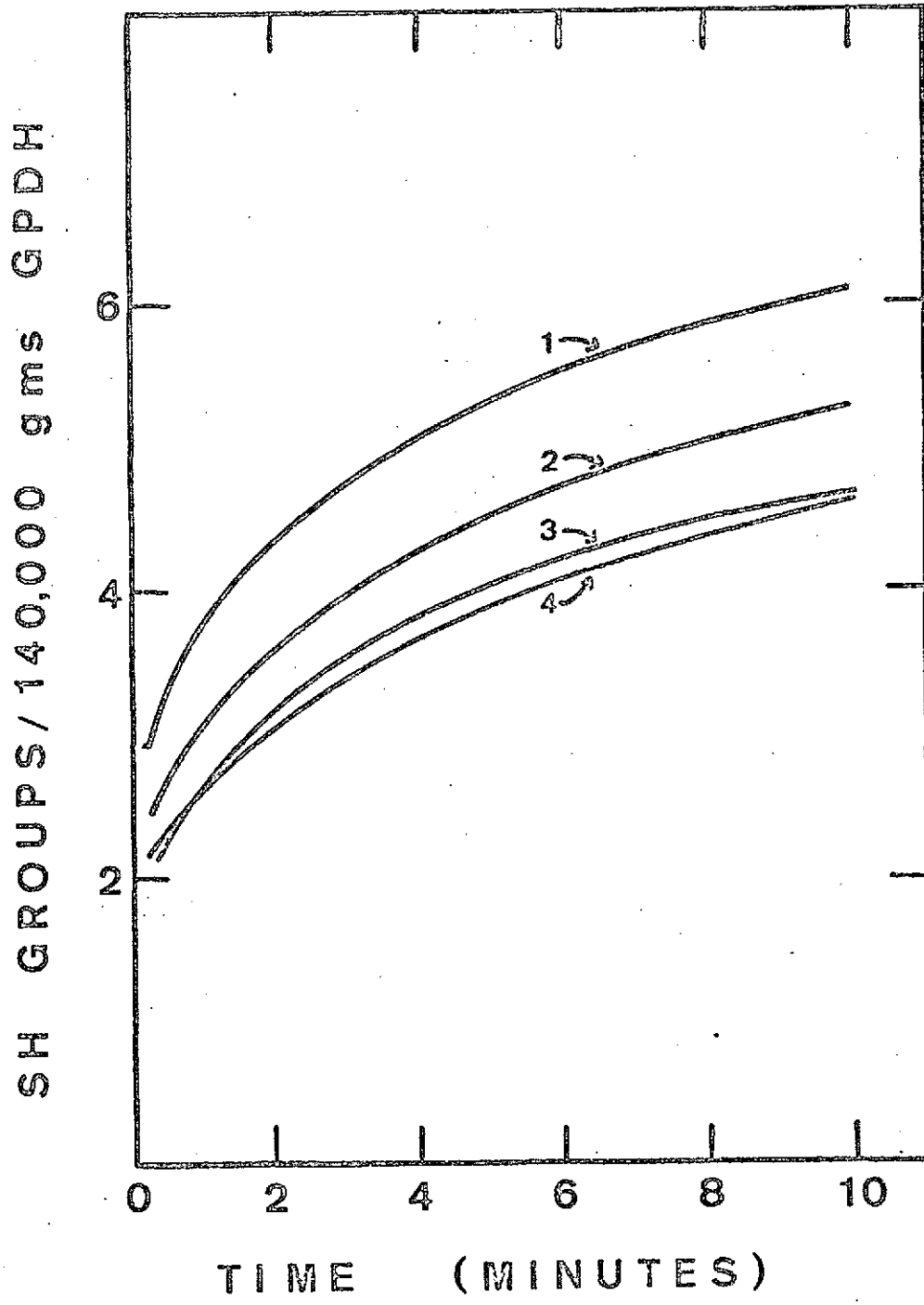


Figure 4

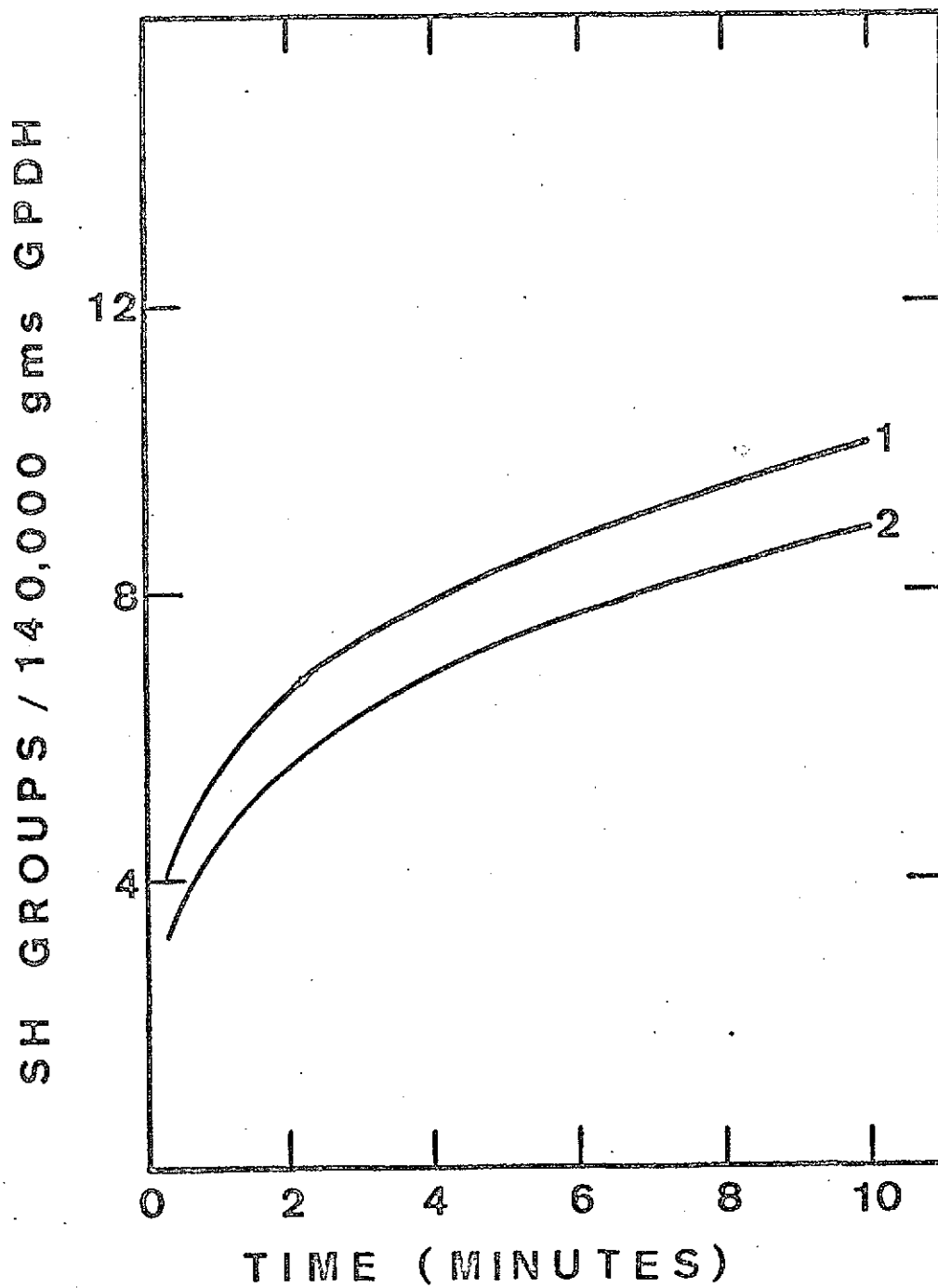


Figure 5

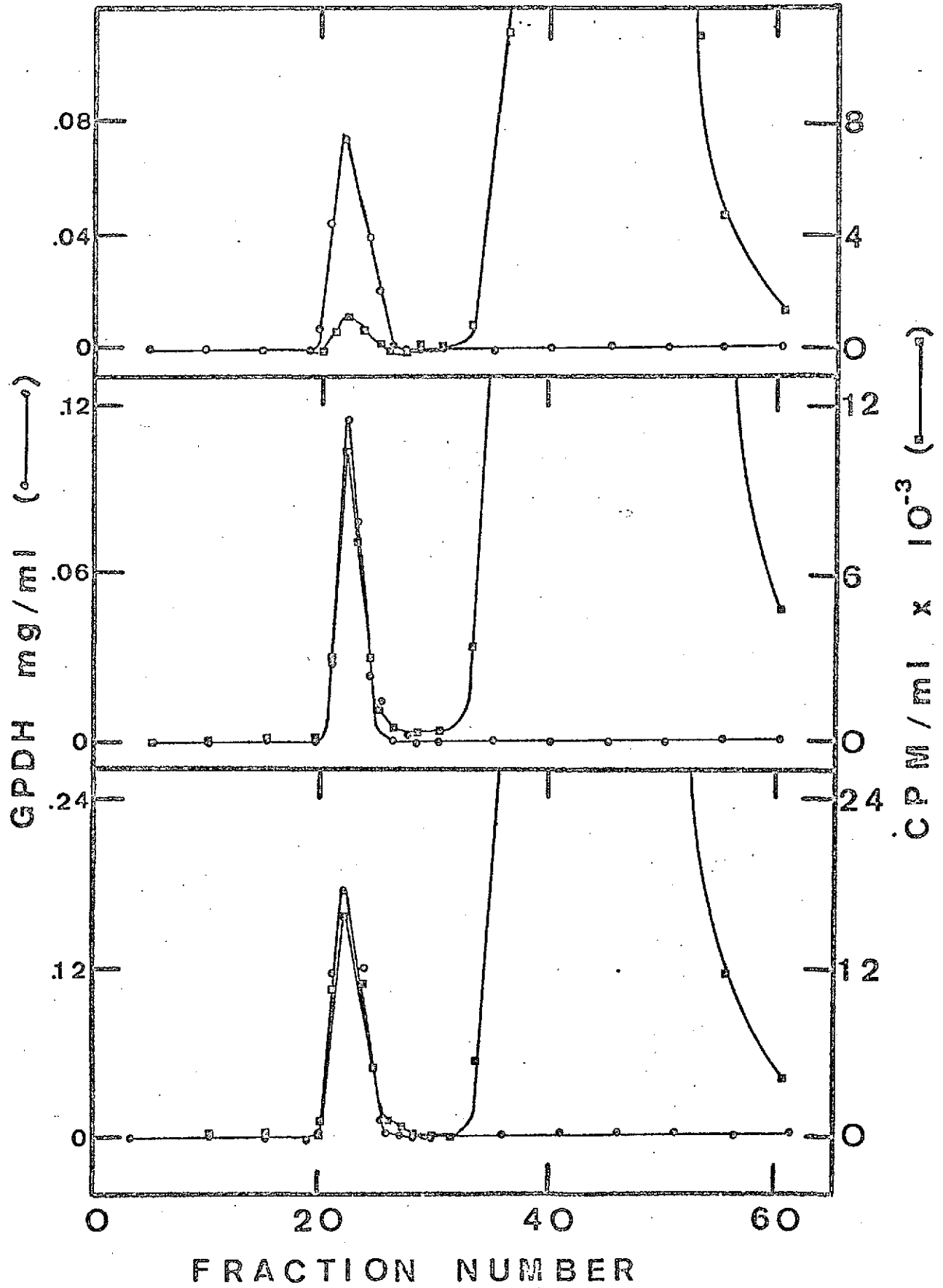


Figure 6

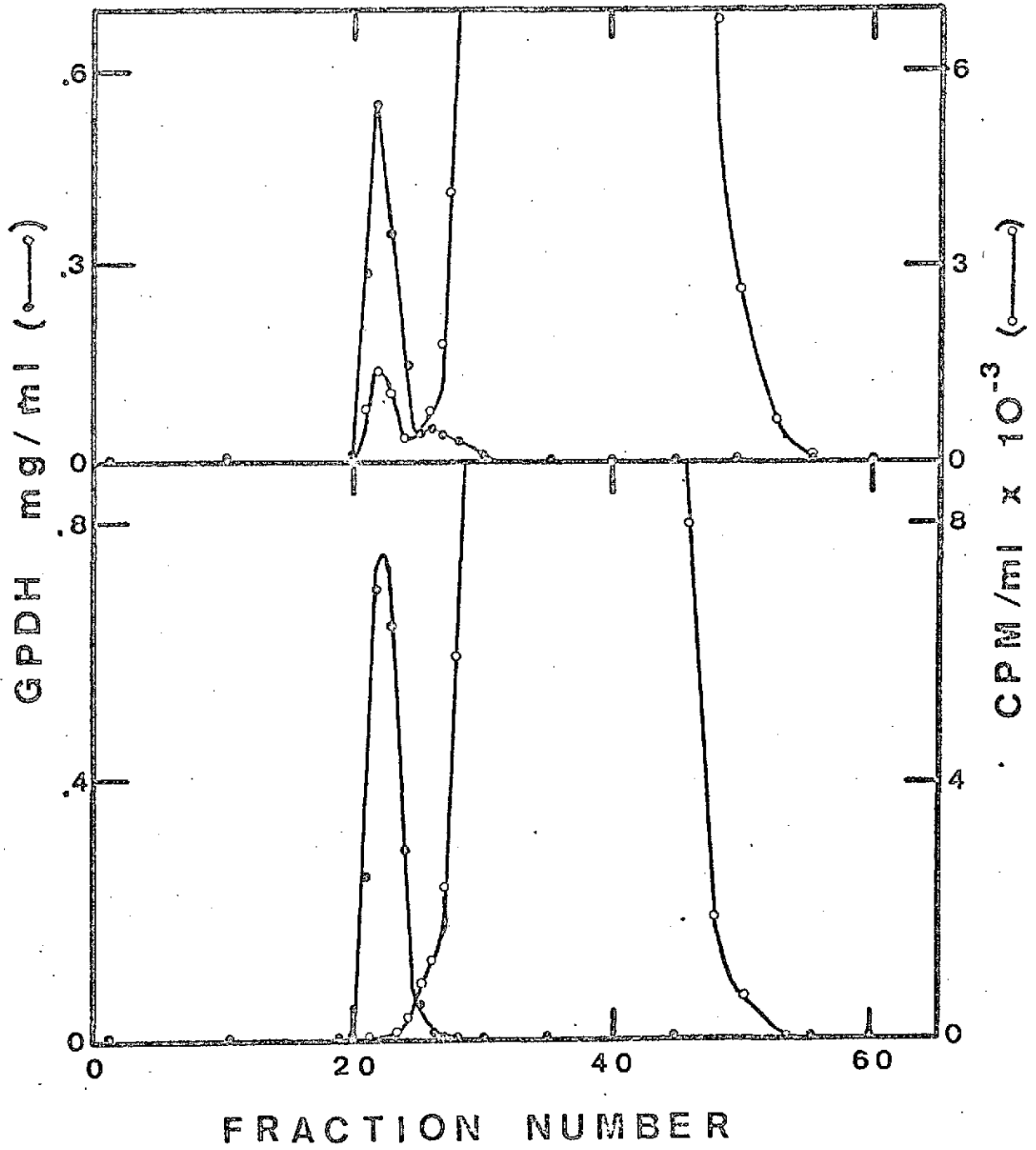


Figure 7

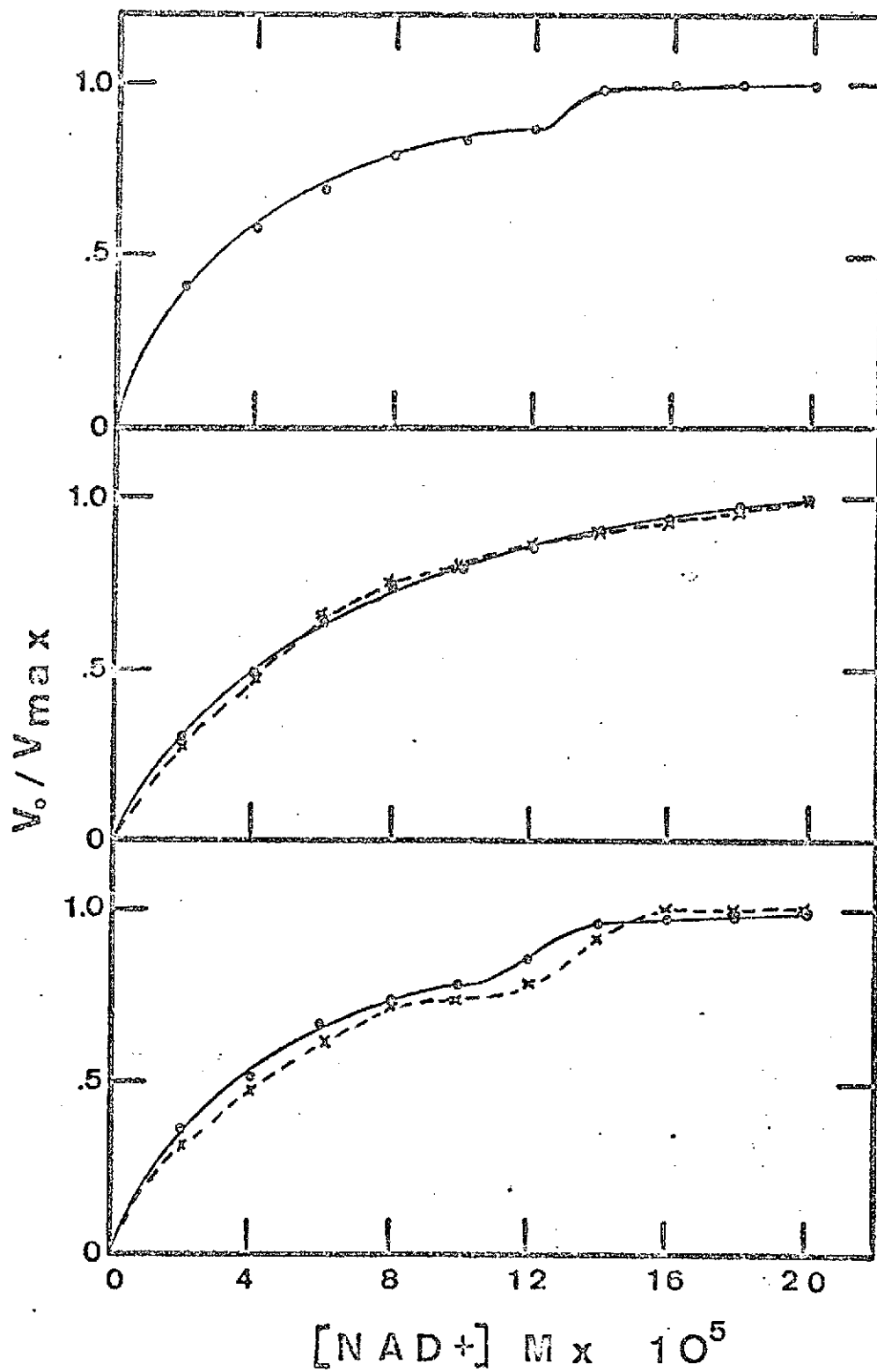


Figure 8

