

AN ABSTRACT OF THE THESIS OF

GEOFFREY PETER CHEUNG for the Ph. D. in Chemistry
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Title ENZYMATIC STUDIES OF THE PRENATAL CHICK WITH
SPECIAL REFERENCE TO THE MULTIPLE FORMS OF
LIVER SOLUBLE NADP-LINKED ISOCITRIC DEHYDRO-
GENASE

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Levels of activity of glucose-6-phosphate- , 6-phospho-
gluconate- , and isocitric- dehydrogenase were measured in the
soluble and particulate fractions of heart, brain and liver at
several ages throughout the development of the chick embryo.
Compared to isocitric dehydrogenase, levels of glucose-6-
phosphate- , and 6-phosphogluconate- dehydrogenase are generally
low. This suggests that the majority of the NADPH in the prenatal
chick arises from the NADP-isocitric dehydrogenase. The results,
however, say nothing of the possibility of different pools of NADPH
arising from the different enzymes. A NADPH-NAD transhydro-
genase activity was found in the digitonin-treated mitochondrial
fractions of the same organs, and its levels were also determined.
They were found to be very low, making it unlikely that the majority
of the NADPH generated by the isocitric dehydrogenase is shunted

into the electron transport chain of the mitochondria.

Multiple forms of the NADP-linked isocitric dehydrogenase were shown to be present in the soluble fractions from liver of 18-day old embryos, using differential rates of heat denaturation. Partial purification of the soluble liver enzyme yielded two forms, one stable and the other labile to heat. It was found that these two enzymes have different sedimentation properties, pH optima and reaction rates with NADP. The dimeric, heat stable form may be dissociated into the monomeric, heat labile, form by a lowering of the ionic strength. This dissociation is readily reversible, and the newly formed dimer appears to be similar with the native dimer.

On the basis of experiments presented and comparison with the behavior of similar enzymes in other organisms, a mode of interaction of the multiple forms is postulated. Soluble NADP-linked isocitric dehydrogenase of liver may play a role in the regulation of cellular metabolism during development.

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SPECIAL REFERENCE TO THE MULTIPLE FORMS OF
LIVER SOLUBLE NADP-LINKED
ISOCITRIC DEHYDROGENASE

by

GEOFFREY PETER CHEUNG

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APPROVED:

Redacted for privacy

Professor of Chemistry
In Charge of Major

Redacted for privacy

Chairman of the Department of Chemistry

Redacted for privacy

Dean of Graduate School

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Typed by Gwendolyn Hansen

To

My parents and my wife

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LIST OF ABBREVIATIONS USED IN THIS THESIS

NADP, NADPH:	the oxidized and reduced forms of nicotinamide-adenine dinucleotide phosphate
NAD, NADH:	the oxidized and reduced forms of nicotinamide-adenine dinucleotide
TRIS-:	2-amino-2-hydroxymethylpropane-1, 3, diol
EDTA:	ethylene diamine tetraacetic acid
AP(NAD):	3-acetylpyridine analogue of NAD
AP(NADP):	3-acetylpyridine analogue of NADP
DNA:	deoxyribonucleic acid
H _s , B _s , L _s :	the soluble (cytoplasmic) fractions of heart, brain and liver and the enzymes obtained therefrom
LS or LS-enzyme:	the soluble isocitric dehydrogenase from 18-day old chick embryo liver
S-enzyme:	the heat stable form of soluble isocitric dehydrogenase
L-enzyme:	the heat labile form of soluble isocitric dehydrogenase

ENZYMATIC STUDIES OF THE PRENATAL CHICK WITH
SPECIAL REFERENCE TO THE MULTIPLE FORMS OF
LIVER SOLUBLE NADP-LINKED
ISOCITRIC DEHYDROGENASE

PART I. LEVELS OF SOME NADP-LINKED ENZYMES
DURING DEVELOPMENT

INTRODUCTION

Classical studies of embryology involved primarily descriptions of morphological change. Such studies are of value in understanding the differentiation of a diploid cell into a multicellular organism in biological terms. Of equal value is the necessity of understanding the changes occurring at the molecular level. The chick embryo offers an excellent system for such studies aiming at the ultimate description of cellular differentiation in terms of metabolic or genetic control. The enzymatic makeup of the embryo may be conveniently measured and related to differentiation. It is anticipated that such determinations may reflect the actual change in molecular content of cells or cell fractions accompanying development. Furthermore, enzymes catalyze definite metabolic changes and their presence and levels of activity serve as indicators of metabolic activity. Integrated metabolic activity eventually shows up as gross morphological change.

Many recent attempts have been made to correlate observed morphological changes with measured changes in enzymic activity,

including studies on the chick embryo. Mahler, Wittenberger and Brand (46) demonstrated the activity levels of most of the enzymes of glycolysis and of the Krebs cycle in 4-day embryos and 10-day livers. Brand and Mahler (7) demonstrated a rise in liver mitochondria in both NADH oxidase and NADH diaphorases. Okuno (56) measured levels of hepatic phosphorylase, while Silber, Huennekens and Gabrio (65) measured that of formate-activating enzyme and tetrahydrofolate reductase. Solomon (67, 68, 69) carefully noted levels of lactic-, glutamic-, and malic-dehydrogenases in chick embryo, mitochondria and supernatant of heart, brain and muscle. Strittmatter (70) recorded levels of electron transport enzymes in both mitochondria and microsomes of the liver at selected ages. Enzymes involved in NADPH generation are of interest, since it has been speculated that this pyridine nucleotide plays a role in metabolic control mechanisms (49).

Radiorespirometric experiments involving the use of glucose-1, -2, or -6- C^{14} showed that in homogenates of chick embryo hearts, the phosphogluconate pathway of glucose catabolism predominates over the glycolytic pathway (17, p. 32). This persists until about the fifth or seventh day, when the reverse occurs. Burt and Wenger (8) found high levels of glucose-6-phosphate dehydrogenase in early chick embryo brain extracts. These authors found a close correlation between glucose-6-phosphate dehydrogenase levels and the

mitotic index and suggested that variations in the enzyme level may reflect corresponding variations in cellular proliferation and differentiation. They found that the levels of glucose-6-phosphate dehydrogenase drop off on the fifth day. Thus both radiorespirometric and enzymatic data indicate that at a time when nucleic acid synthesis is greatest (during the first few days of development), the phosphogluconate pathway is predominant. It appears that this pathway of glucose breakdown serves also to furnish the five-carbon units necessary for nucleic acid synthesis. Newburgh and co-workers (55), using early chick embryo explants, confirmed the glucose-6-phosphate dehydrogenase data of Burt and Wenger, but found that the NADP-linked isocitric dehydrogenase also occurs at significantly high levels. On the basis that the correspondence is closer between DNA accumulation and isocitric dehydrogenase, it was suggested that isocitric dehydrogenase functioned to supply NADPH used in biosynthesis. That this is extremely likely, is shown in the later work of Baker and Newburgh (5) who measured the levels of isocitric dehydrogenase in heart, brain, and liver of the developing chick and based these levels both on DNA and protein nitrogen. Activity of this enzyme remains significantly high in both supernatant and mitochondria of all three organs studied. Isocitric dehydrogenase therefore appears to be present in significant amounts both before and after the seventh day of

development.

Although Burt and Wenger carried their measurements of brain glucose-6-phosphate dehydrogenase to the 18th day, no comprehensive paper has yet reported any similar measurements in heart or liver of chick embryos. Furthermore, levels of activities of the closely-related NADPH-generating enzyme, 6-phosphogluconate dehydrogenase have not been reported. If isocitric dehydrogenase is used to furnish NADPH in early embryo development when enzymes of the phosphogluconate pathway themselves also produce NADPH, the requirement for this reduced pyridine nucleotide throughout life must be considerable. It would be conceivable, therefore, that activities of both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase might be present throughout embryonic life, especially in the liver, where most lipid synthesis and detoxifications (all requiring NADPH) take place. The measurement of the levels of activity of both these enzymes during development appears to be in order.

Indications are that NADPH in the cell cytoplasm (soluble fraction) is utilized for biosynthetic purposes, particularly lipogenesis (4, p. 58). At the same time that soluble isocitric dehydrogenase activity increases, there is also a rise in the phosphatides of the whole embryo and the brain. The latter is thought to be related to myelin formation (6). Wallace has shown

that levels of isocitric dehydrogenase increases at the time that neurolation occurs in Rana pipiens (72). Kingsburg (39) reports that between the 12th and 16th day of chick embryo liver development rapid adipose deposition occurs. This is the same time that soluble isocitric dehydrogenase activity increases. Nevertheless the fate of the NADPH generated in mitochondrial fractions remains obscure. Since the embryo survives on only that amount of food substance which is in the yolk, more efficient energy production might be derived if NADPH were transhydrogenated to NADH which in turn is oxidized. This has been shown to be the case in rat liver mitochondria which contains 99% NADP-linked isocitric dehydrogenase as determined spectrophotometrically by Purvis (59, 60) and polarographically by Chappell (12). The presence of a Kaplan-type (36) NADPH-NAD transhydrogenase has never been demonstrated in chick embryos.

Part I of this thesis describes the measurement of the three dehydrogenases in chick embryo: glucose-6-phosphate-, 6-phosphogluconate-, and isocitric-dehydrogenase. The levels of a transhydrogenase in the mitochondrial preparations is reported.

MATERIALS AND METHODS

Fertile Eggs

Hy-Line 950-A fertile eggs were purchased from Jenk's Hatchery in Tangent, Oregon. The eggs were incubated in a Jamesway Model 252 B incubator with an automatic turner. The eggs were turned every two hours and incubated at a wet bulb temperature of 86° F and a dry bulb temperature of 99° F until used.

Chemicals

NADP, DL-isocitrate, glucose-6-phosphate 6-phosphogluconate, AP-(NAD), NADH, cytochrome c, bovine serum albumin and Tris- were all obtained from Sigma Chemical Company of St. Louis, Mo.

Cell Fractionation

Cell fractionation was essentially that of Carey and Greville (9, 10). The incubated eggs were dissected at the desired ages and their hearts, brains and livers removed. The excised organs were immediately placed in separate beakers, placed over ice until enough material has accumulated (usually from between 5 to 10 dozen

embryos). Each gram of material was immersed in 10 ml of homogenizing medium containing 0.25 M sucrose, 4 mM EDTA, in 0.05 M Tris-HCl buffer, pH 7.40. Homogenization was carried out in a motor-driven Potter-Elvehjem type homogenizer provided with a loose-fitting Teflon pestle. In some heart preparations of older embryos, it was necessary to cut the cardiac tissue into smaller strips before homogenizing. The resulting homogenate was centrifuged at 600 x g for 15 minutes to sediment the cellular debris, unbroken cells and nuclei, all of which were discarded. The supernatant was then recentrifuged at 10,000 x g for 30 minutes to precipitate the heart, brain or liver mitochondria (H_M , B_M , L_M) as the case may be. The supernatants over the mitochondrial fractions were labelled respectively H_s , B_s , and L_s . The mitochondrial preparations were then resuspended and washed in a 10% w/v of homogenizing medium. This was resedimented at 10,000 x g for 10 minutes. The washed mitochondria samples were then dissolved in glass distilled water (water: original wet weight = 1:1). Lysis of the mitochondria is effected by osmotic shock.

All operations were carried out in the cold ($0-4^{\circ}$ C). Centrifugations were done in a Servall type RC-2 automatic superspeed centrifuge with type ss-34 rotor.

Protein Determinations

Protein was determined by the microbiuret method of Itzhaki and Gill (33). Bovine serum albumin was used as the working standard.

Enzyme Assays

All enzyme assays were performed at room temperature. Enzyme activities were determined by following the optical density changes using a Gilford optical density converter attached to a model DU Beckman spectrophotometer and a Leeds and Northrup recorder. The Gilford cuvette positioner was set so that the optical density in each cuvette was observed for five seconds before the next one is moved into place. This allowed for the simultaneous running of controls, which contain no added coenzyme or substrate. Enzymic activity was calculated from the linear portion of the curve.

In the measurement of the oxidase activities, oxygen uptake was recorded polarographically by the model KM oxygraph by Gilson Medical Electronics.

All reactions were started by the addition of the enzyme. Specific activities of isocitric, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases and transhydrogenase were

expressed as $\mu\text{moles of product formed/min/mg protein}$. The extinction coefficient of NADPH at 340 $\text{m}\mu$ was taken to be $6.22 \times 10^6 \text{ cm}^2 \text{ M}^{-1}$ (32).

Succinic oxidase

Activity of this enzyme was measured using 90 μmoles Tris-HCl buffer, pH 7.4 together with 120 μmoles succinate (sodium salt), 1mg cytochrome c and 0.1 ml of the preparation, in a final volume of 2.2 ml. Specific activity was expressed as $\mu\text{moles O}_2$ taken up/min./mg protein/2.2 ml.

NADH-cytochrome c reductase

The activity of this enzyme was measured in the Beckman DU by following cytochrome c reduction at 550 $\text{m}\mu$. The reaction mixture consisted of 0.1 ml sample, 120 μmoles Tris-HCl buffer (pH 8.5), 1 mg cytochrome c, 4 μmoles KCN and 1.2 μmole NADH, in a final volume of 3 ml. Specific activity was expressed in ΔOD at 550 $\text{m}\mu$ per min per mg protein.

Transhydrogenase

The activity of this enzyme was taken by following the reduction of AP(NAD) at 365 $\text{m}\mu$. In a total volume of 3 ml, the reaction mixture consisted of 0.1 ml sample, 115 μmoles Tris buffer,

pH 7.4, 4 μ moles KCN, 0.8 μ moles NADPH and 0.6 μ mole AP(NAD).

Dehydrogenases

Measurement of activities of the dehydrogenases all involved using the same reaction mixture with the exception of the difference in substrate. A 3 ml volume contains 110 μ moles Tris buffer, pH 7.4, 4 μ moles KCN, 4 μ moles KCN, 4 μ moles MnCl_2 , 2 μ moles NADP and 0.1 ml sample. The amount of substrate used, as the case may be, were, DL-isocitrate, 4 μ moles; glucose-6-phosphate, 1 μ moles; and 6-phosphogluconate; 2 μ moles (as their sodium salts).

RESULTS

Activities of Succinate Oxidase and Cytochrome c Reductase

To show that the supernatant enzyme preparations (also called soluble enzymes) were not contaminated with enzymes of mitochondrial origin, the activities of two respiratory-linked enzymes were measured in typical preparations of both the supernatant fraction and in mitochondria. Table I shows the activity of succinate oxidase on a preparation from 15-day old embryos. Only the mitochondria (freshly prepared) showed any oxygen uptake at all. Since digitonin is known to be an inhibitor of the electron transport chain (54), a digitonin (1%) treated preparation of mitochondria was similarly assayed. No succinate oxidase activity could be detected, indicating that the measured activity is associated with the mitochondrial electron transport apparatus.

Table I. Activity of Succinic Oxidase in 15 day old embryos

	Cellular Fraction						
	H _M	B _M	L _M	L _M ^{D*}	H _s	B _s	L _s
Enzyme Units**	38.8	11.2	7.25	0	0	0	0

*Mitochondria treated with digitonin to give a final concentration of 1%

**Units are expressed in μ moles O₂ reduced/min/mg protein at room temperature and a constant reaction volume of 2.2 ml.

Table II shows the activities of NADH-cytochrome c reductase at selected stages of development. The mitochondrial activity is seen to be at least ten times the soluble activity. Antimycin A is an inhibitor of NADH-cytochrome-c activity (62), and 4 μ gm effectively inhibited all of the mitochondrial activity. However attempts to inhibit the activity in the soluble fractions with Antimycin A failed. It is concluded from this that the soluble NADH-cytochrome c activity stems from microsomal associated diaphorases, which are Antimycin insensitive (50).

Table II. Activity of NADH-cytochrome-c reductase

Age in days	Units ($OD_{550\ m\mu}$ /min/mg protein) in various Cellular Fractions					
	H_M	B_M	L_M	H_s	B_s	L_s
19	0.03	0.022*	0.004	0.003	0	0
15	0.342	0.069	0.079	0.023	0.0121	0.004
8	0.069	0.025	0.130	0	0	0.009

*In the presence of 4 μ gm antimycin A (purchased from Sigma, dissolved in 95% ethanol) sp. act. of mitochondrial fraction is reduced to zero.

On the basis of the data on succinate oxidase and NADH cytochrome c reductase it is concluded that the method used in the preparation of mitochondria and soluble fractions from chick

embryo liver is acceptable.

Levels of NADP-linked Isocitric Dehydrogenase

The levels of activity of this enzyme have been carefully measured and extensively reported by Baker and Newburgh (5). It is by far the most active NADPH-generating enzyme studied so far in the heart, brain and liver of the chick embryo. For the sake of continuity, and to establish good basis for comparison, the activity of this enzyme was routinely measured. The results are shown on Table III. The pattern during development are similar to those reported by Baker and Newburgh. The specific activity of the mitochondrial enzyme from all three organs begins to increase after the 10th day of development and reaches a maximum on the 14th or 15th day. This is followed by a small decrease in activity until hatching, when levels seem to rise again. Correspondingly, the levels of the soluble enzyme show large increases during the latter one-third of in ovo life.

Levels of Glucose-6-phosphate and 6-phosphogluconate Dehydrogenases

Measurable activity was found only in the soluble fraction of heart, brain, and liver (Table IV). Specific activities, although comparable to those published for lamb embryos (21), for chick

embryos (29, 41, 11, 8, 18) and for rat embryos (63), are extremely low. Highest activity is found in the liver. There is an increase in activity up to the 15th day, after which it declines. Fractions from one-day old chicks show no great increase. This is found in all three organs for both enzymes.

Table III. Activity of Isocitric Dehydrogenase*

Days	Units**					
	H _M	B _M	L _M	H _s	B _s	L _s
(hatch) + 1	12.8	29.8	47	-	105.8	286.8
19	58	14.6	89	-	-	-
18	20	46	38	171	110	371
15	274	67	195	219	241	276
10	80.5	87.8	101	8.2	10.1	141
8	7.62	2	18.3	5.8	1.8	58.5

*Corrections have been made for endogenous reduction of NADPH.

**Units are expressed in μ moles NADP reduced min/mg protein.

Levels of NADPH-NAD Transhydrogenase

All attempts to demonstrate the presence of a supernatant NADPH-NAD transhydrogenase met with no success. Some measurable activity, however, was found in the 1% digitonin-treated mitochondrial preparations. The results are shown on

Table V. Levels of this enzyme are significantly less than those of isocitric dehydrogenase in the same preparations indicating that the activities of these two enzymes may not be metabolically interrelated. At least, all of the reduced NADP from the isocitrate dehydrogenase reaction is probably not reoxidized by way of an initial transhydrogenation in either the mitochondria or supernatant.

Table IV. Activity of Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

Days	Units (μ mole NADP reduced/min/mg)					
	Glucose-6-phosphate dehydrogenase			6-Phosphogluconate dehydrogenase		
	H _s	B _s	L _s	H _s	B _s	L _s
+ 1	1.94	0	1.23	2.8	4.8	8.0
19	0	0	0	8.72	3.23	8.2
15	-	-	-	6	3.6	15
14	5.8	0.45	5.7	-	-	-
10	0	0	9.63	8.8	.6	6.5
8	-	-	9.63	0	0	1.84

Table V. Activity of NADPH-NAD
Transhydrogenase

Days	Units*		
	H _M	B	L _M
17	3	1.97	2
15	8	7	17.8
10	6.3	1.4	7
8	2	0	2.8

*Units are expressed in terms of μ moles AP(NADH) formed/min./mg. protein. Control cuvette has no AP(NAD). For details, see text.

DISCUSSION

Levels of Glucose-6-phosphate Dehydrogenase and
6-phosphogluconate Dehydrogenase

The low levels of both these enzymes in all three organs studied, compared with the much higher activity of isocitric dehydrogenase in the same organs, both intra- and extra-mitochondrial, indicate that these two enzymes may not be the major source of NADPH. The peak activity occurring around the 15th day of both enzymes, followed by a decline until hatching (especially in liver soluble fractions), is probably real and not a facet of experimental technique, since values are consistently higher at that age. Further indication of this is shown by the fact that in chick embryos, a few other enzymes peak on, or close, to this time, then subsequently drop. Among these are glucose-6-phosphatase (66), hepatic phosphorylase (56), dihydrofolic reductase and tetrahydrofolic reductase (65) lactic- and malic-dehydrogenases (68, 69). A plot of Burt and Wenger's measurements of glucose-6-phosphate dehydrogenase, however, showed a gradual decrease in activity to almost nil at 19 days (8), but it must be remembered that these authors were looking at the brain alone. Comparison of the specific activities on Table IV with similar measurements in rat foetus show that activities in the latter

are also similarly low (3, 63). Since the overall metabolic activity is also at a maximum at this time the level of these enzymes may be related to this. However, mere measurement of enzyme levels will not elucidate this.

Levels of Isocitric Dehydrogenase

Allowing for differences in the measurement of protein, the results on Tables III and IV are very similar to those reported in the literature (5). This enzyme, in the mitochondrial fraction of heart and liver also come to a maximum around the 15th day. Brain mitochondria activity decreases steadily beginning on the 10th day. Two of the soluble enzymes (viz. H_s and B_s), behave in a similar manner. i. e., peak at the 15th day while the other (Ls) rises continuously until hatching. High activities of particulate isocitrate dehydrogenase suggest generation of high concentrations of reduced NADP. It was supposed that this situation might be related to an active transhydrogenase as well as other metabolic activities.

Levels of NADPH-NAD Transhydrogenase

As seen in Table V specific activities of this enzyme are low (even in heart mitochondria), when compared to isocitric dehydrogenase. This suggests that very little of the NADPH formed in the isocitric dehydrogenase reaction is reoxidized by way

of the electron transport chain, as previously supposed by the author and others (60, 41, 12).

Fates of Extramitochondrial and Mitochondrial NADPH

All efforts to show polarographically the presence of a supernatant NADPH oxidase failed, although a NADPH-cytochrome c reductase (60) is present. Extramitochondrial NADPH, therefore, is not likely to be terminally oxidized by oxygen. It has been widely reported, however, that a likely pathway of reoxidation of NADPH is by its involvement in reductive synthesis of fatty acids. Closer examination of this possibility by other workers indicate the contrary. Murad and Freedland (52) show that in diabetic and in starved rats, when fatty acid synthesis is severely depressed, no changes were found in the soluble NADP-linked isocitric dehydrogenase activity. Their results were supported by those of Young et al. (73) under similar conditions. Depression of fatty acid synthesis caused by pancreatectomy also does not change the levels of isocitric dehydrogenase, as reported by Abraham et al. (1). Similar conclusions were reached by Kallen and Lowenstein (34) who had previously suggested that as much as two-thirds of the reducing hydrogens ending in fatty acids may arise from lactate in liver mitochondria by way of a substrate intermediated trans-hydrogenation through the malate enzyme (44). This was based on

CONCLUSIONS AND SUMMARY

1. Levels of glucose-6-phosphate and 6-phosphogluconate dehydrogenase were measured in soluble extracts of heart, brain and liver of ages ranging from 8 days to hatching.
2. The relatively low specific activities of both enzymes indicate that they play a negligible role in furnishing reduced NADP when compared to isocitric dehydrogenase.
3. Similarly low levels of the NADPH-NAD transhydrogenase in particulate preparations show that reoxidation of NADPH in the mitochondria via this route is very small, if any, under the conditions used.
4. The fate of extramitochondrial NADPH is controversial, and remains obscure. Mitochondrial NADPH might be reoxidized by way of a NADPH-specific oxidase.

PART II. MULTIPLE FORMS OF LIVER SOLUBLE NADP-LINKED ISOCITRIC DEHYDROGENASE

INTRODUCTION

There exists a wide gap between the classical morphological study of embryology and the present attempts at a chemical and molecular approach. Part I of this thesis has been one such chemical approach. Actual definitive correlation is still lacking, however, between changes in enzyme levels and anatomical developments. Such correlations are mandatory for the further understanding of differentiation and of malignant growth. A beginning has already been made along these exact lines with lactic dehydrogenase with knowledge of its isozymic and multiple molecular forms by Fine and Kaplan (22). It was found that during the embryonic development of the rat heart, there is a gradual shift from the M-type units to the H-type units. It was felt that this shift might be caused by or at least a good indicator of, major metabolic changes.

Since the NADP-linked isocitric dehydrogenase persists in such high levels throughout development and the NAD linked enzyme is consistently low (4), the role played by the former enzyme may be important. The NAD-linked isocitric dehydrogenase also has been shown to be allosteric, and hence, regulatory (64, 28). Indications of the existence of multiple forms of soluble liver NADP isocitric

dehydrogenase have been presented (30, 13). These multiple forms, if present, might play important roles in cellular metabolism of the liver during development.

Part II of this thesis describes the efforts made in searching for a possible role for NADP-linked isocitric dehydrogenase during development. This search consists of demonstrating the existence of multiple molecular forms of the enzyme together with some of their properties.

MATERIAL AND METHODS

Materials

Most of the materials used have previously been described in Part I. The Hooded Cobra (Naja Naja) snake venom were purchased from either Sigma or from the Ross Allen Reptile Farm of Silver Springs, Florida. Ovalbumin (recrystallised) was a gift from Dr. A. H. Nishikawa. Alcohol dehydrogenase and α -chymotrypsin were purchased from Worthington Biochemicals. Bovine serum albumin and NAD were obtained from Sigma. 3-AP-NADP was obtained from Pabst Laboratories and enzyme-grade ammonium sulphate from Mann Chemicals.

Methods

Enzyme Preparation and Assay

This has already been reported in Part I. The crude enzyme material used here for the snake venom experiments and for further purification is the same as the L_s enzyme of Part I.

Heating Procedure

Unless otherwise stated, heating is carried out in a constant temperature water bath at 46° C. The heated material is removed

at the desired time and immediately placed or transferred into an ice bath. After having reached melting ice temperatures, it is then assayed at room temperature. Usually not more than 0.1 ml of any enzyme solution is used per assay. Specific activity, unless otherwise stated, is expressed in $\Delta\text{OD}/\text{min.}/\text{mg}$ protein. Protein is determined by the 260/280 $\text{m}\mu$ method of Layne (42).

Gel Filtration Experiments

Sephadex G-200 (Pharmacia Co., Uppsala, Sweden) and commercial Sephadex columns were used. For purification and for molecular weight estimation of the stable enzyme, the same column was used. The column size was 37 x 2.5 cm. and the bed volume was approximately 181 cm^3 . The flow rate was 9.1 ml per hour, and the void volume as estimated by an equivalent volume of 2% Blue Dextran was 54 ml. An automatic drop/fraction collector was used, and 55 drops, (3.0 ml) fractions, were collected. In Figure IV, (purification) the calcium phosphate gel eludates (from Table X) were divided into equal halves, and each half was eluted separately to avoid placing too large a volume into the column. The density of the sample was increased by bringing it to 20% saturation with respect to sucrose. This caused no change in specific activity in the sample and facilitated its layering onto the column. Protein recovery from the column was 87% and the activity recovered was

close to 100%. In Figure VI (molecular weight determination of the S - enzyme,) 10 mg of each of the marker proteins or of the S - enzyme were dissolved in 5 ml of 0.4 M $(\text{NH}_4)_2\text{SO}_4$ and 0.2 M Tris-HCl buffer, pH 7.4. ("Buffer A"). Each one was brought to 20% sucrose concentration and individually layered on the top of the column using a small syringe and a thin rubber tubing. Each protein was eluted by "Buffer A." Figure VII shows the Sephadex calibration curve for molecular weight determination of the L - enzyme. The column was equilibrated by, and eluted with, 0.05 M Tris-HCl, pH 7.4. ("Buffer B"). The same marker proteins were used as above at the same concentration, except that they were dissolved in Buffer B and brought to 10% sucrose saturation before being applied onto the column. The column size was 43 x 2.5 cm, and the bed volume was 211 cm³. The flow rate was 12.7 ml/hour, and V_0 , as determined with Blue Dextran under the same conditions, was 45 ml. 55-drop fractions were collected, and this consisted of 3.25 ml/fraction.

Sucrose Gradient Centrifugations

This was carried out as described by Martin and Ames (48), using 0.4 ml of each of the enzymes and marker protein. Figure IX, X, and XI, are the sedimentation patterns for alcohol dehydrogenase, the L-enzyme and the reconstituted S-enzyme respectively.

50-ml fractions were collected from each centrifuge tube, with approximately 0.01 ml in each fraction. Protein was estimated in each fraction by diluting each with 0.5 ml of water and reading the absorbance at 280 m μ in a 1.5 ml cuvette. Where activity assays were performed, the whole diluted fraction was used.

RESULTS

Indications of Two Enzymatic Forms

Qualitative indications of two active enzyme forms from soluble fractions of liver homogenates have been shown by Henderson (30) using starch gel electrophoresis. Baker (4, p. 62), studying differential rates of heat inactivation, suggested that there is a heat stable form and a heat labile form.

Preliminary experiments performed in connection with this thesis on the soluble preparation of 18-day old chick embryo liver (abbreviated "LS") showed 4 or 5 enzymatically active bands by polyacrylamide gel electrophoresis. Heat stability differences of the two enzymatic forms were pursued further. Figure I (curve a), shows a typical decay rate of soluble isocitric dehydrogenase of liver when heated at 46^o C for the lengths of time shown. It is clear that on dilution (1:1) of the enzyme with water the rate of decay is increased by approximately 2-fold (curve b). An interesting observation is noted on curves c, d, showing the decay rates when the enzyme is not recooled (to 0^o C) prior to assay. It appears that quick cooling of the enzyme aliquot after heating "freezes" its molecular orientation (probably in a more inactive state); whereas immediate assay without cooling allows the enzyme molecule to

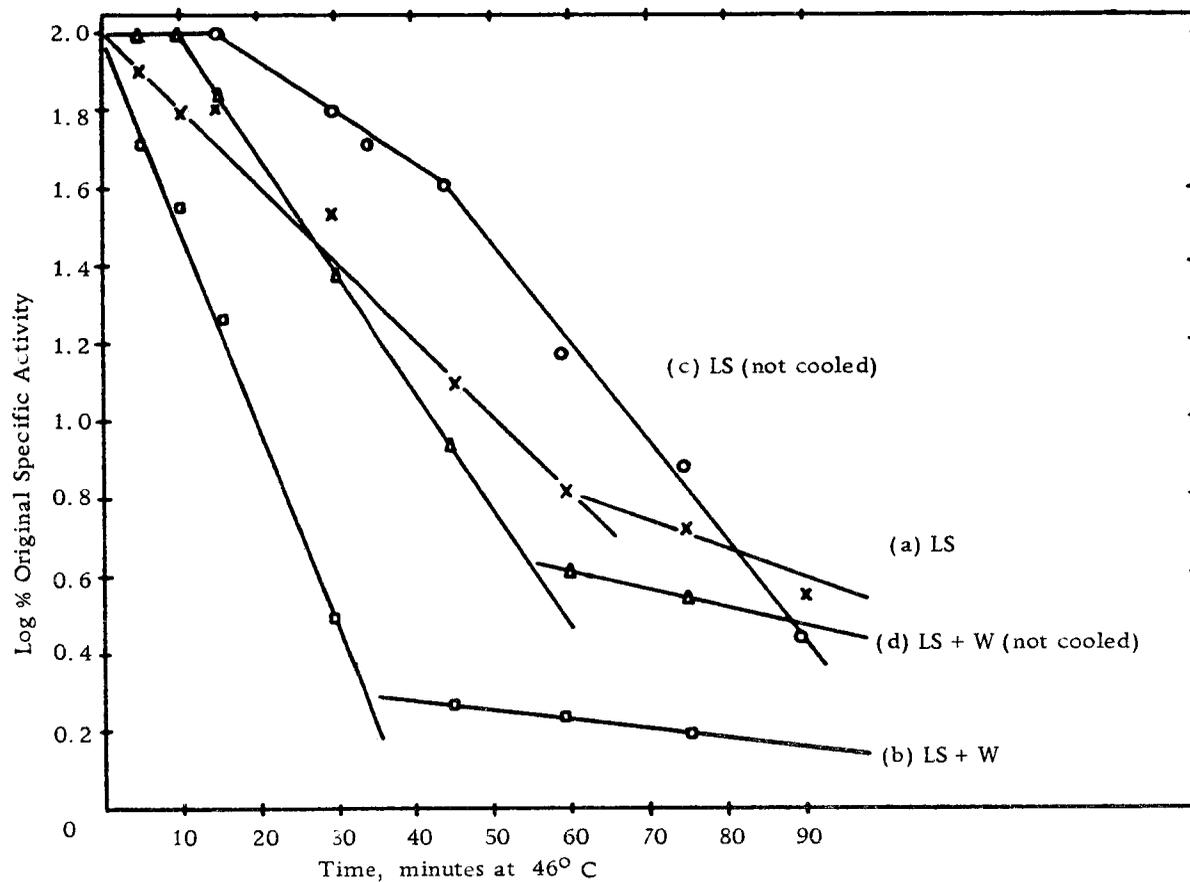


Figure I. Decay rates of soluble isocitric dehydrogenase

4-ml samples were heated in 46° C waterbath. 0.2 ml aliquots were removed at the times indicated and placed in pre-cooled tubes in ice (a, b) 0.1 ml is assayed after cooling. In (c, d), aliquots were assayed immediately after removal from water bath. All assays were at room temperature. For reaction mixture, see "Methods." LS = soluble enzyme of liver. LS + W = enzyme + equal volume of water.

readjust itself to its more active state. Similar curves have been observed by de Flora et al. (23) who studied the thermostability of this same enzyme in rat liver. Curves of the type such as Figure I (a, b) might also indicate that the rate of denaturation was solely dependent upon total enzyme protein present, as pointed out by Reiner (61, p. 275) due to protection by protein-protein interactions. That this is not the case is shown by Figure II and Table VI.

Table VI. Effect of Naja Naja venom concentration on the heat stability of LS enzyme

Concentration of venom used, mg/ml	Specific activity of equal volume mixture after heating
100	0.111
50	0.120
25	0.148
10	0.027
1	0.0023
0.1	0.0018
LS alone, no venom	0.0020

Equal volumes of LS enzyme and venom were mixed. 0.01 ml samples of the mixtures were assayed after heating for 15 minutes at 46° C. (It was necessary to keep this heating period relatively short so that measurable activity would remain in the LS control after heating.) Specific Activity is based on protein of LS enzymes, not total protein. Length of time that venom was preincubated with the enzyme at room temperature does not affect effectiveness of venom for heat protection. (Snake venom alone has no dehydrogenase activity.)

Both of these experiments show that rates of heat denaturation are not linearly proportional to the concentration of Naja Naja venom.

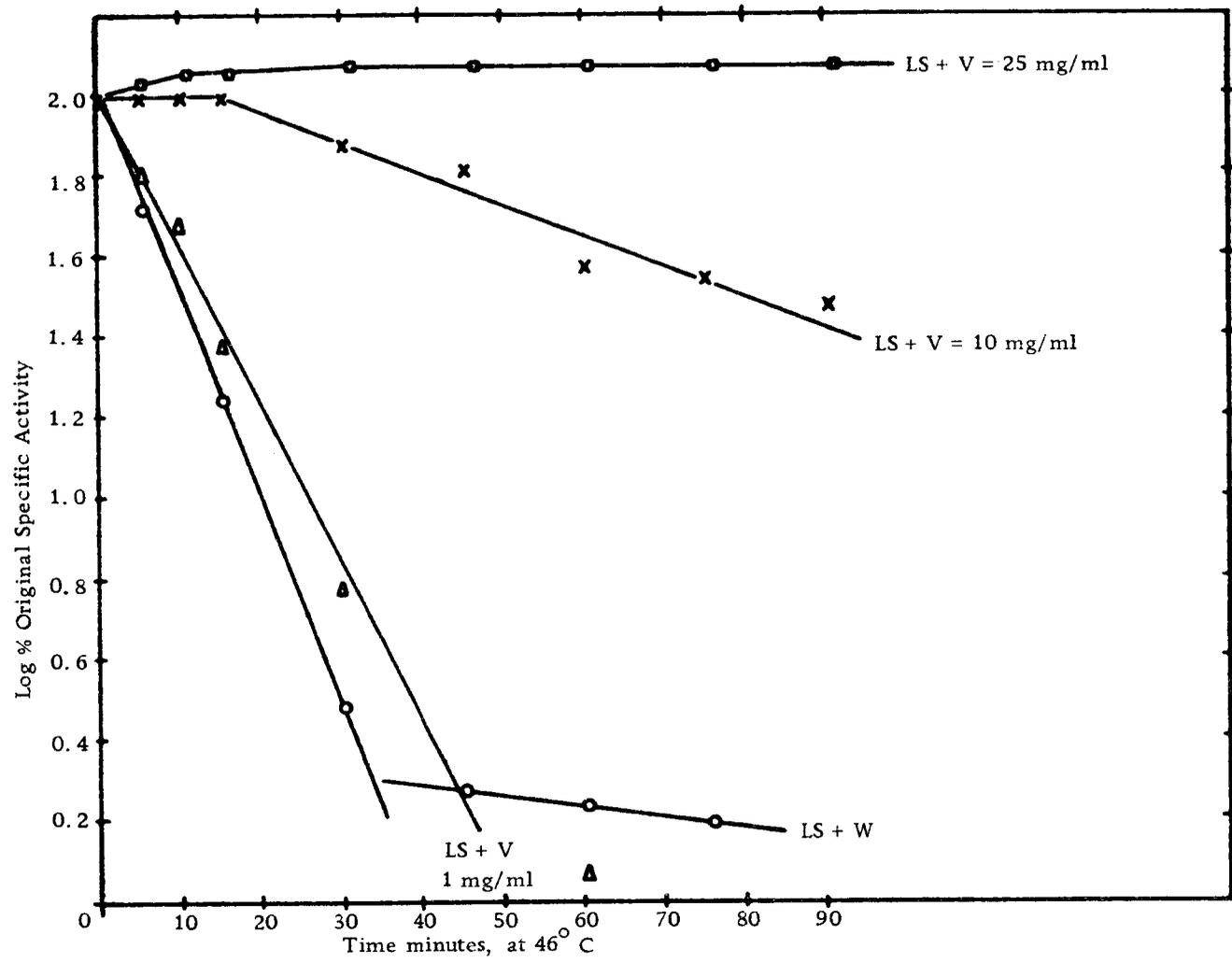


Figure II. Effect of concentration of Hooded Cobra (*Naja Naja*) venom on LS activity

Procedure as in "Methods." Equal volumes of water (W) or venom (V), were mixed with the LS enzyme. Concentrations of the venom were 1, 10, and 25 mg/ml.

Furthermore, Table VII indicates that addition of bovine serum albumin or egg albumin up to four times the concentration of the venom, has little effect. In fact, at levels up to 50 mg/ml (mixed in equal volume with the enzyme), there is less activity remaining after heating than if water were added instead. All this suggests that snake venom may exert a more specific effect on the heat stability of the LS enzyme, causing it to be far less labile and, on occasions, (Table VIII, compare samples 1 and 2) to even enhance its activity considerably.

Table VII. Effect of indifferent protein on the heat stability of the LS enzyme

Conc. bovine serum albumin used, mg/ml	Specific Activity of equal volume mixture after heating	Conc. Ovalbumin used, mg/ml	Specific Activity of equal volume mixture after heating
100	.046	100	.092
55	.016	50	.020
25	.016	25	.020
10	.017	10	.022
1	.015	1	.020
0.1	.015	0.1	.019
LS + W*	.026	LS + W*	.026

Conditions were the same as those in Table VI. Specific Activity was expressed as Δ OD/min/0.1 ml of mixture.

*Equal volume mixtures of LS enzyme and water, heated under same conditions.

Table VIII. Effect of a boiled Naja Naja venom on the heat stability of LS enzyme

Sample Number	Contents, mixed in equal volumes	Specific Activity
1	LS + W unheated	0.085
2	LS + V ₀ unheated	.170
3	LS + V _b unheated	.164
4	LS + V _b heated	.170
*5	LS + W heated	.026

Concentration of venom (V₀), used was 25 mg/ml. Boiled venom, (V_b) was prepared by placing a sample of V₀ in a boiling water bath for 10 minutes. Precipitate was removed by centrifugation at 600 x g for 10 minutes. Samples #4, #5, were heated for 15 minutes at 46° C, cooled, then assayed. 0.1 ml of the indicated material was used in the assays. Specific activity based on Δ OD/min/0.1ml.

*Equal volume mixture of water and LS enzyme.

The effect of boiled venom on the heat stability of the LS enzyme is shown on Table VIII. Activities in experiments number 3 and 4 are almost the same for both the boiled and the unboiled venom, and both values are considerably above that of experiment 5, the water diluted control which has been treated in the same manner. The active constituent of venom which imparts heat stability to the LS enzyme appears not only to be heat stable itself as shown in Table VIII, but also non-dialysable, as shown by Table IX. Heating of the enzyme with and without treatment with the dialysed venom show little changes in activity, while a similarly

treated sample with only water added lost considerable activity.

Table IX. The effect of dialysed Naja Naja venom on the heat stability of LS enzyme

Sample Number	Contents	$\Delta OD/\text{min}/0.1 \text{ ml.}$	
		Expt. A	Expt. B
1	LS + W	0.172	0.224
2	LS + V_0	0.180	0.180
3	LS + V_0 , heated	0.140	0.188
4	LS + W, heated	0.100	0.115

3.0 ml venom (25 mg/ml) was dialysed against 4 liters of glass distilled water in dialysis bag #8, for 3 days. The dialysed venom, (V_0) was then added in equal volume with LS enzyme, and heated for 13 mins. at 46°C . (Samples 3 and 4) After cooling, material was assayed for activity. LS + W is an equal volume mixture of water and LS enzyme.

The action of Naja Naja venom related to the heat stability of the LS enzyme remains speculative, and the actual component in the venom responsible is obscure. One explanation of the former is that the venom causes the enzyme to associate or dissociate into a more heat stable form. That this actually is the case is first indicated by a sucrose gradient centrifugation (5 to 20%) of the LS enzyme. 0.5 ml. of LS was layered onto 4.4 ml of the 5 to 20% sucrose gradient and centrifuged for 19 hours at 37,000 rpm in the Beckman SW-39 swinging bucket head. A hole was then punched in the bottom of the centrifuge tube, and small droplets collected by means of an inverted hypodermic needle, as described by Martin

and Ames (48). A total of 15 fractions were collected, each having about 0.33 ml corresponding to 24 drops per fraction. These fractions were then assayed, their protein concentrations determined, heated to 46° C for 30 minutes, and then reassayed. The results are shown on Figure III. The difference in the enzyme activities before and after heating gives the percentage of the labile enzyme in each fraction. It appears that there exist two molecular forms of the LS enzyme, a more heat stable one, and a more labile one. From Figure III it seems that the stable one is heavier in weight, since it sediments in fractions 6 and 7. In an identical experiment, it has been possible to separate the stable and labile enzymes (stable and labile to 46° C for 30 minutes) by pooling tubes 6 and 7, and pooling tubes 9, 10, and 11.

Partial Purification of Soluble Isocitric Dehydrogenase

It is necessary to purify the crude LS enzyme in order to study the actual nature of its two forms. The purification scheme is essentially that of Plaut's (58), with simplifications and modifications (Table X). Between 10 to 15 dozen 18-day old chick embryos were dissected and their livers removed. Extraction of the crude supernatant (LS) enzyme is as described in Part I, under Methods. (Step 1). Assay conditions are also the same. Step 2. The crude enzyme is first centrifuged for 6 hours at 30,000 x g to sediment

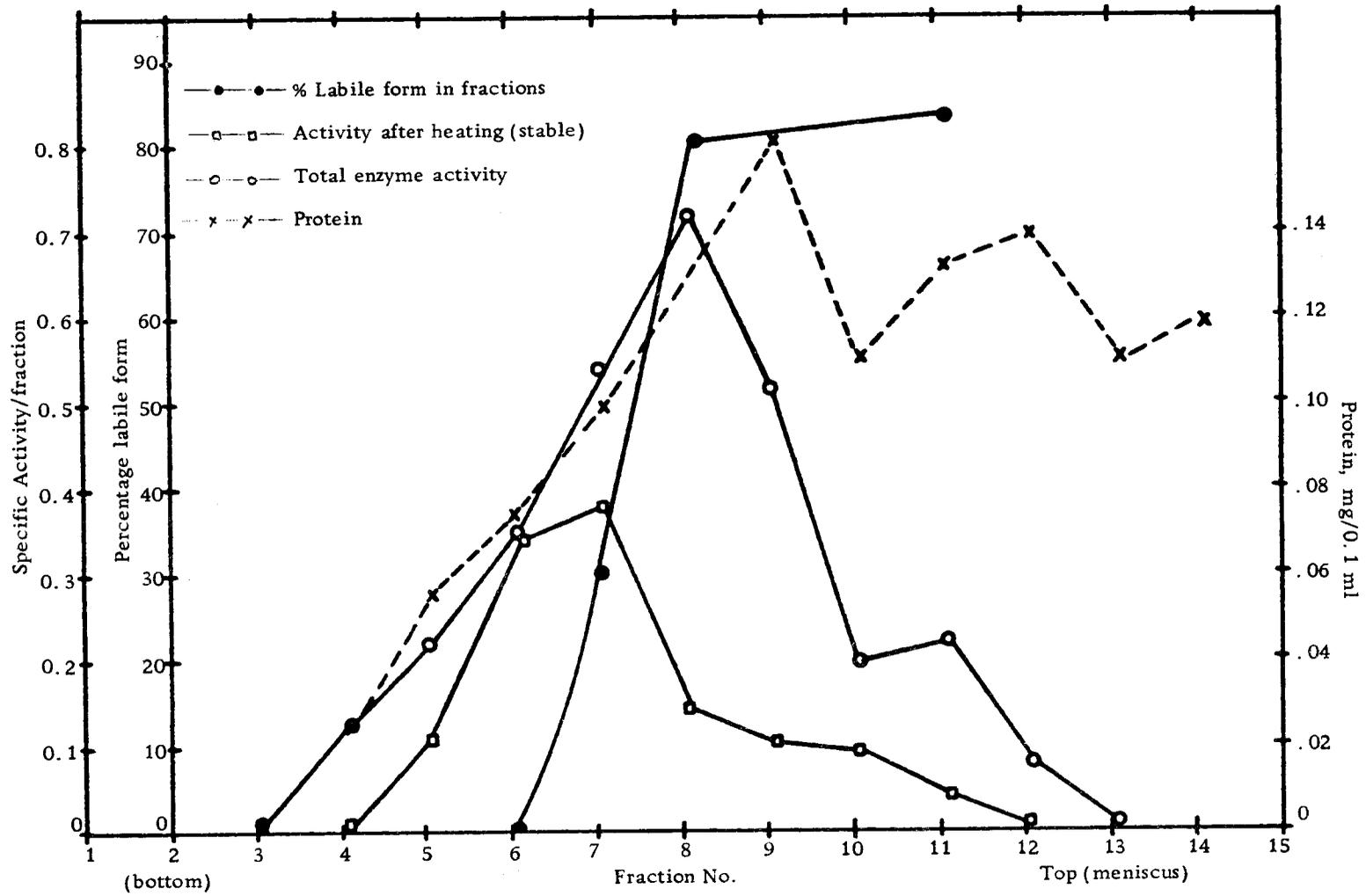


Figure III. Sucrose gradient (5-20%) of LS enzyme

0.5 ml LS enzyme was placed in 4.4 ml of sucrose gradient (5-20%). Protein estimated by OD_{280} . Fractions were assayed, then heated to $46^{\circ}C$ for 30 mins., then reassayed. Difference of the two measurements give the labile form present. (See text)

heavy material. Step 3. To the supernatant of step 2 was added enough solid ammonium sulphate to bring the solution to 40% saturation. After centrifugation and separation the supernatant of this was brought to 75% saturation by the addition of more solid ammonium sulphate, at which point the enzyme protein precipitates. Care was taken to add the salt very slowly each time (over an interval of 90 mins.,) and insoluble material allowed to settle for 18 hours at 0° C before it was removed by centrifugation by 15 mins. at 30,000 x g. The precipitated enzyme was then resuspended in a convenient volume of Tris-HCl buffer, 0.05 M, pH 7.4. Step 4. The enzyme solution was next treated with calcium phosphate gel in three separate treatments. Enough gel solids were respectively added to the enzyme solution to give 0.1 mg-, 1.0 mg-, and 3.5 mg-gel solids per mg of remaining enzyme protein. After each treatment, the precipitate was removed by mild centrifugation and discarded. The resulting solution was deep red. Step 5. To the solution from step 4 was added enough sucrose to give a 20% sucrose solution. No change in specific activity occurred. This was then layered onto a Sephadex G-200 column, equilibrated with Buffer A, 0.4M(NH₄)₂SO₄ in 0.2 M Tris-HCl, pH 7.4 and eluted with the same solution. Figure IV shows that all the activity was concentrated in fractions 25 through 50 (these were combined), and all this activity was stable to heating for 60 mins. at 46° C. Step 6. The enzyme

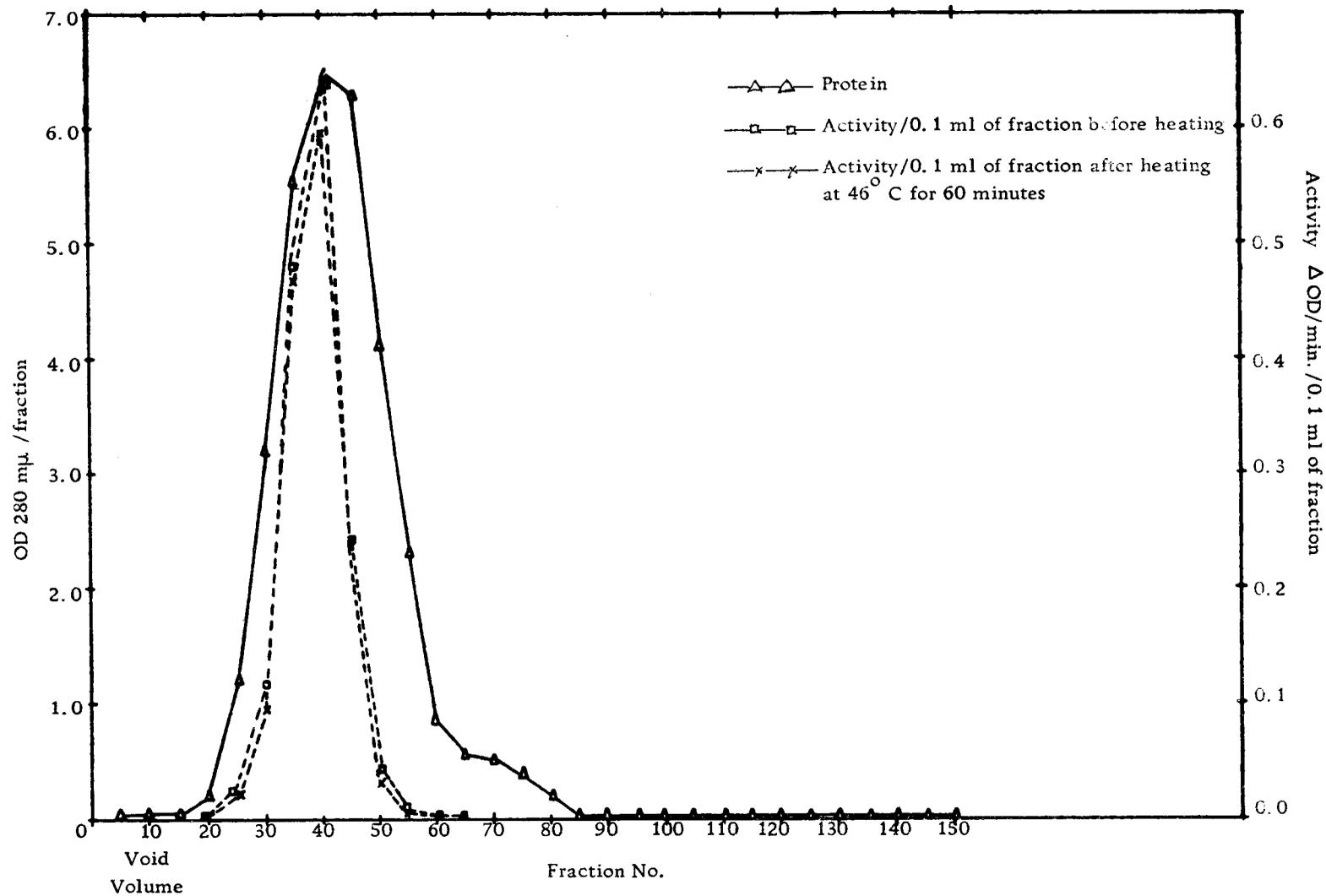


Figure IV. Sephadex G-200 gel-filtration of stable enzyme

protein was reprecipitated from the combined eludates by bringing the latter to 75% $(\text{NH}_4)_2\text{SO}_4$ saturation. The sediment was resuspended in a convenient volume of Buffer A. This solution was then used subsequently as the stable enzyme (S-enzyme). This S-enzyme plane gives a time-course denaturation curve identical to that in Fig. II which is mixed with 25 mg/ml of venom.

Preliminary experiments indicated that in solutions of ammonium sulphate of 10% (0.42 M), or greater, the liver enzyme becomes effectively all the stable or S-form. Dialysis against glass distilled water overnight results in loss of all activity. It was found that the labile, (L), enzyme could be prepared conveniently by dialysis of the S-enzyme against dilute buffer in the cold. Figure V shows that the S-enzyme may be converted into the L-enzyme by dialysis against 0.05 M Tris-HCl buffer, pH 7.4 (buffer B) for 10 hours or greater in the cold. Approximately 75- to 80% of the specific activity is lost in this maneuver, but the remaining activity is entirely of the L-enzyme. Lost specific activity may be reconstituted in most part (indicating reconversion from L- back to the S-form), by adding solid ammonium sulphate back to a final concentration of 0.42 M. This can be seen in Table XI. The reconstituted S-enzyme shows an immediate increase of specific activity, which continues to increase upon heating at 46°C . After 120 minutes at this temperature, 73% of the original specific activity of

Table X. Partial Purification of Soluble Isocitric Dehydrogenase (LS enzyme) From 18-day Old Chick Embryo Liver.

Step	Volume, ml	Protein mg	Total Units ^a	Specific Activity ^b	% Protein Recovered
1. Extract from 52 gm. chick embryo liver	500	10,050	182x10 ³	18.1	100
2. High speed centrifugation	492	4,384	160x10 ^{3c}	36.4	43.5
3. Ammonium sulphate fractionations	32	1,414	117x10 ³	82.5	14
4. Calcium phosphate gel elutions	50	894	281x10 ³	312	8.9
5. Sephadex column eludate	142	670	277x10 ³	413	6.7
6. Final ammonium sulphate precipitation	11	488	233x10 ³	477	4.9

^aUnit activity is defined as ΔOD_{340} per min. under conditions of assay.

^bSpecific activity = units/mg protein.

^cThis increase in total activity is real and was found to be due to the increase in ionic strength on addition of the calcium phosphate gel. It is at this step that all the enzyme is changed into the stable form, as shown in Figure IV. Originally, the crude LS enzyme is >90% labile form (Figure I, curve a). If the precipitates of the $(NH_4)_2SO_4$ step were resuspended in $(NH_4)_2SO_4$ of >10%, (Plaut used 40%)⁴ this sudden jump in total activity will not be observed.

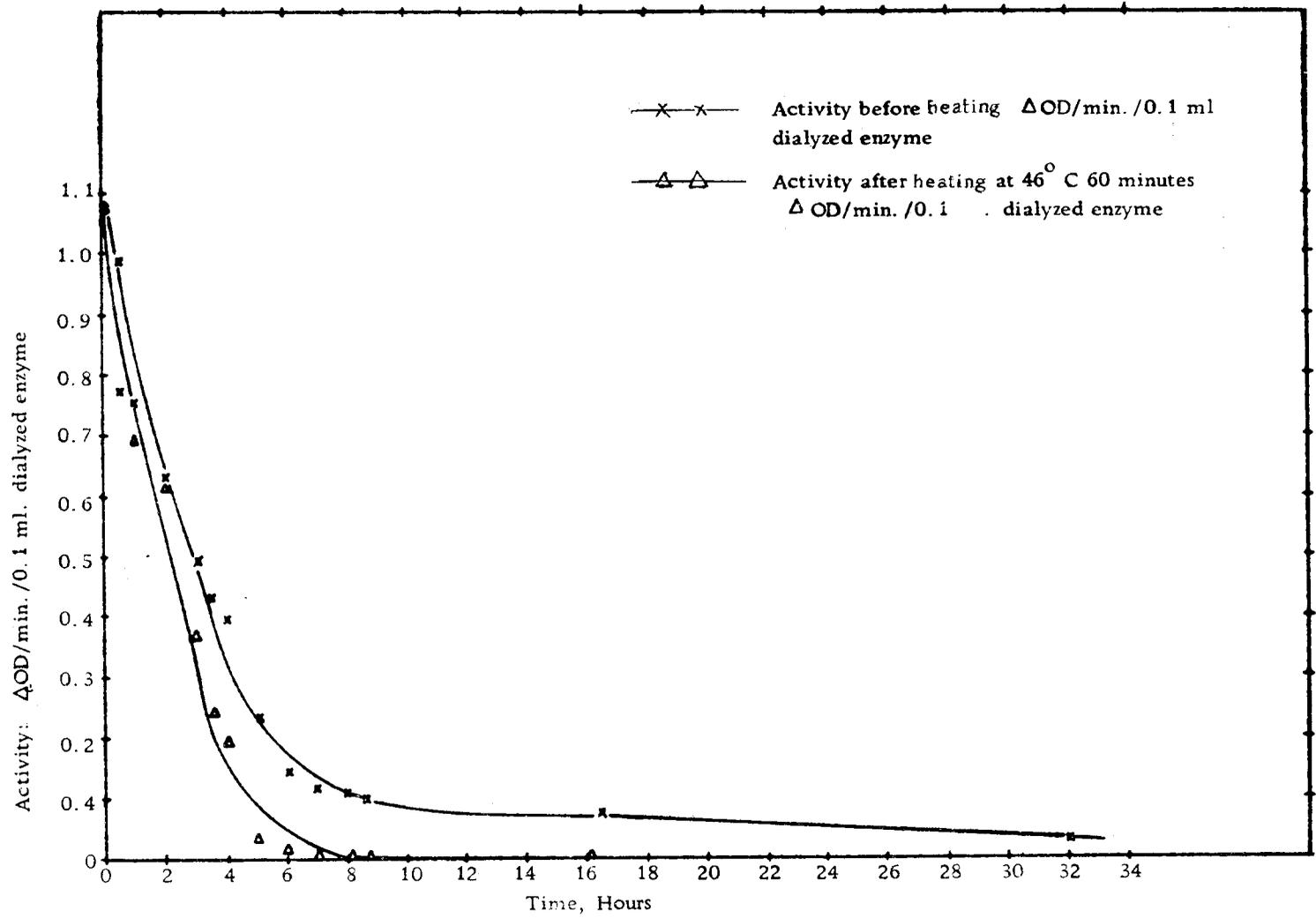


Figure V. Loss of activity of stable enzyme on dialysis against 0.05 M Tris-HCl buffer pH 7.4 (Buffer B)

the S- enzyme is regained. No data is available to indicate if 100% recovery is possible.

Table XI. Interconversions of the S and L forms of the LS enzyme

	Specific Activity: Δ OD/min/mg protein		
	S-enzyme	L-enzyme	Reconstituted S-enzyme
room temperature	0.52	0.1375	0.208
30 min. at 46° C	.52*	--	.342
60 min. at 46° C	.52	0	.322
90 min. at 46° C	.52	0	.342
120 min. at 46° C	-	0	.380

7 ml of S-enzyme of specific activity 0.52 was dialysed against 4 liters of buffer B for 12 hours at 4° C (Union carbide dialysis bag, #8 was used). The L-enzyme obtained has sp. act. = 0.1375. To 0.55 ml of the L-enzyme was added 34.2 mg solid $(\text{NH}_4)_2\text{SO}_4$ to bring the concentration of the latter to 0.466 M. This was called the reconstituted S-enzyme. Relative heat stabilities are shown.

* These values were not actually measured, but their constancy to heat treatment was implied by an identical preparation (from another experiment) which showed no changes to the same treatment.

Some Properties of the S and L Forms of Isocitric Dehydrogenase

Molecular weights of both the S and the L forms have been estimated by gel-filtration using Sephadex G-200, according to the method of Andrews (2). Figure VI shows the calibration curve for a Sephadex G-200 column equilibrated with buffer A for use with

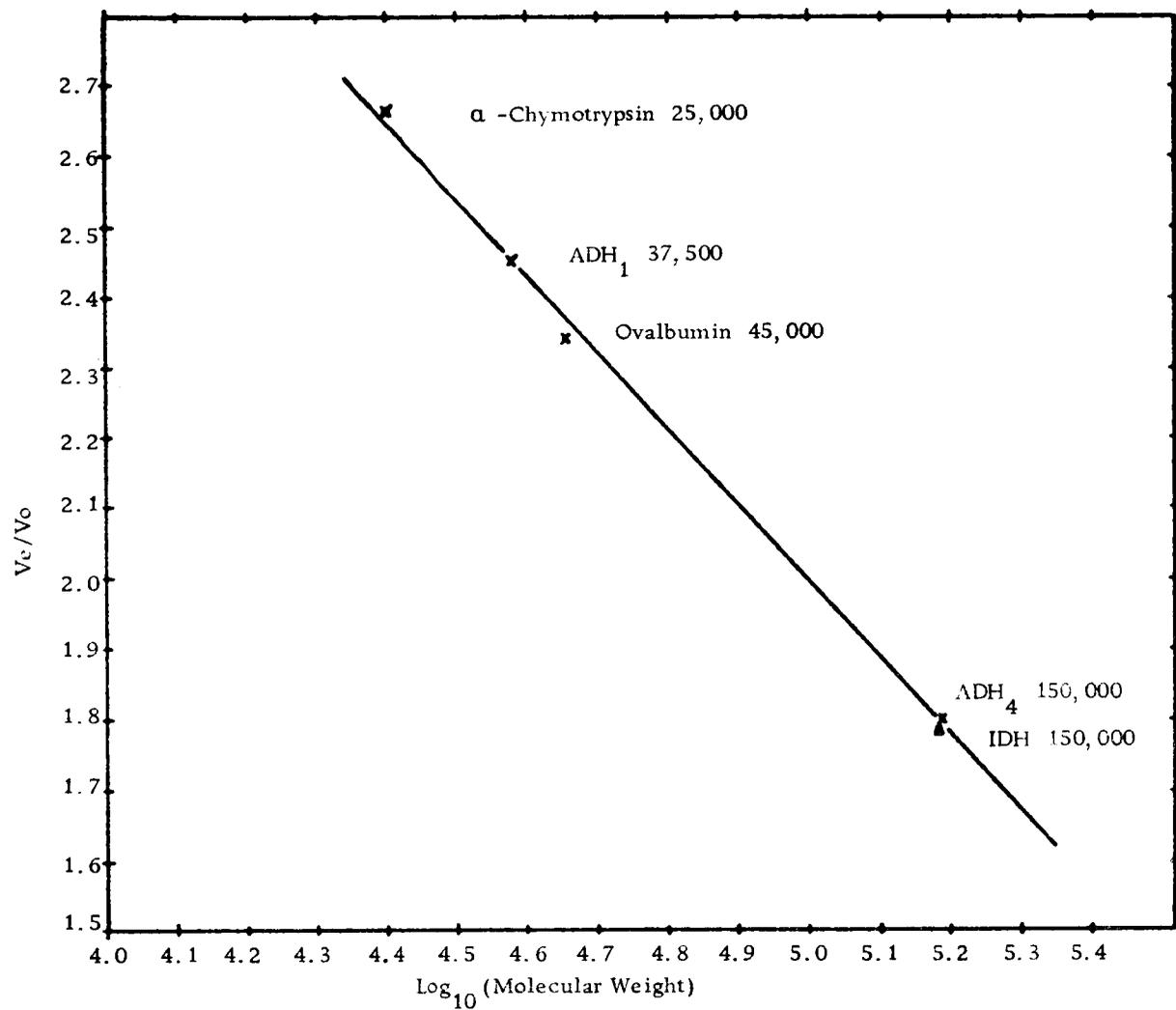


Figure VI. Sephadex calibration curve for molecular weight determination of stable enzyme (for details, see text)

the stable enzyme. Figure VII shows the curve for a similar column equilibrated with buffer B for use with the labile enzyme. The V_e (elution volume) to V_0 (elution volume of Blue Dextran) ratio is plotted against the log of molecular weights of known marker proteins. Employing the same conditions for marker proteins as for the enzyme protein, the molecular weight of the S-form (from Figure VI) was found to be 150,000, while that of the L-form (from Figure VII) was determined to be 85,000. It appears therefore, that the stable form is a dimer of the labile form, both of which are enzymatically active. This possibility has previously been suggested for this enzyme in rat liver by Henderson (30), using only electrophoresis and a line of genetic reasoning.

The S-enzyme (dimer), is considerably more stable to storage at 0 - 4° C than the L-enzyme (monomer). Figure VIII shows the decay of the monomer through a period of time when the dimer shows no change in activity at all.

Molecular weight estimations of the reconstituted S-enzyme was also made by means of sucrose gradient centrifugation, using the method of Martin and Ames (48). Using alcohol dehydrogenase as the marker protein, the centrifugation patterns in 5 to 20% sucrose for 19 hours at 37,500 rpm in the Spinco ultracentrifuge (SW-39 head) are shown in the next three figures. Figure IX shows that of alcohol dehydrogenase monomer and tetramer. Figures

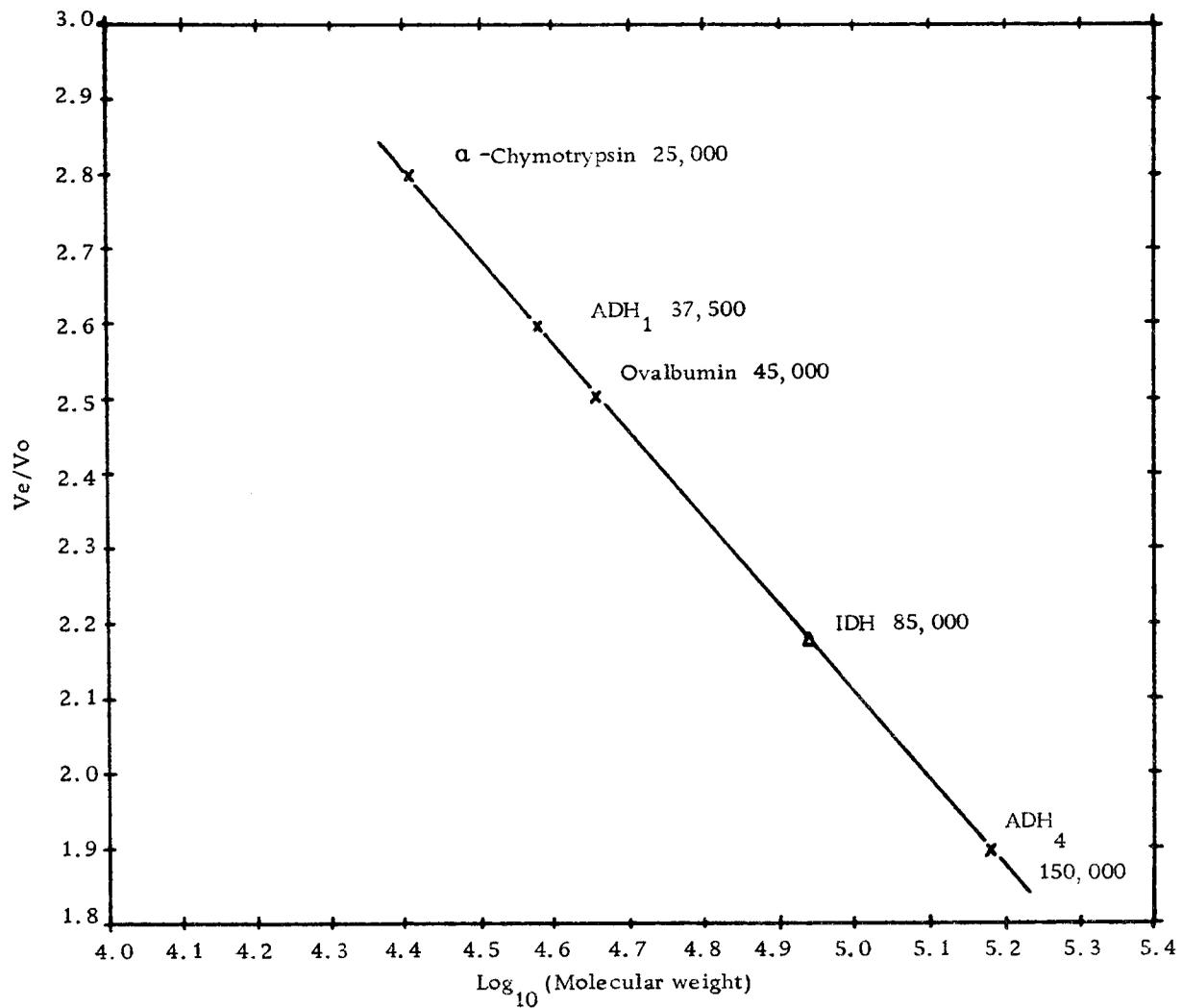


Figure VII. Sephadex calibration curve for molecular weight determination of labile enzyme (for details, see text)

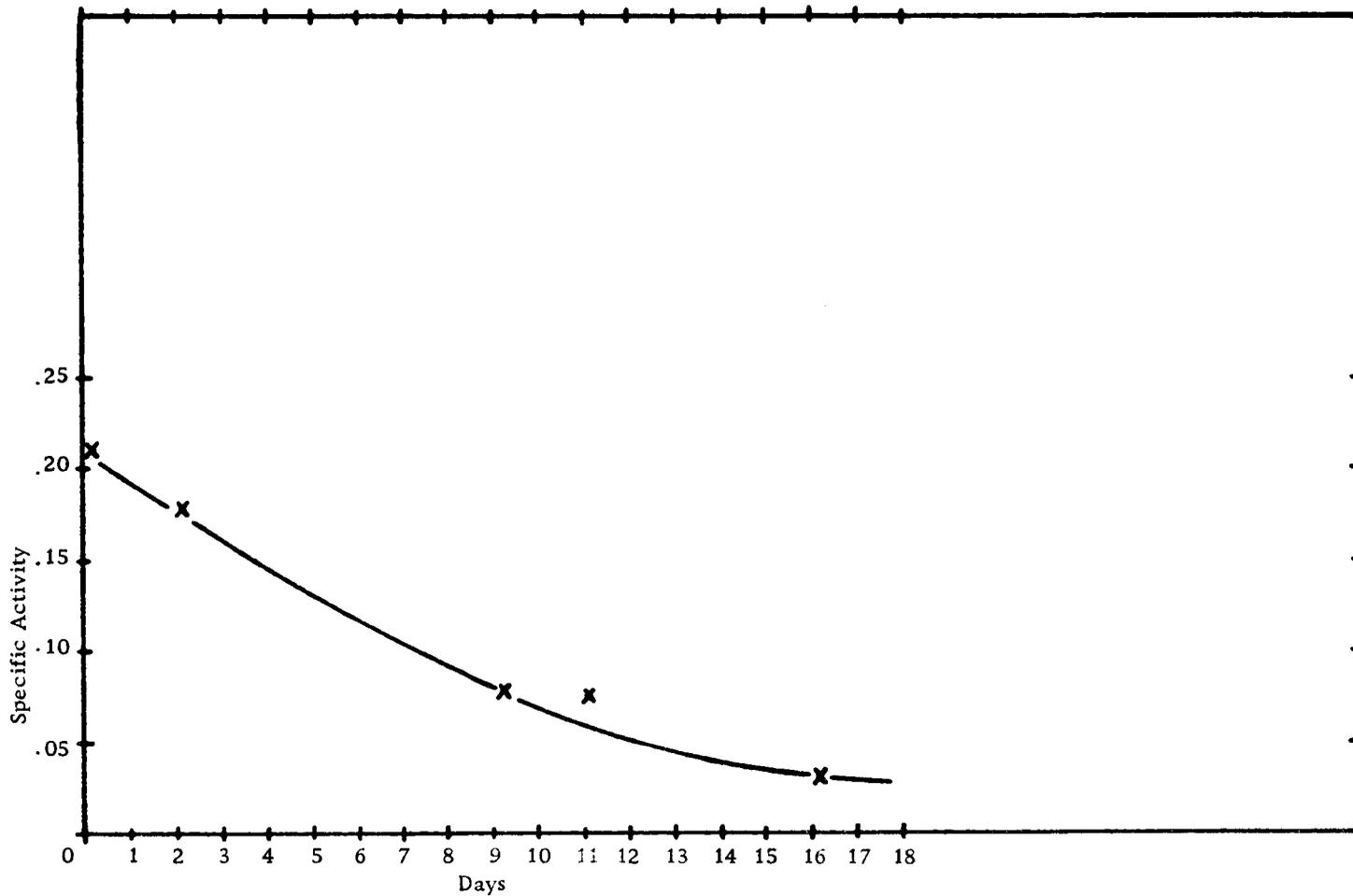


Figure VIII. Decay of labile enzyme on standing at 0-4° C (Activity of stable enzyme during this same period does not change.)

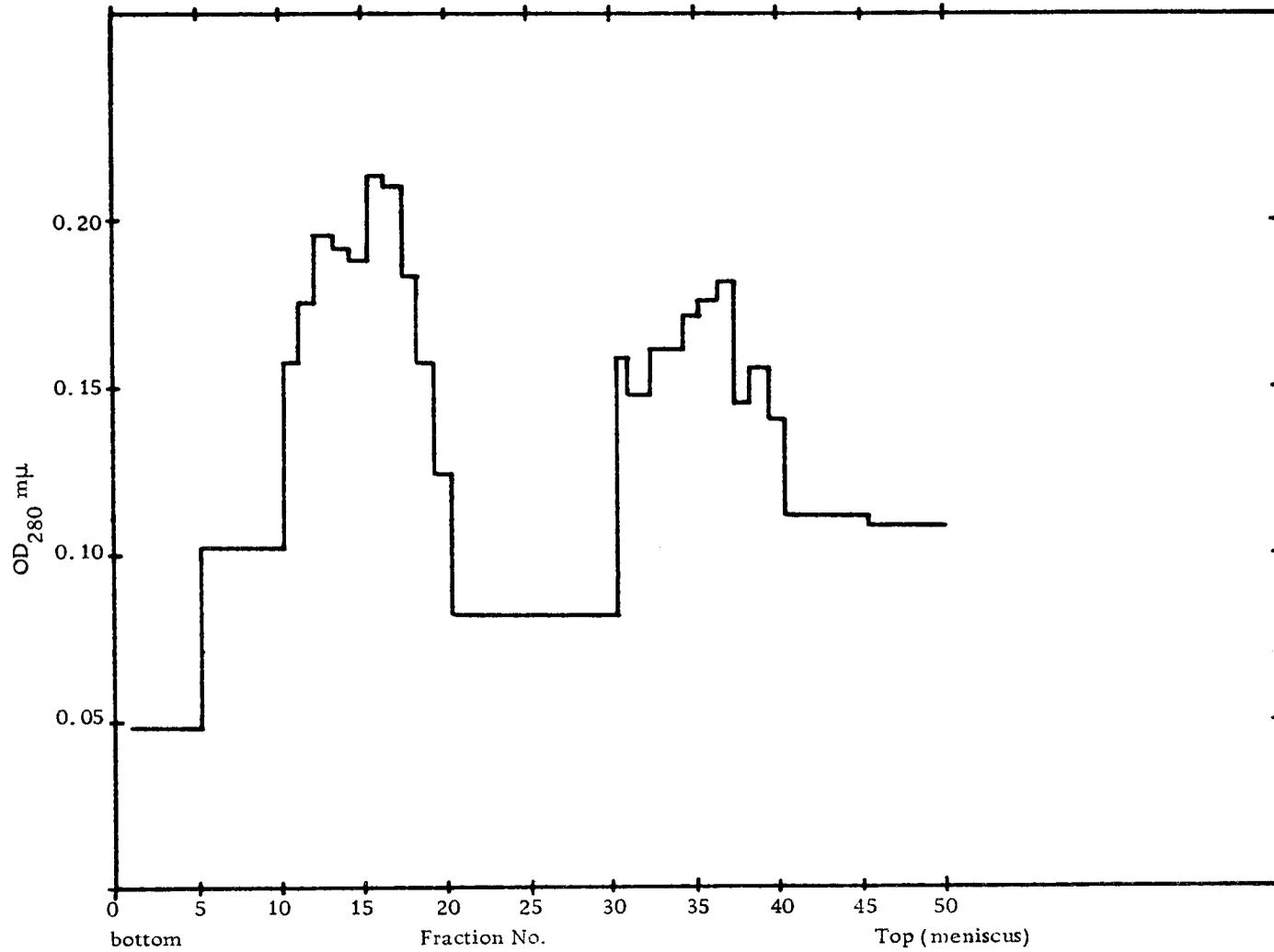


Figure IX. Sedimentation pattern in sucrose gradient of alcohol dehydrogenase

X and XI show the patterns of the L-enzyme and the reconstituted S-enzyme respectively. Conditions were all identical in each of the three cases. For greater accuracy, fifty fractions were collected from each tube, containing 0.01 ml per fraction. The method of Martin and Ames (48) was used in estimating molecular weights:

$$\left(\frac{MW_1}{MW_2}\right)^{2/3} = \frac{S_1}{S_2}$$

where S_1 , S_2 , are the sedimentation coefficients of the two molecules of presumed identical partial specific volumes.

Also, if

$$R = \frac{\text{distance travelled from meniscus by unknown}}{\text{distance travelled from meniscus by standard}}$$

then

$$R = \frac{S_1(\text{unknown})}{S_2(\text{standard})}$$

Calculations indicate that the molecular weight of the L-enzyme was 92,000 (Figure X). Enzymatic assays proved the protein to be still active. Figure XI gave two peaks of protein for the reconstituted S-enzyme, and both were found to be active after assays. The first peak corresponded to a weight of 163,000, and the second lighter peak to a molecular weight of 98,000. Since alcohol dehydrogenase is known to have a molecular weight of 150,000, while that of its monomer is 37,500, the values obtained above for the L-enzyme is assumed to be high (and that obtained by gel filtration closer to the real value). This is based on the fact that the second (monomeric)

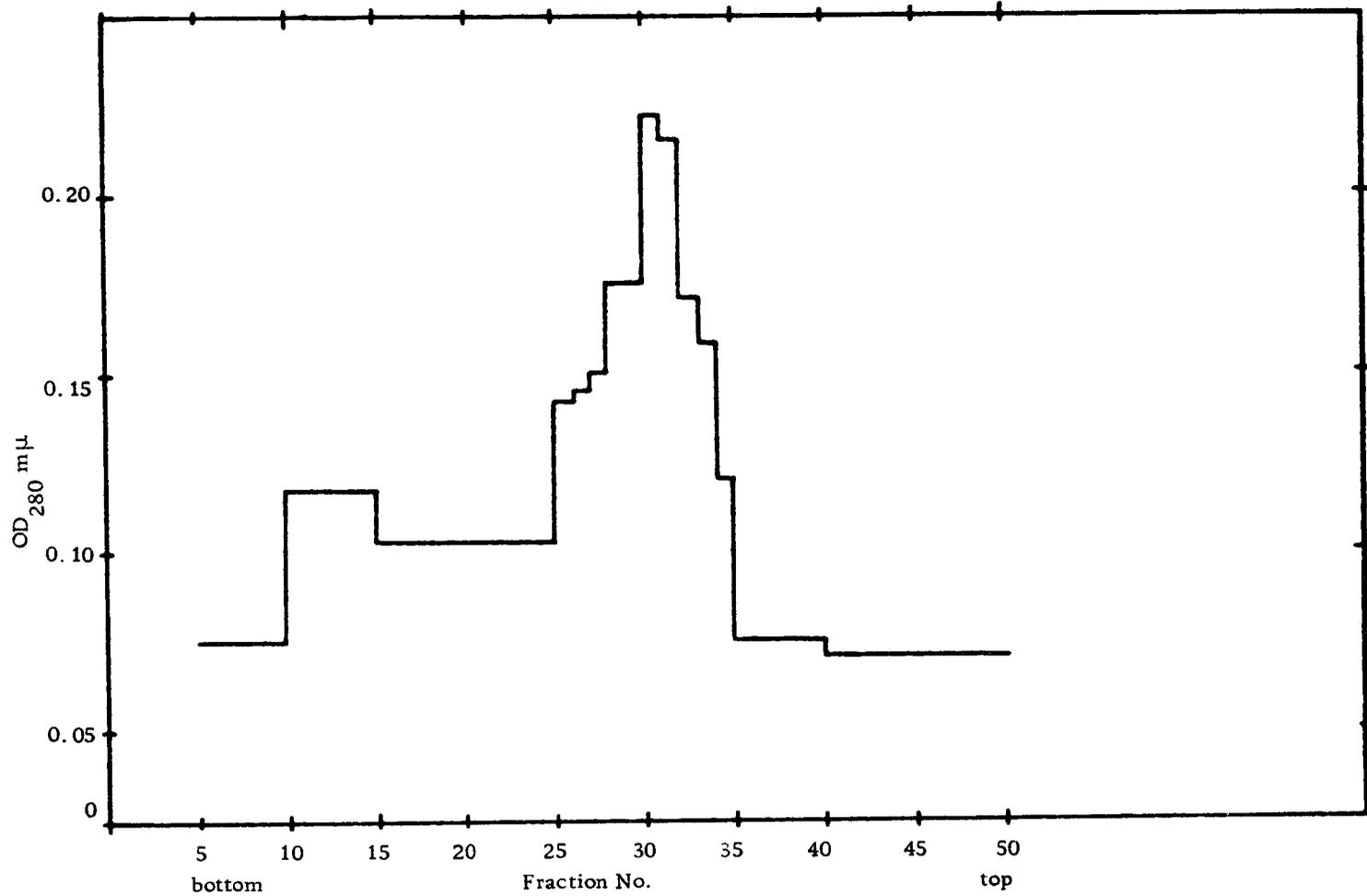


Figure X. Sedimentation pattern in sucrose gradient of labile enzyme

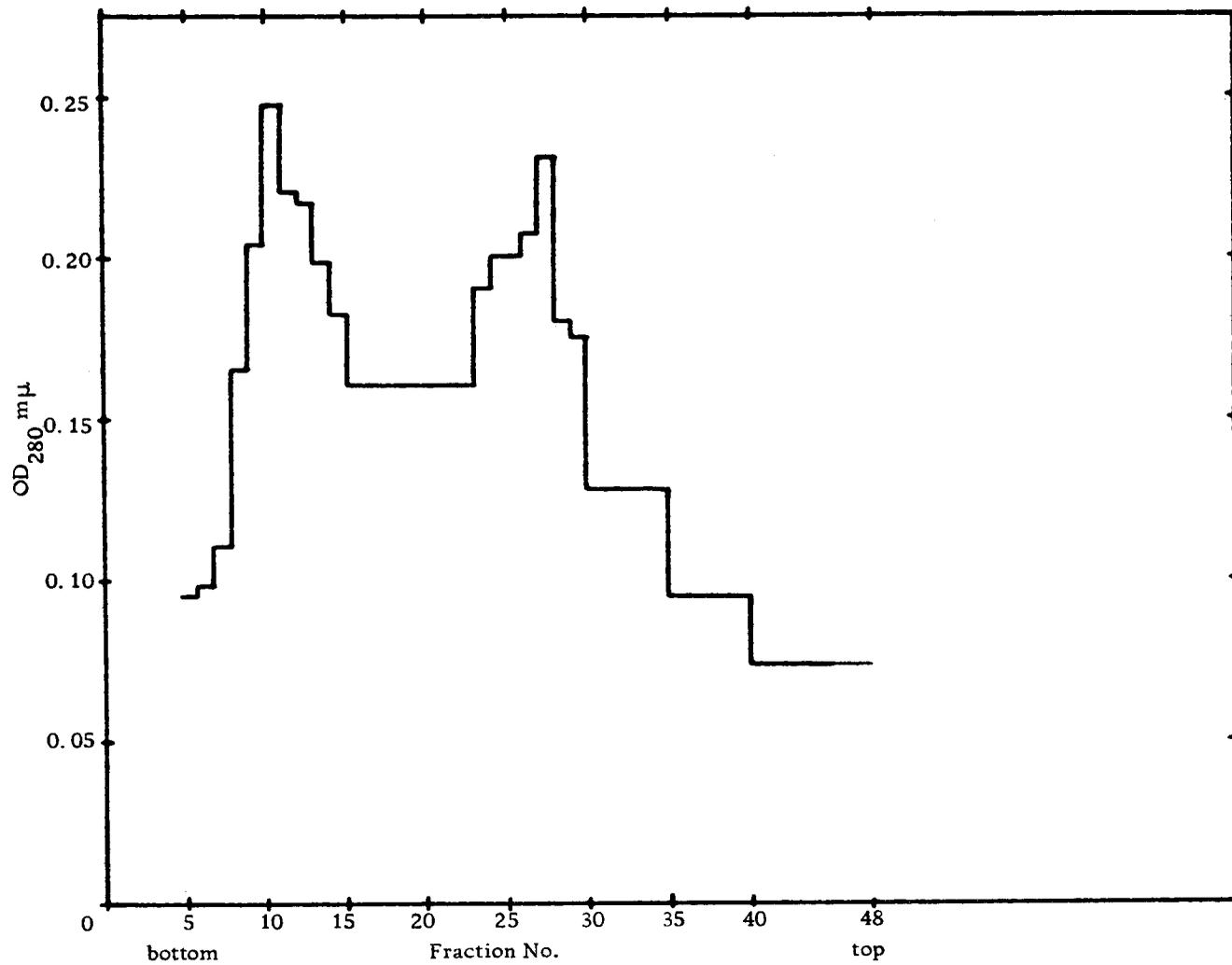


Figure XI. Sedimentation pattern in sucrose gradient of reconstituted stable enzyme

peak of Figure IX for alcohol dehydrogenase gave a value of 42,000, instead of its true value of 37,500. The reason for this discrepancy is not clearly understood.

Figures XII and XIII show the activity-pH curves for the S and the L enzymes respectively for Tris-maleate buffer, 0.05 M. The S enzyme is seen to have a broad range of pH from 6.8 to 8.4. The maximum for the L-enzyme appears to be lower, at 6.0

An attempt was made to determine if any kinetic differences of the two forms of isocitric dehydrogenase existed with respect to their natural substrates and an analogue of NADP, namely 3-acetylpyridine-NADP. Lineweaver-Burk plots for varying concentrations of D-isocitrate for the S-enzyme (Figure XIV) and for the L-enzyme (Figure XV) gave the following values:
 $K_m(S) = 166 \times 10^{-5} \text{ M}$, $K_m(L) = 1.19 \times 10^{-5} \text{ M}$, at a constant concentration of NADP of 0.66 mM. It is assumed that these values are within experimental error and that there are no differences in the actions of the S-enzyme and the L-enzyme for isocitrate under the conditions of the assays.

Figures XVI and XVII show similar plots with varying concentrations of AP-NADP at a constant level of DL-isocitrate of 1.33 mM. Under the conditions of the assays, the $K_m(S)$, and $K_m(L)$ appear to be respectively $1.66 \times 10^{-4} \text{ M}$ and $4.45 \times 10^{-5} \text{ M}$.

Lineweaver-Burk plots with varying concentrations of NADP

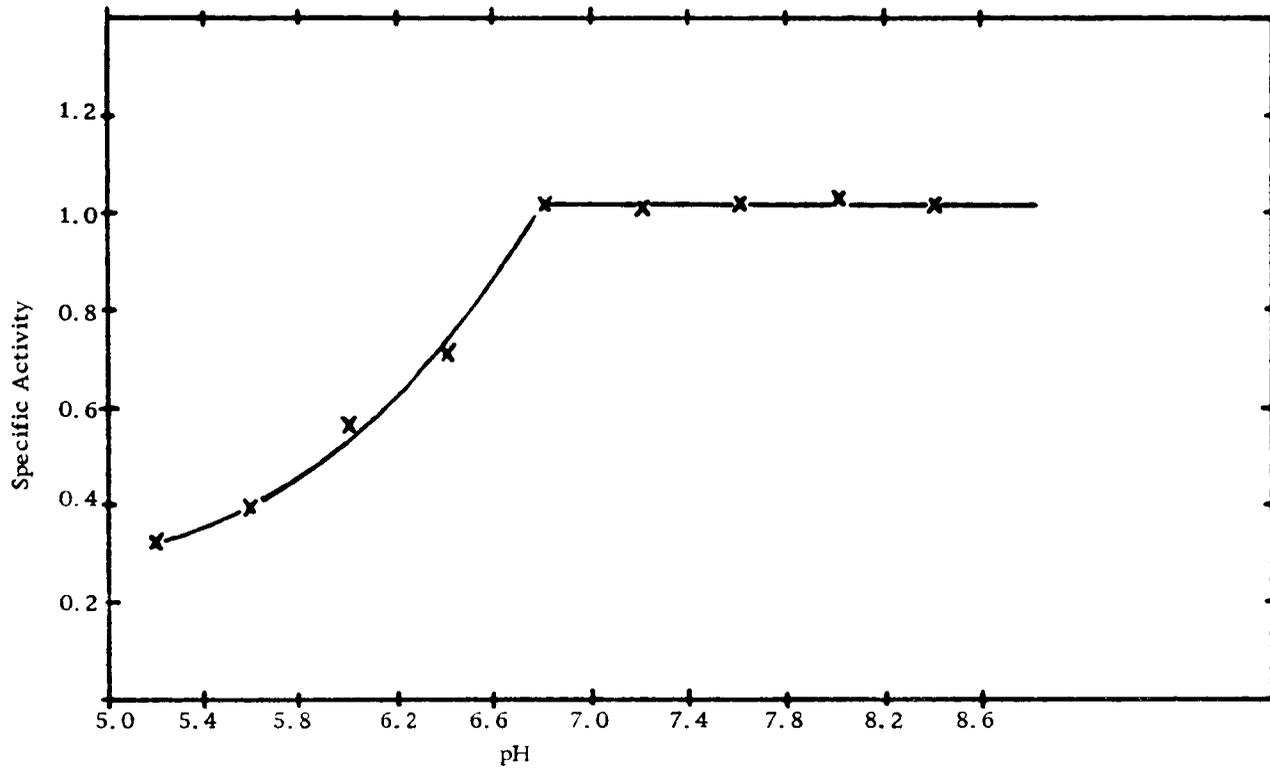


Figure XII. Effects of pH on the activity of the stable enzyme

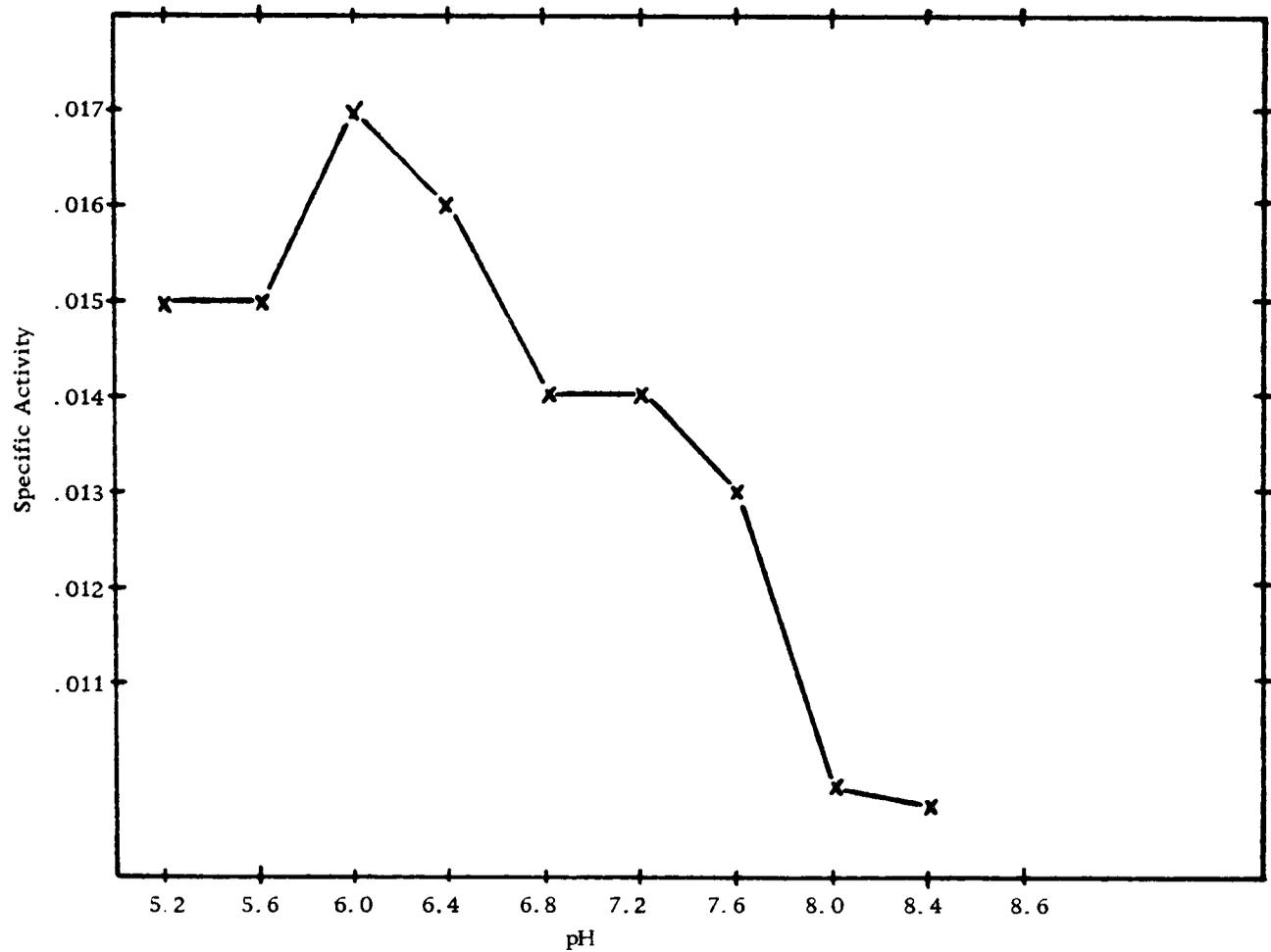


Figure XIII. Effects of pH on the activity of the labile enzyme

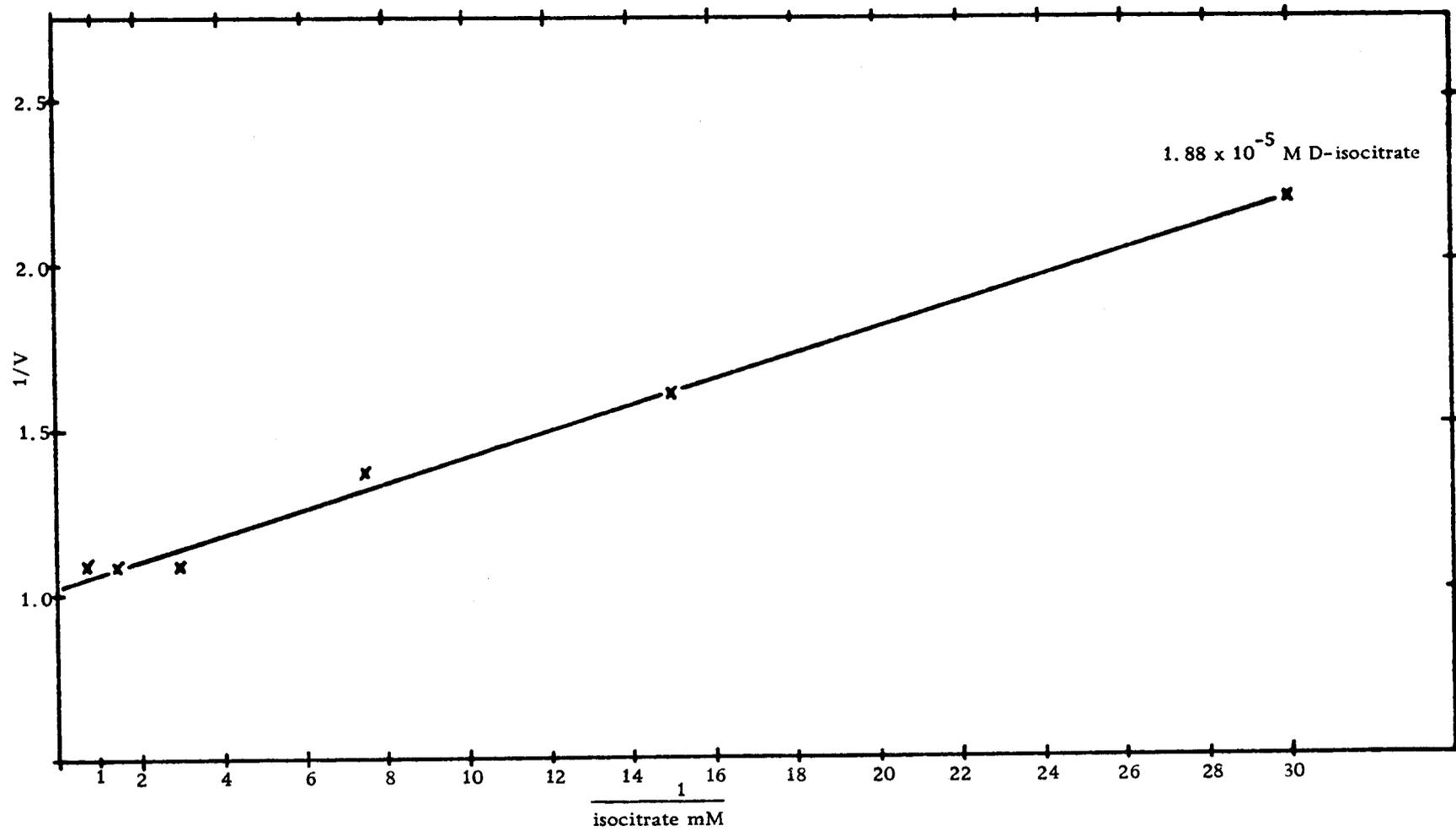


Figure XIV. Lineweaver-Burk plot for varying concentrations of D-isocitrate for the stable enzyme

A constant level of 2 μ moles NADP was used. Velocity, $V = \Delta OD_{340}/\text{min.}$

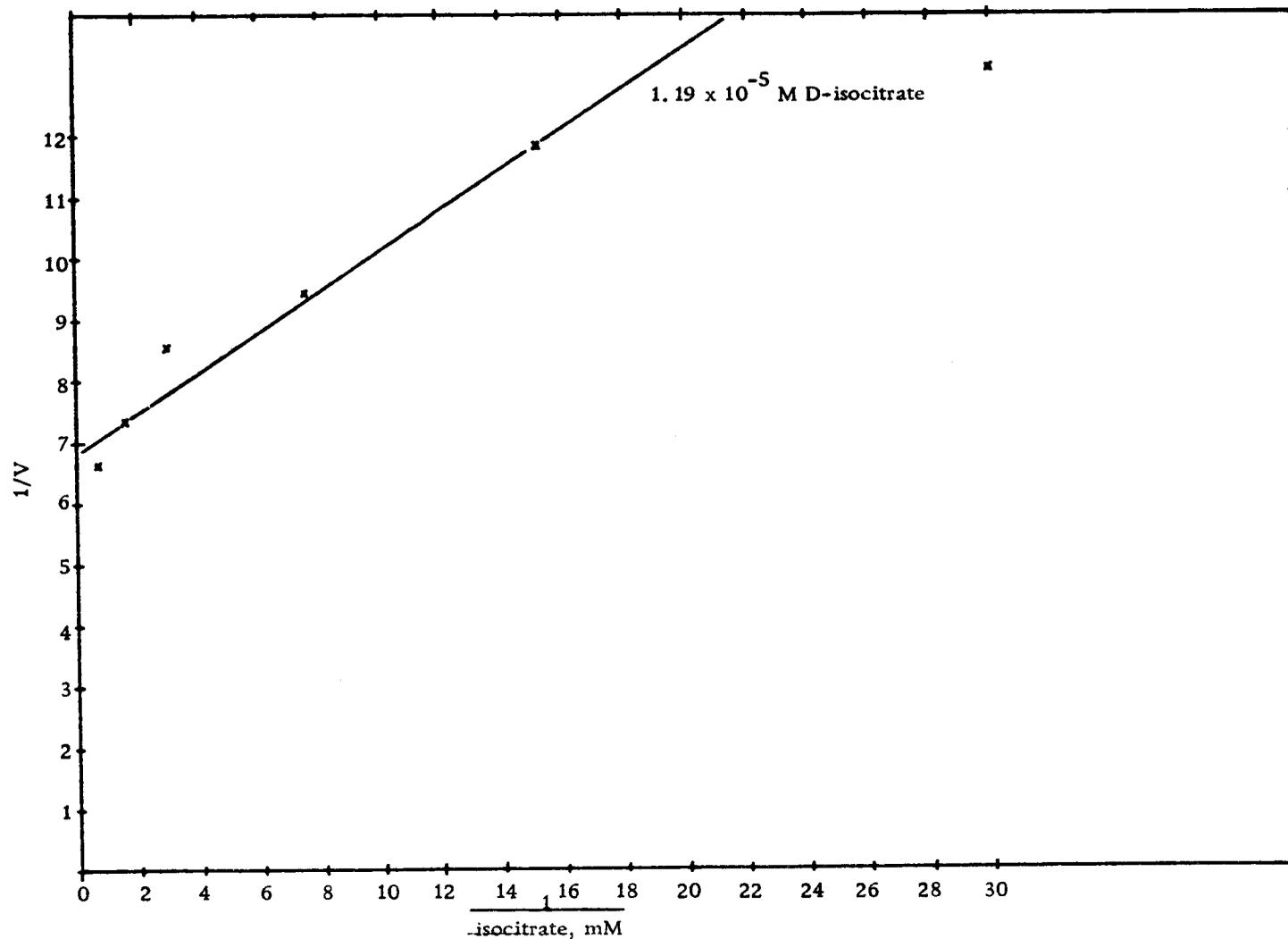


Figure XV. Lineweaver-Burk plot for varying concentrations of D-isocitrate for the labile enzyme

A constant level of 2 μ moles NADP was used. Velocity $V = \Delta OD_{340}/\text{min}$.

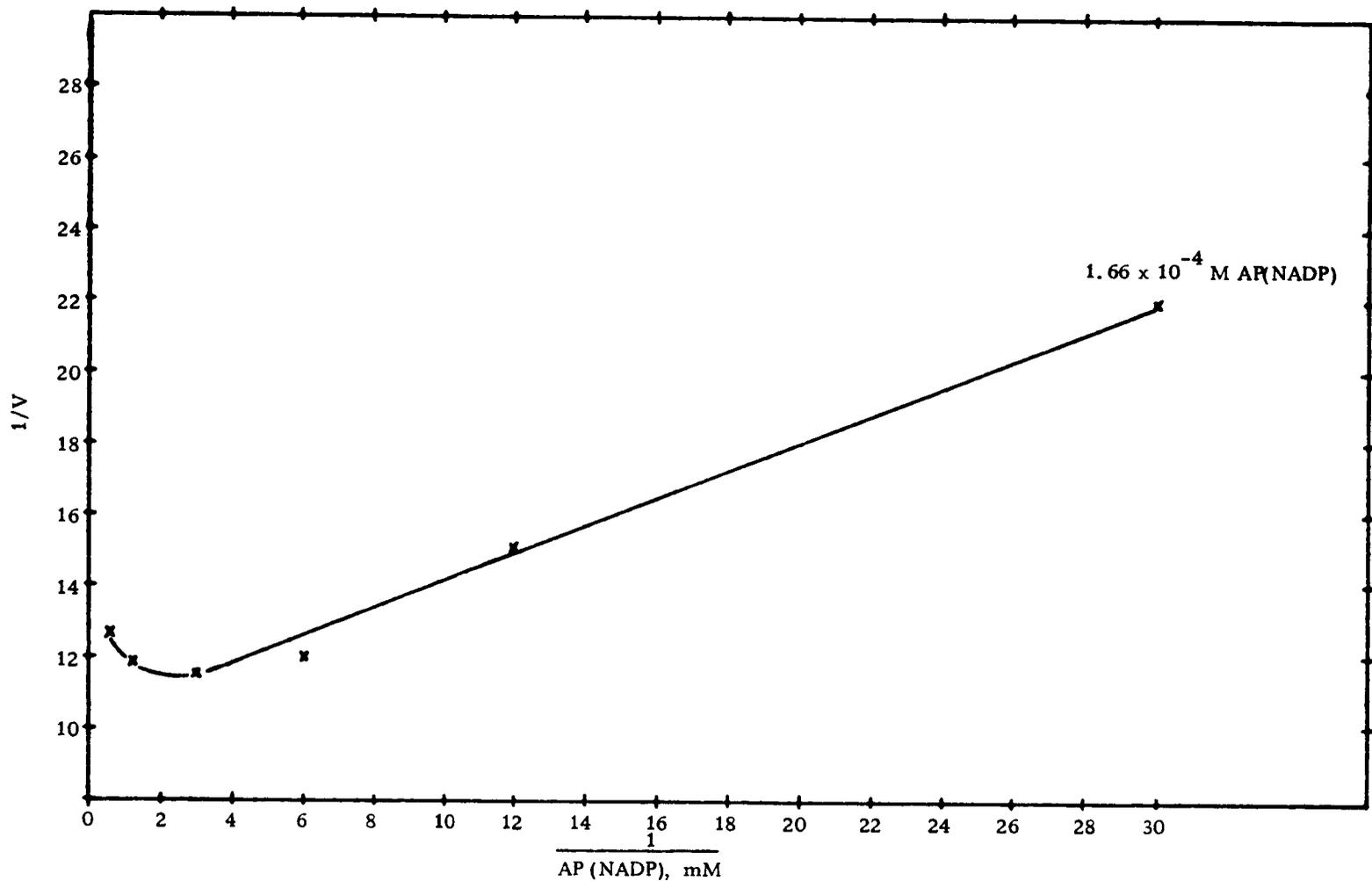


Figure XVI. Lineweaver-Burk plot for varying concentrations of AP(NADP) for the stable enzyme

A constant concentration of 2 μ moles D-isocitrate was used. Velocity, $V = \Delta OD_{340}/min.$

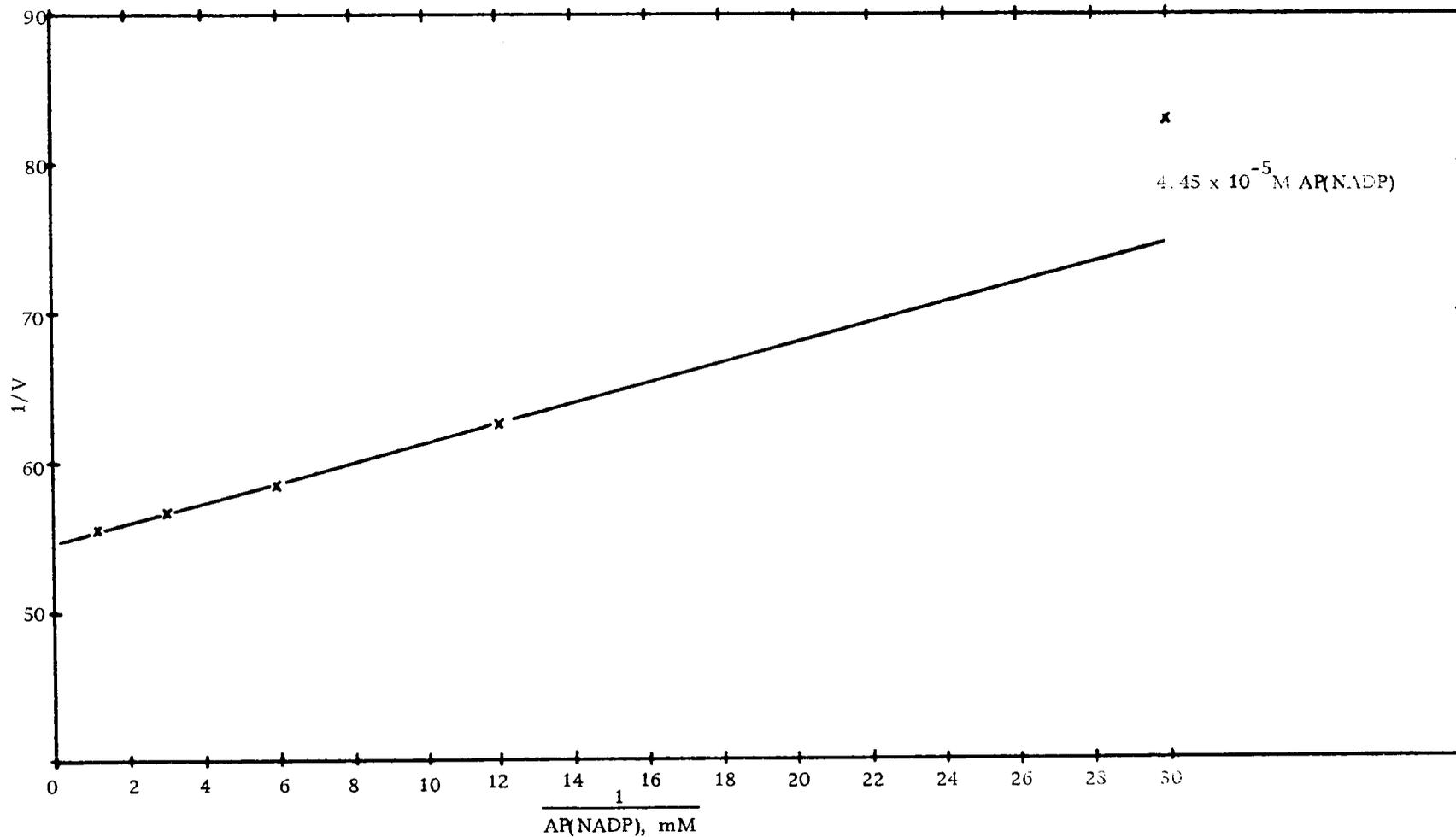


Figure XVII. Lineweaver-Burk plot for varying concentrations of AP(NADP) for the labile enzyme

A constant concentration of 2 μ moles D-isocitrate was used. Velocity $V = \frac{\Delta CO_{340}}{\text{min}}$.

(Figures XVIII and XIX) for a constant level of DL-isocitrate of 1.33 mM are not of the regular form. The apparent K_m for the S-enzyme is 5.89×10^{-5} M, and that for the L-enzyme is 4×10^{-4} M, for the level of isocitrate used. These irregular Lineweaver-Burk plots may indicate different alternatives. They may indicate substrate inhibition by NADP. They may also indicate allosteric interactions of the enzyme molecule with a substrate, or with another protein molecule. More detailed analysis is required to differentiate between these possibilities.

Preliminary experiments using NAD as coenzyme indicate that the L-form is far more reactive than the S-form (final concentration of NAD at 0.66 mM). For the S-enzyme, the specific activity ratio for the two coenzymes, NADP/NAD is 388. (Final concentration of NADP is also 0.66 mM). The same ratio for the L-enzyme is 10.

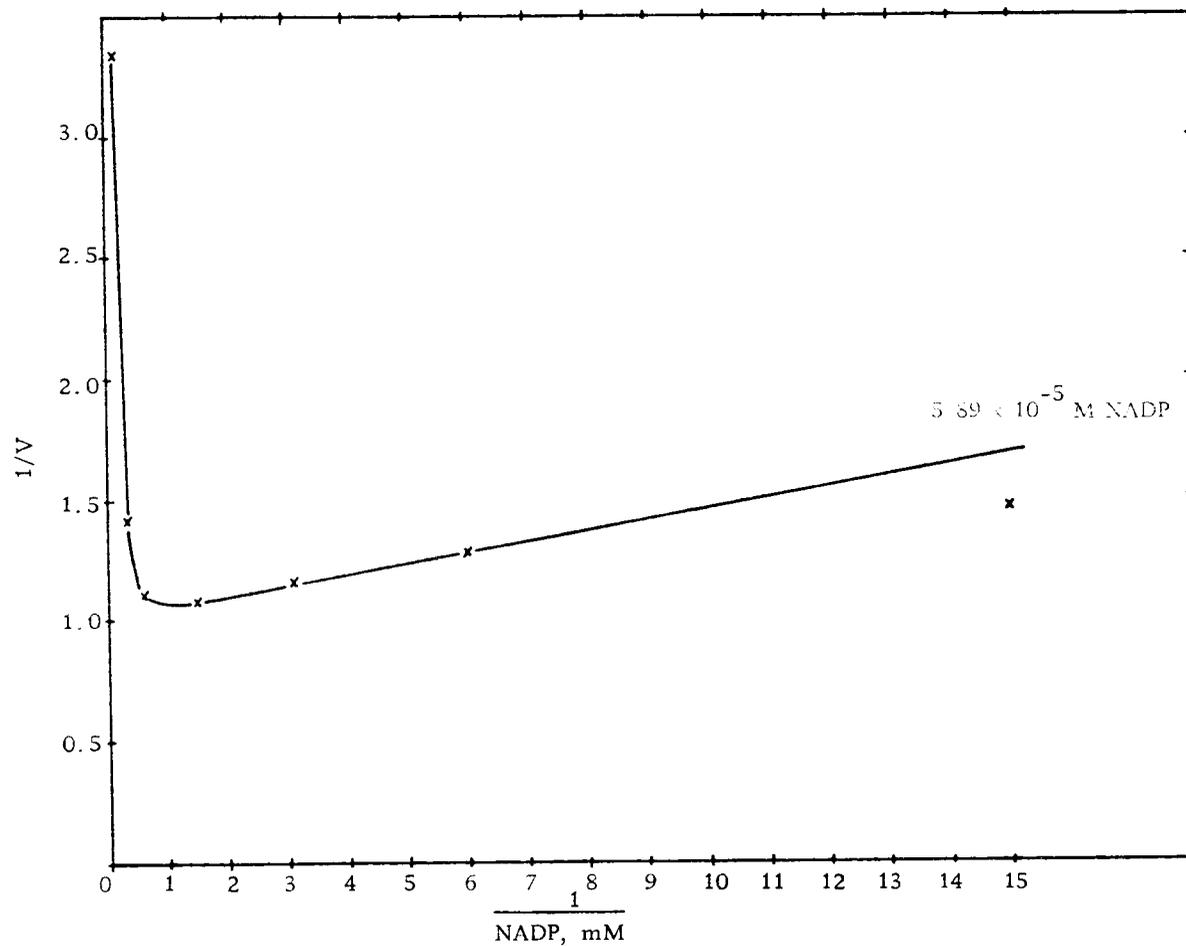


Figure XVIII. Lineweaver-Burk plot for varying concentrations of NADP with the stable enzyme

A constant concentration of 2 μ .moles D-isocitrate was used. Velocity, $V = \Delta\text{OD}_{340}/\text{min}$.

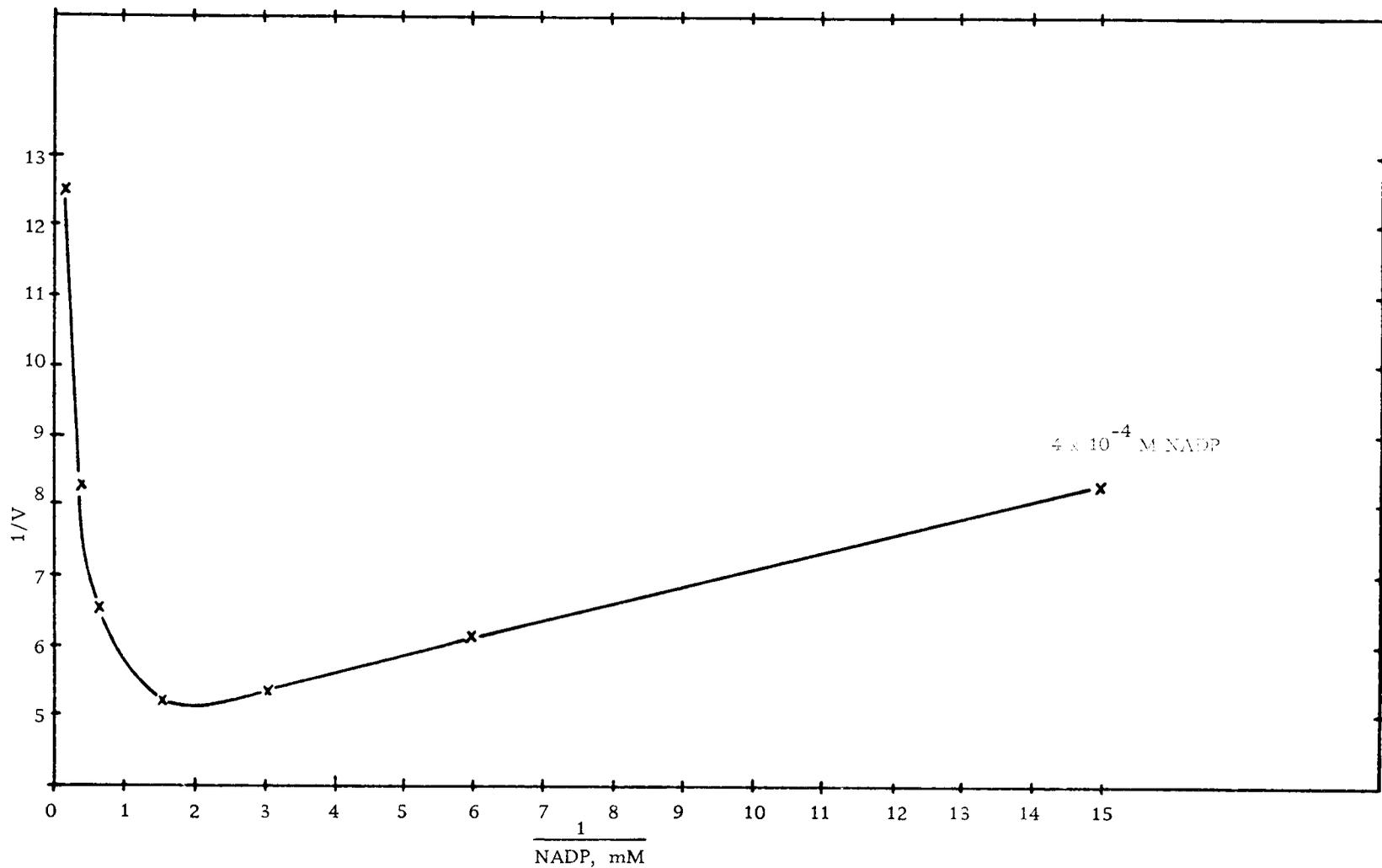


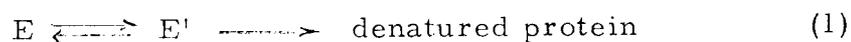
Figure XIX. Lineweaver-Burk plot for varying quantities of NADP with the labile enzyme

A constant concentration of 2 μ moles D-isocitrate was used. Velocity, $V = \Delta\text{OD}_{340}/\text{min}$.

DISCUSSION

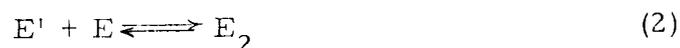
Evidence presented in this thesis suggest strongly that the extramitochondrial form of the NADP-linked isocitric dehydrogenase is composed of subunits, which can be reversibly dissociated in vitro, and possess some distinct differences. It is clear that the stable enzyme is an aggregate of two smaller counterparts each with the same molecular weight. However, it is not known whether these subunits are identical or not, and evidence presented so far is not conclusive. However, there is little doubt that union of two subunits give an overall increase in specific activity. To use the terminology of Monod et al. (5, p. 89) since there is an obvious "co-operative" interaction during dimerization (increase in activity), this union may be either "homotropic" (interactions between identical subunits) or "heterotropic," (interaction between different subunits). Qualitative indications in this thesis appear to favor the latter case, namely, heterotropic, i. e., that the subunits, although identical in molecular weight, might actually be different in that one is enzymatically active, and the other is not. A combination of one of each kind will lead to a dimer with twice the activity of the originally active monomer. Since up to 73% of the original S-enzyme can be reconstituted one might suppose that given the right conditions, all of the original activity could be

reconstituted. If this were true, then one must assume that there is a reversible interconversion between the two subunits, E, and E':



where E' is the enzymatically inactive subunit, which could easily be irreversibly denatured by heat,

and that E and E' must also exist in equilibrium with E₂:

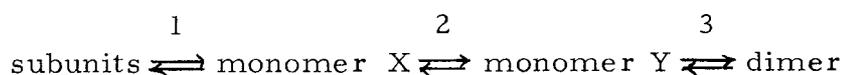


Since experiments indicated that it takes 120 minutes at 46° C to regain 73% of the original S activity, it seems that the equilibrium is far to the left in the first equation (1).

Other qualitative indications agree with this hypothesis. The combination of E with E' not only results in twice the activity of the original E, but also imparts heat stability to both E and E', since a denaturation by heat is irreversible, and a combination is mutually protective. This can be extended to other denaturing agents. Another advantage of this arrangement is its potential property of control. A substance (possibly a metabolite), may affect either one of the steps as shown in (1) or (2), and control the level of active enzyme present in the cell fraction. Plausibility of this extension of the fact is given in the case of Part I of this thesis. As a NADP-linked enzyme, isocitric dehydrogenase exist in far greater activity units (in both soluble and particulate fractions of the cell) than either

one of the other NADP-linked dehydrogenases. Since there is never found more of the NAD-linked enzyme than 10% in the chick embryo, (4), and since the NAD-linked enzyme is known to play a role in cellular metabolism (28, 64), one might then suppose that the NADP enzyme substitutes for this function. The regulatory metabolite or agent in vivo might be postulated to be NADP or NADPH. Further experiments into the reaction kinetics of both the labile and stable forms are required to prove or disprove this. For the time being, the above may serve as a working hypothesis.

Levy, Raineri and Nevaldine (43) found that, in lactating mammary glands, the enzyme glucose-6-phosphate dehydrogenase plays a regulatory role in exactly the same manner as that proposed for isocitric dehydrogenase above. Their enzyme was found to dissociate reversibly into non-identical monomers (with different reactivities toward NAD), and that these monomers are inter-convertible one into the other:



The molecular weights of monomer X and monomer Y are alike. NADPH was found to affect the equilibrium at point 2. Fritz (26, 27) recently described a similar regulatory role for an isozyme of lactic dehydrogenase: LDH-5, activated by citrate. This isozyme was found to have 5 to 8 electrophoretic bands, indicating

that it was formed from non-identical subunits.

Reports within the past two years indicate that most of the dehydrogenases studied so far have parallel properties to those suggested for isocitric dehydrogenase. Levy (43) described the presence and interactions of subunits of glucose-6-phosphate dehydrogenase. Similar situations were found by Kazazian (38) on 6-phosphogluconate dehydrogenase and Kitto (40) on malic dehydrogenase. Freiden (24, 25) has shown the regulatory role of nucleotides in glutamic dehydrogenase, and his results were confirmed by Fahien (20). Sanwal (64) described the allosteric properties of NAD-linked isocitric dehydrogenase. From the laboratory of Kaplan have come reports of the effects of salts and urea on lactic dehydrogenase (19), its reversible inactivation by heat and by salt (16), the factors influencing its reversible dissociation (15) and the mechanisms of its hybridization (14). This same laboratory has since extended their studies to triosephosphate dehydrogenase, glycerophosphate dehydrogenase, alcohol dehydrogenase, and malic dehydrogenase (16). With only one exception (30), no report has appeared indicating the existence of multiple forms of soluble NADP-linked isocitric dehydrogenase. No description of the different properties of the multiple forms has yet appeared. This thesis attempts to fill this gap.

Additional work must be done on the isozymes (mitochondrial

and soluble), and multiple forms of soluble isocitric dehydrogenase along the lines described here on other ages of the chick embryo so that a good insight into the chemistry of the developing organism may be obtained. Such work has already been started using lactic dehydrogenase (22, 37, 31).

CONCLUSIONS AND SUMMARY

1. Multiple forms of soluble NADP-linked isocitric dehydrogenase were found in the liver of the 18-day old chick embryo. A partial purification of the enzyme has been described. Evidence is given that of the two enzymatically active forms, one is a monomer and the other is a dimer.
2. The monomer and dimer are found to differ in heat denaturation, pH optima, and reaction rates with NADP, NAD and AP-NADP.
3. Molecular weights were estimated to be approximately 150,000 for the dimer and 85,000 for the monomer.
4. Dissociation is favored in low ionic strength, and association in high ionic strength. The forms are freely interconvertible by adjustment of the salt concentration of the medium. The reaggregated molecules have the same molecular weight as the origin S-enzyme.
5. Dissociation is accompanied by a loss in specific activity, which could be regained in part upon reaggregation.
6. From the above observations, a working hypothesis is presented concerning the qualitative interactions of the multiple forms of soluble NADP-linked isocitric dehydrogenase.

BIBLIOGRAPHY

1. Abraham, S. et al. The relation of lipogenesis to reduced triphosphopyridine nucleotide generation and to certain enzyme activities in the liver of the totally depancreatized rat. *Biochimica et Biophysica Acta* 62:27-34. 1962.
2. Andrews, P. Estimation of the molecular weights of proteins by sephadex gel-filtration. *Biochemical Journal* 91:222-232. 1964.
3. Bagdasarın, G. and D. Halanicka. Changes of mitochondrial glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during brain development. *Biochimica et Biophysica Acta* 99:367-369. 1965.
4. Baker, Wilbur Winston. Studies of soluble and particulate isocitrate nicotinamide-adenine dinucleotide phosphate-oxidoreductase activities during development of the chick embryo. Ph.D. thesis. Corvallis, Oregon State University, 1964. 75 numb. leaves.
5. Baker, Wilbur Winston 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.
6. Bieber, Allan T., Vernon H. Cheldelin and R. W. Newburgh. Phospholipid patterns of the developing chick embryo. *Biochemistry* 1:532-536. 1962.
7. Brand, Ludwig and H. R. Mahler. Biochemical studies of the developing avian embryo. III. The oxidation of reduced pyridine nucleotides. *Journal of Biological Chemistry* 234: 1615-1624. 1959.
8. Burt, Alvin M. and Byron S. Wenger. Glucose-6-phosphate dehydrogenase activity in brain of developing chick. *Developmental Biology* 3:84-95. 1961.

9. Carey, N. H. and G. D. Greville. Mitochondria from embryonic tissue of the chick. I. Preparation, characterization and some enzymatic properties. *Biochemical Journal* 71:159-166. 1959.
10. _____ . Mitochondria from embryonic tissue of the chick. II. Metabolic activities. *Biochemical Journal* 71: 166-176. 1959.
11. Carzola, A. and E. S. G. Barron. The formation of glutathione and the development of some enzymes for carbohydrate metabolism during the development of chicken embryo. *Experimental Cell Research* 14:68-79. 1958.
12. Chappell, J. B. The oxidation of citrate, isocitrate and cis-aconitate by isolated mitochondria. *Biochemical Journal* 90:225-237. 1964.
13. Cheung, Geoffrey and R. W. Newburgh. Soluble and particulate NADP-linked oxidoreductase of chick embryo liver. *Federation Proceedings* 25:760. 1966.
14. Chilson, Oscar P., L. A. Costillo and Nathan O. Kaplan. Studies on the mechanism of hybridization of lactic dehydrogenases in vitro. *Biochemistry* 4:271-281. 1965.
15. Chilson, Oscar P., G. Barrie Kitto and Nathan O. Kaplan. Factors influencing reversible dissociation of dehydrogenases. *Proceedings of the National Academy of Sciences* 53:1006-1014. 1965.
16. Chilson, Oscar P. et al. Reversible inactivation of dehydrogenases. *Journal of Biological Chemistry* 241:2431-2445. 1966.
17. Coffey, Ronald Gibson. Pathways of glucose utilization and ribose synthesis in chick embryo. Ph.D. thesis. Corvallis, Oregon State University, 1964. 123 numb. leaves.
18. Davidson, Joseph. Activity of certain metabolic enzymes during development of the chick embryo. *Growth* 21:287-295. 1957.

19. DiSabato, Giovanni and Nathan O. Kaplan. The denaturation of lactic dehydrogenase. II. The effect of urea and salts. *Journal of Biological Chemistry* 240:1072-1076. 1965.
20. Fahien, L. A., B. D. Wiggert and P. P. Cohen. Effect of nucleotides and coenzymes in frog liver glutamic dehydrogenase. *Journal of Biological Chemistry* 240:1091-1095. 1965.
21. Filsell, O. H. et al. Nicotinamide nucleotide coenzymes and glucose metabolism in the livers of foetal and newborn lambs. *Biochemical Journal* 89:92-100. 1963.
22. Fine, I. H., Nathan O. Kaplan and D. Kuffinec. Developmental changes of mammalian lactic dehydrogenases. *Biochemistry* 2:116-121. 1963.
23. Flora, de A., M. A. Mangiarotti and I. Torenzoni. Inattivazione delladeidrogenasi NADP-dipendenti in preparazioni dializzate di fegato di ratto. *Bollettino della Societa' Italiani di Biologia Sperimentale* 41:1252-1255. 1965.
24. Frieden, C. Glutamic dehydrogenase. I. Effect of coenzyme on the sedimentation velocity and kinetic behavior. *Journal of Biological Chemistry* 234:809-814. 1959.
25. _____ . Glutamic dehydrogenase. II. Effect of various nucleotides on the association, dissociation and kinetic properties. *Journal of Biological Chemistry* 234:815-820. 1959.
26. Fritz, Paul J. Rabbit muscle lactic dehydrogenase-5. A regulatory enzyme. *Science* 150:364-366. 1965.
27. Fritz, Paul J. and K. B. Jacobson. Multiple molecular forms of lactic dehydrogenase. *Biochemistry* 4:282-289. 1965.
28. Goebell, Harald und Martin Klingenberg. DPN-spezifische Isocitrat-Dehydrogenase der Mifochondrien. I. Kinetische Eigenschaften Vorkommen und Funktion der DPN-spezifischen Isocitrat-Dehydrogenase. *Biochemische Zeitschrift* 340:441-464. 1964.

29. Goodwin, B. C. and I. Sizer. Histone regulation of lactic dehydrogenase in embryonic chick brain tissue. *Science* 148:242-244. 1965.
30. Henderson, N. Isozymes of isocitric dehydrogenase. Subunit structure and intracellular location. *Journal of Experimental Zoology* 158:263-273. 1965.
31. Hinks, Mary and C. J. Masters. Developmental changes in ruminant lactic dehydrogenase. *Biochemistry* 3:1789-1792. 1964.
32. Horecker, B. L. and A. Kornberg. The extinction coefficients of the reduced band of pyridine nucleotides. *Journal of Biological Chemistry* 175:385-390. 1948.
33. Itzhaki, R. F. and D. M. Gill. A microburet method for estimating proteins. *Analytical Biochemistry* 9:401-412. 1964.
34. Kallen, Ronald G. and John M. Lowenstein. Stimulation of fatty acid synthesis by isocitrate and malonate. *Archives of Biochemistry and Biophysics* 96:188-190. 1962.
35. Kaplan, Nathan O. and Margaret M. Ciotti. Chemistry and properties of the 3-acetylpyridine analogue of DPN. *Journal of Biological Chemistry* 221:823-832. 1956.
36. Kaplan, Nathan O., Sidney P. Colowick and Elizabeth F. Neufeld. Pyridine nucleotide transhydrogenase. III. Animal tissue transhydrogenases. *Journal of Biological Chemistry* 205:1-15. 1958.
37. 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.
38. Kazazian, H. H., Jr., W. J. Young and B. Childs. X-linked 6-phosphogluconate dehydrogenase in *Drosophila* subunit associations. *Science* 150:1601-1602. 1965.
39. Kingsburg, Janet W., Margaret Anderson and Esta S. Kornstein. The development of the liver in the chick. *Anatomical Record* 124:165-187. 1956.

40. Kitto, G. B. et al. Multiple forms of mitochondrial malate dehydrogenases. *Biochemical and Biophysical Research Communications* 22:75-87. 1966.
41. Lang, C. A. Respiratory enzymes in heart and liver of pre-natal and postnatal rat. *Biochemical Journal* 95:365-371. 1965.
42. Layne, E. Spectrophotometric and turbidometric methods for measuring proteins. In: *Methods in enzymology*, ed. by S. P. Colowick and N. O. Kaplan. Vol. 3. New York, Academic Press, 1957. p. 447-454.
43. Levy, H. Richard, R. R. Raineri and B. H. Nevaldine. On the structure and catalytic function of mammary glucose-6-phosphate dehydrogenase. *Journal of Biological Chemistry* 241:2181-2187. 1966.
44. Lowenstein, J. M. Pathway of hydrogen in biosynthesis. I. *Journal of Biological Chemistry* 236:1213-1216. 1961.
45. _____ . Pathway of hydrogen in biosynthesis. II. *Journal of Biological Chemistry* 236:1217-1219. 1961.
46. Mahler, H. R., Mary H. Wittenberger and Ludwig Brand. Biochemical studies of the developing avian embryo. *Journal of Biological Chemistry* 233:770-782. 1958.
47. Market, C. L. and F. Møller. Multiple forms of enzymes. Tissue, ontogenic and species specific patterns. *Proceedings of the National Academy of Sciences* 45:753-763. 1959.
48. 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.
49. Matthes, K. J., S. Abraham and I. L. Chaikoff. Fatty acid synthesis from acetate by normal and diabetic rat liver homogenate fractions. *Journal of Biological Chemistry* 235:2560-2568. 1960.
50. Modirzadeh, Jamal and Henry Kamin. Reduction of microsomal cytochromes by pyridine nucleotides. *Biochimica et Biophysica Acta* 99:205-226. 1965.

51. Monod, J., J. Wyman and J. P. Changeux. On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology* 12:88-118. 1965.
52. Murad, S. and R. A. Freedland. The in vivo effect of throxine on citrate cleavage enzyme and NADP linked dehydrogenase activities. *Life Sciences* 4:527-531. 1965.
53. Murthy, M. R. V. and D. A. Rappoport. Biochemistry of the developing rat brain. III. Mitochondrial oxidation of citrate and isocitrate and associated phosphorylation. *Biochimica et Biophysica Acta* 74:328-339. 1963.
54. Nason, Alvin, Reginald A. Garrett and P. P. Nair. Inhibition by digitonin of bouine heart muscle. DPNH-cytochrome-c reductase and its specific reversal by the tocopherols. *Biochemical and Biophysical Research Communications* 14: 220-226. 1964.
55. Newburgh, R. W., B. Buckingham and Heinz Herrmann. Levels of reduced TPN generating systems in chick embryos in ovo and in explants. *Archives of Biochemistry and Biophysics* 97:94-99. 1962.
56. Okuno, Giichi et al. Development of hepatic phosphorylase in chick embryo. *Proceedings of the Society for Experimental Biology and Medicine* 117:524-526. 1964.
57. Pande, S. V. et al. NADP specific dehydrogenases in relation to lipogenesis. *Biochimica et Biophysica Acta* 84:239-250. 1964.
58. Plaut, G. W. E. Isocitric dehydrogenase (TPN-linked) from pig heart (revised procedure). In: *Methods in enzymology*, ed. by S. P. Colowick and N. O. Kpalan. Vol. 3. New York, Academic Press, 1957. p. 645-651.
59. Purvis, J. L. Pathway of oxidation of isocitrate by mitochondria. *Biochimica et Biophysica Acta* 30:440-441. 1958.
60. _____ . Pathway of oxidation of isocitrate by mitochondria and the role of the transhydrogenase reaction. *Biochimica et Biophysica Acta* 52:148-155. 1961.

61. Reiner, John M. Behavior of enzyme systems. Minneapolis, Burgess Publishing Company, 1959. 317 p.
62. Richardson, S. H. and L. R. Fowler. Studies on the electron transfer system. II. Binding of cytochrome c by reconstituted DPNH oxidase. Archives of Biochemistry and Biophysics 100:547-553. 1963.
63. Roux, Jeanne-Marie et Claude Tordet-Caridroit. Variations de la glucose-6-phosphate déshydrogénase dans le foie a la période périnatale chez le rat. Bulletin de la Société de Chimie biologique 47:1269-1272. 1965.
64. Sanwal, B. D. and R. A. Cook. Effect of adenylic acid on regulatory NAD specific isocitric dehydrogenase. Biochemistry 5:886-894. 1966.
65. Silber, Robert, F. M. Huennekens and Beverly W. Gabric. Patterns of dehydrofolic reductase and tetrahydrofolate dependent enzymes in the developing chick embryo. Archives of Biochemistry and Biophysics 99:328-333. 1962.
66. Simbonis, Stanley S. and Raymond A. McBride. Intracellular distribution of glucose-6-phosphatase and glycogen content in the developing chick embryo liver. Developmental Biology 12:347-357. 1966.
67. 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.
68. _____ . Lactic and malic dehydrogenases in organs of the developing chick embryo. Biochemical Journal 68: 30 p. 1958.
69. _____ . Lactic and malic dehydrogenases in the developing chick embryo. Biochemical Journal 70:529-535. 1958.
70. Strittmatter, C. F. Differentiation of electron transport systems in mitochondria and microsomes during embryonic development. Archives of Biochemistry and Biophysics 102:293-305. 1963.

71. Vignais, P. V. and P. M. Vignais. Role des di-et tri-phosphopyridine nucleotides dans l'oxydation mitochondriales de l'isocitrate. *Biochimica et Biophysica Acta* 47:515-528. 1961.
72. Wallace, Robin A. Enzymic patterns in the developing frog embryo. *Developmental Biology* 3:486-515. 1961.
73. Young, Jerry W., Earl Shrago and Henry A. Lardy. Metabolic control of enzymes involved in lipogenesis and gluconeogenesis. *Biochemistry* 3:1687-1692. 1964.