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An Ls-isocitrate:NADP oxidoreductase has been isolated and purified from embryonic chicken liver. The enzyme was purified 200 fold by means of ammonium sulfate fractionation, gel filtration chromatography on Sephadex G-200, ion-exchange chromatography on Bio-Gel CM-100 and adsorption chromatography on hydroxylapatite gel.

The purified enzyme was nearly homogeneous as determined from sedimentation velocity and sedimentation equilibrium ultracentrifugation. The enzyme had an apparent sedimentation coefficient of about 6.15 S and an apparent molecular weight of approximately $1.15 \times 10^5$. The presence of 8 mM isocitrate or 0.42 M ammonium sulfate stabilized the enzyme against inactivation by heating, but did not alter the molecular weight of the enzyme. In the presence of 8mM isocitrate the molecular weight of the enzyme was not altered by an increase in temperature from 4° C to 20° C. 0.42 M ammonium

sulfate containing 5 mM NADP also did not affect the molecular weight of the enzyme. There was no indication of a chemical equilibrium between the enzyme and an enzyme aggregate.

These data indicate that the native supernatant NADP-isocitrate dehydrogenase from embryonic chick liver has a molecular weight of $1.15 \times 10^5$ and that stability against heat inactivation is not the result of enzyme aggregation.

# Partial Purification and Characterization of an Ls-NADP:Isocitrate Oxidoreductase from Embryonic Chick Liver

by

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# PARTIAL PURIFICATION AND CHARACTERIZATION OF AN Ls-NADP: ISOCITRATE OXIDOREDUCTASE FROM EMBRYONIC CHICK LIVER

## INTRODUCTION

The enzyme isocitrate dehydrogenase catalyzes the oxidative decarboxylation of Ls-isocitrate when in the presence of a divalent cation ($Mg^{+2}$ or $Mn^{+2}$) and an oxidant (NAD or NADP). The overall reaction probably proceeds in a sequential manner involving two separate reaction mechanisms. Presumably the initial reaction would be the oxidative dehydrogenation of Ls-isocitrate to form oxalosuccinate. The oxalosuccinate would then be decarboxylated to form $\alpha$-ketoglutarate as the final product (17, 18, 19, 46, 60). There is evidence that if oxalosuccinate is formed as an intermediate, then it remains bound to the enzyme until after the decarboxylation reaction (44, 54, 60, 70).

There are three electrophoretically distinguishable isocitrate dehydrogenase enzymes present in animal tissue (6, 40, 42). These enzymes differ in their intracellular localization as well as their cofactor requirement. One enzyme is found exclusively in the mitochondria and requires NAD as its cofactor (6, 25, 49). This enzyme differs from the other two isocitrate dehydrogenase enzymes in: (1) Km value of the enzyme-substrate complex, (2) pH optimum and (3) stability (49). The NAD enzyme no doubt functions primarily in the tricarboxylic acid cycle to furnish the NADH that is required

for the synthesis of ATP (25).

The other two isocitrate dehydrogenase enzymes require NADP as their cofactor and are found both in the mitochondria and the supernatant fractions (6, 49). These enzymes differ in their tissue and intracellular localization (6, 31).

The main function of the mitochondrial NADP-isocitrate dehydrogenase enzyme is not known. It has been suggested that in certain cases the mitochondrial NADP-enzyme may supply the reducing capacity necessary for the synthesis of ATP (51). This would require a method to utilize NADPH for the reduction of NAD, for example a transhydrogenase enzyme (51, 66). The NADH could then be used for the oxidative phosphorylation of ADP.

There are bacteria which contain high levels of a transhydrogenase enzyme (52). The primary role of the NADP-isocitrate dehydrogenase in these bacteria may be to maintain a sufficient supply of NADPH for use in the production of ATP.

The major function of the supernatant isocitrate dehydrogenase enzyme also is not completely understood (1). It has been postulated that this enzyme is a source for NADPH used in certain important reductive synthetic processes, such as lipid synthesis (1, 4, 39, 46, 69).

Thus the isocitrate dehydrogenase enzymes play an important role both in energy production and in the generation of the reduced pyridine nucleotide coenzymes. The control of the activity of these enzymes may therefore be essential in order to maintain metabolic

stability. This possibility has stimulated several investigations of the physical structure of these enzymes, particularly with respect to potential mechanisms of control (10, 17, 56).

The NAD enzyme has been purified and characterized from a variety of different sources (10, 49, 50, 55, 56, 57). It is subject to the complex allosteric control of a number of metabolically important substances, including citrate, AMP and ATP (2, 50, 55, 56). This would be expected for an enzyme which was postulated to play a key role in metabolic regulation (20, 30, 55, 56, 61).

The initial interest in the isocitrate dehydrogenase enzymes focused mainly on the NAD enzyme and few of the NADP enzymes were purified and characterized. The preliminary investigations of the NADP-isocitrate dehydrogenase enzymes had been mainly kinetic analyses of crude tissue homogenates or partially purified enzyme preparations at best.

Until recently, only the pig heart NADP-isocitrate dehydrogenase had been purified to such an extent that its molecular weight could be estimated reliably. A molecular weight of about $6.0 \times 10^4$ for the native enzyme was confirmed by several investigators (17, 45, 59) although they made no attempts to dissociate the enzyme into smaller molecular weight units.

The first indication that the NADP-isocitrate dehydrogenase enzymes might be composed of subunits came as the result of a

systematic electrophoretic analysis of mouse supernatant NADP-isocitrate dehydrogenase enzymes (31). Tissue homogenates were examined from several different strains of mice and it was found that the enzyme existed in two electrophoretically different forms. The forms were strain specific so that the mice could be divided into two groups according to which form of the enzyme they possessed. When mice with different forms of the enzyme were cross-bred, their progeny had three electrophoretically distinguishable forms. There was one form corresponding to that of each parent and one intermediate form. This led to the proposal that the enzyme was composed of two electrophoretically identical subunits.

This suggestion of a subunit structure for the mouse enzyme may have prompted the recent purification and structural characterization of a number of NADP-isocitrate dehydrogenases (12, 14, 34, 35, 41). The enzyme isolated from pig heart was one of the enzymes of interest (41). It was again confirmed that the native enzyme indeed had a molecular weight of $6.0 \times 10^4$, but in addition it was shown that the enzyme was dissociated into smaller molecular weight units when in the presence of 6.5 M urea or 1% sodium dodecyl sulfate. In 6.5 M urea the enzyme had an apparent minimal molecular weight of about $3.2 \times 10^4$. In 1% sodium dodecyl sulfate it had an apparent sedimentation coefficient of about 2.3 S. These data led to the conclusion that the native pig heart enzyme was composed of two

hydrodynamically identical subunits.

Another NADP-isocitrate dehydrogenase enzyme has been highly purified from the bacteria Bacillus stearothermophilus (34). It has an apparent molecular weight of about $9.0 \times 10^4$ in its native form with an apparent sedimentation coefficient of about 5.5 S. In the presence of 6 M guanidine hydrochloride containing 6 mM dithiothreitol, this enzyme was dissociated into smaller molecular weight units of about $4.5 \times 10^4$. It was suggested that this enzyme also was composed of two hydrodynamically identical subunits.

Another bacterial NADP-isocitrate dehydrogenase, which was isolated and purified from Azotobacter vinelandii, had a molecular weight of about $8.0 \times 10^4$ (14). Exhaustive attempts to dissociate this enzyme in 6 M guanidine containing 10 mM dithiothreitol failed. It was concluded that in this case, the enzyme existed as a single polypeptide chain with a molecular weight of $8.0 \times 10^4$.

Yet another NADP-isocitrate dehydrogenase enzyme, for which a subunit structure was suggested, was partially purified from embryonic chick liver (12). The purified enzyme had a specific activity of five to ten units (specific activity = change in optical density at 340 mμ per minute per mg of protein). This represented about a 25-fold purification of the enzyme.

Characterization of this enzyme by sucrose density-gradient centrifugation and by gel filtration indicated the existence of two

interconvertible molecular forms. These forms possessed marked differences in enzymatic activity, stability toward heating and molecular size.

In the presence of a "high ionic strength" buffer (0.2 M Tris-HCl buffer, pH 7.4 containing 0.42 M ammonium sulfate) the enzyme had an apparent molecular weight of 1.5 to 1.6 x $10^5$ (as determined by gel filtration) and a specific activity of five to ten units. The enzyme in this buffer system was stable to heating for one hour at $46^\circ$ C.

When the enzyme was dialyzed against a "low ionic strength" buffer (0.05 M Tris-HCl buffer, pH 7.4) the apparent molecular weight of the enzyme decreased to 8.5 to 9.8 x $10^4$ and its specific activity was decreased to one to two units. Heating the enzyme for one hour at $46^\circ$ C in this low ionic strength buffer resulted in a total loss of activity. The enzyme could be protected against heat inactivation by the additon of 8 mM isocitrate to the buffer (24).

If ammonium sulfate was added to the dialyzed, unheated enzyme solution (to a final concentration of 0.42 M), the specific activity immediately doubled and the enzyme was stabilized against heat inactivation. The addition of the ammonium sulfate also increased the relative amount of larger molecular weight components of the solution (as determined by sucrose density-gradient ultracentrifugation). It was presumed therefore that some of the enzyme had been

reconverted to the larger molecular weight form by the increase of the ionic strength of the buffer.

The techniques which were used to obtain these molecular weight estimates relied upon the substrate specificity of the enzyme. Although these techniques are useful for the characterization of enzymes in heterogeneous protein mixtures, the reliability of the results is sometimes questionable.

Physiologically, the function of this embryonic NADP-isocitrate dehydrogenase enzyme may be, at least in part, the production of NADPH for use in fatty acid synthesis (4). If this should be the case, then the control of the activity of the enzyme may be necessary for the normal development of the embryo.

Enzymatic control in embryonic development may be expressed merely as a change in enzyme level or, more dramatically, as a complete change of enzyme pattern (3, 9, 27, 33, 36, 42, 63). An interesting example of enzymatic control during embryonic development has been shown for the lactate dehydrogenase enzyme of the embryonic rat heart (27, 33, 36). It was found that during the embryonic development of the heart there was a gradual shift in isoenzyme pattern from the M-type to the H-type lactate dehydrogenase. This shift may be caused by or at least reflect the metabolic changes which occur during the development of the rat heart.

A possible control mechanism for the embryonic chick liver

supernatant NADP-isocitrate dehydrogenase was suggested by the previous author (12). He had shown that the molecular weight as well as the specific activity of the enzyme was a function of the ionic strength of the buffer. Thus the activity of the enzyme could be controlled through the ionic strength of the buffer. It was speculated that under physiological conditions a small molecule, perhaps the enzyme's substrate, could similarly control the enzymatic activity.

The following model may be proposed which would permit the rapidly responsive environmental control of the enzymatic activity of this enzyme under physiological conditions. The enzyme would be synthesized as the low molecular weight form, or subunit. These subunits would establish a rapid, reversible equilibrium with the high molecular weight form, or aggregate. This equilibrium would be in favor of the dissociated subunits until such time that large amounts of NADPH were required, during nerve myelination for example. At this time an increased level of a metabolite, etc., would cause the subunits to coalesce into the more active aggregates. These active aggregates would then supply the potential to produce the necessary increased levels of NADPH. Similar models for enzymatic regulation have been suggested for the glucose-6-phosphate dehydrogenase enzyme found in lactating mammary glands (38) and for some of the lactate dehydrogenase enzymes (29, 30).

It is the purpose of this thesis to attempt further to define the physical properties of the embryonic chick liver supernatant isocitrate dehydrogenase enzyme. This was accomplished primarily by refined molecular weight determinations of the enzyme in the presence of both high and low ionic strength buffers. In these buffers the enzyme was reported to be predominately in a high or low molecular weight form.

The enzyme was purified further, to a specific activity of 45-50 units (specific activity = the change in optical density at 340 mμ per minute per optical density unit at 280 mμ). Molecular weight estimates were then obtained from sedimentation equilibrium and sedimentation velocity ultracentrifugation experiments carried out in an analytical ultracentrifuge.

# MATERIALS

## Eggs and Embryos

Hy-Line 950-A eggs were obtained from Jenks Hatchery, Tangent, Oregon. The eggs were incubated in a Jamesway Model 252B incubator with an automatic turner which was set for two hour intervals. The eggs were incubated at wet and dry bulb temperatures of 86° F and 99° F, respectively. All embryos came from eggs that had been incubated for 18 consecutive days.

## Chemicals

NADP (sodium salt) and DL-isocitric acid (trisodium salt) were obtained from Sigma Chemical Company. Hydroxylapatite gel and Bio-Gel CM-100 were from Bio-Rad Laboratories. Crystalline ammonium sulfate (enzyme grade) was from Mann Research Laboratories. All other chemicals were of "reagent" grade and were obtained from commercial sources.

# METHODS

## Buffer Systems

The 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer consisted of 6.05 grams of Tris dissolved in one liter of glass distilled water adjusted to pH 6.8 containing 55.5 grams of ammonium sulfate. The 0.05 M Tris-HCl buffer was prepared in the same manner as the 0.05 M Tris-HCl: $(NH_4)_2SO_4$ buffer except for the omission of the ammonium sulfate.

The 0.02 M Phosphate buffer consisted of 2.91 grams of $NaH_2PO_4 \cdot H_2O$ dissolved in one liter of glass distilled water and adjusted to pH 7.3. The 0.04, 0.08, 0.09, 0.10 and 0.14 M Phosphate buffers were similar to the 0.02 M Phosphate buffer except they contained 5.64, 10.94, 12.42, 13.8 and 20 grams of $NaH_2PO_4 \cdot H_2O$ respectively.

## Column Chromatography

The Sephadex G-200 was allowed to swell in glass distilled water at 90-95° C for at least five hours. After cooling to room temperature, the gel was equilibrated with the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer at 2-4° C. The column was packed and used according to the manufacturer's recommendations (48), with the hydrostatic head maintained at 10-15 cm throughout the packing and elution of the

column. The average flow rate for the column was about 10 ml per hour. The Sephadex G-25 was treated in a similar manner except that the hydrostatic head was adjusted (as dictated) to maintain a flow rate of about 10 ml per hour.

The Bio-Gel CM-100 was prepared by swelling the gel in the 0.02 M Phosphate buffer under a vacuum at room temperature for about 48 hours. The gel was then equilibrated with the 0.02 M Phosphate buffer at 2-4° C. The column was packed according to the manufacturer's suggestions (7), with a hydrostatically induced flow maintained at 4-8 ml per hour throughout packing and elution. This column was not reused due to the shrinkage of the gel in solutions of high ionic strength.

The hydroxylapatite was equilibrated with the 0.08 M Phosphate buffer at 2-4° C under vacuum for about 24 hours. The columns were packed and used according to the manufacturer's directions (7), using hydrostatic pressure to maintain a flow rate of about 5-15 ml per hour. A large amount of the 0.08 M Phosphate buffer was passed through the columns immediately prior to the application of the samples.

Enzyme Purification

The enzyme was isolated from the livers of approximately 30 dozen chick embryos. Each liver, with its associated gall bladder,

was carefully removed so that the gall bladder was not ruptured. The gall bladder was immediately separated from the liver, intact, and discarded. The remaining liver was immersed in ice-cold homogenizing medium where the tissue was collected until the dissections were completed.

The liver was then drained over cotton gauze for about three minutes prior to homogenization. It was homogenized in small amounts by adding five grams of liver (wet weight) to 50 ml of ice-cold homogenizing medium and homogenizing in a Potter-Elvejhem type glass homogenizer, using a motor driven, loose fitting teflon pestle. The homogenization was for about one minute at a moderate speed. The homogenizing medium consisted of a 0.05 M Tris-HCl buffer, pH 6.8 containing 0.25 M sucrose and 4 mM EDTA.

The homogenates were combined and centrifuged at 600 x g for 30 minutes to remove the unbroken cells, cell debris and cell nuclei. The resultant supernatant was centrifuged for 30 minutes at 10,000 x g to sediment the mitochondria. The remaining supernatant was then centrifuged for at least two hours at 30,000 x g using a SS-34 rotor. The sediment was discarded.

All preparative centrifugations were done with a Sorvall Model RC-2 automatic superspeed refrigerated centrifuge at a temperature of 2-4° C. Subsequent centrifugations were for 15 minutes each using the SS-34 rotor at 30,000 x g. All of the remaining fractionation

procedures were conducted in a cold room at 2-4° C.

Crystalline ammonium sulfate was then added to the 30,000 x g supernatant to bring the final concentration to 40% saturation. The salt was added in small, equal amounts (approximately 7% of the total) over a period of two to three hours. The solution was stirred constantly but no attempt was made to control the pH of the solution. The percent of ammonium sulfate saturation refers to the percent saturation at 25° C, although the fractionation was performed at 2-4° C. This method of adding ammonium sulfate was used whenever the ammonium sulfate concentration of a solution was increased.

The 40% saturated suspension was centrifuged, the precipitate discarded and ammonium sulfate added to the supernatant to a final concentration of 75% saturation. The resultant precipitate was removed from solution by centrifugation and its total volume was estimated by comparison with water in an identical centrifuge tube. The precipitate was then suspended in a sufficient volume of the 0.05 M Tris-HCl buffer to give a solution 10% saturated with ammonium sulfate, assuming that the original volume of precipitate was equivalent to a 75% saturated solution. This amounted to about 200 ml of buffer.

Ammonium sulfate was again added to the enzyme solution to a final concentration of 40% saturation. The salt concentration was then raised by 5% increments until the enzyme barely began to

precipitate (approximately 50% saturation). Enzyme solubility was monitored by centrifuging one ml aliquots of the solution and determining the residual enzymatic activity of the supernatants.

The entire suspension was then centrifuged, the sediment discarded and the ammonium sulfate concentration of the supernatant again increased by 5% increments until most of the enzyme was precipitated (about 65% of saturation). The suspension was centrifuged once again, the supernatant discarded and the precipitated enzyme solubilized in 20-30 ml of the 0.05 M Tris-HCl buffer. This solution was dialyzed against two liters of the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer for at least 24 hours.

After dialysis, the enzyme solution was passed through a 1.9 x 24 cm Sephadex G-25 column to remove particulate and lipid material. The enzyme was collected from the column as a bulk fraction with minimum dilution and it was stored in this solution at 2-4° C until it could be purified further. If an appreciable precipitate developed on storage, the solution was clarified by centrifugation prior to further fractionation.

A 5-10 ml aliquot of this solution was next applied to a 2.8 x 75 cm Sephadex G-200 column and the enzyme was subsequently eluted with the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer at a rate of 9-12 ml per hour. The eluant was collected in 3-4 ml fractions. The fractions were assayed and the most enzymatically active were pooled. The

fractions from all of the columns were collected with an Isco Model A fraction collector using the "time" mode of operation.

The maximum volume which could be conveniently fractionated by this Sephadex column was about 10 ml, therefore the enzyme solution was divided into two or more approximately equal aliquots. The active fractions from each aliquot were saved until all could be combined and concentrated together. The solution was concentrated by adding ammonium sulfate to a final concentration of 65% saturation, removing the precipitated enzyme by centrifugation and solubilizing the precipitate in 6-10 ml of the 0.05 M Tris-HCl buffer.

This solution was dialyzed against two liters of the 0.05 M Tris-HCl buffer for two hours, followed by two to four hours dialysis against two liters of the 0.02 M Phosphate buffer. After dialysis, the enzyme solution was diluted to 20-30 ml with the 0.02 M Phosphate buffer and immediately applied to a 2.4 x 45 cm Bio-Gel CM-100 column.

Approximately 100 ml of the 0.02 M Phosphate buffer were then passed through the column followed by about 100 ml of the 0.04 M Phosphate buffer, both of which were collected as bulk fractions. Excess buffer was removed from above the column bed before new buffer was added, care being taken not to disturb the top of the bed. The enzyme was then eluted from the column with the 0.08 M Phosphate buffer and the eluant collected in 3-5 ml fractions.

The enzymatically active fractions from the Bio-Gel CM-100 column were pooled and applied directly to a 1.9 x 24 cm hydroxylapatite gel column. Approximately 100 ml of the 0.08 M Phosphate buffer were passed through the column followed by about 100 ml of the 0.09 M Phosphate buffer, both of which were again collected in bulk. The enzyme was eluted from the column with the 0.10 M Phosphate buffer and the eluant collected in 2-4 ml fractions.

The most enzymatically active fractions from the column were pooled and dialyzed against two liters of the 0.02 M Phosphate buffer for about 24 hours. The enzyme solution was then concentrated by applying the solution to a 1.1 x 6 cm hydroxylapatite column. The enzyme was eluted from the column with the 0.14 M Phosphate buffer and collected in small fractions (0.25 to 0.50 ml) which were assayed and the most enzymatically active pooled. The pooled fractions were dialyzed two hours against the 0.05 M Tris-HCl buffer followed by several hours dialysis against the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer containing 4 mM EDTA. The enzyme at this state of purity had a specific activity of 45-50 units and was the enzyme which was used exclusively in all the structural studies.

## Enzyme Assay

Enzymatic activity was detected as a change in optical density which accompanied the reduction of the NADP in the assay solution.

The optical density changes were measured at 340 mμ with a Beckman Model DU spectrophotometer in conjunction with a Gilford Model 220 optical density converter attachment. The Gilford was equipped with an automatic cuvette changer set for five second intervals. The optical density changes were recorded with a Leeds and Northrup Speedomax H recorder.

The assay solution consisted of 2.9 ml of a 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 4 μmoles of DL-isocitrate, 2 μmoles of $MnCl_2$ and 2 μmoles of NADP. The reaction was initiated by the addition of 0.1 ml of the enzyme solution and was allowed to continue for about two minutes. The enzymatic activity was calculated from the initial rate of NADP reduction and was expressed as the change in optical density at 340 mμ per minute per optical density unit at 280 mμ. All assays were performed at room temperature.

Ultracentrifuge Techniques

Sedimentation equilibrium and sedimentation velocity ultracentrifugations were used to estimate the molecular weight of the enzyme in a variety of buffer systems at temperatures of 4° C and 20° C. The buffer systems included the 0.05 M Tris-HCl buffer, the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer, the 0.05 M Tris-HCl buffer containing 8 mM isocitrate, the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer

containing 5 mM NADP and a 0.05 M Tris-HCl buffer (pH 7.4 at room temperature containing 8 mM isocitrate). This last buffer was used exclusively for the ultracentrifugations which were run at a temperature of 20° C.

The enzyme was dialyzed against the appropriate buffer at the appropriate temperature for at least 12 hours immediately prior to centrifugation. The corresponding dialysates were used as reference solutions in the centrifugations.

The ultracentrifugations were done in a Spinco Model E analytical ultracentrifuge which was equipped with a RTIC temperature control unit, an electronic speed control and a photoelectric scanning system. The optical systems were previously aligned and focused (21) and no adjustments were made. The light source was on a pivot which allowed convenient and rapid conversion between interference and Schlieren optics. Sapphire windows were used routinely for all centrifugations.

Sedimentation equilibrium experiments were performed according to the methods of Yphantis (64, 67), using a six-channel short-column equilibrium type centerpiece (5). Initial protein concentrations varied from about 0.08 to 0.02% and a column height of about 3 mm was used (0.1 ml of solution). The initial protein concentrations in a given compartment were kept as constant as possible for all of the equilibrium centrifugations. The interference optical system was

used to observe the protein distribution and the inclusion of a base fluid in the sample compartments was deemed undesirable.

The centrifugations were run at speeds of 20,000 and 24,000 rpm at temperatures of 4 and 20° C. In order to attain equilibrium more rapidly, especially at the lower temperature, the technique of "overspeeding" was often utilized (32).

A Kodak Type 77A filter was placed directly above the light source to isolate monochromatic light and the interference fringe patterns were recorded on Kodak Type II-G spectroscopic glass plates. A "baseline" photograph was normally taken before the centrifuge reached running speed and was used to correct for fringe displacements caused by aberrations in the optical system. Another photograph was taken about 20 hours after the initiation of the experiment and at 4-6 hour intervals thereafter until the protein distribution attained equilibrium.

The fringe displacements resulting from the protein concentration were measured on a Nikon Model 6C microcomparator following the method of Yphantis (67). The data were analyzed with the aid of a Control Data Corporation 3300 computer (22). The values of $\ell n\, j(r)$ and $(r^2 - r_m^2)$ were calculated for each set of data points, where $(r_m)$ and $(r)$ were the radii from the axis of rotation to the meniscus and to the point where the fringe deflection, $j(r)$, was measured. The $j(r)$'s were expressed in terms of net fringe displacement and

$(r^2 - r_m^2) = \triangle r^2$ in square centimeters. The weight-average molecular weight, Mw(r), the number-average molecular weight, Mn(r), and the z-average molecular weight Mz(r), were then computed at each radius (r) and plotted as a function of the relative protein concentration, j(r), at these radii (Figures I to IV).

The Mn(r), Mw(r) and Mz(r) were obtained by a computer program which gave the number-, weight- and z-average molecular weights at any point in the solution column, using a quadratic fit over a pre-set region around each point (r) to obtain these averages.

$$Mn(r) = \text{Sigma} \cdot \frac{j(r)}{\int j(r)dr^2 + k}$$

where $$k = \text{Sigma} \cdot \frac{[3\ Mw(a) - Mz(a)]}{2}$$

and the integral is taken from point (a) to point (r)

$$Mw(r) = \text{Sigma} \cdot \frac{d\ [\ell n\ j(r)]}{d(\triangle r^2)}$$

$$Mz(r) = Mw(r) \cdot \left[1 + \frac{d\ [\ell n Mw]}{d\ [\ell n\ j(r)]}\right]$$

$$\text{Sigma} = \frac{2RT}{(1 - \bar{v}p)w^2}$$

The partial specific volume, $(\bar{v})$, was assumed to be equal to 0.74 and the density of the solution (p) was taken to be equal to the density of the solvent. (R) was the universal gas constant, $8.314 \times 10^7$,

(T) was temperature in degrees Kelvin and (w) was the angular velocity in radians per second.

Sedimentation velocity centrifugations were run at a speed of 60,000 rpm at 20° C in the 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 8 mM isocitrate. A standard 12 mm, double sector, epon-type centerpiece was used, with the initial protein concentrations ranging between 3.0 and 10.0 mg per ml. A Kodak Type 77A filter was again placed above the light source and the Schlieren optical system was used to observe the sedimentation of the protein. The Schlieren refractive index gradient curves were recorded on Kodak metallographic photographic glass plates. Photographs were taken automatically at four or eight minute intervals until the boundary had traversed at least one half of the cell.

The Schlieren concentration gradient curves were measured with the Nikon microcomparator. Each gradient was measured at 20-40 points, which were approximately equally spaced along the radial axis and which were " representative" of the gradient curve.

The data were again analyzed with the aid of the computer (23). The second moment of each gradient curve was calculated by numerical integration using "Simpson's Rule."

$$(r')^2 = \frac{\int r^2 dc/dr}{\int dc/dr}$$

The values of (dc/dr) were obtained directly from the gradient curves after correction for magnification, (r) was the distance from the axis of rotation in centimeters and (r') was the value of the second moment of the gradient curve about the axis of rotation measured in centimeters.

The sedimentation coefficient (S) was obtained from the slope of the line fit to the $\ell n (r')$ vs. (t) plot (Figure VII)

$$S = \frac{1}{w^2} \cdot \frac{d\{\ell n(r')\}}{d(t)}$$

Sedimentation velocity centrifugations were also conducted with the dilute solutions, approximately 0.8 mg per ml, which had supplied the material for the equilibrium centrifugations. These solutions were kept at the temperature used for the corresponding equilibrium centrifugation for the duration of the equilibrium experiment. The sedimentation velocity centrifugation was conducted as soon as possible after the termination of the corresponding equilibrium centrifugation at the same temperature used for the equilibrium experiment. The initial protein concentrations used for these velocity experiments was equivalent to the highest concentration employed in the comparable equilibrium experiment.

These sedimentation velocity centrifugations were also run at 60,000 rpm using the 12 mm double sector centerpiece. The

migration of the protein was observed and recorded as an adsorption boundary at 280 mμ using the photoelectric scanning system. Scans were taken automatically at four or eight minute intervals, at the intermediate scan speed at a chart speed of 5 cm per minute.

Sedimentation coefficients were obtained from the slopes of the $\ell n(r)$ vs. (t) plots, where (r) was the distance from the axis of rotation to the center of the adsorption boundary in centimeters and (t) was the time in minutes (Figure V) (58). The center of the adsorption boundary was taken as the midpoint between the maximum and minimum adsorption (11).

The observed sedimentation coefficients were corrected to water at $20^{\circ}$ C, using the viscosity corrections $n/n_{H_2O} = 1.01$ and $n_{20}/n_4 = 1.7$ (23). These $S_{20,w}$ values were tabulated in Table II together with the observed (S) values.

# RESULTS

The recovery and purification at each step of a generalized scheme of enzyme purification are given in Table 1. All of the steps in the purification procedure resulted in the distribution of the protein throughout a large number of fractions. This distribution was a function of the net charge, solubility, adsorption characteristics, size, etc. of the proteins. Since the enzyme protein was not distributed equally among the fractions, only those fractions which contained a sufficiently large amount of enzymatic activity were pooled and saved for further fractionation. Therefore a decision had to be made concerning the level of enzymatic activity which was sufficiently large to be saved. This decision was based on several considerations and was different for each enzyme preparation. Hence Table 1 is merely an approximation of the degree of purification and percent recovery which may be expected from each purification step.

Results from the sedimentation equilibrium experiments are given in Figures I, II, III and IV. The Yphantis technique of high speed equilibrium centrifugation provided a sensitive test for protein homogeneity as well as supplying an estimate of the minimum molecular weight protein in the enzyme preparation. Figures I and II represent the weight-average molecular weight, $M_w(r)$, as a function of the relative protein concentration, $j(r)$, for the enzyme in the five

Table 1. Enzyme purification

| | Purification Step | Volume ml | Protein mg | Specific activity |
|---|---|---|---|---|
| 1. | 10,000 x g supernatant | 1,000 | 18,000 | 0.20 |
| 2. | 30,000 x g supernatant | 980 | 8,500 | 0.40 |
| 3. | First ammonium sulfate fractionation | 250 | 1,500 | 0.80 |
| 4. | Second ammonium sulfate fractionation | 20 | 1,000 | 1.0 |
| 5. | Sephadex G-200 column eludate | 70 | 300 | 3.0 |
| 6. | Bio-Gel CM-100 column eludate | 50 | 10 | 30.0 |
| 7. | Hydroxylapatite column eludate | 40 | 5 | 40.0 |
| 8. | Hydroxylapatite concentrated eludate | 1 | 5 | 40.0 |

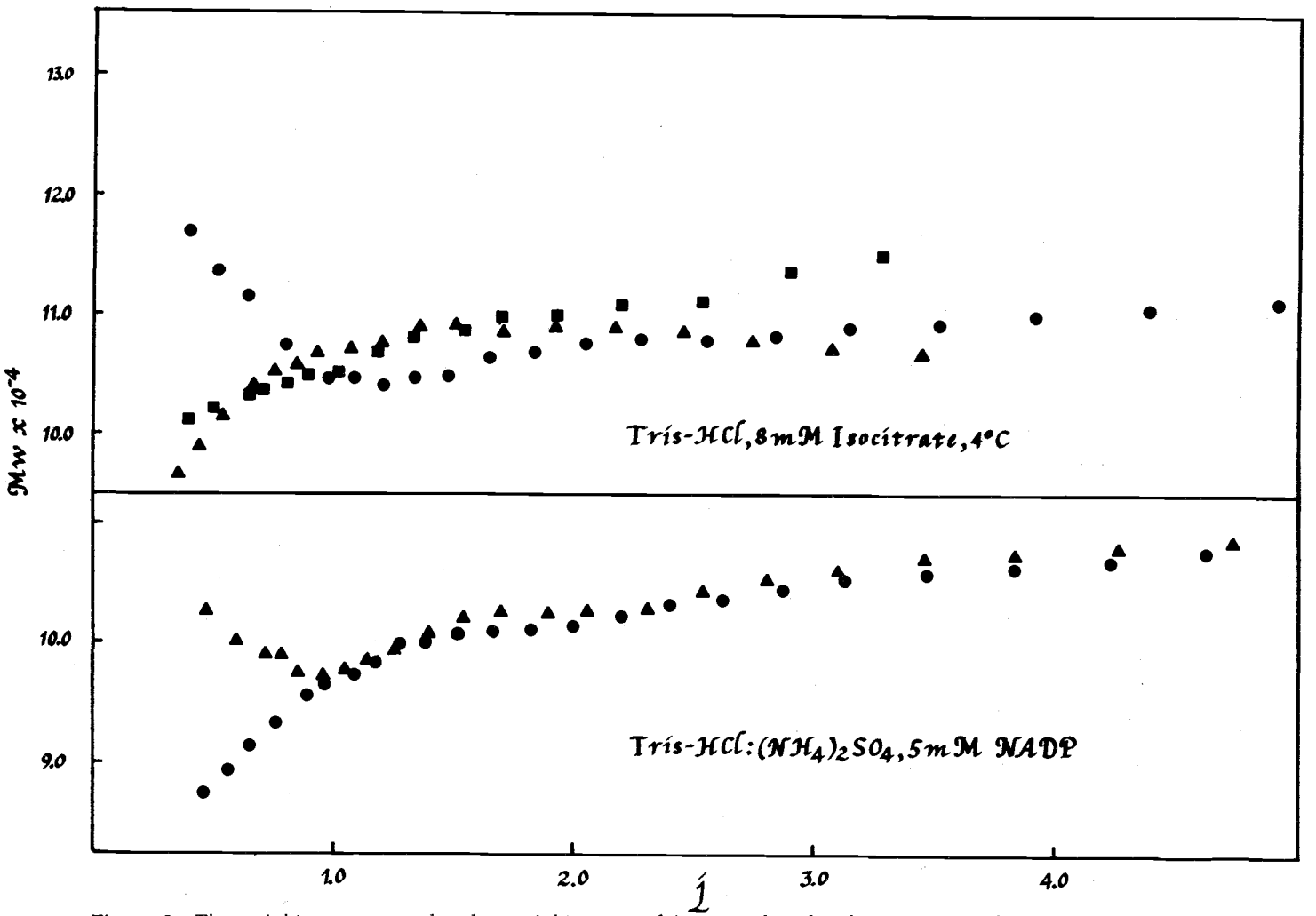


Figure I The weight-average molecular weight versus fringe number for the enzyme in the Tris-HCl-8mM isocitrate and the Tris-HCl:$(NH_4)_2SO_4$-5 mM NADP buffers. Initial enzyme concentrations were approximately 0.8 mg per ml (●), 0.4 mg per ml (▲) and 0.2 mg per ml (■), 0.4 mg per ml (▲) and 0.2 mg per ml (■).

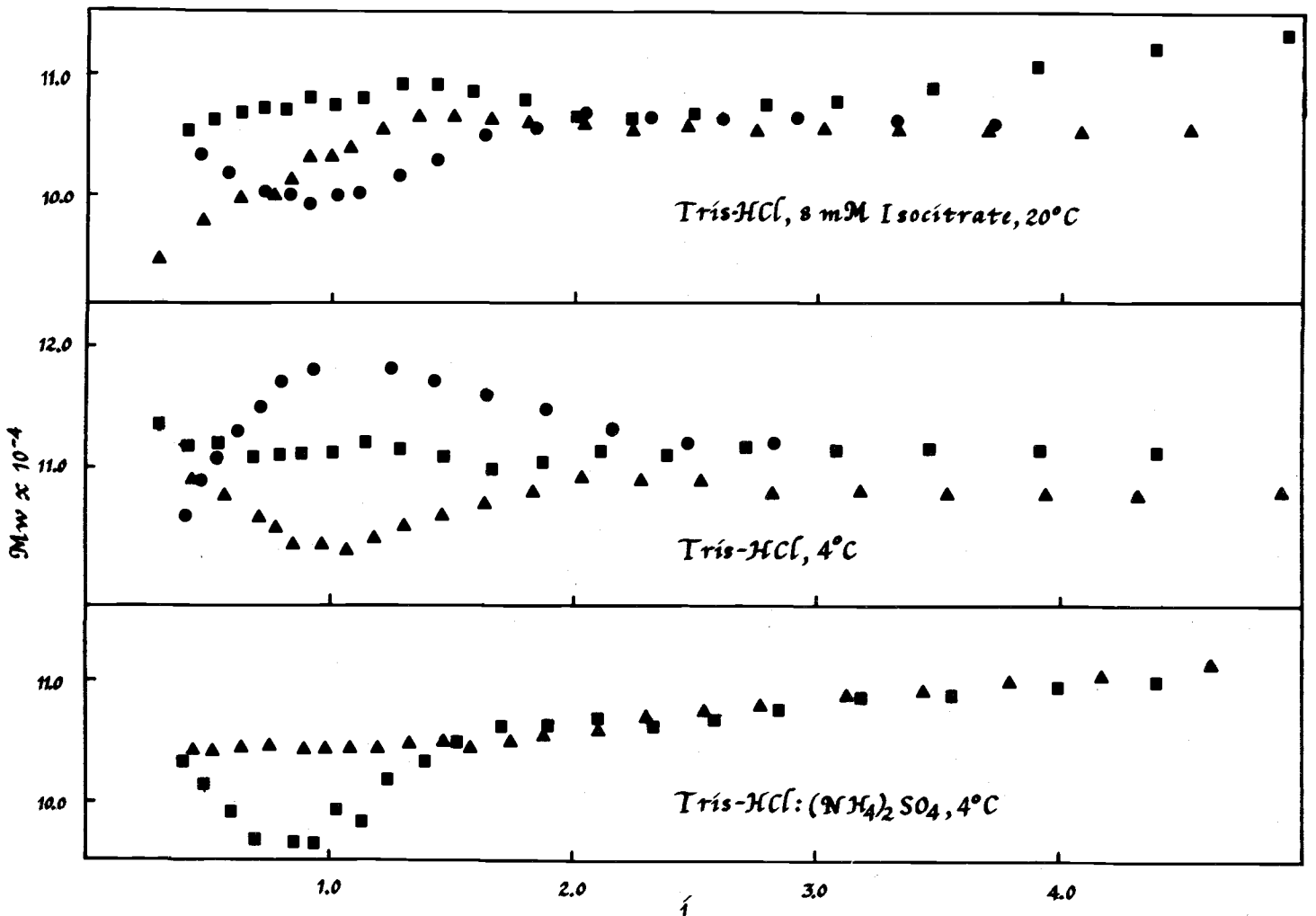


Figure II The weight-average molecular weight versus fringe number for the enzyme in the Tris-HCl, Tris-HCl:$(NH_4)_2SO_4$ and the 20° C Tris-HCl buffers. Initial enzyme concentrations were approximately 0.8 mg per ml (●), 0.4 mg per ml (▲) and 0.2 mg per ml (■).

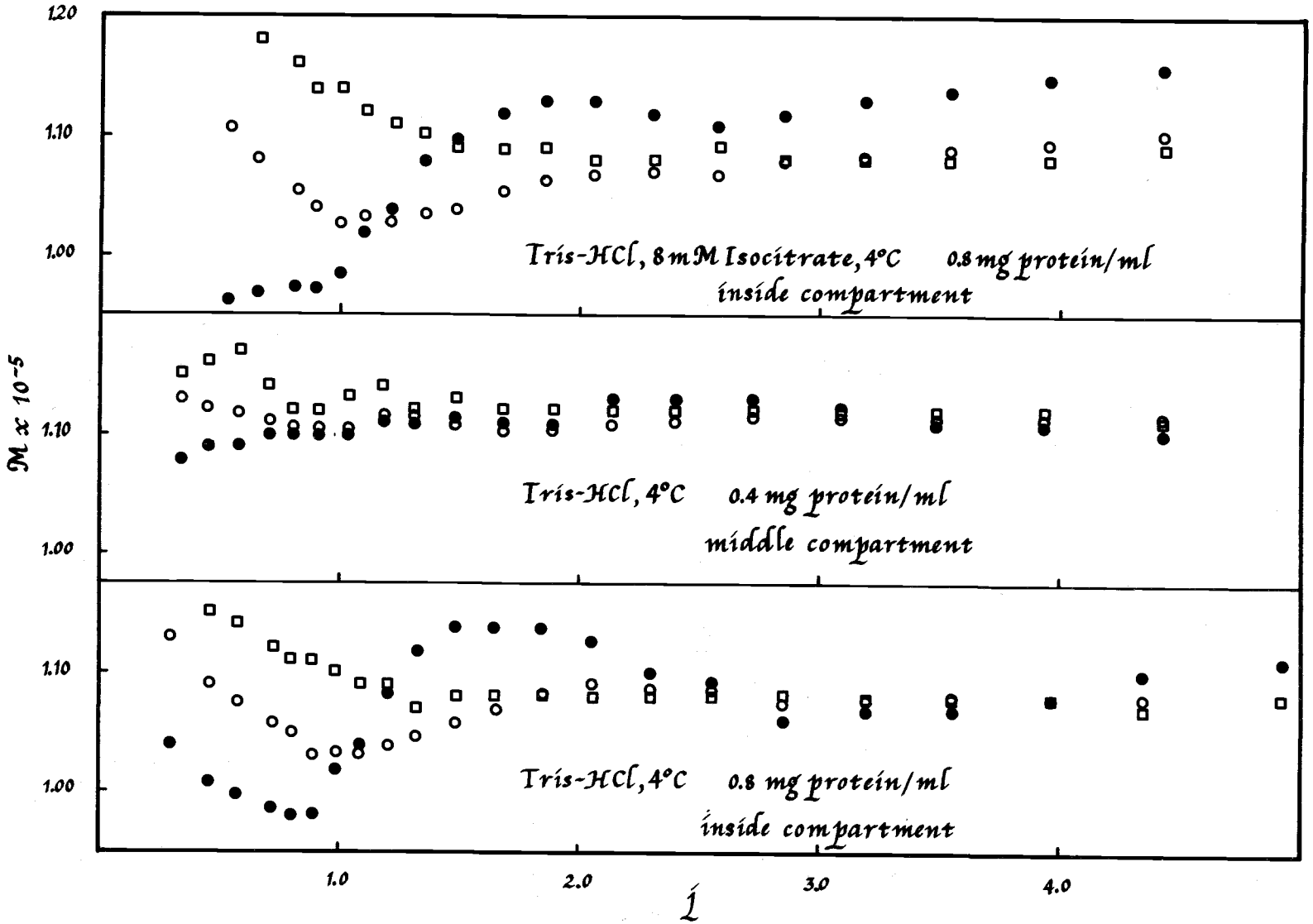


Figure III The weight-(O), number-(□) and z-average (●) molecular weight versus fringe number for the enzyme in the Tris-HCl-8 mM isocitrate and the Tris-HCl buffers.

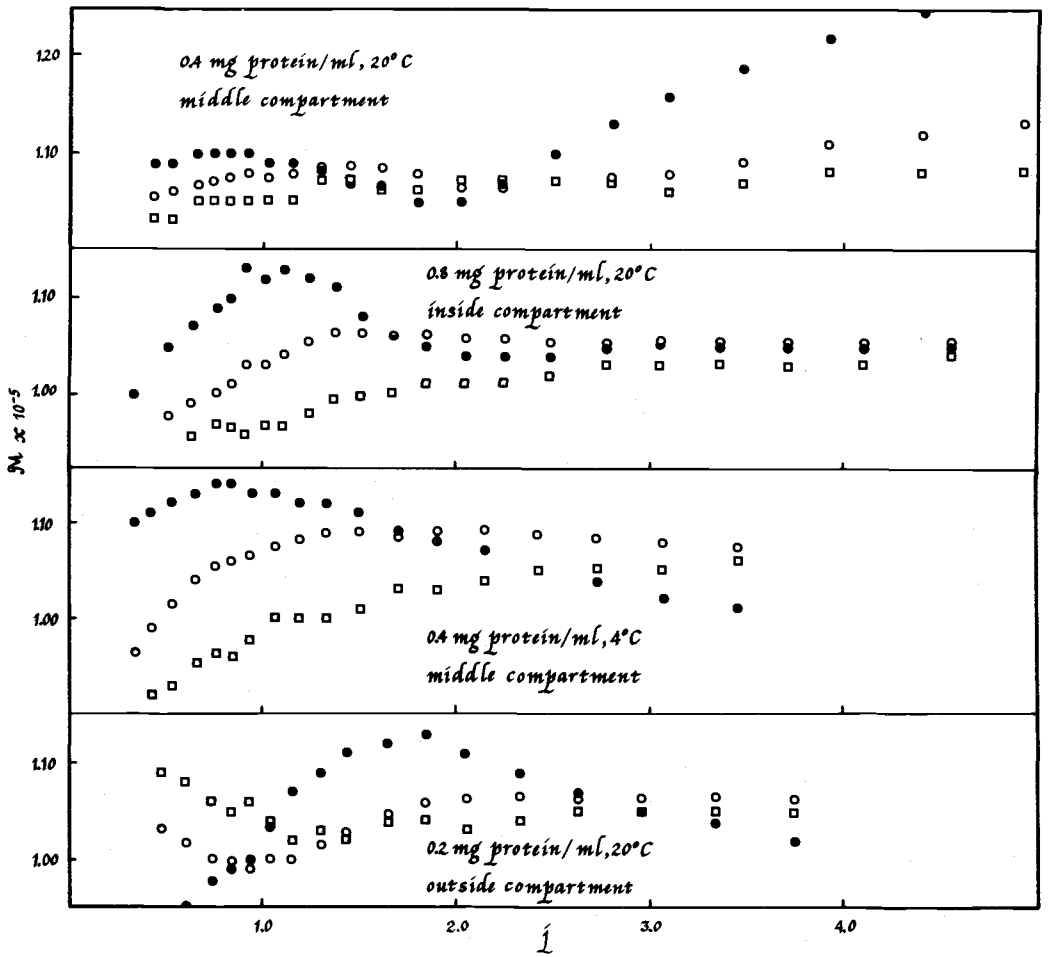


Figure IV The weight- (O), number (□) and z-average (●) molecular weight versus fringe number for the enzyme in the Tris-HCl-8mM isocitrate buffer.

different buffer systems employed. Three different enzyme concentrations were examined in each buffer system. Although the enzyme displayed some heterogeneity as evident from the dependence of Mw(r) on j(r), this variation was small ($9.0 \times 10^4$ to $1.15 \times 10^5$).

Figures III and IV present the weight-, number-, and z-average molecular weights, Mw(r), Mn(r) and Mz(r), again as a function of the relative protein concentration, j(r). The disparity between these different molecular weight averages for a given enzyme concentration and buffer system is quite small, lending further credence to the postulation that the enzyme preparation was nearly homogeneous. These molecular weight distributions were also independent of the temperature and buffer systems examined.

The data obtained from the photoelectric scanner are summarized in Figure V as $\ell n(r)$ vs. (t) plots, where (r) was the distance from the axis of rotation to the center of the adsorption boundary in centimeters and (t) was the time in minutes. These data were used primarily to estimate the size of the major component of the enzyme solution in the buffer systems which were used for the equilibrium ultracentrifugations. The observed, uncorrected sedimentation coefficients ranged from 3.28 to 6.12 S for the buffer systems and temperatures which were employed. The relative homogeneity of the samples could also be estimated from these data.

Figure VI represents "typical" photoelectric scans taken about

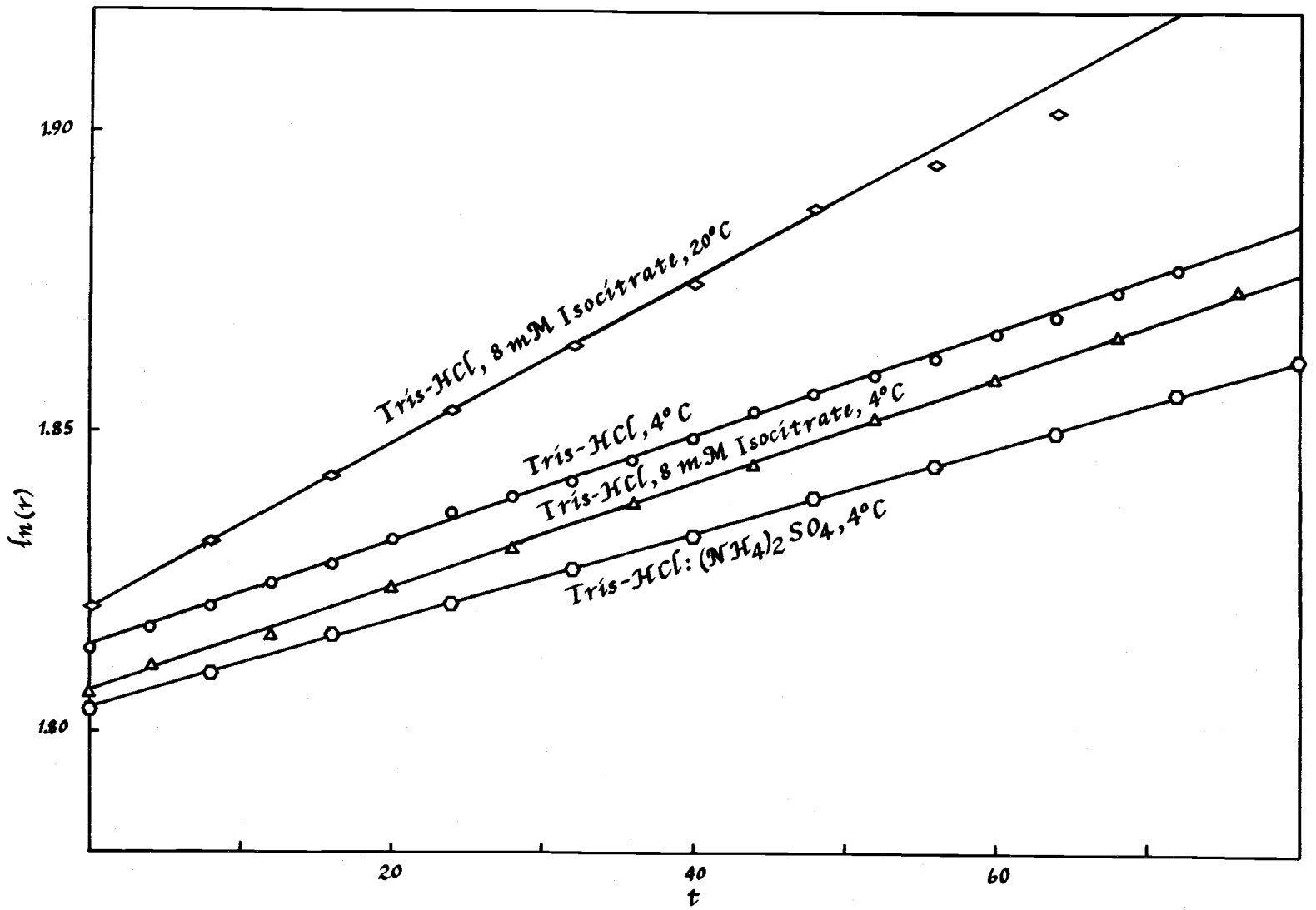


Figure V Sedimentation velocity of the enzyme as a function of the buffer systems. Initial protein concentrations were approximately 0.8 mg per ml.

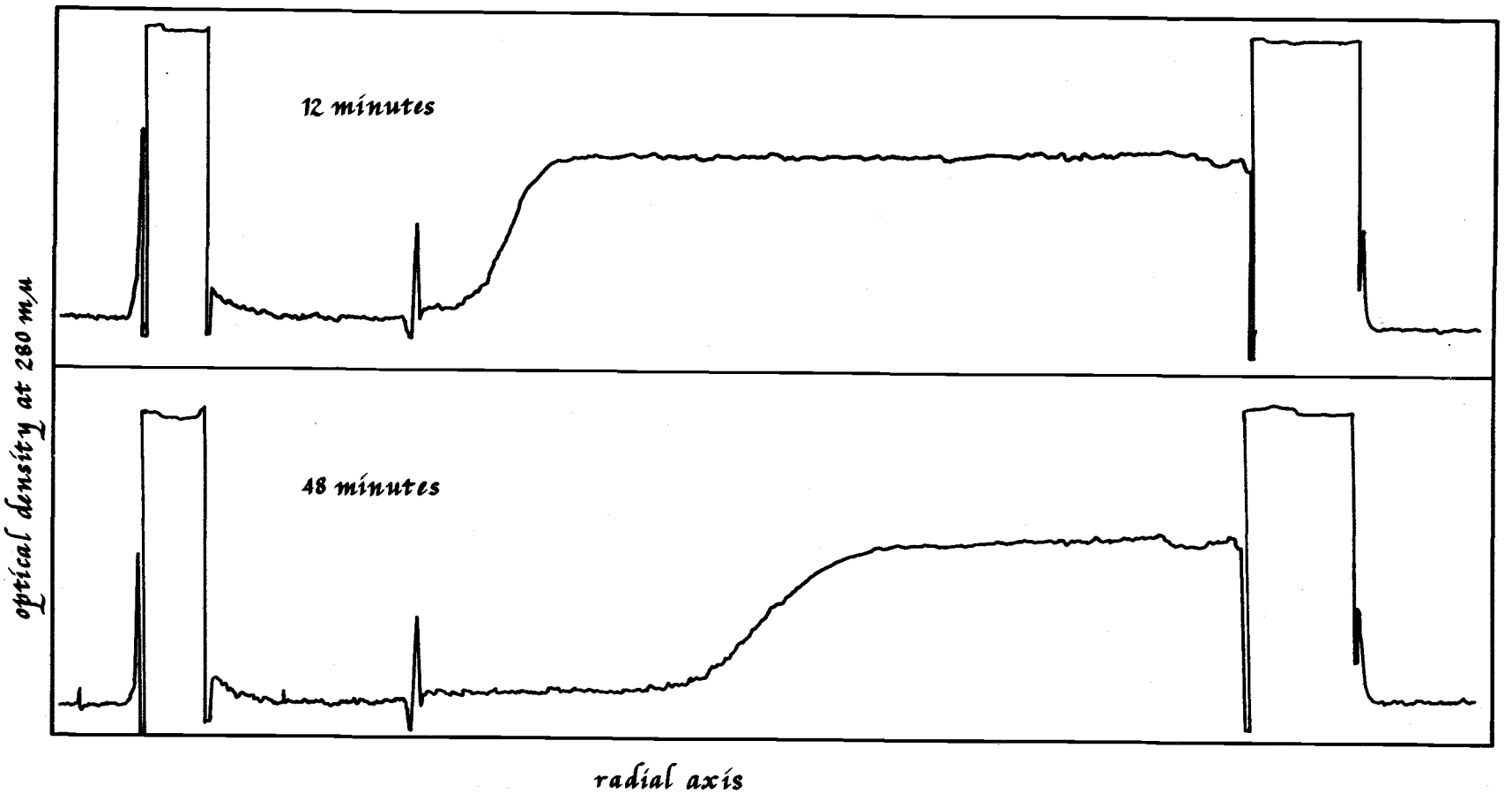


Figure VI Sedimentation velocity data obtained from the photoelectric scanner after 12 and 48 minutes of centrifugation.

eight and 48 minutes after the centrifuge had reached running speed. In all instances examined, there was only one discernible boundary. This would preclude the presence of large amounts of material which had sedimentation coefficients significantly different from that of the majority of proteins in the solution.

The results obtained from the Schlieren gradient curves are presented in Figure VII as $\ell n (r')$ vs. (t) plots, where (r') was the value of the second moment of the gradient curve about the axis of rotation in centimeters and (t) was again the time in minutes. The observed, uncorrected sedimentation coefficients ranged from 5.61 to 6.00 S for the protein concentrations which were employed. Figures VIII and IX respectively depict the data obtained from the concentration gradient curves for the 10 mg per ml protein concentration at about 12 and 60 minutes after the centrifuge had reached running speed. The Schlieren "peaks" were nearly symmetrical and again there was only one discernible "peak" present for all of the conditions examined, attesting to the relative homogeneity of the enzyme protein.

The observed sedimentation coefficients which were calculated from Figures V and VII are tabulated in Table 2. The sedimentation coefficients corrected to the viscosity of water at $20^{o}$ C, ($S_{20, w}$) had a range from 6.12 to 6.38 and are included in Table 2. The concentration dependency of the sedimentation coefficient is illustrated in Figure X as a plot of the $S_{20, w}$ vs. the protein concentration

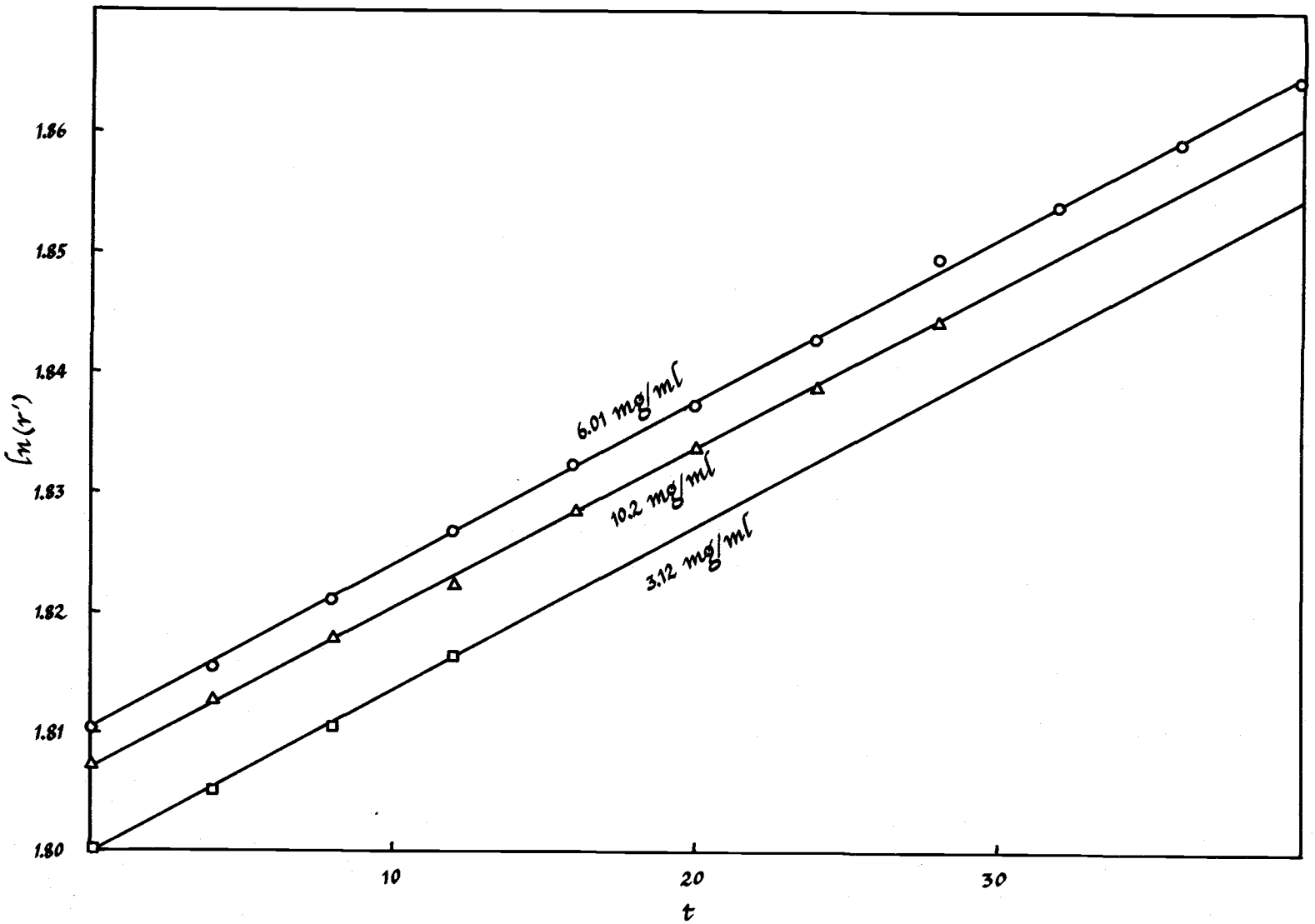


Figure VII Sedimentation velocity of the enzyme as a function of protein concentration.

Table 2. Sedimentation coefficient

Scanner Data

| Buffer | Observed (S) | $S_{20, w}$ |
|---|---|---|
| Tris-HCl at 4° C | 3.90 | 6.24 |
| Tris-HCl containing 8 mM Isocitrate at 4° C | 3.99 | 6.38 |
| Tris-HCl:$(NH_4)_2SO_4$ | 3.28 | ---- |
| Tris-HCl containing 8 mM Isocitrate at 20° C | 6.12 | 6.12 |

Schlieren Data

| Buffer | Protein Concentration | $S_{20, w}$ |
|---|---|---|
| Tris-HCl containing 8 mM Isocitrate at 20° C | 3.12 mg per ml | 6.00 |
| Tris-HCl containing 8 mM Isocitrate at 20° C | 6.01 mg per ml | 5.74 |
| Tris-HCl containing 8 mM Isocitrate at 20° C | 10.2 mg per ml | 5.61 |

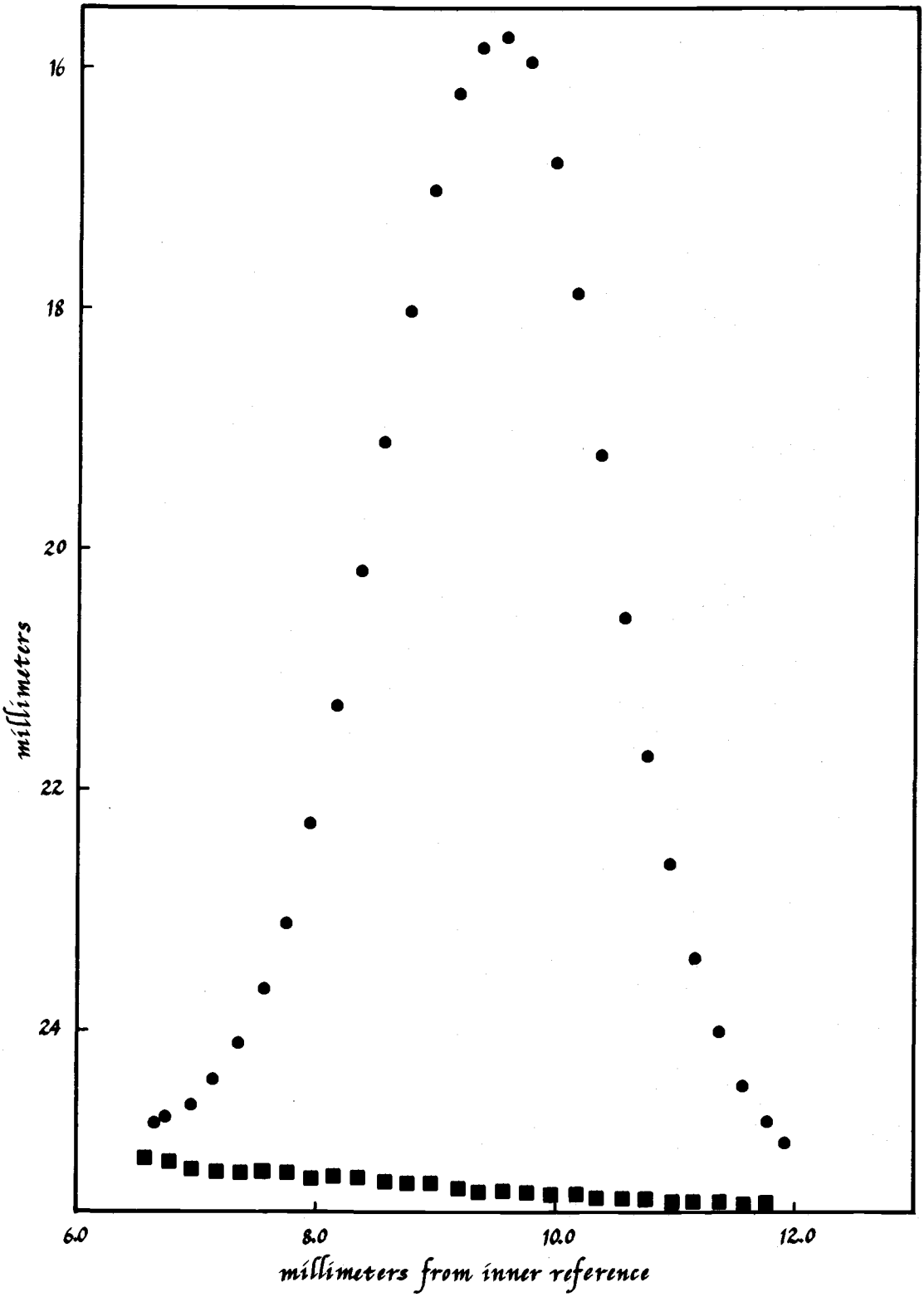


Figure VIII Sedimentation velocity data obtained from the Schlieren concentration gradient curve for the solvent (■) and solution (●) after about 12 minutes of centrifugation.

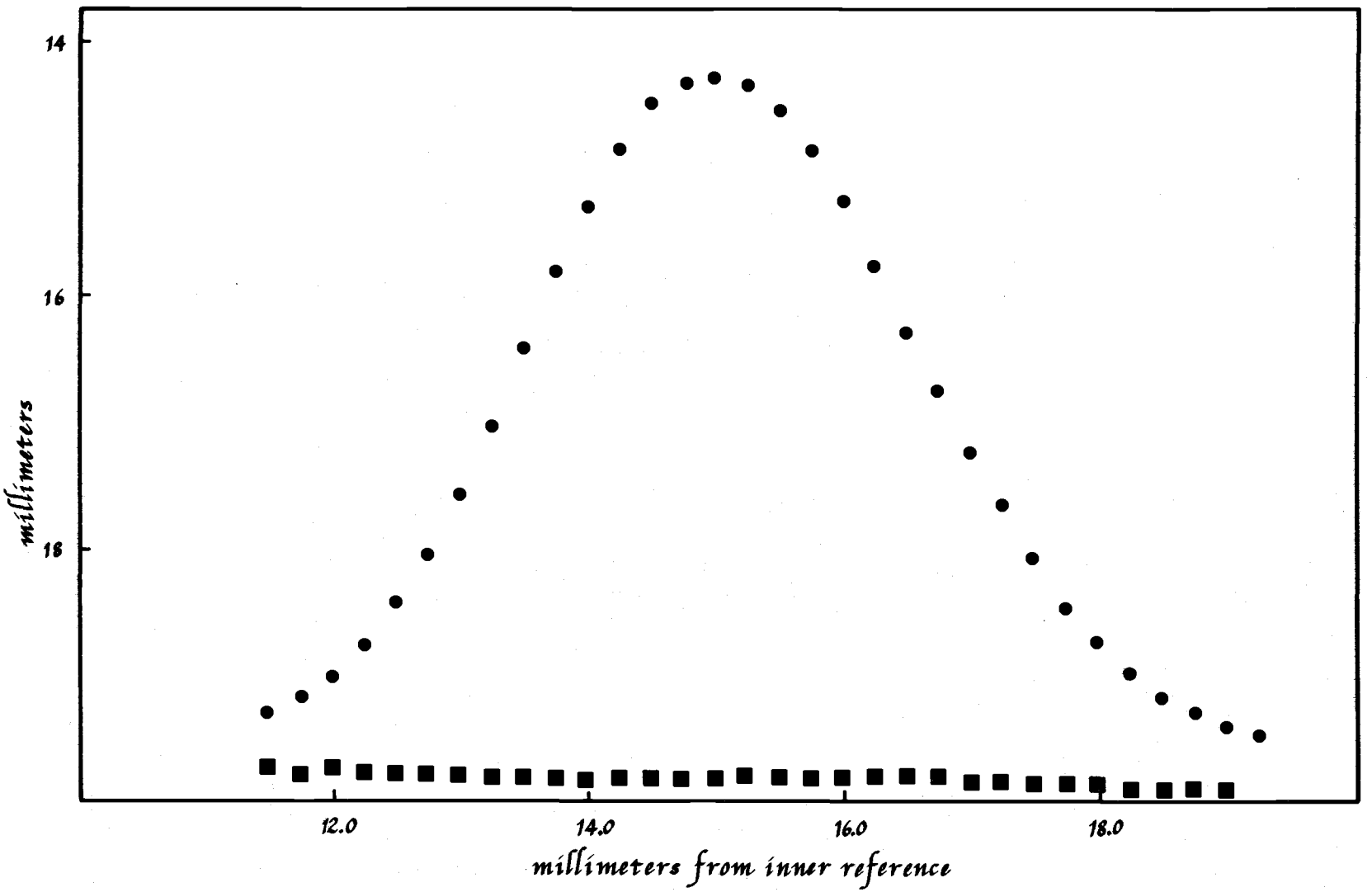


Figure IX Sedimentation velocity data obtained from the Schlieren concentration gradient curve for the solvent (■) and solution (●) after about 70 minutes of centrifugation.

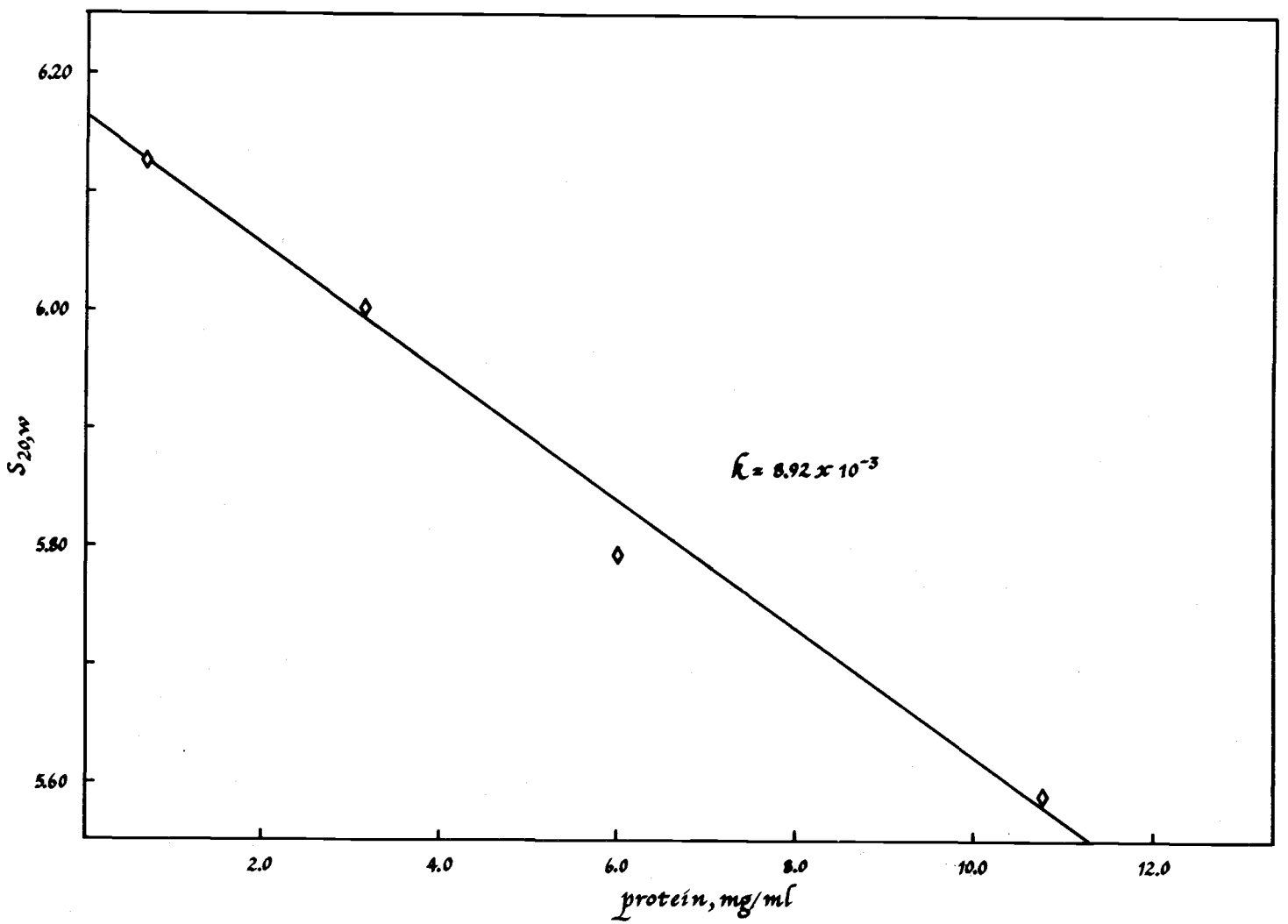


Figure X The concentration dependence of the sedimentation coefficient.

measured in optical density units at 280 mμ). An $S^{o}_{20, w}$ of 6.15 was obtained by the extrapolation of Figure X to infinite protein dilution.

Table 3 is a summary of heat inactivation experiments in some of the different buffer systems. If the enzyme was subjected to 46° C for one hour, the enzyme lost approximately 50% of its activity when it was in the 0.05 M Tris-HCl buffer. No activity was lost, however, when the enzyme was in the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer, the 0.05 M Tris-HCl buffer containing 8 mM isocitrate or the 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 8 mM isocitrate.

Since only small aliquots of the enzyme were used for the heat inactivation experiments, apparent increases in activity as a result of heating were likely due to a slight amount of evaporation from the aliquots.

Table 3. Heat inactivation

| Buffer | Specific activity | Specific Activity after heating |
|---|---|---|
| Tris-HCl containing 8 mM Isocitrate at 4° C | 52.6 | 59.2 |
| Tris-HCl containing 8 mM Isocitrate at 20° C | 46.5 | 37.9 |
| Tris-HCl at 4° C | 35.7 | 10.4 |
| Tris-HCl:$(NH_4)_2SO_4$ at 4° C | 41.9 | 52.3 |

# DISCUSSION

It has been previously reported that the embryonic chick liver supernatant NADP-isocitrate dehydrogenase enzyme may exist in two, separable, interconvertible molecular forms (12). When the enzyme was in a "high ionic strength" buffer (0.2 M Tris-HCl buffer, pH 7.4 containing 0.42 M $(NH_4)_2SO_4$), it was heat stable and had an apparent molecular weight of approximately $1.6 \times 10^5$. Removal of the ammonium sulfate by dialysis against a "low ionic strength" buffer (0.05 M Tris-HCl buffer, pH 7.4) converted the enzyme into a heat labile form which had an apparent molecular weight of approximately $9.0 \times 10^4$. It could be protected against heat inactivation by the addition of 8 mM isocitrate to this low ionic strength buffer (24).

A model was subsequently proposed to account for this behavior. It was postulated that the active form of the enzyme was the heat stable form with a molecular weight of $1.6 \times 10^5$ and that this form could dissociate into less active, heat susceptible subunits, with molecular weights of $8.0 \times 10^4$. It was assumed that a rapid, reversible, concentration dependent equilibrium would be established between the subunits and the aggregated forms. Furthermore, this equilibrium could be displaced by changing the ionic strength of the buffer system or perhaps by the presence of a small organic molecule, e.g., one of the enzyme's substrates. This model then

represents an effective means whereby the activity of the enzyme is rapidly responsive to its environment.

The main purpose of this thesis research was further to clarify the physical structure of the enzyme, its different forms and their interactions.

Initial enzyme preparations were prepared by the procedure as reported by the previous author (12) and were apparently heterogeneous preparations. Characterization of the enzyme in these preparations was necessarily restricted to experimental techniques that were applicable to heterogeneous enzyme preparations. These techniques included sucrose density-gradient ultracentrifugation (43) and active enzyme ultracentrifugation (15, 16). These methods proved to be unsatisfactory and were hence abandoned. It was therefore decided to purify the enzyme to such an extent that more fundamental and, hopefully, more reliable ultracentrifugation techniques could be utilized.

In the present thesis research the Yphantis (67) technique of high speed equilibrium sedimentation was used to estimate the molecular weight of the enzyme. This technique was selected because equilibrium could be reached in a relatively short time (about 24 hours) and only a small amount of protein was required (approximately 0.2 mg). The ability to examine small amounts of protein was mandatory due to the limited supply of purified enzyme protein available

(usually less than 5 mg per enzyme preparation).

The convenience of the Yphantis technique was offset to some extent by its sensitivity to heterogeneity. Heterogeneity was readily detectable, although the number and molecular weight of the different proteins present could not be determined from this equilibrium data alone. Heterogeneous preparations were characterized by a broad weight-average molecular weight range which was a function of both the amount and the molecular weight of the different proteins present.

In order to obtain molecular weight estimates of the major components in heterogeneous samples, additional knowledge about the sample is required (67). As a supplement to the equilibrium data, the number and relative amounts of the different proteins may be estimated from sedimentation velocity ultracentrifugation experiments.

Sedimentation velocity ultracentrifugation experiments were done in five different buffer systems: (1) the 0.05 M Tris-HCl buffer, (2) the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer, (3) the 0.05 M Tris-HCl buffer containing 8 mM isocitrate, (4) the 0.05 M Tris-HCl:$(NH_4)_2SO_4$ buffer containing 5 mM NADP and (5) a 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 8 mM isocitrate. All of these buffers contained 3 mM EDTA which was added before the pH was adjusted. The initial protein concentration for these velocity experiments was about 0.8 mg per ml and only one discernible boundary

was observed in each experiment. The sedimentation coefficient, $S_{20,w}$, had a range from 6.1 to 6.4 in these buffer systems (Table 2).

The sedimentation equilibrium data which correspond to these sedimentation velocity experiments are presented in Figures I, II, III and IV. These data show slight increases in the molecular weight averages, Mn(r), Mw(r) and Mz(r) as the relative protein concentration, j(r), increases. As has been mentioned, this increase results from the presence of a protein or proteins in the sample with a molecular weight larger than that of the average protein.

The protein concentrations which were examined in these equilibrium experiments ranged from 0.3 to 5.0 interference fringes. This may have been below the concentration for which larger molecular weight components would make a significant contribution to these averages. Hence the amount and molecular weight of the high molecular components present in the enzyme preparation cannot be determined from this sedimentation equilibrium data alone.

The inconsistency of the molecular weight averages observed at low j(r) values is likely due to errors in measuring the fringe deflections. The reliability of the Yphantis technique is less at low protein concentration since the fringe deflections are small and systematic measurement errors are thus proportionally more significant (67). The sample, however, no doubt contained some protein with a molecular weight less than $1.15 \times 10^5$.

Nevertheless, this data in conjunction with the sedimentation velocity data (which showed a single discernible protein boundary) implies that the molecular weight range for most of the proteins in this enzyme preparation was rather narrow. Therefore the molecular weight of the enzyme protein was assumed to be approximately $1.15 \times 10^5$.

These equilibrium and velocity data also indicate that the molecular weight of the protein was not significantly altered by the buffer systems nor temperatures which were used. Neither was there indication of a rapid, reversible, concentration dependent association reaction within the concentration range which was studied (67). It is obvious from Table 3 that susceptibility of the enzyme to heat inactivation was not directly associated with the molecular size of the enzyme.

The dependence of the $S_{20,w}$ on concentration was evaluated from the data presented in Table 2 and Figure X. These data are from sedimentation velocity experiments using different initial enzyme concentrations (3.0 to 10.0 mg per ml) in the 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 8 mM isocitrate. Figure X shows a slight increase in the observed sedimentation coefficient as the protein concentration decreases. Figure X was extrapolated to an $S^{o}_{20,w}$ of approximately 6.15 at infinite protein dilution.

An analysis of these sedimentation velocity data is rather difficult. The velocity experiments at the higher protein concentrations were run at 20° C, in the 0.05 M Tris-HCl buffer, pH 7.4 at room temperature containing 8 mM isocitrate. The comparable sedimentation equilibrium experiment demonstrates an appreciable amount of nonideality in the region of high relative protein concentration, i.e. large j(r). This is manifest by a decrease in the observed Mw(r) at the larger j(r) values.

Although this nonideal behavior may not invalidate the sedimentation velocity experiments, those experiments in which high protein concentrations were examined (Figure VII and Table 2) must be interpreted with due caution. The consistency of the results from the photoelectric scanner in a variety of different buffer systems (Figure V and Table 2) would indicate that these results were more reliable. The scanner experiments involved lower protein concentrations so that nonideality of the solutions should have been less significant.

It is difficult to compare the results of the present investigation with those mentioned previously (12). Perhaps the different behavior of the enzyme in the two investigations may be attributed, in part at least, to the difference in purity of the two enzyme preparations. Failure of the current investigation to observe an alteration of the molecular weight of the enzyme when the ionic strength of the buffer was changed may be the result of many factors. Extensive

purification may have caused an irreversible conformational change in the enzyme subunits so that they could no longer aggregate. A structural deformation of the enzyme could have occurred as a direct result of one of the procedures used in the purification of the enzyme. Or a small organic molecule may have been responsible for maintaining conformational stability of the subunits (10, 13, 65). Most organic molecules of this nature should have been removed from the enzyme solution during the purification of the enzyme.

Alternatively there may have been an enzymatically inert protein in the less purified preparations which was capable of association with the enzyme. If this association phenomenon was possible only in high ionic strength buffers without inhibiting enzymatic activity, then the apparent molecular weight of the enzyme would be a function of the ionic strength of the buffer system. As the enzyme was purified by ion-exchange chromatography in low ionic strength buffers, this inert protein could be removed from the enzyme solution.

From the data obtained in the current investigation it is apparent that the purified NADP-isocitrate dehydrogenase exists in the active state as a protein with a molecular weight of about $1.15 \times 10^5$. There is no evidence that the enzyme is in chemical equilibrium with either a smaller or larger molecular form. It is thus unlikely that the activity of this enzyme is mediated through gross molecular

alteration under normal physiological conditions. Attempts to disrupt the enzyme into subunits by substrates, high ionic strength buffers and elevated temperatures ($20^{o}$ C) all failed.

It is concluded then that: (1) the NADP-isocitrate dehydrogenase of embryonic chick liver has a molecular weight of $1.15 \times 10^{5}$ as measured by high speed equilibrium sedimentation, (2) the protection against heat inactivation afforded by isocitrate and ammonium sulfate does not result from the formation of a heat stable enzyme aggregate and (3) the increase in enzymatic activity induced by ammonium sulfate is not accompanied by enzyme aggregation.

# BIBLIOGRAPHY


1. Abraham, S. et al. The relation of lipogenesis to reduced triphosphopyridine nucleotide generation and to certain enzyme activities in the liver of 'totally' depancreatized rats. Biochimica et Biophysica Acta 62:27-34. 1962.

2. Atkinson, Daniel E., James A. Hathaway, and Eddie C. Smith. Kinetics of regulatory enzymes. Kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. Journal of Biological Chemistry 240:2682-2690. 1965.

3. Bagdasarian, G., and D. Julanicka. Changes of mitochondrial glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during brain development. Biochimica et Biophysica Acta 99:367-369. 1965.

4. Baker, W. W. and R. W. Newburgh. Intracellular distribution of isocitrate-nicotinamide-adenine dinucleotide phosphate-oxidoreductase activity during development of the chick embryo. Biochemical Journal 89:510-515. 1963.

5. Beckman Instruments, Spinco Division. Technical Bulletin E-TB-015B Instructions for using multichannel short-column equilibrium centerpieces in the Model E. Palo Alto, California, December, 1964. 9 p.

6. Bell, Joyce L., and D. N. Baron. Intracellular distribution of isocitrate dehydrogenases. Biochemical Journal 90:8P. 1964.

7. Bio-Rad Laboratories. Price List T. Richmond, California, July, 1968. 56 p.

8. Bitensky, Mark W., K. Lemone Yielding, and Gordon M. Tomkins. The effect of allosteric modifiers on the rate of denaturation of glutamate dehydrogenase. Journal of Biological Chemistry 240:1077-1082. 1965.

9. Burt, Alvin M., and Byron S. Wenger. Glucose-6-phosphate dehydrogenase activity in the brain of the developing chick. Developmental Biology 3:84-95. 1961.

10. Chen, Raymond F., D. M. Brown, and G. W. E. Plaut. DPN-linked isocitrate dehydrogenase of bovine heart: Structural changes associated with activation by ADP. Biochemistry 3: 552-559. 1964.

11. Chervenka, C. H. A manual of methods for the analytical ultracentrifuge. Palo Alto, California, Beckman Instruments, Spinco Division, 1969. 100 p.

12. Cheung, Geoffrey Peter. Enzymatic studies of the prenatal chick with special reference to the multiple forms of liver soluble NADP-linked isocitrate dehydrogenase. Ph. D. thesis. Corvallis, Oregon State University, 1966. 74 numb. leaves.

13. Chilson, Oscar P. et al. Reversible inactivation of dehydrogenases. Journal of Biological Chemistry 241:2431-2445. 1966.

14. Chung, Albert E., and James S. Frazen. Oxidized triphosphopyridine nucleotide specific isocitrate dehydrogenase from Azotobacter vinelandii. Isolation and characterization. Biochemistry 8:3175-3184. 1969.

15. Cohen, Rene. Sur un nouveau mode d'utilisation des centrifugeuses analytiques. Comptes Rendus Hebdomadaires des Seances de L'Academie des Sciences 256:3513-3515. 1963.

16. Cohen, R., B. Giruad, and A. Messiah. Theory and practice of the analytical centrifugation of an active substrate-enzyme complex. Biopolymers 5:203-225. 1967.

17. Colman, Roberta F. Effect of modification of a methionyl residue on the kinetic and molecular properties of isocitrate dehydrogenase. Journal of Biological Chemistry 243:2454-2464. 1968.

18. Colman, Roberta F. The role of sulfhydryl groups in the catalytic function of isocitrate dehydrogenase. I. Reaction with 5, 5'-dithiobis(2-nitrobenzoic acid). Biochemistry 8:888-898. 1969.

19. Colman, Roberta F., and Rita Chu. Deuterium solvent isotope effects in reactions catalyzed by isocitrate dehydrogenase. Biochemical and Biophysical research Communications 34:528-535. 1969.

20. Coultate, T. P., and D. T. Dennis. Regulatory properties of a plant NAD:isocitrate dehydrogenase. The effect of inorganic ions. European Journal of Biochemistry 7:153-158. 1969.

21. Dyson, Robert D. A simple alignment test for the interference optics of the ultracentrifuge. Analytical Biochemistry 33:193-199. 1969.

22. Dyson, Robert D. Assistant Professor, Oregon State University, Dept. of Biochemistry and Biophysics. An unpublished personal computer program. Corvallis, Oregon. 1969.

23. Ellerton, David, Research Associate, Oregon State University, Dept. of Biochemistry and Biophysics. Unpublished research on the viscosity of buffer systems as a function of temperature. Corvallis, Oregon. 1969.

24. Epstein, Arnold. Graduate Research Assistant, Oregon State University, Dept. of Biochemistry and Biophysics. Unpublished research on the heat stability of the soluble isocitrate dehydrogenase of embryonic chick liver. Corvallis, Oregon. 1968.

25. Ernster, Lars, and Alvin J. Glasky. On the mitochondrial oxidation of isocitrate. Biochimica et Biophysica Acta 38: 169-170. 1960.

26. Fahien, Leonard A., Barbara O. Wiggert, and Philip P. Cohen. Effects of nucleotides and coenzymes on frog liver glutamate dehydrogenase. Journal of Biological Chemistry 240:1091-1095. 1965.

27. Fine, I. H., N. O. Kaplan, and D. Kuftinec. Developmental changes of mammalian lactate dehydrogenases. Biochemistry 2:116-121. 1963.

28. Frieden, Carl, and Roberta F. Colman. Glutamate dehydrogenase concentrations as a determinant in the effect of purine nucleotides on enzymatic activity. Journal of Biological Chemistry 242:1705-1715. 1967.

29. Fritz, P. J., and Bruce Jacobson. Multiple molecular forms of lactate dehydrogenase. Biochemistry 4:282-289. 1965.

30. Fritz, Paul J. Rabbit muscle lactate dehydrogenase 5: A regulatory enzyme. Science 150:364-366. 1965.

31. Henderson, Nanine S. Isozymes of isocitrate dehydrogenase: Subunit structure and intracellular location. Journal of Experimental Zoology 158:263-274. 1965.

32. Hexner, P. E., L. E. Radford, and J. W. Beams. Achievement of sedimentation equilibrium. Proceedings of the National Academy of Sciences 47:1848-1852. 1961.

33. Hinks, Mary, and C. J. Masters. Developmental changes in ruminant lactate dehydrogenase. Biochemistry 3:1789-1791.

1964.

34. Howard, Robert L., and R. R. Becker. Isolation and some properties of the $TPN^+$ isocitrate dehydrogenase from Bacillus stearothermophilus. Journal of Biological Chemistry (in press). 1970.

35. Hubbard, Jerry S., and Alan B. Miller. Purification and reversible inactivation of the isocitrate dehydrogenase from an obligate halophile. Journal of Bacteriology 99:161-168. 1969.

36. Kar, Nirmal C., and Carl M. Pearson. Developmental changes and heterogeneity of lactic and malic dehydrogenases of human skeletal muscles and other organs. Proceedings of the National Academy of Sciences 50:995-1002. 1963.

37. Kazazian, H. H., W. J. Young, and B. Childs. X-linked 6-phosphogluconate dehydrogenase in Drosophilia: Subunit associations. Science 150:1601-1602. 1965.

38. Levy, H. Richard, Ronald R. Raineri, and Barbara H. Nevaldine. On the structure and catalytic function of mammary glucose-6-phosphate dehydrogenase. Journal of Biological Chemistry 241:2181-2187. 1966.

39. Lowenstein, John M. The pathway of hydrogen in biosynthesis. II. Extramitochondrial isocitrate dehydrogenase. Journal of of Biological Chemistry 236:1217-1219. 1961.

40. Lowenstein, John M., and Sandra R. Smith. Intra- and extramitochondrial isocitrate dehydrogenases. Biochimica et Biophysica Acta 56:385-387. 1962.

41. Magar, Magar E., and John E. Robbins. The subunits of porcine heart TPN-linked isocitrate dehydrogenase. Biochimica et Biophysica Acta 191:173-176. 1969.

42. Markert, Clement L., and Freddy Møller. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. Proceedings of the National Academy of Sciences 45:753-763. 1959.

43. Martin, Robert G. and Bruce N. Ames. A method for determining the sedimentation behavior of enzymes: Application to protein mixtures. Journal of Biological Chemistry 236:1372-1379. 1961.

44. Moyle, Jennifer. Some properties of purified isocitric enzyme. Biochemical Journal 63:552-558. 1956.

45. Moyle, Jennifer, and M. Dixon. Purification of the isocitric enzyme (triphosphopyridine nucleotide-linked isocitric dehydrogenase-oxalosuccinic carboxylase). Biochemical Journal 63:548-552. 1956.

46. Ochoa, Severo. Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. Journal of Biological Chemistry 174:133-157. 1948.

47. Pande, S. V., R. Parvin Khan, and T. A. Venkitasubramanian. Nicotinamide adenine dinucleotide phosphate-specifice dehydrogenases in relation to lipogenesis. Biochimica et Biophysica Acta 84:239-250. 1964.

48. Pharmacia Fine Chemicals. Sephadex - gel filtration in theory and practice. Piscataway, New Jersey, June 1967, 56 p.

49. Plaut, Gerhard W. E. Isocitrate dehydrogenase. In: The Enzymes, ed. by Paul D. Boyer, Henry Lardy, and Karl Myrbäck. Vol. 7. New York, Academic Press, 1959. p. 105-126.

50. Plaut, G. W. E., and T. Aogaichi. Purification and properties of diphosphopyridine nucleotide-linked isocitrate dehydrogenase of mammalian liver. Journal of Biological Chemistry 243:5572-5583. 1968.

51. Purvis, J. L. Pathway of oxidation of isocitrate by mitochondria and the role of the transhydrogenase reaction. Biochimica et Biophysica Acta 52:148-155. 1961.

52. Ragland, T. E., T. Kawasaki, and J. M. Lowenstein. Comparative aspects of some bacterial dehydrogenases and transhydrogenases. Journal of Bacteriology 91:236-244. 1966.

53. Reeves, Henry C., Bette Ann Brehmeyer, and Samuel J. Ajl. Multiple forms of bacterial NADP-specific isocitrate dehydrogenase. Science 172:359-360. 1968.

54. Rose, Zelda B. Studies on the mechanism of action of isocitrate dehydrogenase. Journal of Biological Chemistry 235: 928-933. 1960.

55. Sanwal, B. D., M. W. Zink, and C. S. Stachow. Nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase. A possible regulatory protein. Journal of Biological Chemistry 239:1597-1603. 1964.

56. Sanwal, B. D., and C. S. Stachow. Allosteric activation of nicotinamide adenine dinucleotide specific isocitrate dehydrogenase of Neurospora. Biochimica et Biophysica Acta 96:28-44. 1965.

57. Sanwal, B. D., and R. A. Cook. Effect of adenylic acid on the regulatory nicotinamide-adenine dinucleotide specific isocitrate dehydrogenase. Biochemistry 5:886-894. 1966.

58. Schachman, Howard K. Ultracentrifugation in biochemistry. New York, Academic Press, 1959. 272 p.

59. Siebert, Gunther, et al. The preparation of isocitrate dehydrogenase from mammalian heart. Journal of Biological Chemistry 226:965-975. 1957.

60. Siebert, Gunther, M. Carsiotis, and G. W. E. Plaut. The enzymatic properties of isocitrate dehydrogenase. Journal of Biological Chemistry 226:977-991. 1957.

61. Stadtman, E. R. Allosteric regulation of enzyme activity. In: Advances in Enzymology and Related Subjects of Biochemistry, ed. by F. F. Nord. Vol. 28. New York, Interscience, 1966. p. 41-154.

62. Sund, Horst. The pyridine nucleotide-dependent dehydrogenases. In: Biological Oxidations, ed. by Thomas P. Singer. New York, Interscience, 1968. p. 641-649.

63. Solomon, J. B. Changes in the distribution of glutamic, lactic, and malic dehydrogenases in liver cell fractions during development of the chick embryo. Developmental Biology 1:182-198. 1959.

64. Van Holde, K. E. Sedimentation equilibrium. In: Fractions. No. 1. Palo Alto, California, Beckman Instruments, Spinco Division, 1967. p. 1-10.

65. Vesell, Elliot S. (ed.). Multiple molecular forms of enzymes. Annals of the New York Academy of Sciences, Art. 1. New York, 1968. 689 p.

66. Vignais, P. V. and P. M. Vignais. Role des di- et triphosphopyridine nucleotides dans l'oxydation mitochondriale de l'isocitrate. Biochimica et Biophysica Acta 47:515-528. 1961.

67. Yphantis, David A. Equilibrium ultracentrifugation of dilute solutions. Biochemistry 3:297-317. 1964.

68. Yielding, K. Lemone, Grodon M. Tomkins, and Diana S. Trundle. Metal-ion requirement for pyridine nucleotide-induced disaggregation of glutamate dehydrogenase. Biochimica et Biophysica Acta 77:703-705. 1963.

69. Young, Jerry W., Earl Shrago, and Henry Lardy. Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. Biochemistry 3:1687-1692. 1964.

70. Zalkin, H., and D. B. Sprinson. An investigation of imine formation in the isocitrate dehydrogenase reaction. Journal of Biological Chemistry 241:1067-1071. 1966.