PURIFICATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-SPECIFIC GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA AND PARTIAL CHARACTERIZATION OF ITS PHYSICAL, KINETIC, AND IMMUNOLOGICAL PROPERTIES.

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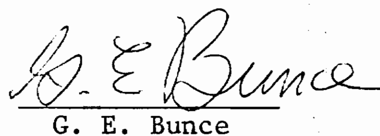

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LIST OF ABBREVIATIONS

GDH .... Glutamate dehydrogenase

NAD^+ .... Nicotinamide adenine dinucleotide

$NADP^+$ .... Nicotinamide adenine dinucleotide phosphate

Tris .... Tris (hydroxymethyl) aminomethane

MDH .... Malate dehydrogenase

SDS .... Sodium dodecyl sulfate

DTNB .... 5,5'-dithiobis (2-nitrobenzoic acid)

INTRODUCTION

Enzyme induction is ubiquitous to all living organisms. A wide variety of enzymes and other proteins (e.g., permeases) have been observed to have their rates of synthesis and/or degradation influenced by exogenous or endogenous small molecules (1-3). These molecules may be inducers (or corepressors) related structurally to the substrate(s) of the enzyme or, as discussed by Tomkins (4), symbols which code for the general metabolic state of the organism. Although rapid progress has been made in recent years on the mechanism of enzyme induction (5-7) and derepression (8) in procaryotes, the greater genetic and structural complexity of higher eucaryotes has limited progress in these organisms to hormone inducible enzymes (9, 10) and to proteins which are produced in large excess for export (11, 12) or other specialized functions (13-15).

There are very few small molecules, other than hormones, which have been observed to induce the synthesis of enzymes in higher eucaryotes. For this reason, a number of inducible enzymes in eucaryotic microorganisms (16, 17) are being used as models to study the mechanism of enzyme induction in eucaryotic cells. One such model system, under study in this laboratory, is the ammonium inducible NADP-GDH (18, 19) in Chlorella sorokiniana. This organism also contains a constitutive NAD-specific isozyme (20) of GDH that is synthesized in either ammonium- or nitrate- containing medium. Although both isozymes catalyze the synthesis and degradation of L-glutamate, their

role in ammonia assimilation is unclear. Current experimental evidence is consistent with the inference that the coupled reactions of glutamine synthetase and glutamate synthase represent the primary route of ammonia assimilation into Chlorella (21), higher plants (22), and bacteria (23). Thus, the role of the GDH isozymes in Chlorella and in other plant and microbial cells might be to regulate the levels of glutamate and/or α-ketoglutarate which are involved in coupled reactions in amino acid metabolism and the tricarboxylic acid cycle.

In this laboratory, Israel et al. (24) recently observed that the activities of both GDH isozymes change dramatically during the cell cycle and during shifts in the nitrogen nutritional status of Chlorella cells. To elucidate the molecular mechanisms responsible for these changes in enzyme activity, it is necessary to be able to measure directly the rates of synthesis and turnover of these isozymes independent of activity measurements. Radio-immunological techniques (25, 26) have been used for the direct measurement of rates of synthesis and turnover of specific enzymes. Since these techniques require the use of antibody prepared against purified enzyme, the purpose of the research described in this thesis has been to purify the NADP-GDH from Chlorella and to prepare antibody against it. Moreover, to understand more clearly the in vivo changes in activity of this enzyme, the purified enzyme has been partially characterized both physically and kinetically.

Although this thesis reports only the research on purification and partial characterization of the NADP-GDH isozyme from Chlorella,

the author was also involved in research on the *in vivo* induction and turnover of this isozyme. His research contributions were recognized in two publications which he coauthored (24, 27).

LITERATURE REVIEW

L-glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating) EC 1.4.1.2-4) is an important enzyme linking nitrogen and carbon metabolism. Recent reviews have given extensive coverage to the structural features, ligand binding characteristics, and the mechanism of reaction of the bovine GDH (28, 29). Since Smith *et al*. (30) have given an excellent summary of the research on GDH from animals, plants, and microbes up to 1974, this literature review will concentrate on more recently available publications. Because the work described in this thesis is on the unicellular plant *Chlorella sorokiniana*, the author also will discuss mainly research performed on eucaryotic microorganisms and higher plants.

Great diversity exists in the regulation of glutamate dehydrogenase activity in various fungal genera (31). Since the main pathway of ammonium assimilation in many fungi is through the action of GDH, the regulation of GDH has been studied primarily in relation to nitrogen metabolism (23). The *Candida utilis* NADP-GDH is a 276,000 molecular weight protein with 6 subunits, each of 47,000 molecular weight (32). The *in vivo* activity of the enzyme has been studied by N^{15} incorporation into amino acids, and has been shown to be controlled by feedback inhibition by the level of intracellular amino acids (33).

Two glutamate dehydrogenases from *Dictyostelium discoideum* have recently been characterized and are allosterically affected by various nucleotides (34). These findings and others led Langridge *et al*. (34)

to propose that the GDHs of *Dictostelium* may be regulated by the *in vivo* "energy charge" (35) as described by Atkinson.

Recent partial characterization of the 2 GDHs from *Coprinus cinereus* (36, 37) indicates that the levels of the enzymes may be controlled *in vivo* both by the carbon source and nitrogen source of the culture (38). Germinating spores of *Geotrichium candidum* contain only an NADP-GDH (39) and its regulation is apparently by repression of synthesis of the enzyme by NH_4^+. Similar repression of synthesis of GDH by NH_4^+ has been reported in *Schizophyllum commune* (40).

Early work on the regulation of the NAD- and NADP-GDHs of *Neurospora crassa* (41) showed that NH_4^+ and amino acids elevate the level of the NAD-GDH and repress the level of NADP-GDH. D-amino acids elicit identical effects, indicating that the amino acids themselves and not their metabolic products are the active agents. Good carbon sources (ie., glucose, sucrose etc.) inhibit the induction and repression (42) of the NAD- and NADP-GDHs, possibly indicating catabolite control of enzyme synthesis as seen in *Escherichia coli* (5). The NADP-GDH has been sequenced (43-46) and shows some homology with the bovine liver and chicken liver enzymes (30). The 290,000 molecular-weight native enzyme (47) is composed of 6 identical subunits, with a molecular weight of 48,000, and has been shown to have essential lysine (47), tyrosine (48), and arginine (49) residues. A deactivating conformational change induced by NADPH has also been demonstrated (50). The NAD-GDH has been purified and has a native molecular weight of 480,000 and is composed of 4 identical 116,000 dalton subunits (51).

Several mutations in the protein for NADP-GDH (i.e. *am*^{1} - *am*^{19}) have been characterized immunologically (52) and physically (53). The mutations in *am*^{1}, *am*^{2}, *am*^{3}, *am*^{7} and *am*^{19} are all single amino acid replacements and produce proteins which cross react immunologically with the native enzyme. Mutant 14 in the *am* gene produces no detectable NADP-GDH activity or cross reactive material when cultured in the usual fungal medium, but, when mated with other *am* mutants, it can complement them and produce active NADP-GDH. Fincham and Baron (54) demonstrated that *am*^{14} produces a highly unstable GDH subunit which is normally broken down so rapidly that it is not detectable. When present in a heterozygous state with a stable NADP-GDH subunit, they showed it can form hexameric GDH with normal enzyme activity. If grown on medium supplemented with 1 M glycerol, which acts to stabilize the enzyme *in vivo* and *in vitro*, the *am*^{14} mutant can form catalytically normal GDH. Two revertants of *am*^{14} have been isolated with amino acid substitutions at residue 20 (leu in wild type). This finding indicates that leu 20 is the site of mutation in *am*^{14}, and is extremely important in maintenance of the *in vivo* stability of the enzyme. The availability of mutants in the *Neurospora* NADP-GDH will greatly fascilitate the determination of amino acid residues necessary for the function of the enzyme.

NADP-GDH seems important in the regulation of NH_4^+ assimilation in *Aspergillus nidulans*. The NADP-GDH is found at high levels in cells growing on inorganic nitrogen and at low levels in cells cultured on amino acids (55). This enzyme decreases in activity during carbon starvation (56), and this decrease is blocked by addition of cycloheximide

(57), indicating control of the enzyme by both carbon and nitrogen sources. Mutants which lack NADP-GDH are slow growers on NH_4^+ (58) as sole nitrogen source, and are derepressed for several NH_4^+ repressible enzyme systems (59). Immunological studies have shown that 3 of 5 gdhA mutants examined contain cross-reacting material to NADP-GDH, and several other mutants had altered kinetic and stability characteristics (60). A total of 41 mutants (gdhA1 - 41) have been studied which lack NADP-GDH and all mutations map in the gdhA locus, indicating gdhA to be the locus of the structural gene for NADP-GDH.

NAD-GDH of *A. nidulans* is apparently not regulated in the same manner as the NADP-GDH from *Aspergillus*. Instead, its regulation may be controlled by the carbon source only. Little change is detected in the level of the enzyme in the presence of either NH_4^+ or amino acids, but severe depression of NAD-GDH activity is seen on growth on glucose as the sole carbon source (57). As discussed earlier, the GDHs of *N. crassa* also showed catabolite repression. Mutants of NAD-GDH in *Aspergillus* have been obtained which show altered Km values for substrates and increased thermal lability (61). These mutants are unlinked to gdhA locus and are designated gdhB mutants. A double mutant gdhA1, gdhB1 can still grow, albeit slowly, on low concentrations of NH_4^+ indicating the possible existence of glutamate synthase in *Aspergillus*. Another mutant, gdhC1, releases NAD-GDH from glucose repression (62) and may provide insight into the molecular mechanism of action of glucose repression in fungi.

In Saccharomyces cerevisiae the NAD- and NADP-GDHs appear to be controlled in an opposite manner to those in N. crassa. In Saccharomyces the NADP-GDH activity is high and the NAD-GDH activity is low in cells cultured on NH_4^+ (63). There is a reciprocal relationship between the specific activities of the two enzymes in cultures of NH_4^+. Increasing concentrations of NH_4^+ result in higher levels of the NADP enzyme, and in lower levels of the NAD enzyme. An increase in the specific activity of the NADP-GDH, along with a decrease in the NAD enzyme, was also seen with the non-metabolizable compound, methylamine, in the medium. These data indicate a direct role for NH_4^+ in the control of these enzyme levels. The gene coding for the structure of the NADP-GDH has been mapped with several mutants which yield modified or inactive enzymes (64). Several of these mutants ($gdhA^-$) also relieve the NH_4^+ repression of arginase, serine dehydratase and other enzymes (65). This observation led Dubois et al. (66) to propose that the NADP-GDH of Saccharomyces acted as an aporepressor, which when combined with NH_4^+ and α-ketoglutarate, acts to repress a large number of catabolic enzymes. The stipulation that intracellular NH_4^+ and α-ketoglutarate pools must both be high to elicit NH_4^+ repression, places severe restrictions on when NH_4^+ repression can occur. However, it also provides a route to link nitrogen catabolite repression with carbon catabolite repression. Two enzymes, amino acid permease and allophanate hydrolase, which were originally thought to be under NH_4^+ repression via NADP-GDH, have been shown under certain conditions not to require NADP-GDH for control. Bossinger and Cooper (67) have shown that, although NH_4^+ repression of

allophanate hydrolase is not functional in a gdhA$^-$ mutant, repression of the enzyme still occurs with the nitrogenous compounds, glutamate, glutamine, aspartate and asparagine. Roon et al. (68) in a different gdhA$^-$ mutant have shown that the loss of NH_4^+ repression of the general amino acid permease seen in this mutant can be reestablished in a double mutant, gdhA$^-$, gdhCR, which contains 20-50 times the normal amount of NAD-GDH (69). This finding led the authors to conclude that, since the NH_4^+ repression in the mutant lacking NADP-GDH, could be reestablished by an excess of NAD-GDH, then the NADP-GDH was only important in its role of NH_4^+ conversion and not at a direct repressor molecule. These results are not, however, totally inconsistent with the proposals of Waime and his associates (66). It can be proposed that the excess NAD-GDH in this double mutant may perturb the intracellular α-ketoglutarate pool sufficiently to elicit NH_4^+ repression via an NADP-GDH which may be catalytically inactive yet maintain repressing ability. Until immunological studies can show the presence or absence of catalytically inactive, cross reacting material in gdhA$^-$ mutants of Saccharomyces, it will be difficult to determine the precise role of the NADP-GDH in nitrogen repression.

Until recently, the NADP-GDH of Saccharomyces, was thought to be strictly cytoplasmic in nature. However, Camardella et al. (70) have shown that there are actually two NADP-GDHs, one which is the cytoplasmic enzyme studied previously, and a second NADP-enzyme found in the nucleus of Saccharomyces. The second NADP-GDH has been partially purified (71) and been shown to have different kinetic and electrophoretic properties

from the cytoplasmic enzyme. Further characterization of the nuclear GDH will be required before the possibility that this enzyme is a modified form of the cytoplasmic enzyme can be discounted.

Only a single GDH is found in *Tetrahymena* which can utilize NAD^+ $NADP^+$, NADH, and NADPH (72). Although studies on *in vivo* regulation of the enzyme have not been performed, *in vitro* studies show that this mitochondrial GDH has a 3.5 fold stimulation of activity with NADH by 100 μM ADP. Since the activity with NADPH was unaffected at this concentration of ADP, it appears that this enzyme may be subject to various allosteric control mechanisms *in vivo*.

The NAD- and NADP-GDHs of *Chlorella pyrenoidosa* Pringsheim 82T have been partially characterized by Shatilov *et al*. (73-77). The NAD-GDH has been purified to homogeneity and is a 310,000 molecular weight protein composed of 6 subunits of 49,000 daltons (78). The size and kinetic properties of the enzyme are noticeably different from those of the NAD-GDH purified from *C. sorokiniana*. The NAD-GDH from *C. sorokiniana* is composed of 4 subunits of 45,000 daltons, and has a native molecular weight of 180,000 (20).

Early studies with different nitrogen sources showed an increase in activity of the NADP-GDH of *C. pyrenoidosa* Pr. 82T after growth on NH_4^+ (79). This increase was blocked by cycloheximide and actinomycin D. Talley *et al*. (18) separated the two GDHs of *C. sorokiniana* and showed that NH_4^+ induced the synthesis of NADP-GDH at all times during synchronous growth. This finding indicates that the structural gene for the NADP-enzyme is continuously available during the cell cycle.

Israel et al. (24) have shown that the NADP-GDH of C. sorokiniana is apparently stable in vivo in the presence of NH_4^+ but decays very rapidly under nitrogen starvation. Cycloheximide blocks this in vivo loss in enzyme activity by an unknown mechanism.

The regulation of glutamate dehydrogenases in higher plants has been studied to a far lesser degree than in eucaryotic microorganisms. The molecular weights of GDHs in higher plants have ranged from 210,000 to 250,000 daltons in Pisum sativum (80) and Cucurbita moschata (81), respectively. The report of Fawole et al. (82) on a 51,000 molecular weight subunit of the Vigna unguiculata NAD-GDH is the only information available on the subunit structure of plant GDHs. Two or more isoenzymes of GDH have been reported in mung bean (83), Caulerpa simpliciuscula (84), pumpkin (81), tomato (85), tobacco culture (86), safflower (87), oat (88), rice (89), lettuce (90) and corn (91). The isozyme patterns of GDH have been studied throughout the development of some plants and have been found to change with time. However, caution must be used in interpreting isozyme patterns on polyacrylamide disc gels due to artifactual staining and smearing of enzyme bands. V. unguiculata (82) and P. sativum (80) each appear to have only a single GDH with enzyme activity with both NADH and NADPH. The regulation of some plant GDHs appear to be similar to that of the fungal system where NH_4^+ induces GDH activity. In oat (92), rice (89) and grape leaves (93) the infusion of NH_4^+ causes the level of GDH to increase. In oat leaves and rice roots, the increase in GDH activity is blocked by cycloheximide. In excised P. sativum roots, the amino acids

L-aspartate and L-glutamate cause an increase in GDH activity (94) which can be prevented by cycloheximide; however, excess NO_2^- in the medium causes an activation of the GDH which is not blocked by the antibiotic (95). The GDHs in these organisms may provide good model systems for the induction of enzymes in eucaryotic cells.

MATERIALS AND METHODS

Organism and Growth Conditions - The source of the enzyme for these studies was the thermophilic green alga, *Chlorella sorokiniana* (96), previously described as *C. pyrenoidosa* strain 7-11-05 (97). The cells were cultured asynchronously at 38.5° in ammonium medium (24) and aerated with 4% CO_2-air (0.225ℓ/min) in 5.1 cm glass culture tubes in continuous light (950 f.c./bank) in a constant-temperature water bath (98). Cells from the glass culture tubes were used to inoculate a 35 ℓ Plexiglas culture chamber (99). The growth conditions in this mass culture chamber were similar to those in the glass tubes except the aeration rate was increased to 22ℓ/min. The change in culture turbidity (i.e., absorbance at 550 nm, 1.3 cm diameter colorimeter tubes, Bausch and Lomb Model 340 spectrophotometer) was used to monitor the growth rate of the cells. The initial culture turbidites were adjusted to 0.05 (approximately 4 to 5 x 10^6 cells/ml) and 0.10 in the glass tubes and the Plexiglas chamber, respectively.

Harvest Procedure and Preparation of Cells for Analysis - All manipulations were performed at 4° unless stated otherwise. The pH values are given at the temperatures that each buffer is used.

When the turbidity of a 30 ℓ mass culture reached 1.5 - 2.0, 15 ℓ of cell suspension was harvested, and an equal volume of fresh medium was added to replace this harvest volume. This harvest-dilution procedure was repeated a second time. When the aforementioned turbidity was reached the third time, the entire 30 ℓ culture was harvested. The

cells were harvested with a Sharples centrifuge at 50,000 rpm, resuspended in 2 volumes of 0.01 M Tris-HCl buffer (pH 8.25), and then pelleted at 11,700 x _g_ in a Sorvall RC2-B refrigerated centrifuge (GSA rotor). By centrifugation, the cells were washed twice in 1-volume of 0.01 M Tris-HCl buffer, and resuspended in an equal volume of 0.1 M Tris-HCl buffer (pH 8.25). The cells were then frozen and stored at -20° until time of breakage. The cells, from the total 60 ℓ harvest volume, were thawed and passed through a mechanically driven French pressure cell (model 5-598A, American Instrument Co.) at 18,000 to 20,000 p.s.i., and the preparation was centrifuged at 27,000 x _g_ for 30 min. The resulting pellet was combined with an equal volume of 0.1 M Tris-HCl buffer and passed through the French pressure cell and centrifuged once again. The two supernatants were combined and subjected to 100,000 x _g_ for 1-h in a Beckman L2-65B ultracentrifuge (SW-27 rotor). The approximately 350 ml of 100,000 x _g_ supernatant was used as the starting material for purification of the NADP-GDH.

Assays for Enzyme Activity and Total Protein - The assays of the _Chlorella_ NADP- and NAD-GDH isozymes were modified from those described by Israel _et al_. (24).

The assay mixture for measurement of the aminating reaction of the NADP-GDH was modified to contain 50 mM Tris-HCl buffer, 300 mM ammonium sulfate, 25 mM α-ketoglutarate, 0.35 mM NADPH, and 20 μl of enzyme solution to give a final assay volume of 1.02 ml and a pH of 7.2 at 38.5^{o}. This assay mixture was prepared by additions of the following

stock solutions: 0.44 ml, 0.1 M Tris-HCl buffer (pH 7.65, 22°); 0.20 ml, 1.53 M $(NH_4)_2SO_4$; 0.05 ml, 0.51 M α-ketoglutarate in 0.02 M Tris-HCl buffer (pH 7.0, 22°) titrated with solid KOH; 0.05 ml, 7.14 mM NADPH prepared immediately prior to use; and 0.26 ml, quartz distilled or deionized H_2O. The $(NH_4)_2SO_4$ and NADPH were dissolved in 0.02 M Tris-HCl buffer prepared by a 1:5 dilution of 0.1 M Tris-HCl buffer (pH 7.65, 22°).

The assay mixture for measurement of the deaminating reaction of the NADP-GDH was modified to contain 50 mM Tris-HCl buffer, 200 mM L-glutamate, 1 mM $NADP^+$, and 20 µl of enzyme solution to give a final assay volume of 1.02 ml and a pH of 8.7 at 38.5^o. This assay mixture was prepared by additions of the following stock solutions: 0.44 ml, 0.1 M Tris buffer (pH unadjusted); 0.02 ml, 1.02 M L-glutamate in 0.02 M Tris-HCl buffer (pH 8.9, 22°) titrated with solid KOH; 0.05 ml, 20.4 mM $NADP^+$ in 0.02 M Tris-HCl buffer (pH 6.0, 4°); and 0.31 ml, quartz distilled or deionized H_2O. Enzyme activity was determined from the change in absorbance at 340 nm with time after enzyme addition.

Malate dehydrogenase and catalase were assayed by the procedures of Gregory _et al_. (100) and of Beers and Sizer (101), respectively. One unit of enzyme activity was defined as the amount of enzyme activity required to catalyse the conversion of 1 µmol of substrate per min.

Total protein was measured by the method of Lowry (102). Thiols and other substances which would interfer with the analysis of total protein were removed by ultrafiltration in an Amicon model 12 cell with a PM-10 membrane.

Analytical Electrophoresis - The polyacrylamide gel electrophoresis was performed at 4° with a reservoir buffer concentration fivefold greater than that recommended by Davis (103). In 5 mm diameter tubes, the volumes of the separating and stacking gels were 1.0 and 0.2 ml, respectively. Samples of 10 to 150 µl were applied in 20% sucrose. The bands of NADP-GDH activity and protein were located by a tetrazolium assay system and by Coomassie Blue staining, as previously described (18, 20), respectively. The methods used for gradient gel electrophoresis and SDS polyacrylamide gel electrophoresis are described in a subsequent section of this thesis.

Purification of NADP-GDH - Unless stated otherwise, all purification steps were performed at approximately 4°. The 350 ml of 100,000 x *g* supernatant which had been stored for 5-10 days at -20° was thawed in a beaker of H_2O at 22°.

Step 1. Freeze-Thaw Procedure - The resulting 100,000 x *g* supernatant was refrozen twice, for 2 h at -20° and thawed at 22°, and the precipitate was removed by centrifugation at 16,000 x *g*.

Step 2. Ammonium Sulfate Fractionation - By addition of a saturated solution of $(NH_4)_2SO_4$ (i.e., equilibrated with solid $(NH_4)_2SO_4$ for at least 14 days), the frozen-thawed supernatant was brought to 40% saturation with this salt. After 1-h, the precipitate was removed by centrifugation at 27,000 x *g* for 30 min. The resulting supernatant was brought to 70% $(NH_4)_2SO_4$ saturation. After centrifugation at 27,000 x *g* for 30 min, the supernatant was discarded and the precipitate, which

contained most of the NADP-GDH activity, was dissolved in 60 ml of 0.01 M Tris-HCl buffer (pH 7.4). The resulting solution of proteins, with a pH of 6.2 to 6.5, was allowed to stand for 3 days. At the end of this time period, a large precipitate, containing no NADP-GDH activity, was removed by centrifugation at 27,000 x *g*.

Step 3. Gel Filtration - A 20 ml aliquot, from the 60 ml protein solution from the previous step, was applied to a column (5 x 39 cm) of Sephadex G-200 (40-120 μ) which had been preequilibrated with 0.01 M Tris-HCl buffer (pH 7.4). To stabilize the column bed, it was overlaid with approximately 1 cm of Sephadex G-10 (40-120 μ). The flow rate was maintained at 0.3 ml/min with a pressure head of 15 cm of H_2O, and 5.5 ml fractions were collected. The fractions which contained approximately 80% of the NADP-GDH activity were combined. To process the entire 60 ml of protein solution, this gel-filtration step was repeated two additional times.

Step 4. DEAE-Cellulose Chromatography - The enzyme solution was applied to a column (2.5 x 24 cm) of Whatman DE-52 which had been preequilibrated with 0.01 M Tris-HCl buffer (pH 7.4). After the sample was applied, the column was washed with 300 ml of the preequilibration buffer and then developed with a linear gradient from 0 to 0.4 M NaCl in 0.01 M Tris-HCl buffer (pH 7.4). The flow rate was maintained at 3.2 ml/min with a peristaltic pump (Harvard model 1201) and 6.7 ml fractions were collected.

Step 5. Calcium Phosphate Fractionation - By separate additions of 2.1 M $(NH_4)_2SO_4$ and 100 mM dithiothreitol in 0.1 M Tris-HCl buffer (pH 8.2), the enzyme solution from the previous step was adjusted to 0.1 M $(NH_4)_2SO_4$ and 2 mM dithiothreitol. A suspension of $CaPO_4$ (80 mg/ml in 0.01 M Tris-HCl buffer, pH 7.4) was then added to give a final $CaPO_4$ concentration of 4 mg/ml. This suspension was allowed to stand 1.5 h, and the $CaPO_4$, to which the enzyme was bound, was pelleted by centrifugation at 750 x *g* for 10 min. The $CaPO_4$ pellet was washed 4-times with 20 ml of 0.1 M Tris-HCl buffer (pH 8.2), containing 0.1 M $(NH_4)_2SO_4$ and 2 mM dithiothreitol. At each wash, the pellet was resuspended (with a glass rod) and then centrifuged at 750 x *g*. To elute the NADP-GDH activity, the $CaPO_4$ was resuspended for 10 min in 10 ml of 0.1 M Tris-CH1 buffer (pH 8.2), containing 0.4 M $(NH_4)_2SO_4$ and 2 mM dithiothreitol. After centrifugation, the supernatant was assayed for enzyme activity, and this process was repeated (10-15 times) until 75-85% of the NADP-GDH activity was recovered. The supernatants were combined and subjected to ultrafiltration to concentrate the enzyme and to change to the electrophoresis buffer (0.19 M glycine, 2 mM dithiothreitol, 0.25 M Tris pH 8.4).

Step 6. Preparative Polyacrylamide Gel Electrophoresis - To remove any precipitate formed during ultrafiltration, the enzyme solution from the previous step was centrifuged at 27,000 x *g* for 30 min. By addition of 100 mM dithiothreitol in electrophoresis buffer (pH 8.4), the enzyme solution was adjusted to 10 mM dithiothreitol and then

allowed to stand at 22° for 1 to 3 h. A 7% polyacrylamide separating-gel (1 cm) and a 2.5% stacking-gel (3 cm) were prepared at 22° in a Canalco Prep-Disc apparatus in the P2-320 column by a method similar to that of Stephens *et al.* (104). Before cooling to 4° for electrophoresis, each gel was allowed to polymerize for at least 2-h. The upper and lower resevoirs of the electrophoresis unit contained electrophoresis buffer (pH 8.4) with 2 mM dithiothreitol. By addition of 60% (w/v) sucrose in electrophoresis buffer, the enzyme solution was adjusted to 20% sucrose (w/v) and then layered on the stacking-gel with a long cannula. The sample was overlayed with 10% sucrose and 2.5 μM Bromophenol Blue in 5 ml of electrophoresis buffer, and a current of 20 mA was established. To collect the proteins during electrophoretic elution from the gel, electrophoresis buffer was employed at a flow rate of 1.75 ml/min. After elution of the tracking dye, 3 ml fractions were collected. Those fractions, containing 70-90% of the NADP-GDH activity, were combined, concentrated by ultrafiltration, and resubjected to preparative electrophoresis in the exact manner described above. After this second preparative electrophoresis step, those fractions which contained NADP-GDH activity were subjected to analytical disc gel electrophoresis. These fractions which contained a single protein, as indicated by staining with Coomassie Blue, were pooled and used as the source of purified NADP-GDH in all subsequent studies.

Determination of Molecular Weight of NADP-GDH and Its Subunit - The molecular weight of the purified native enzyme was estimated by sedimentation-equilibrium, gel-filtration, and electrophoresis.

Sedimentation equilibrium was performed by the meniscus depletion method of Yphantis (105). Centrifugation of the enzyme (0.2 mg/ml) was performed at 8,766 rpm at 4.2^{o} in a Beckman-Spinco Model E ultracentrifuge equipped with interference optics.

Gel-filtration was performed with a Sephadex G-200 column (1.5 x 84 cm) preequilibrated with 0.2 M Tris-HCl buffer (pH 8.2) at 4°. The column was calibrated with standards of ovalbumin, aldolase, apoferritin, and catalase by the method of Andrews (106). The protein standards and the NADP-GDH were added to the column in 0.5 ml volumes and eluted at a flow rate of 0.16 ml/min. Elution of the protein standards was monitored at 280 nm, with the exception of catalase which was assayed as described in a previous section. The data are plotted by a modification of the method of Laurent and Killander (107).

Gradient gel electrophoresis was performed, in 2.5 to 27% polyacrylamide slab gels (Gradipore) in 0.01 M Tris, 0.08 M borate, 0.003 M EDTA, by use of a Pharmacia GE-4 electrophoresis apparatus. Samples were applied in a 20% glycerol solution. Electrophoresis was performed at 70 V without buffer circulation for 20 min, and then electrophoresis was continued at 300 V for 4.5 h with buffer circulation. The gel was stained and destained as described earlier (20).

From the migration distance of the native NADP-GDH, during analytical gel electrophoresis in 5, 7, and 9% polyacrylamide gels, a Ferguson plot (108) was used, as discussed by Rodbard and Chambach (109), to estimate the molecular weight of the enzyme.

The molecular weight of the subunit of the dissociated NADP-GDH was estimated by SDS polyacrylamide gel electrophoresis as described by Laemmli (110). The electrophoresis system of Davis (100) was employed with 0.1% SDS in the gels and in the electrophoresis buffer.

Amino Acid Analysis - The amino acid composition of the purified NADP-GDH was measured in part with a Beckman model 121 automatic amino acid analyzer by the procedure of Spackman *et al*. (111). For separation of the basic amino acids, a 0.9 x 23 cm column of Beckman PA-35 resin was eluted with 0.35 N sodium citrate (pH 5.25). For the acidic and neutral amino acids, a 0.9 x 69 cm column of Beckman AA-15 resin was eluted with 0.2 N sodium citrate at pH values 3.25 and 4.25, respectively. The enzyme was hydrolyzed in 6N HCl under N_2 atmosphere for 24 h and dried *in vacuo*.

The tryptophan and tyrosine content of the enzyme was measured by the method of Bencze and Schmid (112). By titration with DTNB in 1% SDS, the total number of sulfhydryl groups in the enzyme was measured as described by Habeeb (113). The enzyme was cleaved at its cysteine residues by the procedure of Stark (114), and the number of and molecular weights of the resulting peptides were measured by SDS electrophoresis in 15% polyacrylamide gels. With the exception that the enzyme was not oxidized, the dansylation method for N-terminal amino acids was performed exactly as recommended by Gray (115). For this latter analysis, standard dansylated amino acids were separated by polyamide thin-layer chromatography with the solvent systems described by Woods and Wang (116).

Immunological Techniques - Preimmune serum and NAD-GDH antiserum were prepared from blood obtained from a female New Zealand White rabbit. The rabbit was bled from a marginal ear vein, and the whole blood was allowed to clot for 30 min at 22°. The clot was separated from the glass test tube wall with a glass stirring rod. The clotted blood was then stored at 4° for at least 8-h, and then centrifuged at 2,000 x *g* for 10 min. The serum was decanted and stored at -20° until use. Prior to immunization of the rabbit with the purified NADP-GDH, approximately 40 ml of blood were collected for preparation of preimmune serum. For the initial injection, 1 mg of NADP-GDH was dissolved in 0.5 ml of 0.04% SDS in H_2O, mixed with 0.5 ml of Freund's complete adjuvant, and then sonicated (Sonifier cell disruptor, model W185D, Heat Systems-Ultrasonics, Inc.; microprobe) for approximately 1-min or until a stable emulsion was obtained. One-half milliter of this emulsion was injected intramuscularly into each thigh. After 3 weeks, the rabbit was injected with 0.5 mg of NADP-GDH prepared and administered as with the initial injection. Equivalent booster injections were given at 3-week intervals thereafter. Beginning with the second injection, 40 ml of blood were collected 2-weeks after each booster injection.

Svendsen buffer (Bio-Rad Buffer III) was used in all immunological procedures. For Ouchterlony double diffusion analysis (117) and "rocket" immunoelectrophoresis (118), a 1% agarose solution was made by dissolving 10 g of agarose in 1 ℓ of Svendsen buffer at 100°. Aliquots of the agarose solutions were dispensed into separate containers and

stored at 4°. For Ouchterlony immunodiffusion analysis, the agarose was redissolved in boiling H_2O, cooled to 50°, pipetted onto 2.5 x 7.5 cm microscope slides, cooled until translucent. Approximately 4 mm diameter wells were punched into the agarose and these were filled with antigens or antiserum. The immunodiffusion was allowed to occur at 22° in a H_2O saturated atmosphere for at least 18-h before the precipitin bands were photographed.

For "rocket" immunoelectrophoresis, the agarose was redissolved in boiling H_2O, cooled to 50°, and maintained at this temperature until addition of serum. Sufficient serum was added to give a final serum concentration of 1 to 3% (v/v). After adequate mixing, this serum-agarose preparation was pipetted onto microscope slides or into a gel-mold (119) and allowed to cool. Approximately 1.5 cm from one edge of the slide or plate, wells were punched into the agarose 6 mm apart (center to center) in a straight line. Telfa wicks were wetted with buffer and used to connect the gel with the buffer reservoirs. Electrophoresis was performed with a Bio-Rad electrophoresis cell (model 1400) at 15 to 25 V/cm for 3 to 5-h with circulation of cooling H_2O at 12°. The agarose gels were then pressed and stained as described by Weeke (119).

Affinity Chromatography - All affinity chromatography columns were prepared in 5 ml plastic syringe barrels with a 2 ml bed volume. The columns were equilibrated with at least 10 bed volumes of the buffer in which chromatography was performed. Samples were applied in 1 ml of equilibration buffer and 10 bed volumes of buffer were passed through

the column before specific elution of NADP-GDH was attempted. Affinity chromatography was performed with the buffers at the temperatures indicated in Results.

Reagents - The dansyl amino acids, Tris, DTNB, SDS, ceruloplasm, apoferritin, and nitro-blue tetrazolium were obtained from Sigma Chemical Co.; $(NH_4)_2SO_4$ (Enzyme Grade), L-glutamate, and phenazine methosulfate from Schwarz/Mann; α-ketoglutarate, dithiothreitol, glycine, and catalase (A Grade) from Calbiochem, Inc.; Sephadex G-200 and G-10, chymotrypsinogen, ovalbumin, and aldolase from Pharmacia Fine Chemicals; DE-52 from Whatman, Inc.; agarose, immunoelectrophoresis buffer III, Coomassie Brilliant Blue-R250, ammonium persulfate, and $CaPO_4$ from Bio-Rad Laboratories; acrylamide, N,N' methylene-bis-acrylamide (recrystallized), and Bromophenol Blue from Canalco Co.; polyamide layers from Pierce; Freud's complete adjuvant from Grand Island Biological Co.; gradient gels from Isolab, Inc.; ultrafiltration membranes from Amicon Corp. The following reagents were gifts: 2',5'-ADP Sepharose 4B and Blue Dextran Sepharose 6B from Dr. Richard L. Easterday, Pharmacia Fine Chemicals; NAD ribosyl-hydrazide Sepharose 4B from Dr. James Yuan, Old Dominion University; Porcine MDH from Dr. E. M. Gregory, V.P.I.&.S.U.; norbornane 2,3-dicarboxylate from Dr. W. G. Niehaus, V.P.I.&S.U.

RESULTS

<u>Mass Culture of Chlorella</u> - The level of the NADP-GDH in Chlorella was observed (18) to be proportional to the growth rate of the cells. Therefore, to insure a high specific activity of the enzyme in cells from which the enzyme was to be purified, the cells were harvested immediately prior to the end of the exponential growth phase of the culture.

To obtain sufficient innoculum for the 35 ℓ Plexiglas chamber used for the mass culture of the cells, the cells were cultured to a turbidity of 1.0 in 5.1 cm glass culture tubes. When the culture turbidity exceeded this value, a rapid decrease in culture pH was accompanied by a loss in exponential growth (Fig. 1,2).

In the 35 ℓ chamber, the growth rate of the culture began to slow in the turbidity range from 1.2 to 2.2 (Fig. 3). Therefore, to maintain the mass culture near its optimum turbidity for yields of cells with highest enzyme specific activity, it was diluted semi-continuously with fresh medium (Fig. 3). At each dilution, sufficient culture (i.e., 50% of total culture volume) was harvested to yield approximately 100 ml packed cell volume.

<u>Purification of the NADP-GDH</u> - This inducible GDH was purified to electrophoretic homogeneity in six steps beginning with a 100,000 x *g* supernatant from a cell homogenate (Table 1). Essentially all of the NADP-GDH activity in cell homogenates was recovered in the 100,000 x *g* supernatant. Analytical polyacrylamide gel electrophoresis was used

to evaluate the degree of homogeneity of the enzyme at different stages of purification (Fig. 4).

The elution patterns for total protein and NADP-GDH activity were used to select fractions with high specific activity of the enzyme from the Sephadex G-200 (Fig. 5) and DE-52 (Fig. 6) columns. Although all of the NAD-GDH isozyme was removed from the crude enzyme preparation in the initial freeze-thaw step, it would have been separated from the NADP-GDH in the DE-52 column chromatography step. The NAD-GDH was shown in early studies to bind to the DE-52 column more tightly than the NADP-GDH. A concentration of 0.24-0.28 M NaCl in 0.01 M Tris-HCl buffer was required to elute the NAD-GDH from the column.

Preparative polyacrylamide gel electrophoresis was the only fractionation method tested, until recently (see later discussion of affinity chromatography), which completely separated the contaminating proteins which remained with the NADP-GDH after the calcium phosphate step. However, because the enzyme preparation (i.e., 17-20 mg) from the calcium phosphate step was comprised of only a few different proteins, the preparative gel electrophoresis system was overloaded and the resolving power of this step was somewhat diminished. Therefore, to obtain electrophoretically pure NADP-GDH, the enzyme preparation from the calcium phosphate step was subjected to two successive rounds of electrophoresis.

With the exception of the preparative gel electrophoresis step, the activity of the NADP-GDH was stable through all of the purification steps in the absence of added stabilizing agents. Losses of 50% or

Fig. 1. Asynchronous growth of *Chlorella sorokiniana* cultured in a 5.1 cm glass tube.

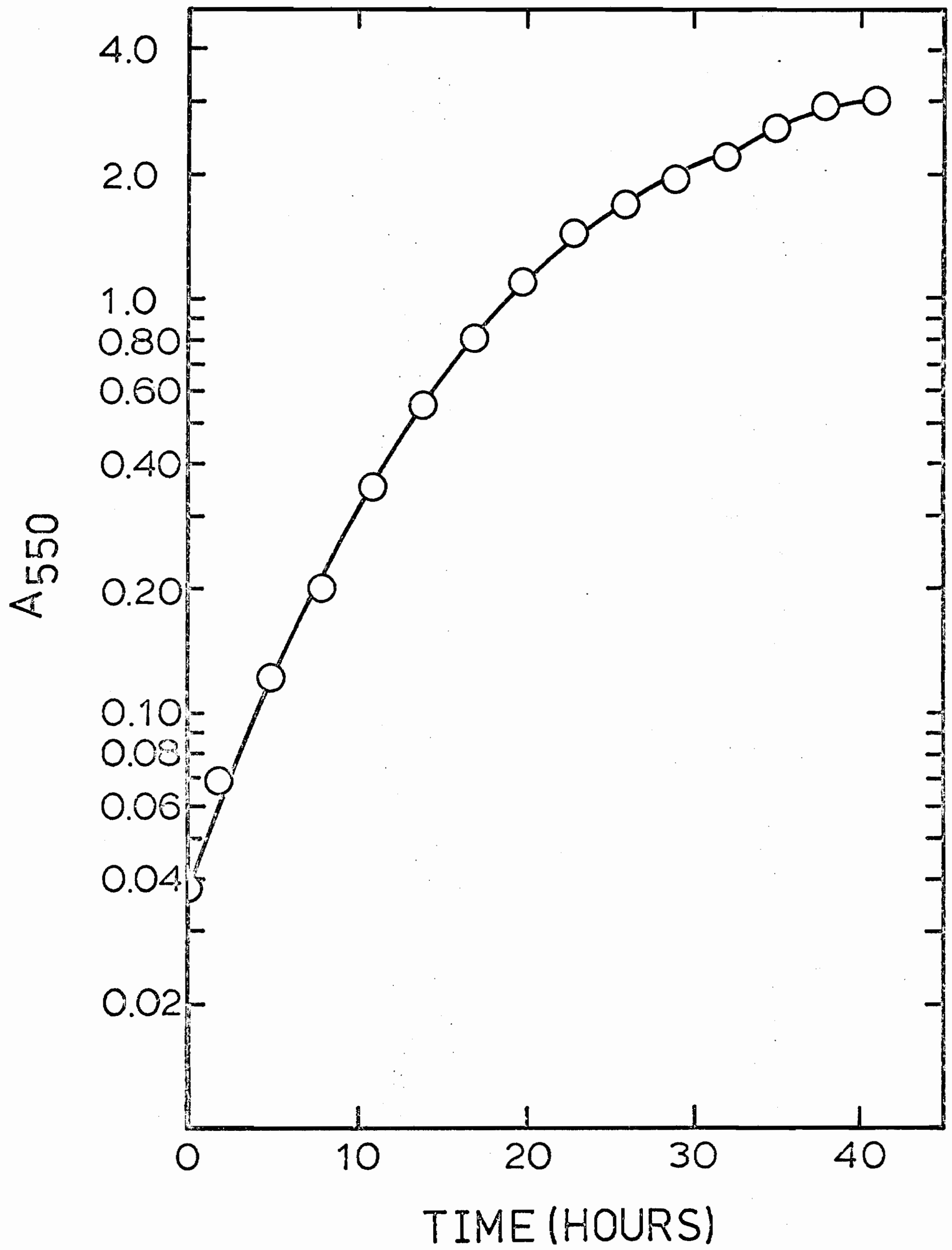

4.0
2.0
1.0
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0.04
0.02
A550
0
10
20
30
40
TIME (HOURS)

Fig. 2. Change in pH of medium during asynchronous growth of *Chlorella sorokiniana* in a 5.1 cm glass tube.

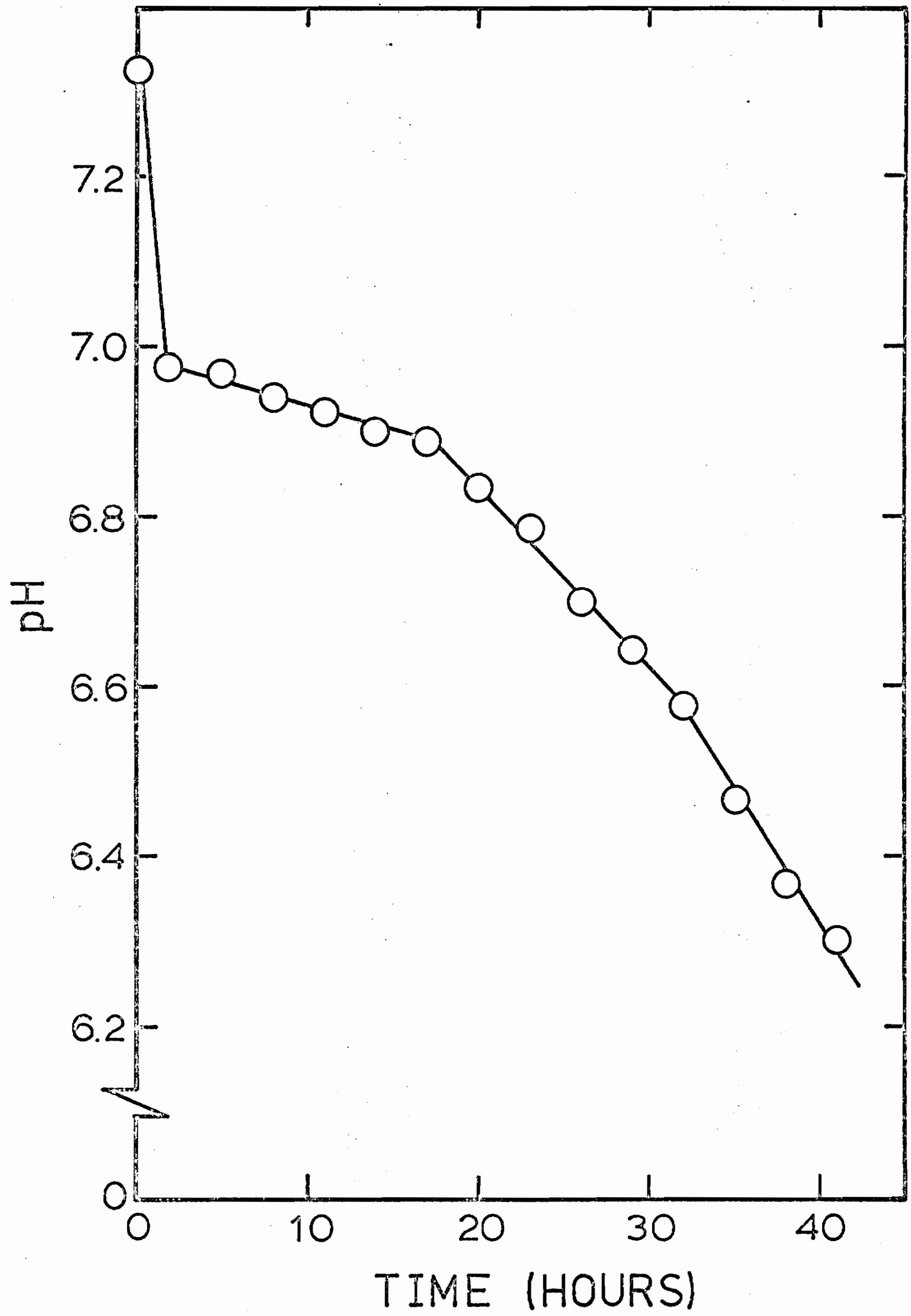

7.2
7.0
6.8
pH
6.6
6.4
6.2
0
0
10
20
30
40
TIME (HOURS)

Fig. 3. Asynchronous growth of *Chlorella sorokiniana* in a 35 liter Plexiglas chamber. The first two arrows indicate times of harvest of 15 liters of culture and addition of an equal volume of fresh medium. The third indicates time of harvest of the entire 30 liter culture volume.

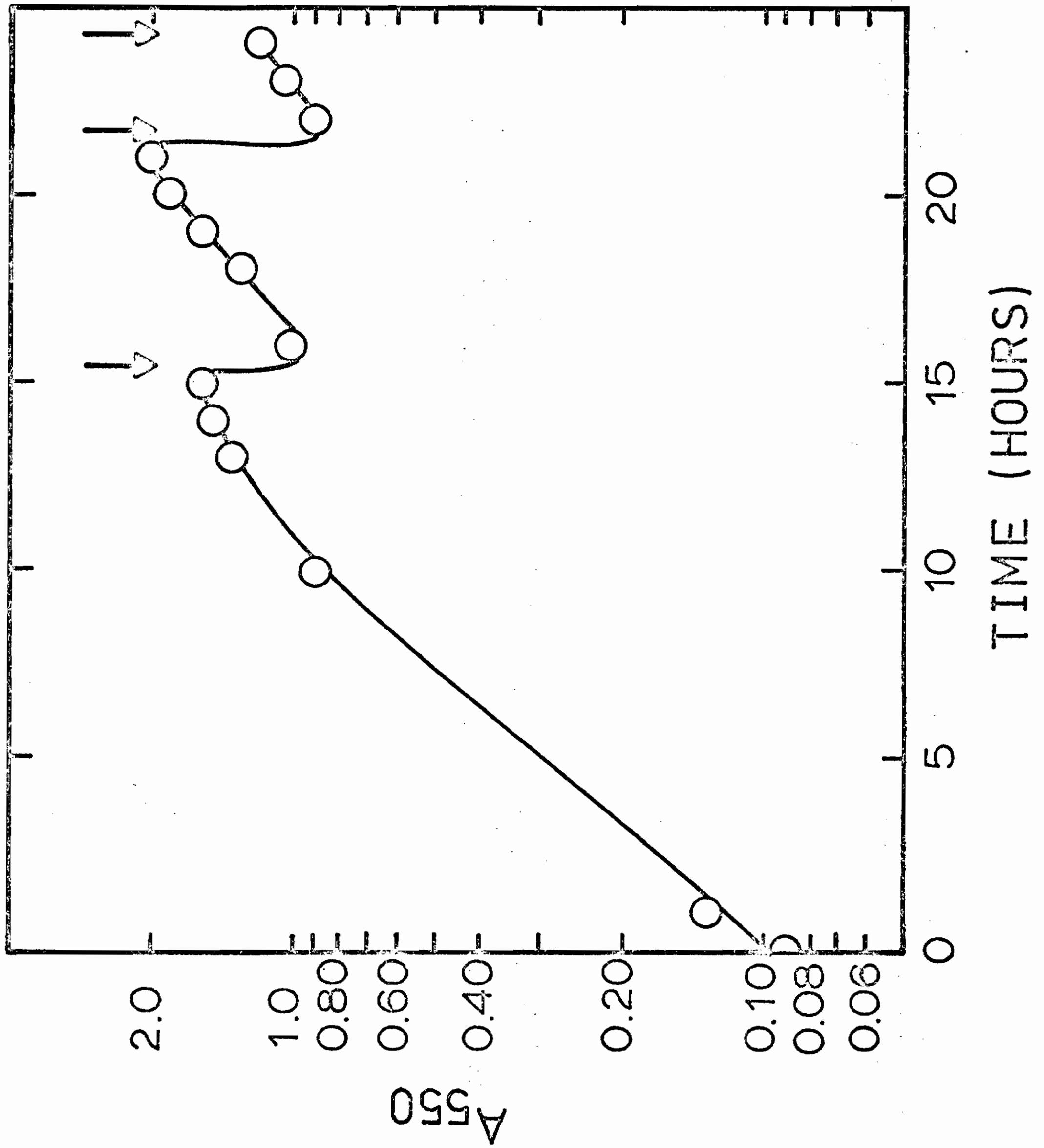

A_{550}
2.0
1.0
0.80
0.60
0.40
0.20
0.10
0.08
0.06
0
5
10
15
20
TIME (HOURS)

TABLE I

PURIFICATION PROCEDURE OF NADP-GLUTAMATE DEHYDROGENASE FROM *CHLORELLA SOROKINIANA*

Step	Total Units[1]	Total Protein (mg)	S.A.[2]	Recovery (%)	Fold Purification[3]
100,000 x *g* Supernatant	9,400	12,000	1.3	100	1
1. Freeze Thaw	8,000	3,300	2.5	85	2
2. Ammonium Sulfate Ppt. (40-70%)	6,400	910	7.0	68	5
3. G-200 Gel Filtration	5,500	350	16	58	12
4. DE-52 Ion Exchange Chromatography	3,400	82	42	36	32
5. $CaPO_4$ gel	2,900	23	130	31	100
6. Preparative Electrophoresis (2-times)	2,400	7	340[4]	25	260

[1]One unit is defined as the enzyme necessary to oxidize 1 µmole of NADPH/min at 38.5°

[2]Specific activity is defined as units/mg protein

[3]Fold purification is defined as the specific activity of step $_x$/specific activity 100,000 x *g*

[4]Specific activity decreases with storage due to loss of catalytic activity

Fig. 4. Polyacrylamide disc gels (7%) of *Chlorella sorokiniana* NADP-glutamate dehydrogenase at different purification steps. Gels B through F are stained for total protein while A and G are stained for glutamate dehydrogenase activity. A, crude cell extract; B, 100,000 x *g* supernatant; C, 40-70% $(NH_4)_2SO_4$ ppt.; D, Sephadex G-200 gel filtration; E, DE-52 chromatography; F, Preparative electrophoresis; G, Preparative electrophoresis.

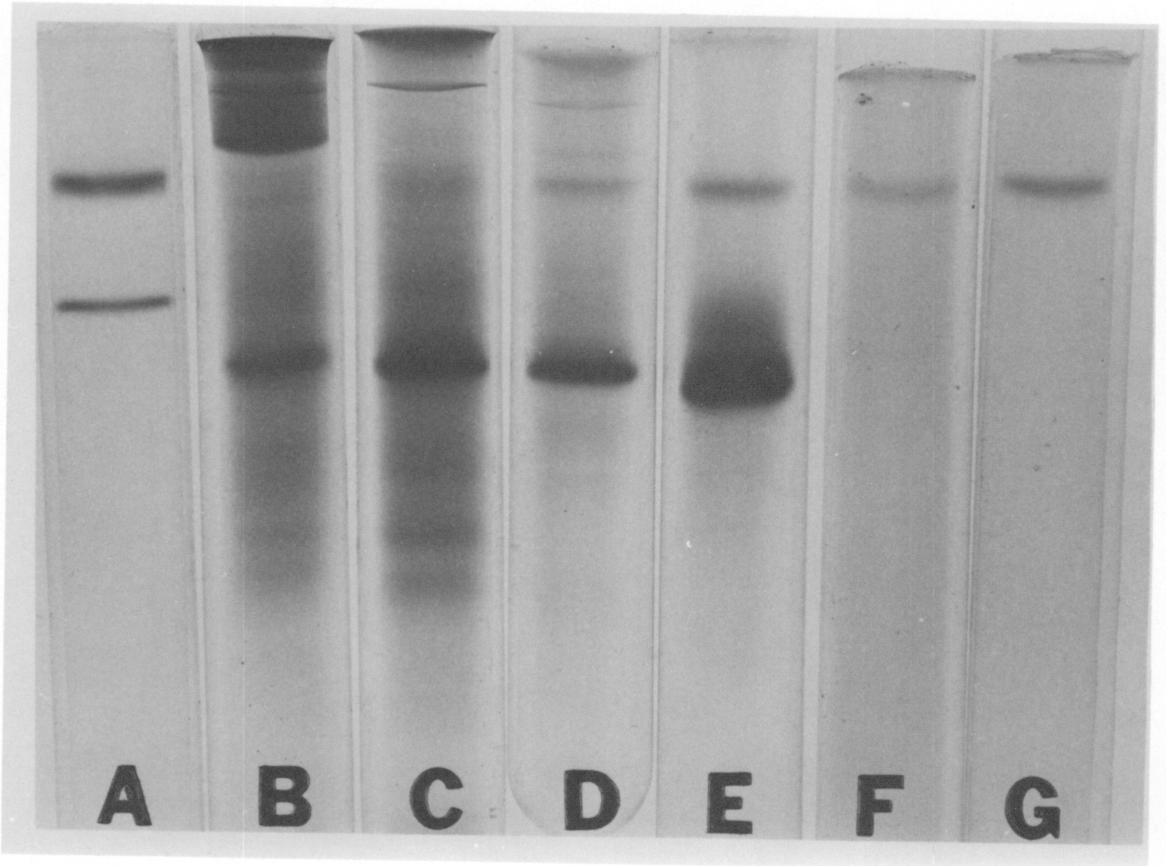

A
B
C
D
E
F
G

Fig. 5. Elution profile of NADP-glutamate dehydrogenase and protein from a Sephadex G-200 column (5 x 39 cm) developed in 0.01 M Tris-HCl (pH 7.4) at 4°. Only the first 25% of the included volume of the column is shown.

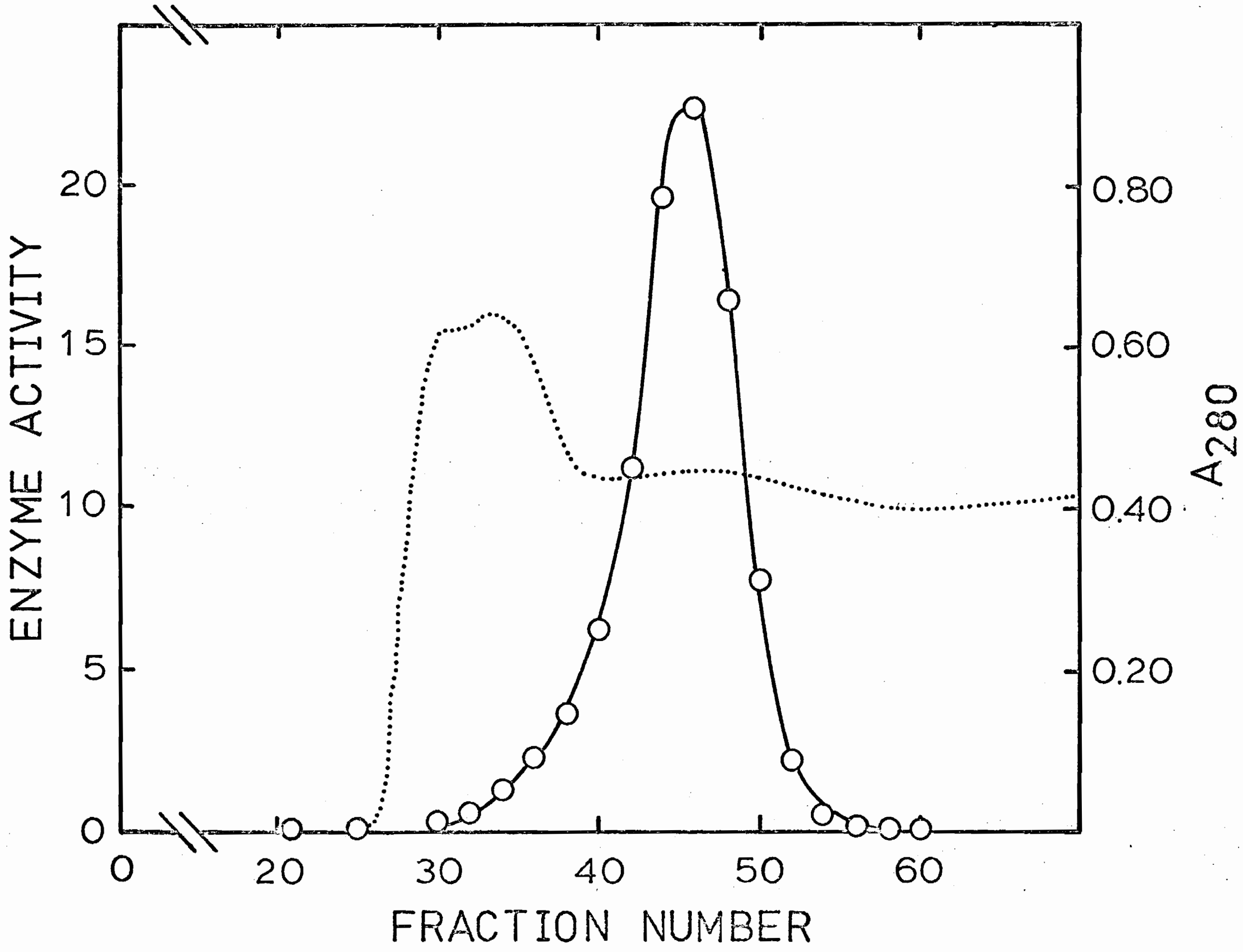
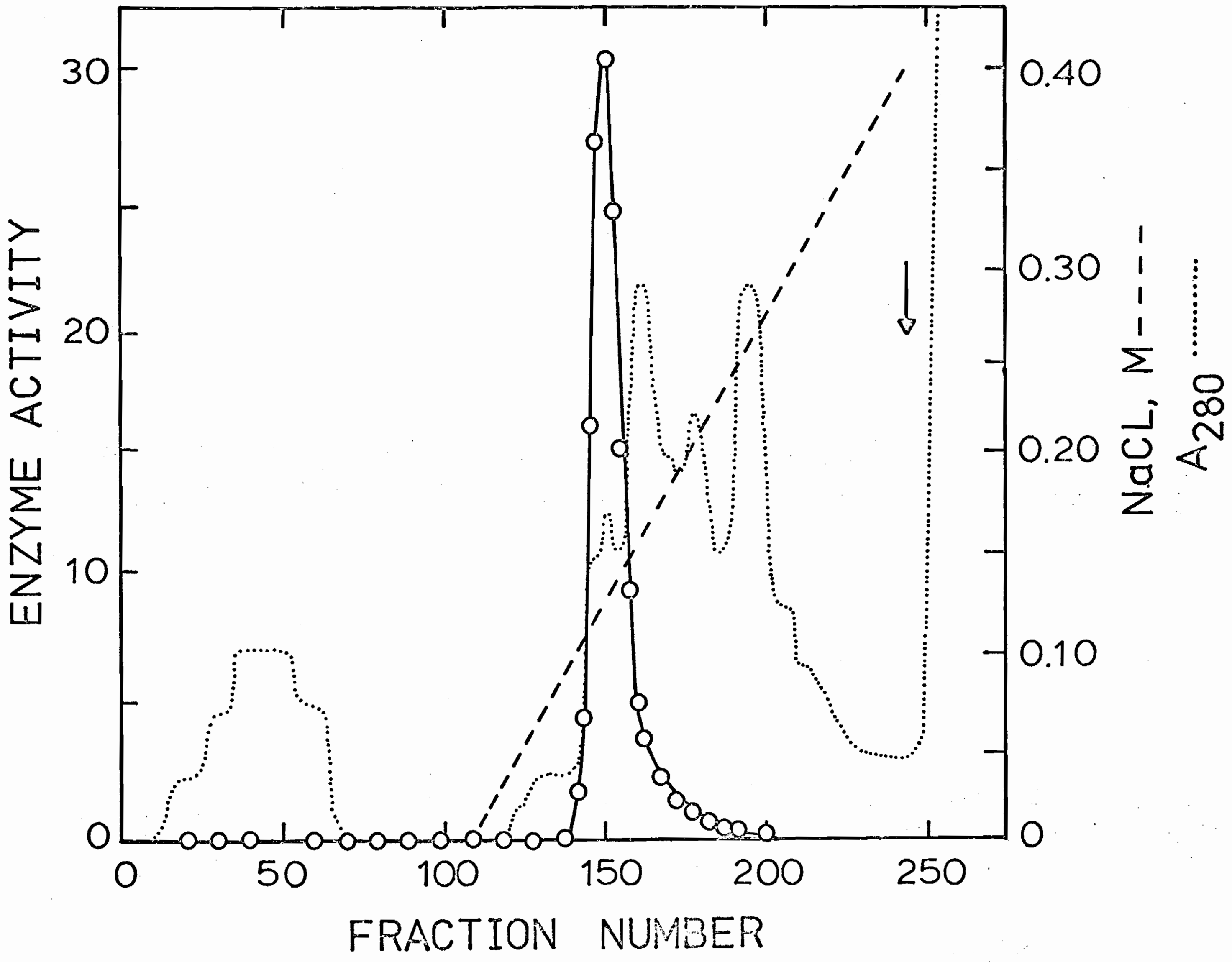

ENZYME ACTIVITY
0
5
10
15
20
FRACTION NUMBER
0
20
30
40
50
60
A_{280}
0.20
0.40
0.60
0.80

Fig. 6. Elution profile of NADP-glutamate dehydrogenase and protein from a DE-52 column (2.5 x 24 cm) preequilibrated with 0.01 M Tris-HCl (pH 7.4), and developed with a linear gradient of 0-0.4 M NaCl in 0.01 M Tris-HCl (pH 7.4). The arrow indicates point of addition of 1 M NaCl to the elution buffer.



ENZYME ACTIVITY
FRACTION NUMBER
NaCL, M ----
A280 ....
0
10
20
30
0
50
100
150
200
250
0
0.10
0.20
0.30
0.40

greater in enzyme activity were initially observed during preparative electrophoresis. By pretreatment of the enzyme preparation with 10 mM dithiothreitol, and the addition of 2 mM dithiothreitol to the buffer reservoirs, 100% of the NADP-GDH activity could be stabilized during preparative gel electrophoresis[1].

Because of the limited capacity of preparative electrophoresis as a last step in a large scale purification procedure, affinity column chromatography was examined as a possible alternative method. Because Blue Dextran Sepharose 6B, 2',5'-ADP Sepharose 4B, and NAD-ribosyl hydrazide Sepharose 4B have been used to purify other dehydrogenases (120-122), the ability of the NADP-GDH to be selectively bound and eluted from these affinity materials was tested.

As a positive control, porcine MDH (100) was shown to bind to Blue Dextran Sepharose 6B in 0.01 M Tris-HCl buffer (pH 7.5 at 22°) and to be specifically eluted with 10 mM NADH. However, the NADP-GDH did not bind to this affinity material under these conditions nor under any other conditions tested (i.e., 0.01 M Tris-Cl, pH 8.25 at 4°, pH 6.90 at 4°; 0.01 M Tris-Cl + 0.2 M L-glutamate, pH 6.5 at 4°). In the presence or absence of L-glutamate, the NADP-GDH also failed to bind to 2',5'-ADP Sepharose 4B in a number of different buffers (i.e., 0.01 M sodium maleate, pH 6.2 at 22°; 0.01 M sodium norbornane 2,3 dicarboxylate, pH 7.2 at 22°). The enzyme did bind to an affinity column of NAD-ribosyl hydrazide Sepharose 4B and could be specifically eluted with 10 mM $NADP^{+}$ (Fig. 7). As a first step in the evaluation of this

[1]A. T. Yeung, personal communication.

latter affinity material in the purification of the NADP-GDH, the enzyme recovered in the 40% to 70% ammonium sulfate fraction was bound to the column. After washing with 10 volumes of buffer, the enzyme was eluted with $NADP^+$. The fractions containing NADP-GDH activity were subjected to analytical disc gel electrophoresis. Although the enzyme was still contaminated with a few other proteins, those proteins which are normally present after the calcium phosphate purification step had been removed. Thus, it is tentatively concluded that, affinity chromatography with this latter material, can replace preparative gel electrophoresis as the last step in the large scale purification of the NADP-GDH.

Because selective heat-denaturation of contaminating proteins has been used as a purification step for certain enzymes, the heat stability characteristics of both the NAD- and NADP-GDH isozymes in crude cell homogenates were tested early in the development of the NADP-GDH purification procedure. Both GDH isozymes were essentially stable at 50° for 15 min. However, because negligible protein was precipitated at this temperature, no significant enzyme purification was achieved. Whereas at 60° for 15 min the NAD-GDH activity was stable, the NADP-GDH activity decayed rapidly (i.e., $t_{\frac{1}{2}}$ = 3-4 min). It should be noted that NADPH enhanced the rate of heat inactivation of NADP-GDH (Table II). Thus, although selective heat denaturation did not prove to be feasible for the purification of the NADP-GDH, the observation, that the NAD-GDH can withstand prolonged high temperature, led Meredith (20) to use a heat treatment step (i.e., 65°, 30 min) in the purification of the NAD-GDH.

Although dithiothreitol stabilized the activity of the NADP-GDH during preparative electrophoresis, this reducing agent (10 mM) caused the loss in activity of the enzyme in the purified state or in crude cell extracts during storage at -20°. In the absence of dithiothreitol, the activity of the enzyme in crude cell extracts was stable at -20°. In the absence of added substrates, the activity of the purified enzyme was stable for several days in 0.1 M Tris-HCl buffer at 4°; however, at -20° for one week, 50% of its activity was lost. NADPH (1 mM) was the only substrate which enhanced the loss in enzyme activity at -20°.

The NADP-GDH was judged to be homogenous by analytical disc gel electrophoresis (Fig. 4), gradient gel electrophoresis (Fig. 8), sedimentation equilibrium centrifugation, and by two different SDS polyacrylamide gel electrophoresis systems (110, 124).

Molecular Weight of NADP-GDH Protomer and Subunit - From the elution volumes of protein standards and of the purified NADP-GDH from a Sephadex G-200 column, the molecular weight of the native enzyme was estimated to be 400,000 (Fig. 9). Polyacrylamide gel electrophoresis by the method of Ferguson (108) and gradient polyacrylamide gel electrophoresis gave molecular weights of 400,000 and 420,000, respectively (Fig. 10, 11).

Because the aforementioned methods are greatly influenced by the shape of a protein molecule, equilibrium centrifugation was also employed to estimate the molecular weight of the native enzyme. By this latter method, the molecular weight of the enzyme was calculated to be 290,000 $\pm$ 20,000. By use of sucrose density centrifugation, Talley *et al.* (18)

Fig. 7. Elution of NADP-glutamate dehydrogenase from a column of NAD-ribosyl hydrazide Sepharose 4B equilibrated in 0.01 M sodium maleate (pH 6.2) at 22°. The first arrow indicates elution with 10 mM $NADP^{+}$ in 0.01 M sodium maleate, and the second arrow indicates elution with 2M NaCl in 0.01 M sodium maleate.

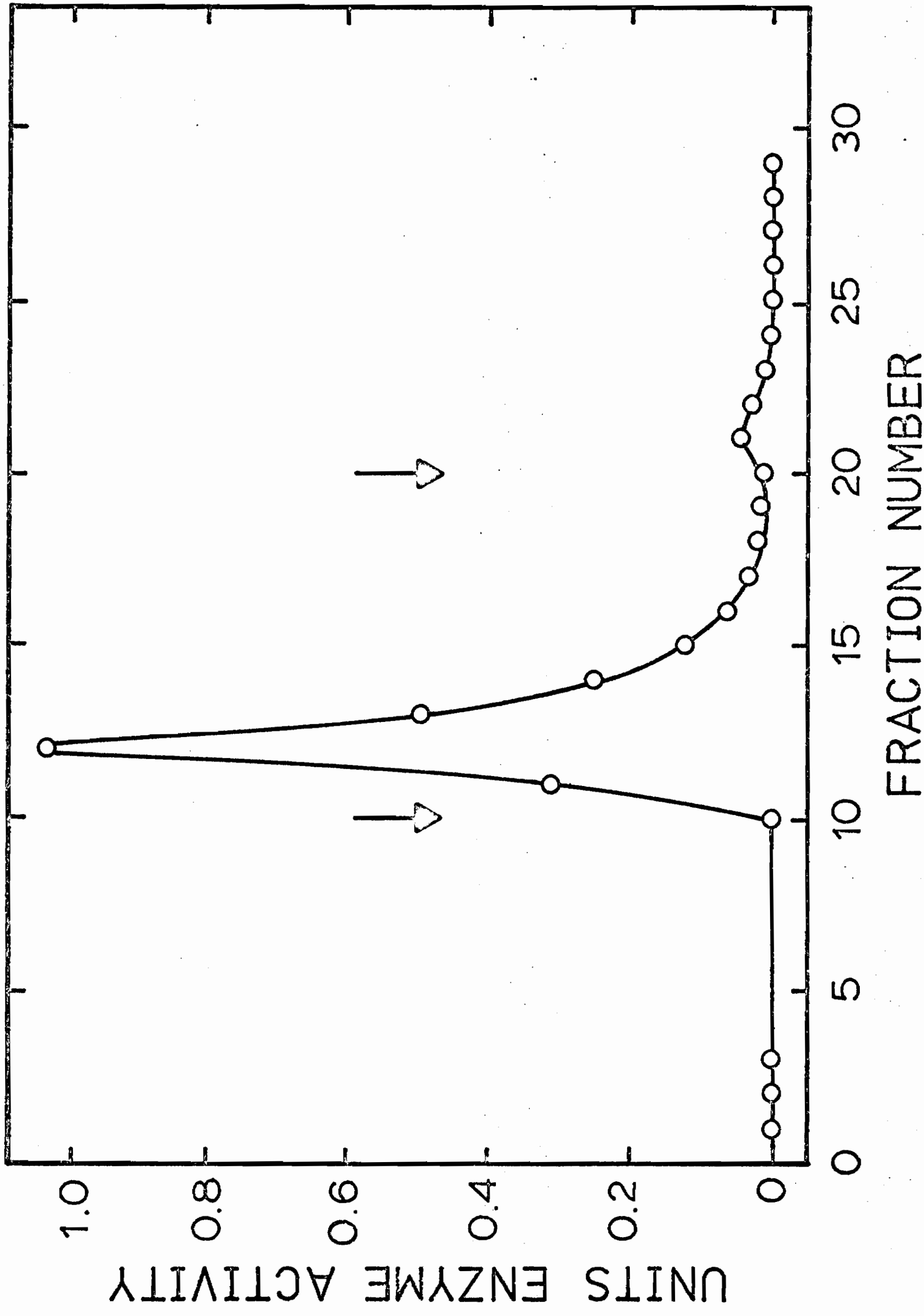
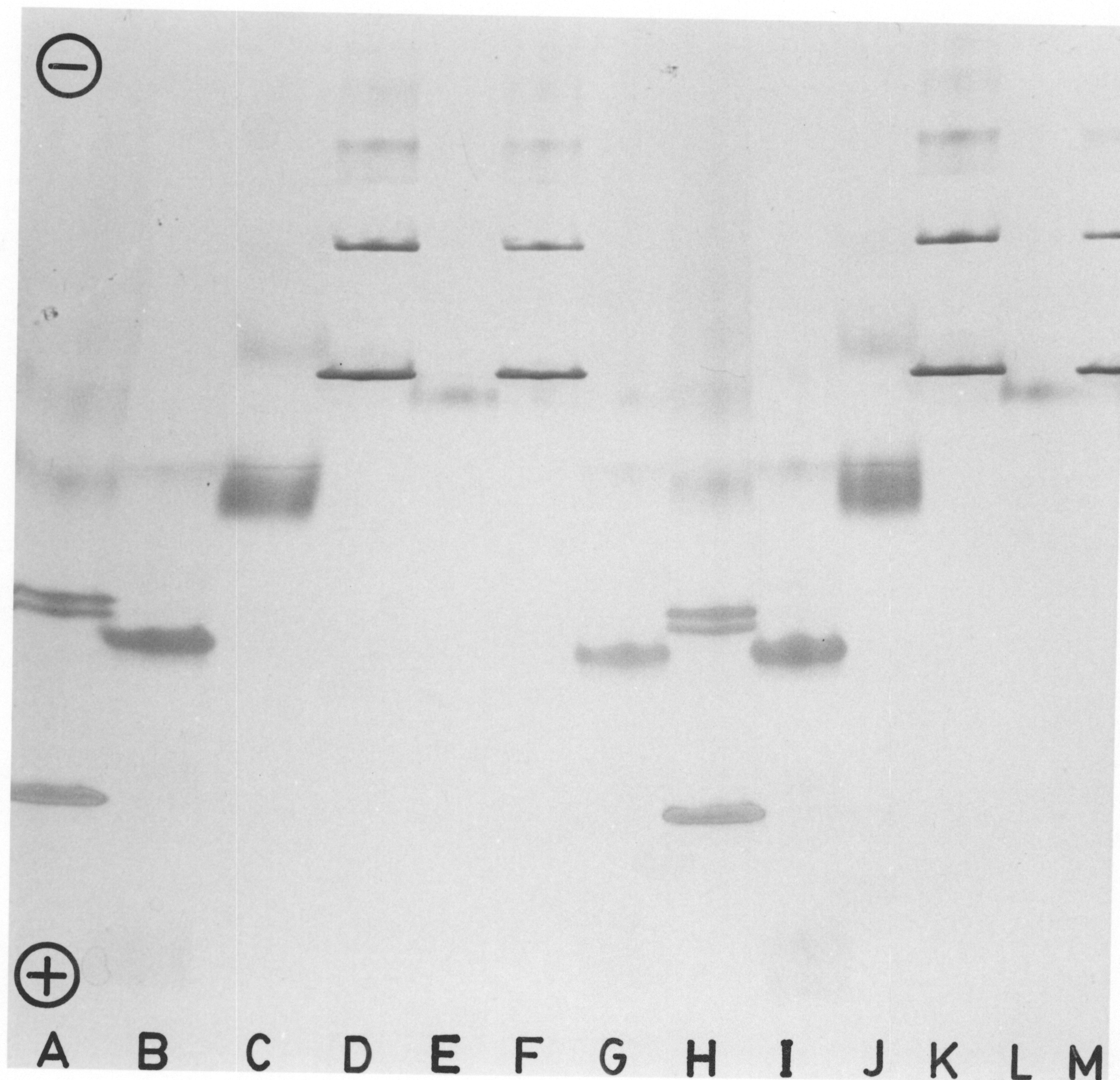

UNITS ENZYME ACTIVITY
1.0
0.8
0.6
0.4
0.2
0
FRACTION NUMBER
0
5
10
15
20
25
30

TABLE II

EFFECT OF NADPH ON DECAY OF NADP-GDH FROM *CHLORELLA SOROKINIANA* DURING HEATING AT 50° FOR 5 MIN

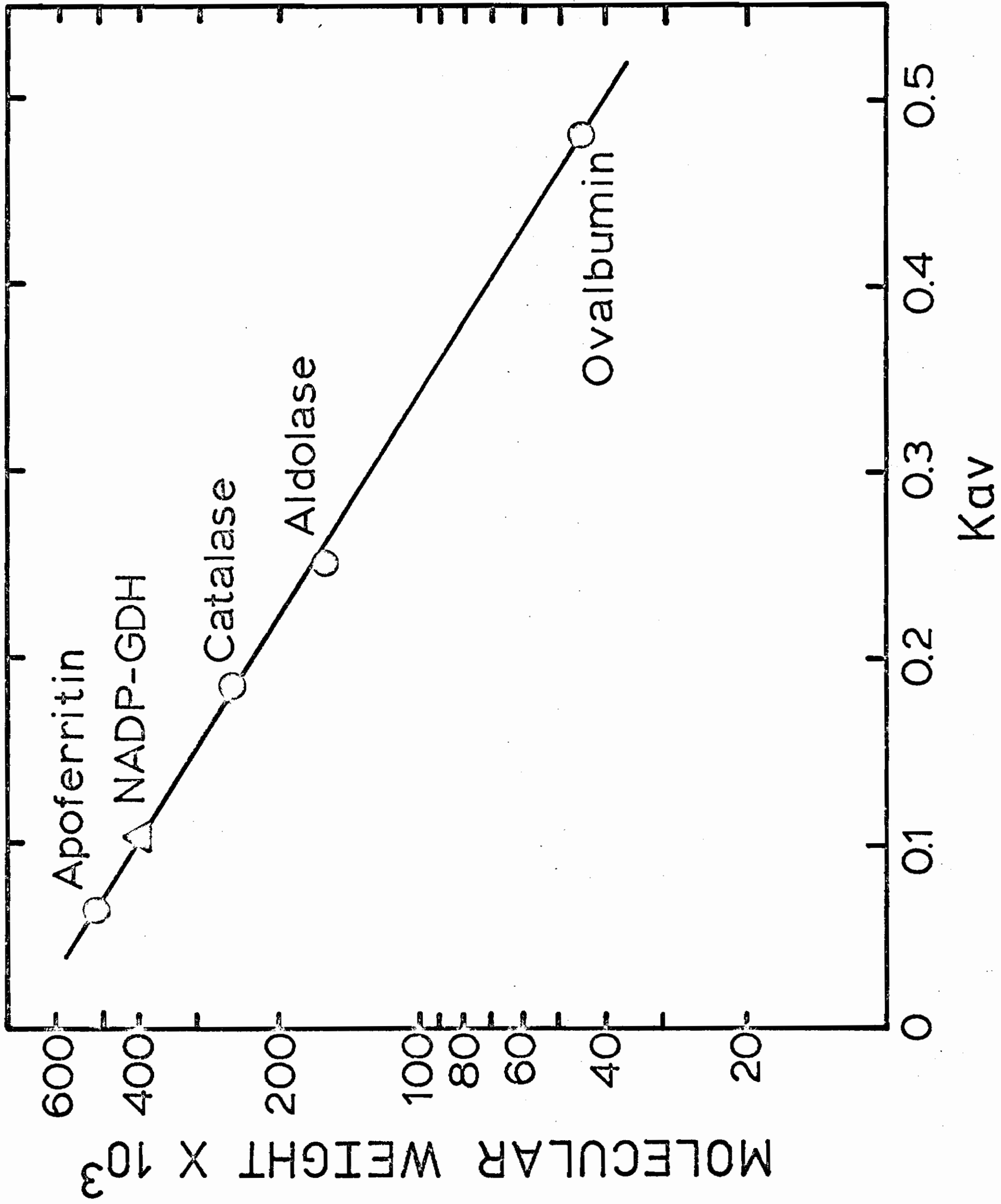
NADP, mM	% Initial Activity
NONE	100
0.33	65
0.73	12

Fig. 8. Gradient polyacrylamide slab gel (2.5 to 27%) of NADP-glutamate dehydrogenase and molecular weight standards. A and H, Bovine serum albumin; B, G and I, Ceruloplasm; C and J, Catalase; D, F, K and M, Apoferritin; E and L, NADP-glutamate dehydrogenase.



⊖
⊕
A B C D E F G H I J K L M

Fig. 9. Determination of the molecular weight of native NADP-glutamate dehydrogenase by chromatography on a column of Sephadex G-200.



Apoferritin
NADP-GDH
Catalase
Aldolase
Ovalbumin
MOLECULAR WEIGHT X 10^3
600
400
200
100
80
60
40
20
Kav
0
0.1
0.2
0.3
0.4
0.5

Fig. 10. Estimation of molecular weight of native NADP-glutamate dehydrogenase from *Chlorella sorokiniana* from the relationship of the retardation coefficients (Kr) of the enzyme with those of standard proteins (Bovine serum albumin monomer (1), dimer (2), and trimer (3), and Apoferritin). The Kr of the standards and NADP-glutamate dehydrogenase were determined from a Ferguson plot.

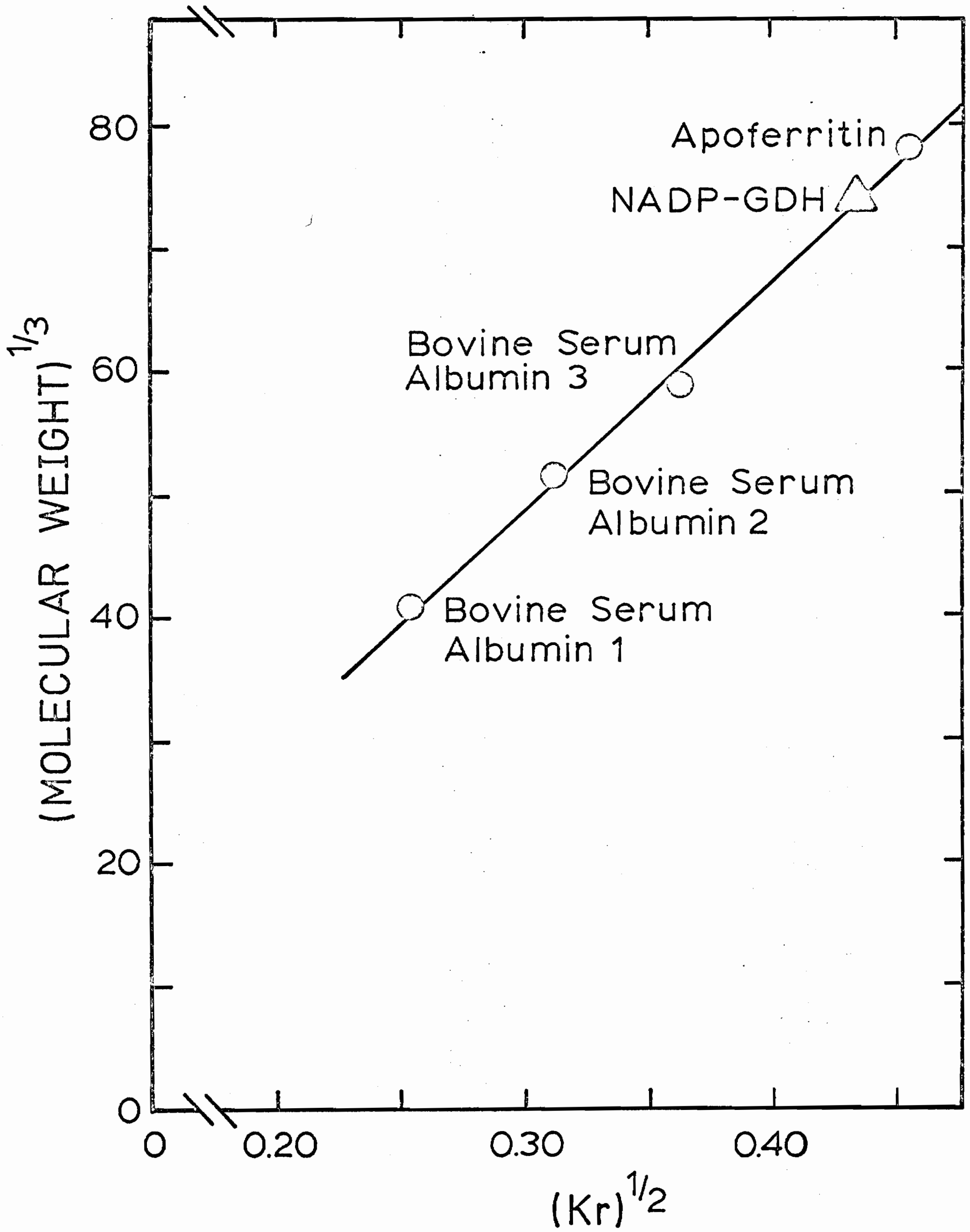
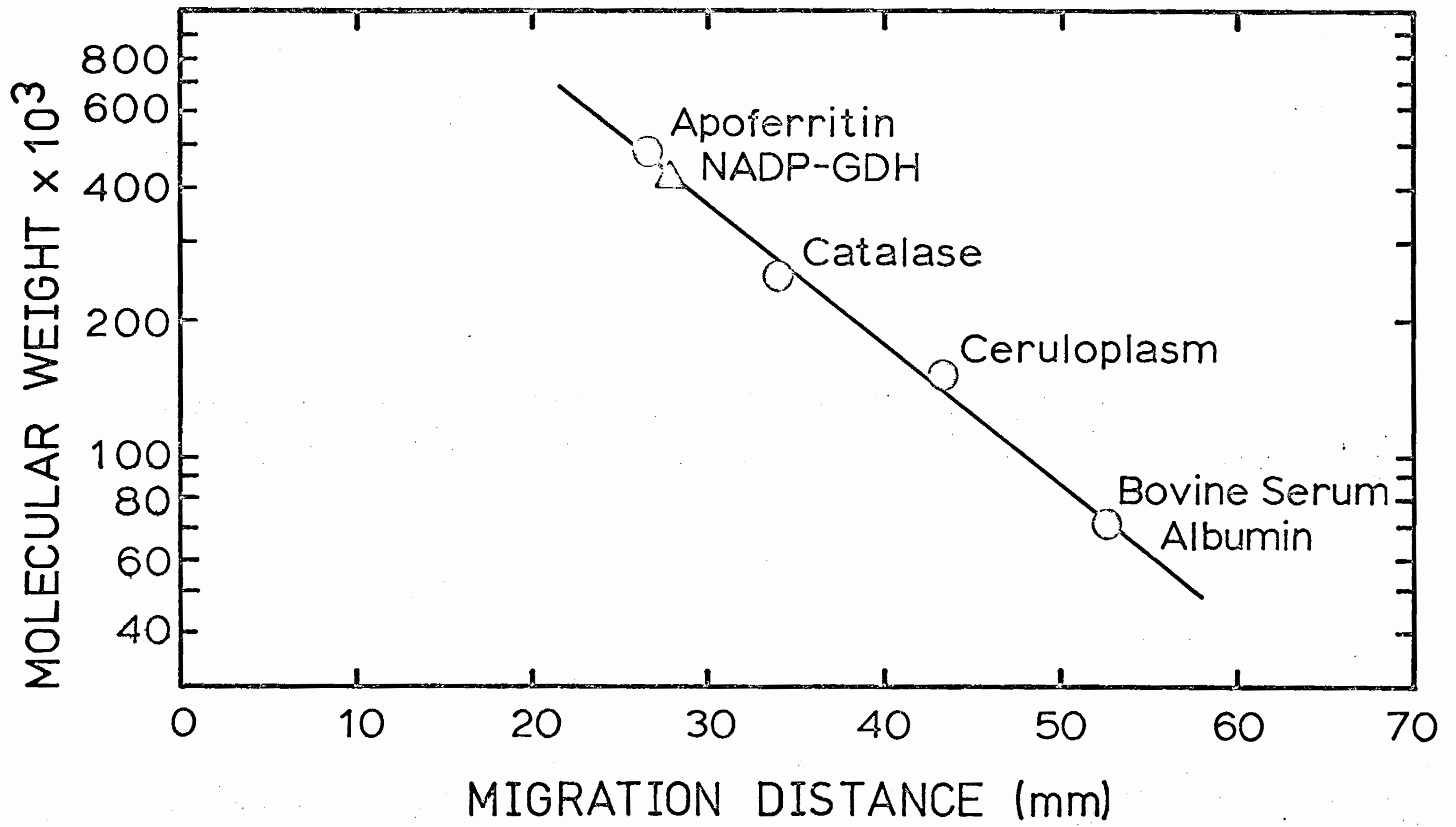

80
60
40
20
0
(MOLECULAR WEIGHT)$^{1/3}$
0
0.20
0.30
0.40
$(Kr)^{1/2}$
Apoferritin
NADP-GDH
Bovine Serum
Albumin 3
Bovine Serum
Albumin 2
Bovine Serum
Albumin 1

Fig. 11. Estimation of molecular weight of native NADP-glutamate dehydrogenase from *Chlorella sorokiniana* by measurement of the migration distance of the enzyme and standards after electrophoresis on a gradient polyacrylamide slab gel (2.5 to 27%).



MOLECULAR WEIGHT × 10^3
800
600
400
200
100
80
60
40
Apoferritin
NADP-GDH
Catalase
Ceruloplasm
Bovine Serum
Albumin
0
10
20
30
40
50
60
70
MIGRATION DISTANCE (mm)

estimated the molecular weight of this same enzyme to be 270,000 ± 20,000.

When similar techniques were used to estimate the molecular weight of Chlorella nitrate reductase, large differences among these estimates were also observed (123). These differences have been proposed (123) to occur with "cigar-shaped" protein molecules.

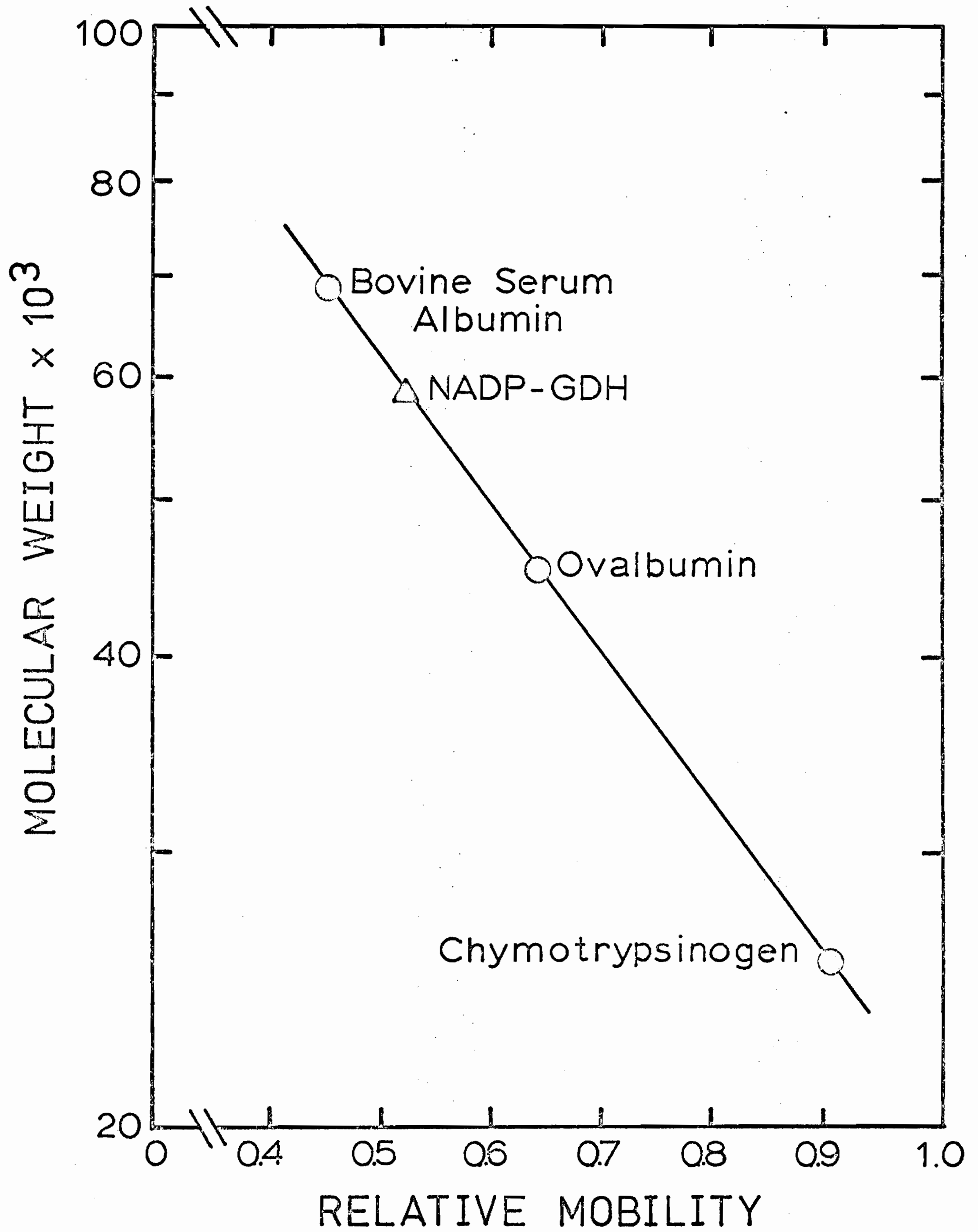
By use of SDS polyacrylamide gel electrophoresis, with two different gel concentrations in two different buffers, the subunit(s) of the dissociated NADP-GDH was shown to migrate as a single molecular weight species of 58,000 ± 3,000 (Fig. 12). Thus, the native NADP-GDH appears to be composed of 5-7 subunits.

Amino Acid Composition of NADP-GDH - The amino acid composition of the NADP-GDH was measured (Table III) and shown to be quite different from the composition reported by Meredith (20) for the NAD-GDH in this same organism. Whereas glycine, glutamate, aspartate, and alanine were in highest concentration in the NADP-GDH, tryptophan, histidine, and cysteine were in lowest concentration in this enzyme.

Because the cysteine content of the NADP-GDH was measured by two independent methods, the value of six cysteines per subunit was used to calculate a subunit molecular weight of 59,500. This molecular weight estimate is in good agreement with the molecular weight (i.e., 58,000 ± 3,000) estimated by SDS polyacrylamide gel electrophoresis (Fig. 12).

In the native NADP-GDH, six sulfhydryl groups could be titrated with Ellman's reagent (113), i.e., 5,5' dithiobis (2-nitrobenzoic acid). Thus, all of the cysteine residues in the enzyme appear to exist in the

Fig. 12. Molecular weight determination of the subunit(s) of NADP-glutamate dehydrogenase by electrophoresis in 0.1% SDS in 9% polyacrylamide gels.



100
80
60
40
20
0
0.4
0.5
0.6
0.7
0.8
0.9
1.0
MOLECULAR WEIGHT × 10^3
RELATIVE MOBILITY
Bovine Serum Albumin
NADP-GDH
Ovalbumin
Chymotrypsinogen

TABLE III

AMINO ACID COMPOSITIONS OF NADP- AND NAD-GLUTAMATE DEHYDROGENASE ISOZYMES FROM *CHLORELLA SOROKINIANA*

Amino Acid	Residues/Subunit[1]	
	NADP-glutamate dehydrogenase	NAD-glutamate[2] dehydrogenase
Lys	38	27
His	6	8
Arg	28	6
Cys	6	6
Asp (Asn)	53	48
Met	15	6
Thr	24	30
Ser	30	74
Glu (Gln)	55	80
Pro	23	38
Gly	54	35
Ala	50	16
Val	38	8
Ilu	23	7
Leu	39	14
Phe	15	6
Tyr	21	6
Trp	5	6

[1]Subunit molecular weights of NADP and NAD enzymes are 59,500 and 45,000

[2]Data from Reference 20

free sulfhydryl form. Another reagent, 2-nitro-5-thiocyanatobenzoic acid, was used to cleave the NADP-GDH at its cysteine residues. Surprisingly, although the enzyme contains six cysteines, only two peptide cleavage products were detectable by SDS polyacrylamide gel electrophoresis. From their very close proximity on the gels, the molecular weights of each of these peptides were estimated to be close to 24,000. Thus, at least one cysteine residue is located near the center of the protein molecule. The SDS electrophoresis system used would not detect peptides with a molecular weight of less than 8,000. Therefore, the cleavage of cysteine residues clustered near the middle or near the ends of the protein molecule would produce peptides which would migrate faster than the tracking dye and be eluted from the gel. The failure to detect other large peptide cleavage products suggests that the cysteines might be clustered near the middle and/or ends of the protein.

The N-terminal amino acid of the NADP-GDH appears to have its α-amino group blocked. The identical dansylation procedure (115), used in this laboratory to show (20) that lysine is the N-terminal amino acid of the NAD-GDH, indicated that the amino group of the N-terminal acid of the NADP-GDH is not available for dansylation. A number of GDHs from other organisms have been reported (44, 47) to have blocked N-terminal amino acids. The failure to detect a free N-terminal amino acid is consistent with the inference that the NADP-GDH consists of a single type of subunit. However, the enzyme could presumably contain different types of subunits each with blocked N-terminal amino acids.

pH Optima and Michaelis Constants for NADP-GDH - The pH optima for the aminating and deaminating reactions of the NADP-GDH were 7.2 and 9.2, respectively (Fig. 13). Because the enzyme underwent inactivation above pH 9.0, the K_m values for the substrates in the deaminating direction were measured at pH 8.7. At the pH optima for the enzyme, the activity with NAD^+ or NADH was only approximately 0.1% of that with $NADP^+$ and NADPH. The K_m values for NH_4^+, α-ketoglutarate, NADPH, L-glutamate, and $NADP^+$ were 68 mM, 12 mM, 0.13 mM, 32 mM, and 0.038 mM, respectively (Fig. 14-18). The K_m values for glutamate and $NADP^+$ were identical at pH 7.8 and 8.7.[2]

At low substrate concentrations, no cooperative effects of the substrates on NADP-GDH activity were observed (Fig. 19). However, substrate inhibition of enzyme activity was observed at high concentrations of α-ketoglutarate.

Although nucleotides have been reported to affect the activity of GDHs in other organisms (30), the activity of NADP-GDH in the aminating direction was unaffected by 1.0 mM AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, or GTP.

Immunological Studies with NADP-GDH - With antiserum developed in rabbits from purified NADP-GDH, Ouchterlony double-diffusion analysis showed that only a single precipitin band forms with either the purified NADP-GDH or with a crude enzyme preparation purified through the freeze-thaw purification step (Fig. 20). Preimmune serum gave no precipitin

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[2]D. W. Israel, unpublished data

bands with these enzyme preparations. The addition of 0.04% SDS to the crude enzyme preparation caused no obvious change in its precipitin pattern. At a protein concentration of 0.25 μg, the NAD-GDH did not form a precipitin band with NADP-GDH antiserum. However, since 0.5 μg of protein was the lower limit of detection for the purified NADP-GDH, a higher concentration of the NAD-GDH must be tested before it can be concluded that NADP-GDH antibody does not cross-react with the NAD-GDH.

Since "rocket" immunoelectrophoresis (118) is a more sensitive technique than Ouchterlony double diffusion, the homogeneity of the purified NADP-GDH was analyzed by this technique. From the rocket patterns, the purified NADP-GDH appears to be immunologically homogenous (Fig. 21). Moreover, the peak heights of the rockets are proportional to the amount of NADP-GDH antigen present and less than 0.25 μg of the enzyme could be detected (Fig. 22).

Fig. 13. pH dependence of maximal velocity of NADP-glutamate dehydrogenase for the amination (-○-) and deamination (-○-) reaction.

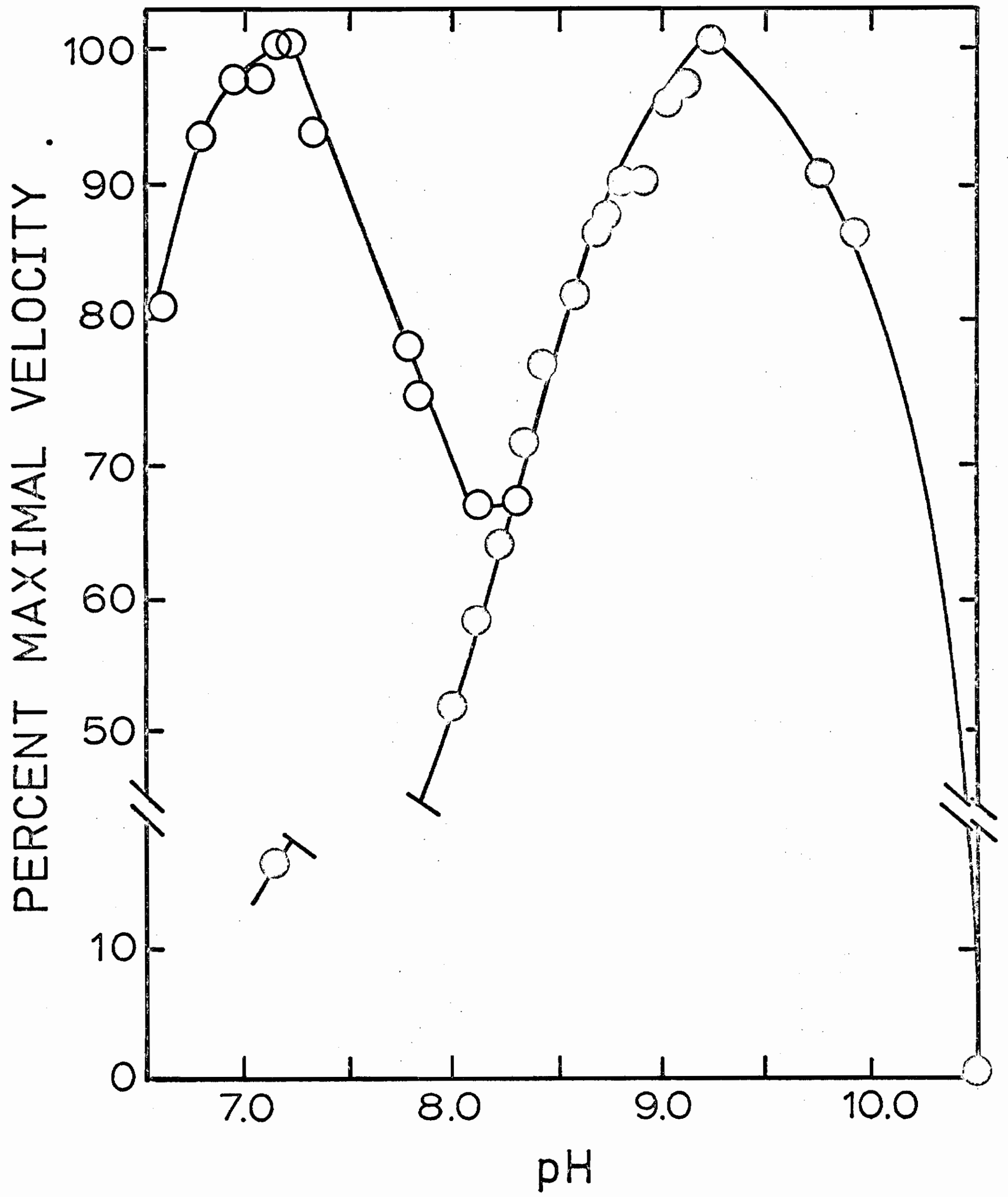
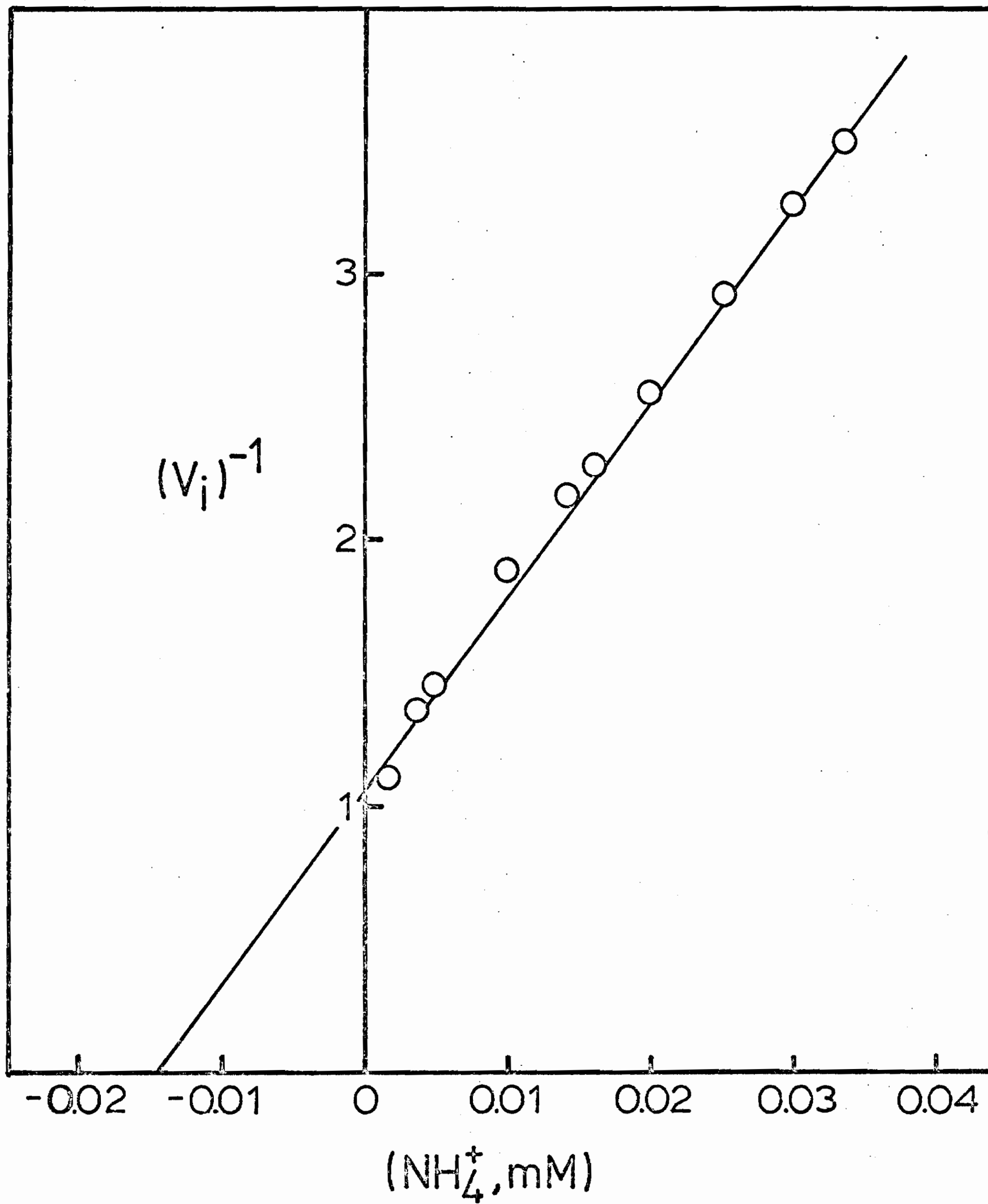

PERCENT MAXIMAL VELOCITY
100
90
80
70
60
50
10
0
7.0
8.0
9.0
10.0
pH

Fig. 14. Lineweaver-Burk plot to determime Km for NH_4^+ for the NADP-glutamate dehydrogenase.



$(V_i)^{-1}$
3
2
1
-0.02
-0.01
0
0.01
0.02
0.03
0.04
(NH_4^+, mM)

Fig. 15. Lineweaver-Burk plot to determine Km for α-ketoglutarate for the NADP-glutamate dehydrogenase.

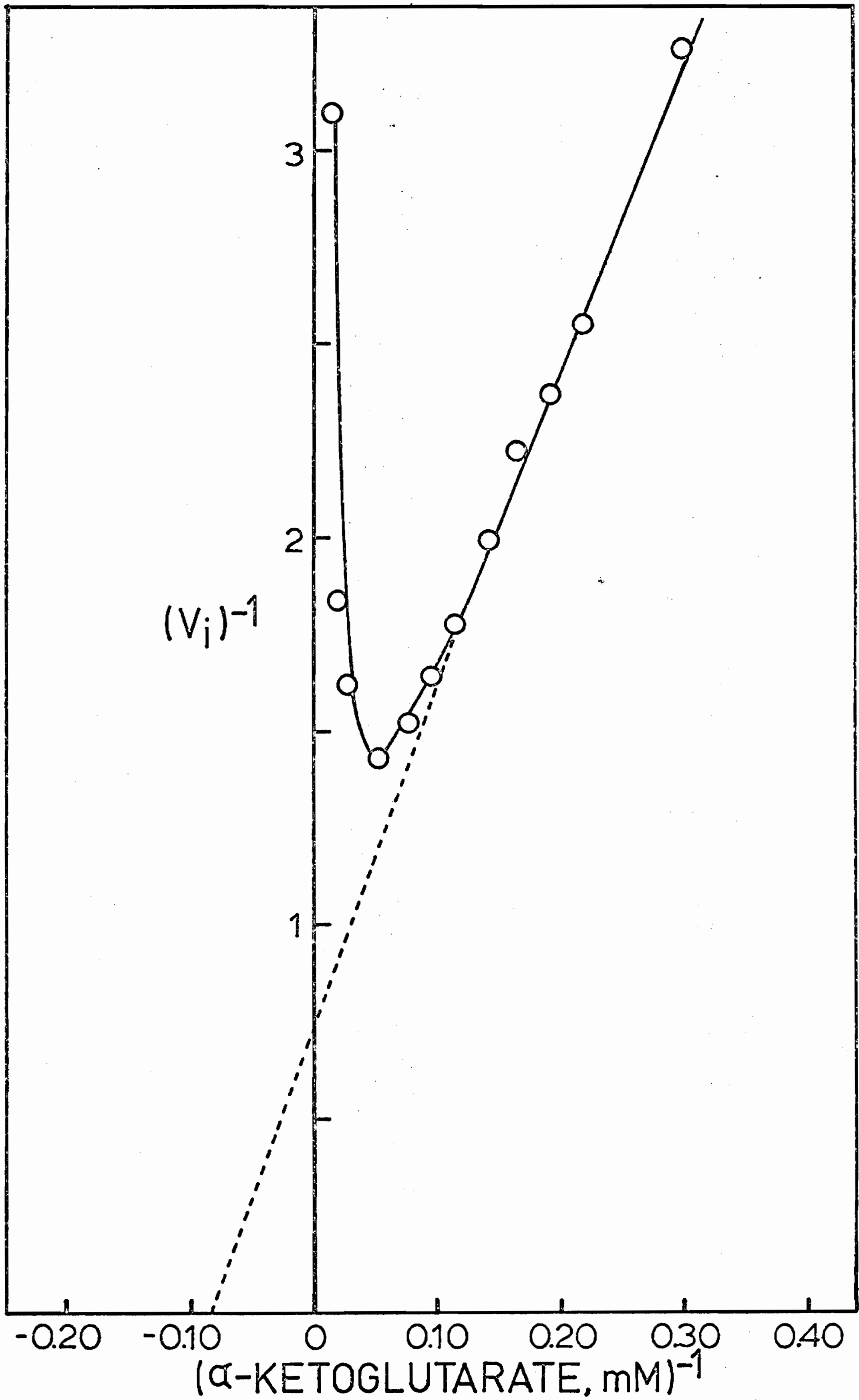

3
2
1
$(V_i)^{-1}$
-0.20
-0.10
0
0.10
0.20
0.30
0.40
(α-KETOGLUTARATE, mM)$^{-1}$

Fig. 16. Lineweaver-Burk plot to determine Km for NADPH for NADP-glutamate dehydrogenase.

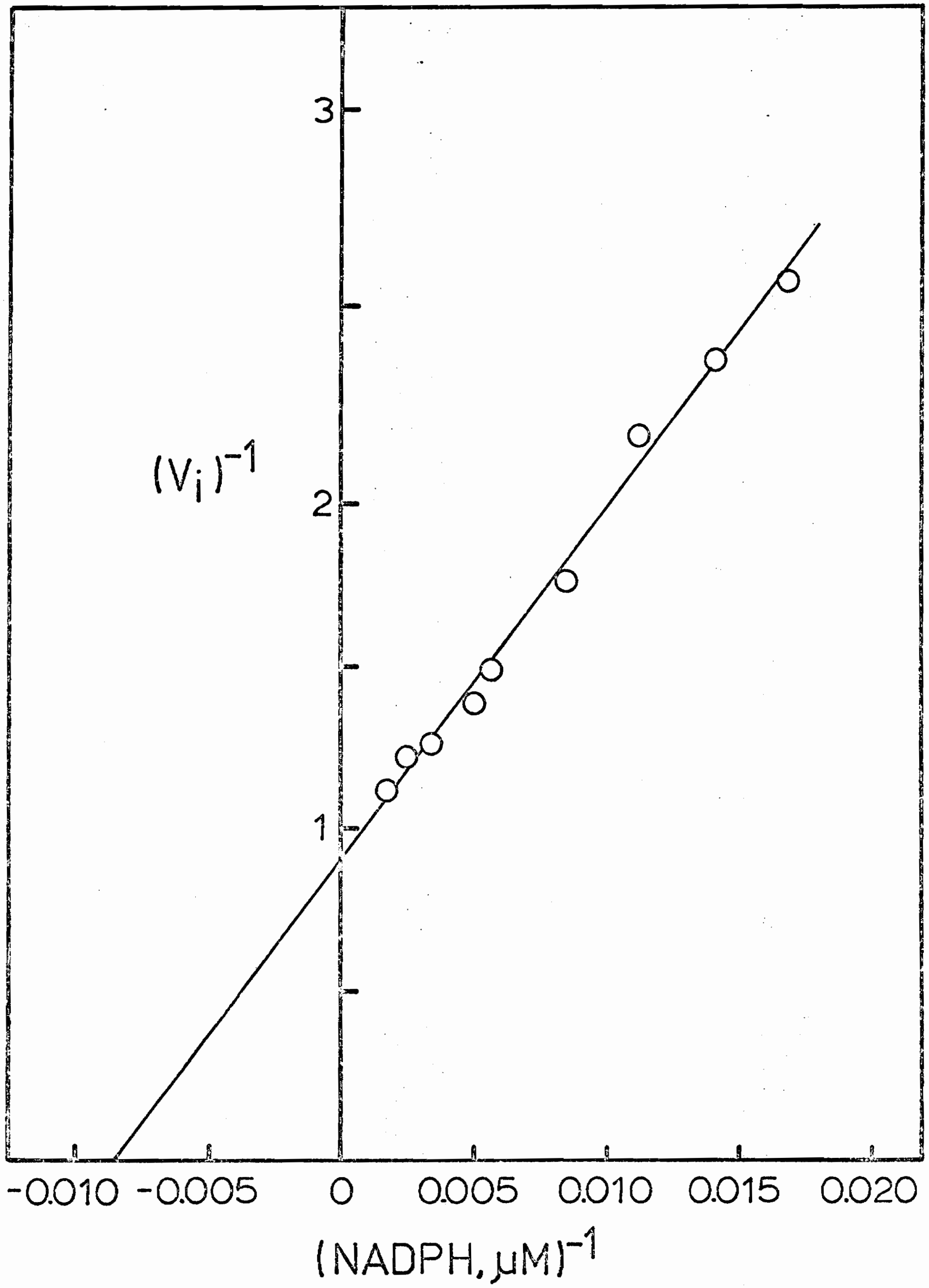
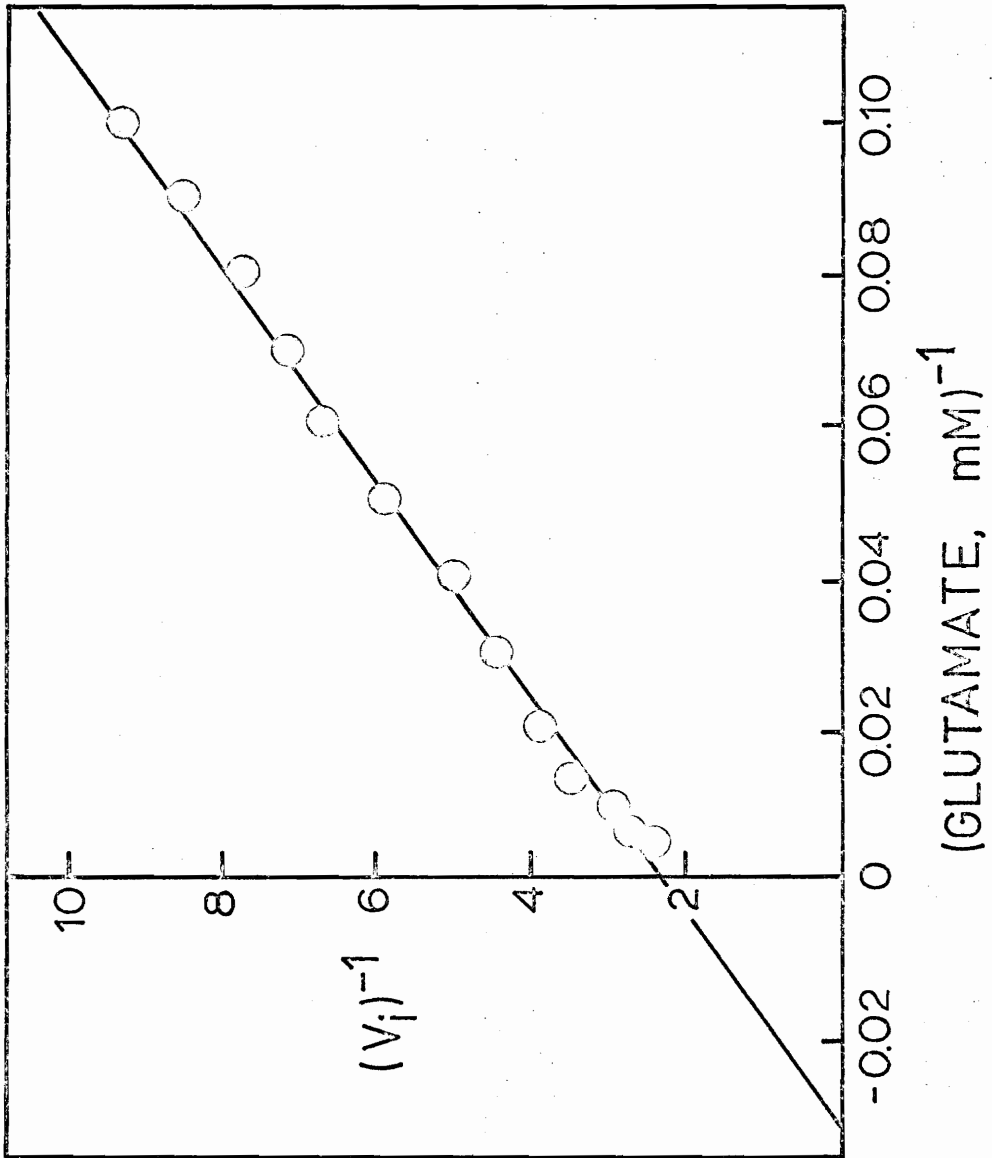

3
2
1
$(V_i)^{-1}$
-0.010
-0.005
0
0.005
0.010
0.015
0.020
$(NADPH, \mu M)^{-1}$

Fig. 17. Lineweaver-Burk plot to determine Km value for L-glutamate for NADP-glutamate dehydrogenase.



$(V_i)^{-1}$
10
8
6
4
2
-0.02
0
0.02
0.04
0.06
0.08
0.10
$(\text{GLUTAMATE, mM})^{-1}$

Fig. 18. Lineweaver-Burk plot to determine Km value for $NADP^+$ for NADP-glutamate dehydrogenase.

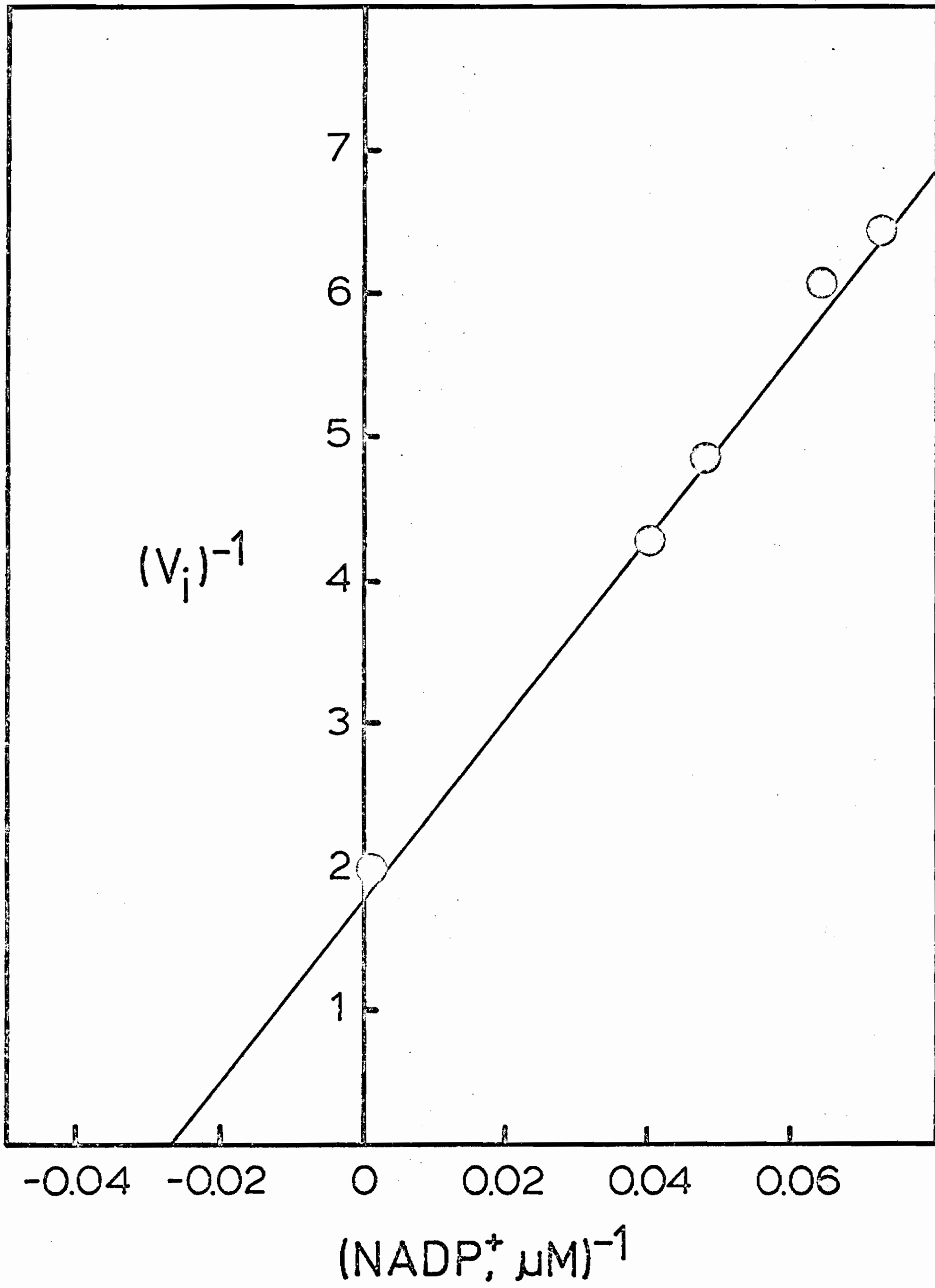
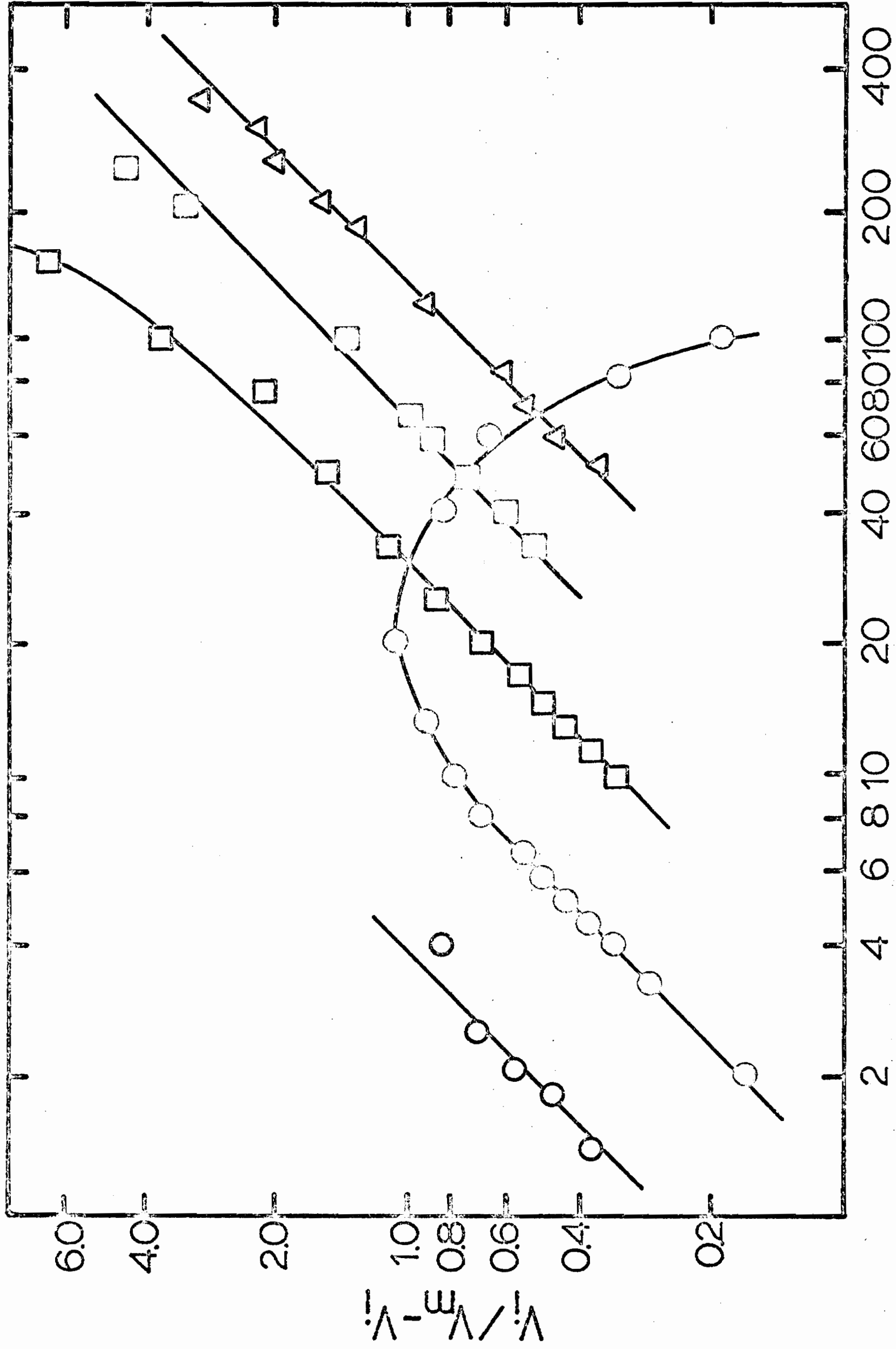

7
6
5
4
3
2
1
$(V_i)^{-1}$
-0.04
-0.02
0
0.02
0.04
0.06
$(NADP^+, \mu M)^{-1}$

Fig. 19. Hill plot to determine the least number of interacting sites for each substrate of NADP-glutamate dehydrogenase. NADP (-○-), α-ketoglutarate (-●-), glutamate (-□-), NH_4^+ (-■-) and NADPH (-△-). Data from Figures 14 to 18.



6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
$V_i/V_m - V_i$
2
4
6
8
10
20
40
60
80
100
200
400
(NADPH, μM)
($NADP^+$, 10×μM)
(NH_4^+, L-GLUTAMATE, α-KETOGLUTARATE, mM)

Fig. 20. Ouchterlony double diffusion analysis in 1% agarose gel. (A) central well, preimmune serum; (B) central well, anti-NADP-glutamate dehydrogenase serum; (A and B) clockwise from upper well, 1 μg pure NADP- glutamate dehydrogenase in 0.04% SDS, 0.5 μg NADP-glutamate dehydrogenase in a crude cell extract, 0.25 μg NAD-glutamate dehydrogenase in a crude cell extract, 0.25 μg pure NADP-glutamate dehydrogenase in 0.04% SDS, 0.25 μg NAD-glutamate dehydrogenase in a crude cell extract in 0.04% SDS, 0.5 μg NADP-glutamate dehydrogenase in a crude cell extract in 0.04% SDS.

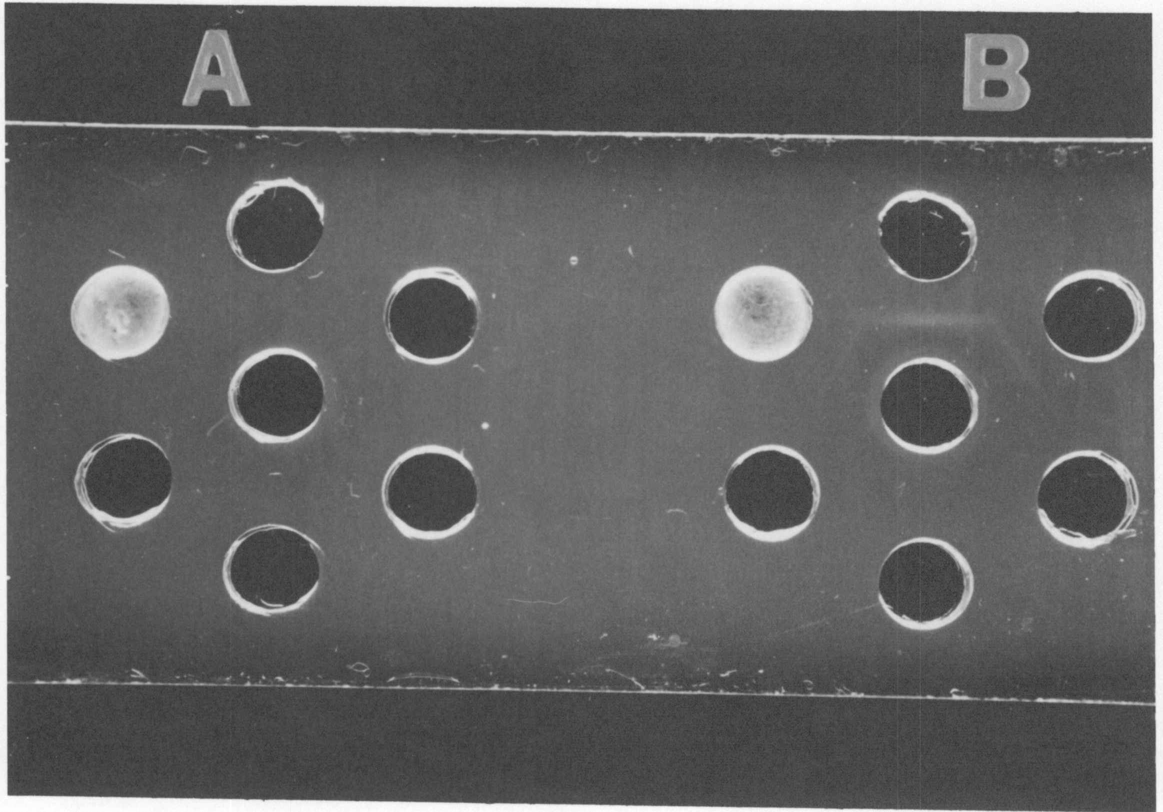

A
B

Fig. 21. "Rocket" immunoelectrophoresis pattern of pure NADP-glutamate dehydrogenase in a 1% agarose gel with 3% antiserum. A, 0.25 μg; B, 0.50 μg; C, 1.0 μg.

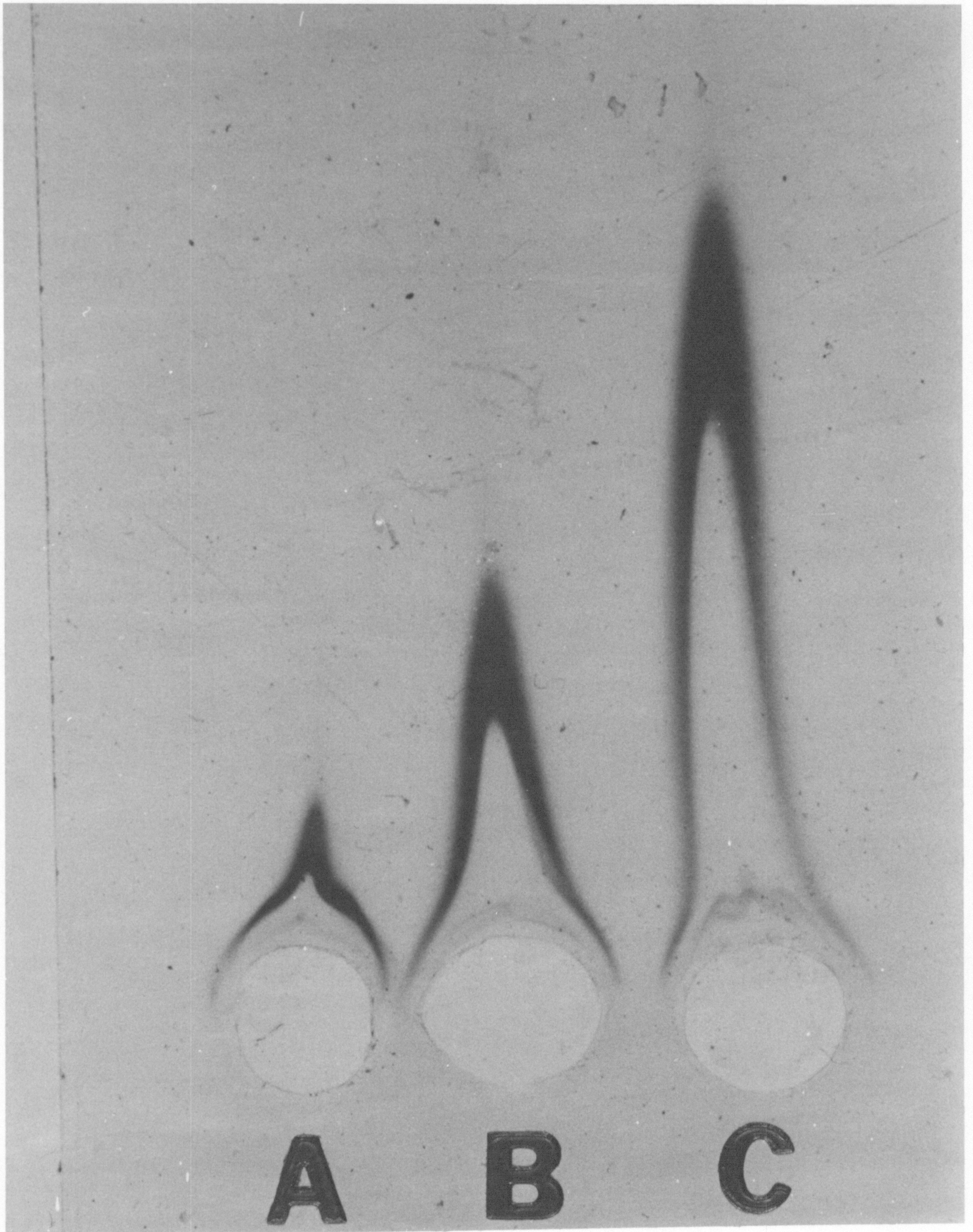
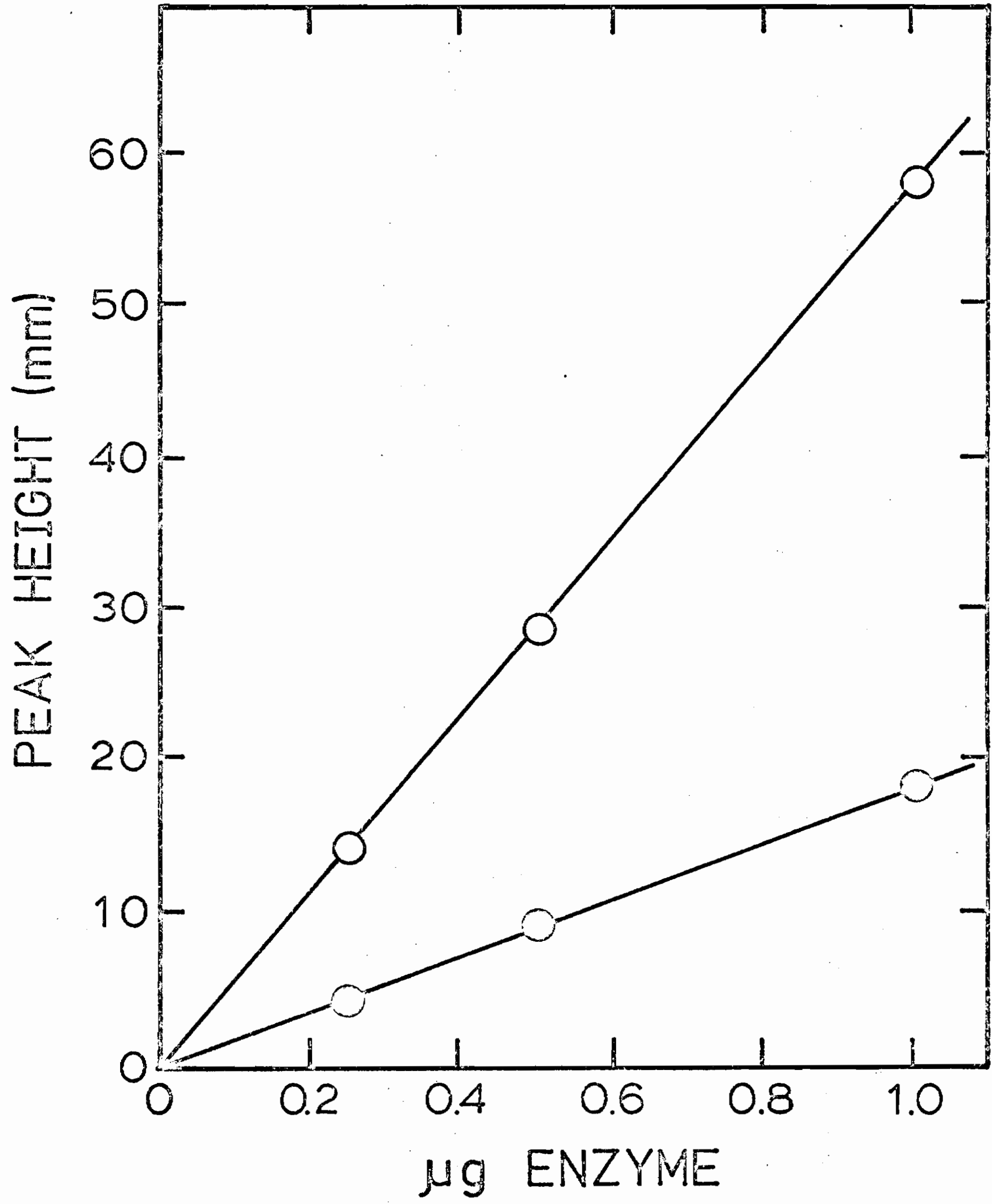

A
B
C

Fig. 22. Peak heights of 0.25 μg, 0.50 μg, and 1.0 μg of NADP-glutamate dehydrogenase after electrophoresis in 1% agarose gels containing 1% agarose gels containing 1% (-○-) or 3% (-○-) antiserum against the purified enzyme.


PEAK HEIGHT (mm)
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
µg ENZYME

DISCUSSION

The NADP-GDH from C. sorokiniana has structural similarities shared with a number of GDHs isolated from other organisms (Table IV). The GDHs from most organisms have been shown to be composed of 6 identical subunits. Notable exceptions to this 6 subunit value are the NAD-GDH isozymes from N. crassa (47) and C. sorokiniana (20) which contain only 4 subunits.

Depending on the method of molecular weight analysis, the NADP-GDH from C. sorokiniana has 5-7 identical subunits with a molecular weight of approximately 58,000-59,000. Because molecular weights estimated by sedimentation-equilibrium are not influenced greatly by the shape of proteins, the molecular weight of 290,000, determined by this method, for the Chlorella NADP-GDH is probably closer to the actual value than the approximately 400,000 value determined by gel-filtration and polyacrylamide gel electrophoresis.

The NADP-GDH from C. sorokiniana is also similar to GDHs in other organisms in that it lacks disulfide bridges and also has a high arginine to lysine ratio. The absence of a free N-terminal group in this enzyme is also observed for both GDH isozymes in N. crassa. In contrast, the NAD-GDH isozyme in C. sorokiniana possesses an N-terminal lysine.

The NADP- and NAD-GDH isozymes from C. pyrenoidosa Pringsheim 82T have been recently partially-purified and purified, respectively, by Shatilov et al. (75). These workers used a different combination of

TABLE IV

CHARACTERISTICS OF GLUTAMATE DEHYDROGENASES ISOLATED FROM VARIOUS SOURCES[1]

Source	Nucleotide Specificity	Native Molecular Weight ($x10^{-5}$)	Subunit Molecular Weight ($x10^{-4}$)	Arginine to Lysine Ratio
Chlorella sorokiniana	NADP	2.9-4.1	5.8-5.9	0.737
Chlorella sorokiniana	NAD	1.8	4.8	0.222
Neurospora crassa	NADP	2.8-3.0	4.84	0.586
Neurospora crassa	NAD	4.5-5.1	11.1-12.1	0.969
Human liver	NAD(P)	nd[2]	nd	0.813
Bovine liver	NAD(P)	3.0-3.3	5.54	0.909
Rat liver	NAD(P)	3.3-3.7	4.3-5.3	0.846
Dogfish liver	NAD(P)	3.1-3.5	nd	0.714
Chicken liver	NAD(P)	3.1-3.5	5.57	0.800
Escherichia coli	NADP	2.4-2.5	4.6	0.818
Clostridium SB_4	NAD	2.7-3.9	nd	0.586

[1]All data except that for *Chlorella sorokiniana* is from reference 30

[2]Not determined

purification procedures than those described in this thesis. A number of physical and kinetic properties of these two isozymes from *C. pyrenoidosa* differ significantly from those observed in *C. sorokiniana* (Table V).

The activities of a number of GDHs in other organisms have been reported (30) to be regulated by nucleotides and other small molecule effectors. In the present study, nucleotides were not observed to affect the activity of the purified NADP-GDH from *C. sorokiniana*. Moreover, Meredith (20) recently showed that the activity of the purified NAD-GDH from this organism was also insensitive to all nucleotides and amino acids tested. It is possible that substrate inhibition by α-ketoglutarate (Fig. 15) of the activity of the NADP-GDH might play some regulatory role *in vivo*.

The apparent lack of modifiers of activity for these isozymes in *C. sorokiniana* might indicate that their regulation occurs at the level of synthesis or degradation. The NH_4^+ induction of the NADP-GDH from *Chlorella* (18) contrasts dramatically with the repression of synthesis of the NADP-GDH by NH_4^+ in some fungi (23). In that the synthesis of the NADP-GDH in a number of bacilli (30) is induced by NH_4^+, the level of this enzyme might be regulated in a manner more similar to procaryotic than to eucaryotic microorganisms.

The induction of the synthesis of the *Chlorella* NADP-GDH by NH_4^+ and the greater activity of the enzyme in the aminating direction (i.e., saturating substrate levels) are consistent with an anabolic function for this enzyme. However, the K_m values for $NADP^+$ and L-glutamate are

TABLE V

COMPARISON OF PHYSICAL AND KINETIC PROPERTIES OF GLUTAMATE DEHYDROGENASE ISOZYMES ISOLATED FROM TWO SPECIES OF *CHLORELLA*

Source and Nucleotide Specificity	Molecular Weight (x 10^{-5})	pH Optimum Amination	K_m values[1] for Substrates, mM			Nucleotide[2]	
			NH_4^+	α-Ketoglutarate	Glutamate	Oxidized	Reduced
C. sorokiniana-NADP	2.9-4.1	7.2	68	12	32	0.038	0.13
C. sorokiniana-NAD[3]	1.8	8.0	40	2.0	60	0.15	0.15
C. pyrenoidosa-NADP[4]	3.0	8.1	18	0.35	11	0.012	0.018
C. pyrenoidosa-NAD	3.0-3.1	8.7	41	1.3	0.5	0.14	0.034

[1]Cooperativity was seen with several substrates with the *C. pyrenoidosa* enzymes

[2]The K_m stated is for the nucleotide of highest activity (i.e., NADP and NADPH for NADP-enzyme)

[3]Data from reference 20

[4]Data from reference 78

lower than those for NADPH and NH_4^+, suggesting that this NADP-GDH might play a catabolic role in metabolism. Thus, the *in vivo* metabolic role of the NADP-GDH in *Chlorella* is still unclear.

The specific antibody which has been prepared against the NADP-GDH will be useful in elucidation of the molecular events associated with the induction of the enzyme and also with the rapid *in vivo* loss in its activity upon NH_4^+ removal from cells.

SUMMARY

The ammonium inducible NADP-GDH from C. sorokiniana has been purified 260-fold to homogeneity. Depending on the technique used, the native enzyme appeared to have a molecular mass of 290,000 to 400,000 daltons and to be composed of subunits with an identical molecular weight of 58,000. Differences in the molecular weight of the native enzyme, as determined by sedimentation equilibrium, Sephadex G-200 gel filtration and gradient polyacrylamide gel electrophoresis, indicate that the native enzyme may be elliptical in shape.

The amino acid composition of the NADP-GDH is high in glycine, glutamate, and aspartate. Moreover, the arginine to lysine ratio is similar to those measured in other glutamate dehydrogenases. The N-terminal amino acid is unavailable to dansylation. All six cysteines in the enzyme are in the free sulfhydryl form.

The enzyme is very specific for the reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate and has less than 0.5% of maximal activity, using the oxidized and reduced forms of nicotinamide adenine dinucleotide. With low concentrations of the substrates, no cooperativity was seen; however, severe substrate inhibition was observed with α-ketoglutarate. Antiserum produced to the subunits of the NADP-GDH yielded a single precipitin band against purified enzyme in Ouchterlony double diffusion analysis. "Rocket" immunoelectrophoresis has been used to quantify the amount of antigen present in samples of the purified enzyme.

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PURIFICATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-SPECIFIC GLUTAMATE DEHYDROGENASE FROM CHLORELLA SOROKINIANA AND PARTIAL CHARACTERIZATION OF ITS PHYSICAL, KINETIC, AND IMMUNOLOGICAL PROPERTIES

by

Richard Mark Gronostajski

(ABSTRACT)

The ammonium inducible nicotinamide phosphate-specific glutamate dehydrogenase from Chlorella sorokiniana has been purified 260-fold to homogeneity. Depending on the technique used, the native enzyme appeared to have a molecular mass of 290,000 to 400,000 daltons and to be composed of subunits with an identical molecular weight of 58,000. Differences in the molecular weight of the native enzyme, as determined by sedimentation equilibrium, Sephadex G-200 gel filtration and gradient polyacrylamide gel electrophoresis, indicate that the native enzyme may be elliptical in shape.

The amino acid composition of the enzyme is high in glycine, glutamate, and asparate. Moreover, the arginine to lysine ratio is similar to those measured in other glutamate dehydrogenases. The N-terminal amino acid is unavailable to dansylation. All six cysteines in the enzyme are in the free sulfhydryl form.

The enzyme is very specific for the reduced and oxidized forms of nicotinamide adenine dinucleotide phosphate and has less than 0.5 percent of maximal activity, using the oxidized and reduced forms of nicotinamide adine dinucleotide. With low concentrations of the

substrates, no cooperativity was seen; however severe substrate inhibition was observed with α-ketoglutarate. Antiserum produced to the subunits of the enzyme yielded a single precipitin band against purified enzyme in Ouchterlony double diffusion analysis. "Rocket" immunoelectrophoresis has been used to quantify the amount of antigen present in samples of the purified enzyme.