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LA THÈSE A ÉTÉ
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ASPARTATE AMINOTRANSFERASE

AND

AROMATIC-AMINO-ACID AMINOTRANSFERASE

IN

ESCHERICHIA COLI

by

WINSON M.S. ORR

Thesis presented to the School of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
University of Ottawa
Ottawa, Canada
1976

ACKNOWLEDGEMENT

I am deeply indebted to Dr. C. Mavrides for his support, encouragement, advice and patience during the completion of this research project.

I would also like to express my thanks to Miss Diane Davis for many amino acid analyses.

Lastly, I would like to thank the Medical Research Council of Canada for financial support.

SUMMARY

Two aminotransferases from Escherichia coli were purified to homogeneity by the criterion of gel electrophoresis. The first enzyme (enzyme A)is active on L-aspartic acid, L-tyrosine, L-phenylalanine and L-tryptophan; the second enzyme (enzyme B) is active on the aromatic amino acids. Both are marginally active with L-methionine and utilize α-ketoglutarate and oxaloacetate as co-substrates. Enzyme A is identical in substrate specificity with transaminase A and is a multispecific asparate aminotransferase (EC 2.6.1.1); enzyme B has never been described in E. coli before and is an aromatic-amino-acid aminotransferase (EC 2.6.1.57). The two enzymes are different in V_{max} and K_m values with their common substrates and pyridoxal phosphate, in heat stability (enzyme A being heat-stable and enzyme B being heat-labile at 55^{o}) and in pH optima with the amino acid substrates. The molecular weights of enzymes A and B were estimated by gel filtration to be about 82,000 and 88,000, respectively. In organisms grown in minimal medium, enzyme B was repressed by tyrosine by 90% whereas enzyme A was unaffected.

These two enzymes show broad similarity in their amino acid composition, each enzyme appears to consist of two subunits of equal size and with apparent molecular weights of 43,000-45,000 estimated by SDS gel electrophoresis. Enzyme B may be converted to enzyme A by controlled proteolysis with subtilisin. The con-

version was detected by the generation of new aspartate amino-transferase activity from enzyme B and was further verified by identification by acrylamide gel electrophoresis of the newly formed enzyme A. The above results suggested that the two enzymes are products of two genes different in a short, probably terminal, nucleotide sequence.

LIST OF ABBREVIATIONS

Chemical Compounds

AA	Anthranilic acid
Asp	Aspartate (or Aspartic acid)
CA	Chorismic acid
CDRP	1-(o-carboxyphenylamino)-1-deoxy-ribulose-5-phosphate
DAHP	3-deoxy-D-arabinoneptulosonic acid-7-phosphate
DCIP	Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethyl cellulose
DHQ	5-dehydroquinic acid
DHS	5-dehydroshikimic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
EP	Erythrose-4-phosphate
EPSAP	3-Enolpyruvylshikimic acid-5-phosphate
InGP	Indoleglycerol phosphate
α-KG	α-Ketoglutarate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
OAA	Oxaloacetic acid
PA	Prephenic acid
Phe	Phenylalanine

LIST OF ABBREVIATIONS (cont'd)

Chemical Compounds

pHPP	p-Hydroxyphenylpyruvic acid
PI	Isoelectric point
PLP	Pyridoxal phosphate
PP	Phenylpyruvic acid
PRA	N-(5'-phosphoribosyl)-anthranilic acid
Prot	Protein
P-protein	Chorismate mutase-P-prephenate dehydratase
T-protein	Chorismate mutase-T-prephenate dehydrogenase
SA	Shikimic acid
SAP	Shikimic acid-5-phosphate
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-Tetramethyl ethylene-diamine
Trp	Tryptophan
Tyr	Tyrosine

Enzymes

AspA	Aspartate aminotransferase
Enzyme A	Aspartate aminotransferase (EC 2.6.1.1)
Enzyme B	Aromatic-amino-acid aminotransferase (EC 2.6.1.57)
PheA	Phenylalanine aminotransferase

LIST OF ABBREVIATIONS (cont'd)

Enzymes

TyrA	Tyrosine aminotransferase

Weights and Measures

g	gram
mg	milligram
µg	microgram
µM	micromole
nm	nanometer
mín	minute
l	litre
ma	milliampere
ml	milliliter

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I. INTRODUCTION

A. Pathways of biosynthesis of aromatic amino acids in microorganisms

The general outlines of the pathways to the aromatic amino acids tyrosine, phenylalanine, and tryptophan have been known for sometime, and they were first reviewed by Umbarger and Davis (1963). Since then, the situation regarding the "branch point" in aromatic biosynthesis has been extensively studied, and much information on the biochemical genetics and control of the biosynthesis of aromatic amino acids has accumulated.

A general outline of the pathways to be discussed consists of a "common aromatic pathway" leading through shikimate to chorismate, after which there is branching to the individual pathways (Fig. 1).

a) Common aromatic pathway.

The common aromatic pathway involves the condensation of two products of carbohydrate metabolism, phosphoenolpyruvate and erythrose 4-phosphate, to give a straight chain seven-carbon compound which is then cyclized and undergoes a number of reactions through shikimate to chorismate (Fig. 2).

In recent research, the main advance has been the clarification of the region of the branch point (Fig. 1), where, from

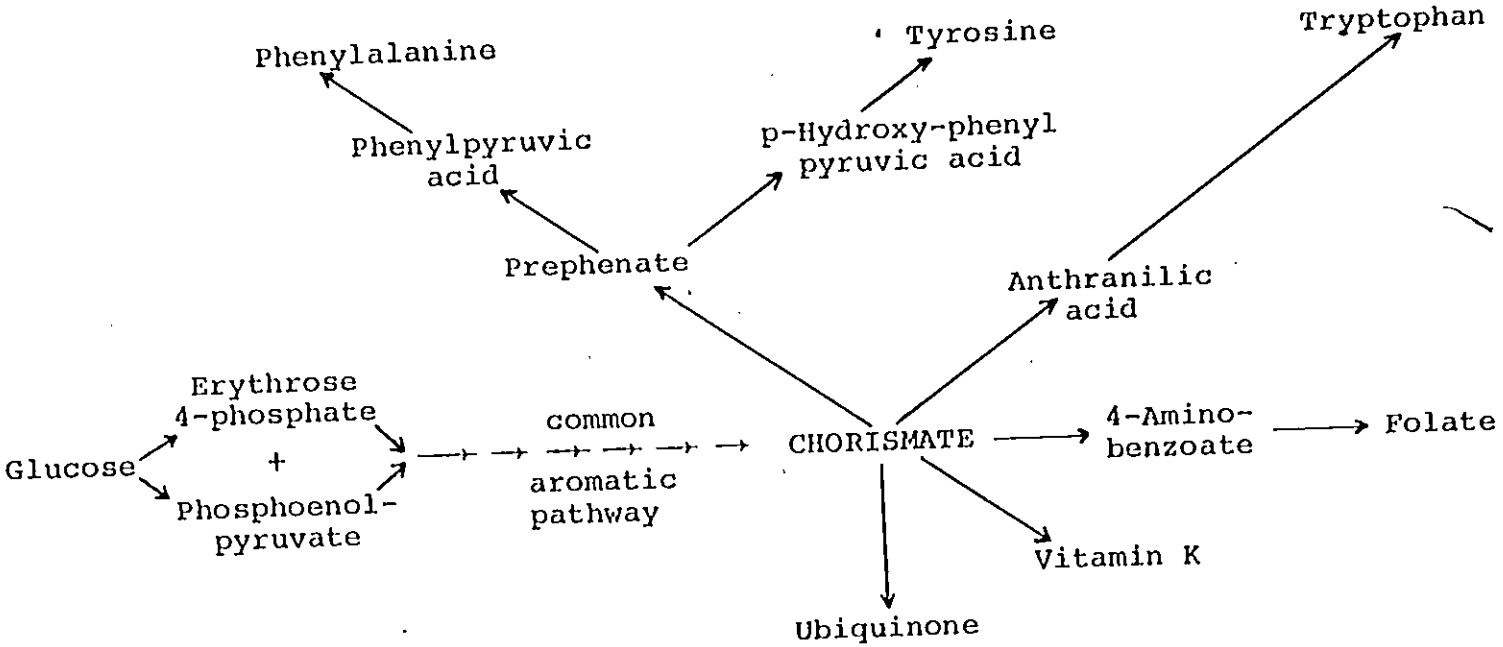


Figure 1

General outline of pathway for the biosynthesis of aromatic compounds formed by E. coli as adapted from Pittard and Gibson (1970).

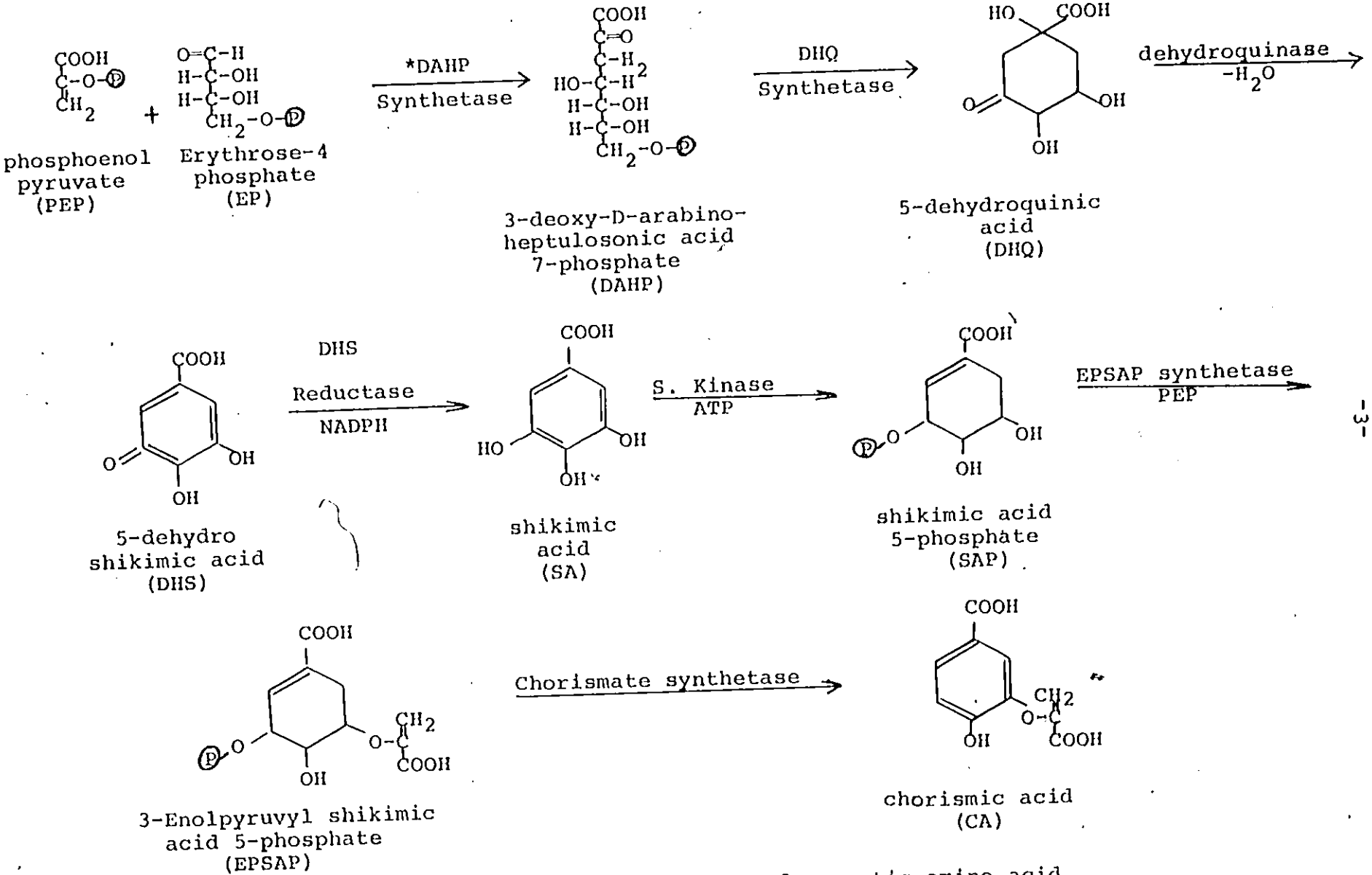


Figure 2. Intermediates in the common aromatic pathway of aromatic amino acid biosynthesis as adapted from Gibson and Pittard (1968). *DAHP synthetase (tyr), DAHP synthetase (phe), DAHP synthetase (trp)

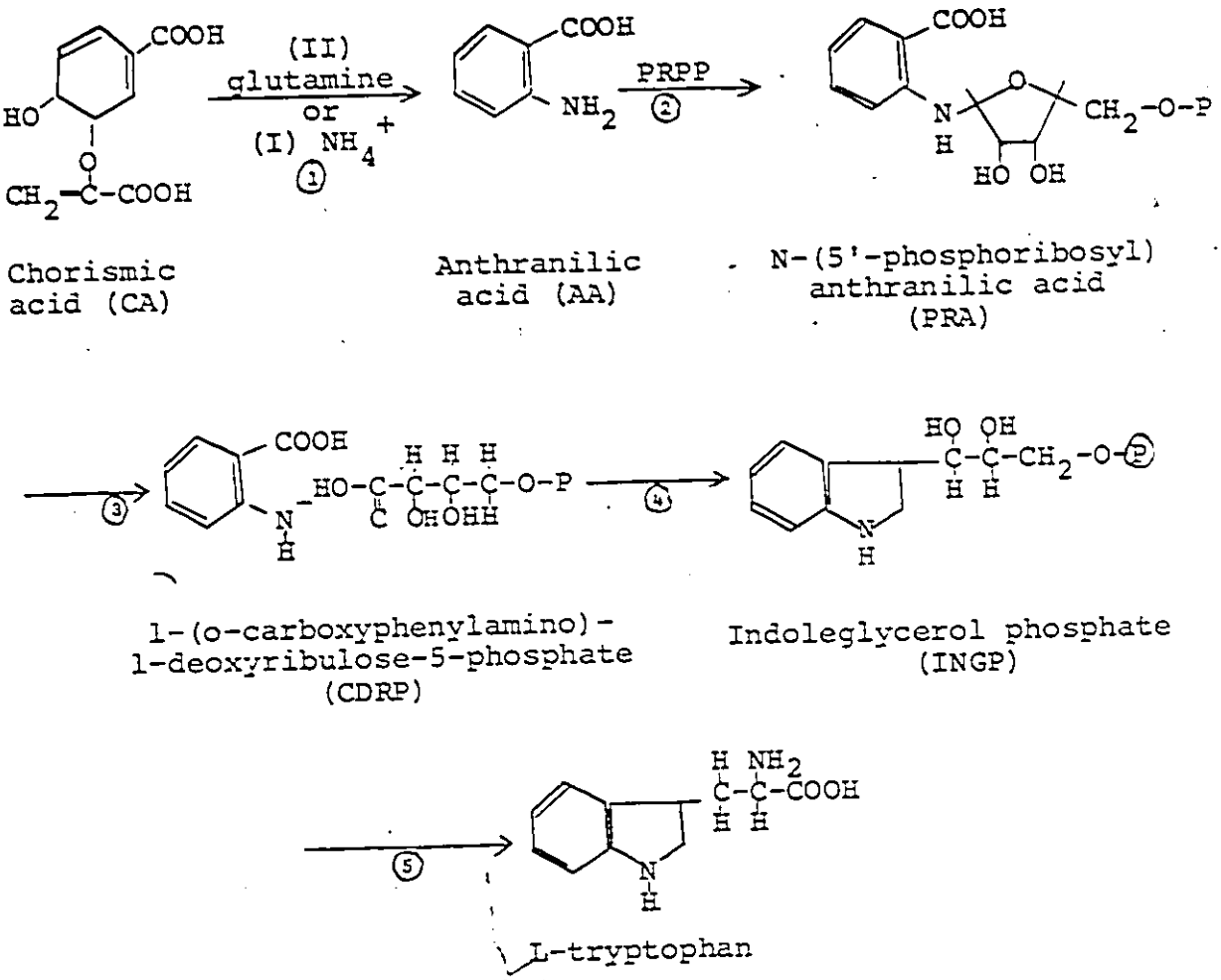
chorismate, a series of individual pathways diverge. After the establishment of 3-enol-pyruvylshikimate-5-phosphate (Fig. 2) as an intermediate on the common aromatic pathway, two groups (Gibson, 1962; Rivera, 1962) studying the conversion of shikimate to anthranilate showed that EPSAP was a precusor of anthranilate, as well as of phenylpyruvate and para-hydroxyphenylpyruvate. Gibson and Gibson (1962) and Gibson and Gibson (1964) suggested that a specific branch point compound was involved, and this compound was sought by examining a mutant in which the pathways to tryptophan, tyrosine and phenylalanine were blocked. This specific branch point compound was named chorismic acid (chorismic meaning separating) which has been isolated from culture fluids of E. coli (Lingens et al. 1967), N. crassa (DeMoss, 1965) and Saccharomyces cerevisiae (Lingens and Müller, 1967) indicating the general role of the compound in aromatic biosynthesis.

b) Tryptophan pathway.

The pathway of tryptophan biosynthesis is as set in Fig. 3, showing the series of enzyme reactions involved in this pathway from chorismic acid to tryptophan.

c) Pathways to phenylalanine and tyrosine

The intermediates between chorismate, phenylalanine, and tyrosine, namely, prephenate, phenylpyruvate and p-hydroxyphenylpyruvate (Fig. 4) have been known for a number of years (Cotton and Gibson, 1965). These intermediates are formed in the phenylalanine



Chorismic acid (CA)

Anthranilic acid (AA)

N-(5'-phosphoribosyl) anthranilic acid (PRA)

1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP)

Indoleglycerol phosphate (INGP)

L-tryptophan

Figure 3

A summary of the trp pathway and the corresponding genes and enzymes of E. coli as adapted from Ito and Crawford (1965) and Margolin (1967).

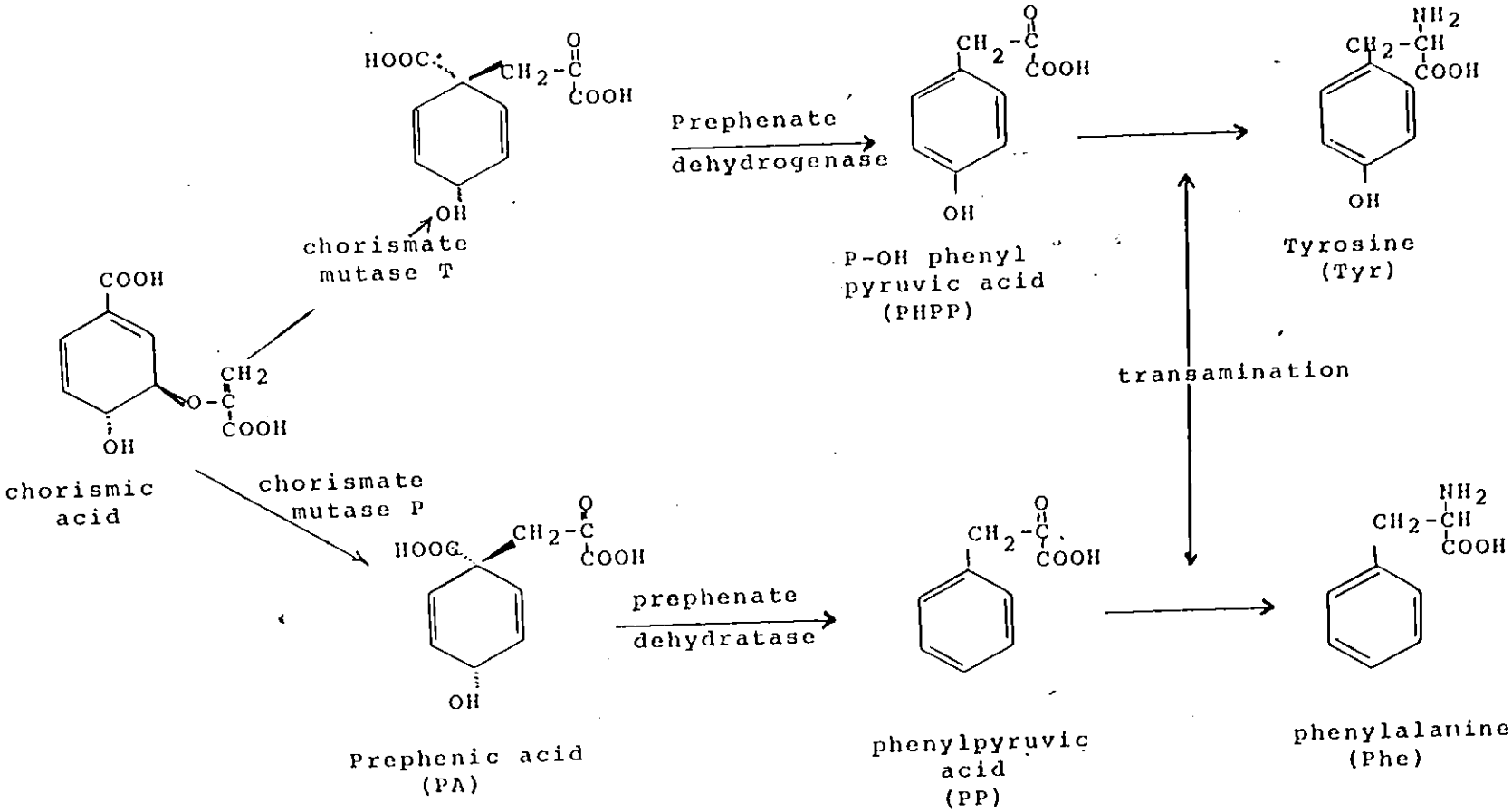


FIG. 4. The conversion of chorismate into phenylalamine and tyrosine as adapted from Cotton and Gibson (1965).

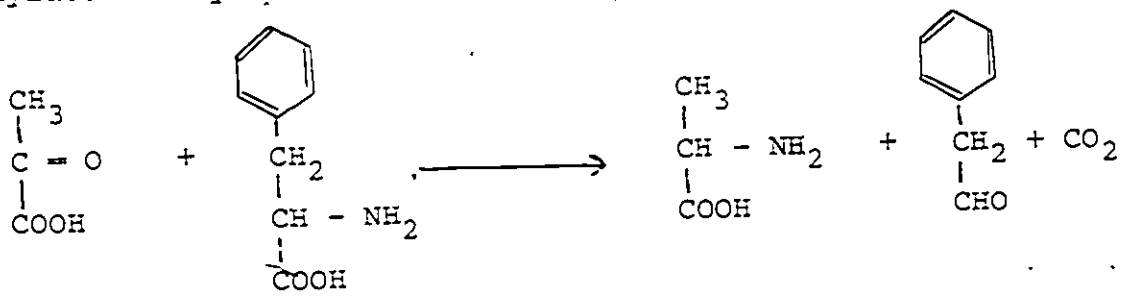
T-protein (chorismate mutase T-prephenate dehydrogenase)
P-protein (chorismate mutase P-prephenate dehydratase)

and tyrosine pathway in E. coli, A. aerogenes, Saccharomyces cerevisiae and N. crassa. Transamination is involved in the last step of the phenylalanine and tyrosine synthesis.

B. Transamination

a) Chemical Transamination

In this reaction an amino group is transferred from one molecule to another without the intermediate participation of free ammonia. Such reactions were first observed by Herbst and Engel (1934) who demonstrated transfer of the α-amino groups of amino acids to α-keto acids in boiling aqueous solution. The reaction between pyruvic acid and phenylalanine gave alanine, phenylacetaldehyde, and carbon dioxide:



pyruvic acid — phenylalanine — alanine — phenylacetaldehyde

b). Enzymatic transamination

Enzymatic transamination was first reported by Braunstein and Kritzman (1937). These workers observed transfer of amino groups to α-keto acids in pigeon breast muscle. The transformation of an amino acid to the analogous α-keto acid, and reversal of this reaction, were observed by Needham (1930). Needham found

that deamination of glutamic acid and aspartic acid could occur in pigeon breast muscle without appearance of ammonia. Further evidence for the existence of the transamination reaction was provided by Szent-Gyorgyi (1936) who reported that the rate at which oxaloacetic acid disappeared was greatly increased when glutamic acid was added to pigeon breast muscle. He later confirmed the findings with an enzyme preparation from the same tissue (Szent-Gyorgyi, 1937).

Braunstein and Kritzamnn (1937) originally reported that α-ketoglutarate and oxaloacetate could accept the α-amino groups of a large number of amino acids. The studies of Cohen (1939) showed that the analytical procedures and techniques employed in the original investigations were inadequate, and subsequent studies by Cohen (1940) and by Braunstein (1947) led to the revised conclusion that only three amino acids (glutamic acid, aspartic acid and alanine) participated significantly in transamination.

The glutamate-alanine and glutamate-aspartate reactions for some time were considered to be the major systems concerned with amino group transfer. It is now recognized that many other α-ketoglutarate-amino acid reactions are catalyzed by preparations of animal and plant tissues, and of micoorganisms. In more recent investigations the occurrence of transamination reactions involving many amino acids has been clearly established. Feldman and Gunsalus (1950) demonstrated the formation of glutamate from

α-ketoglutarate and a number of amino acids with cell suspensions of E. coli and Pseudomones fluorescens. Appreciable glutamate formation was observed with aspartate, valine, leucine, norleucine, tyrosine, phenylalanine, tryptophan, and methionine, while somewhat less activity was noticed with isoleucine, threonine, lysine, histidine, and glycine. Similar results were obtained with Bacillus subtilis although the transamination rates were lower. It is of interest that the α-ketoglutarate-alanine reaction could not be shown in E. coli and proceeded relatively slowly with P. fluorescens. Fractionation of cell extracts of E. coli yield preparations capable of catalyzing reversible transamination between α-ketoglutarate and phenylalanine or tyrosine, and these reactions were accelerated by pyridoxal phosphate.

C. Significance of Transamination in Amino Acid Metabolism.

The widespread occurrence and broad specificity of the transamination reaction suggests that it plays a significant role in the metabolism of animals, plants, and microorganisms. Transamination participates in the process of oxidative deamination of L-amino acids in mammalian tissue and is related to the urea-forming mechanism. The ability of the α-keto acid analogs to replace the corresponding α-amino acids in supporting growth is consistent with the existence of active transaminase systems. In Wood and Cooley's study (1954), young rats were found to grow as rapidly on a diet consisting of ten essential amino acids

plus glutamic acid, as when fed a ration in which five essential amino acids (leucine, isoleucine, valine, phenylalanine, and methionine) were replaced by their α-keto acid analogs and an equivalent source of nitrogen. This finding suggests that the total transaminase activity of the intact rat is great and in view of the necessity for the simultaneous presence of all the amino acids for protein synthesis it may be concluded that transamination of these five α-keto acids occurs rapidly.

Transamination provides a mechanism for the reversible formation of alanine, glutamate, and aspartate from pyruvate and the analogous α-keto acid intermediates of the citric acid cycle. A transamination step is often involved in the biosynthesis of amino acids by microorganisms (e.g. isoleucine, valine, leucine, phenylalanine, and tyrosine). In Escheridria coli, there appeared to be at least three distinct transaminases involved in such reactions (Rudman and Meister, 1953).

The first, designated "transaminase A", was shown to catalyze transamination of tyrosine, phenylalanine, tryptophan and aspartate, as well as, to a lesser extent, methionine and leucine, with α-ketoglutarate or oxaloacetate as cosubstrate. The second, "transaminase B", catalyzed transamination of leucine, isoleucine, and valine with essentially no activity toward aspartate, tryptophan and tyrosine and little activity toward methionine and phenylalanine and also with α-ketoglutarate or oxaloacetate as

cosubstrate. The third enzyme "transaminase C" catalyzed the transamination between α-ketoisovalerate and the amino acids alanine and α-aminobutyric acid. Table I summarizes the transaminases from E. coli according to this study (Rudman and Meister, 1953).

The transaminase A, B, C nomenclature has since commonly been employed in the literature for E. coli.

D. Repression of Transaminase A in E. coli.

There is evidence that the synthesis of transaminases like a number of other enzymes, can be repressed. Repression of transaminase A (assayed as tyrosine and phenylalanine aminotransferases) by tyrosine was first reported by Silbert, Jorgensen and Lin (1963). It was repressed about 70% by tyrosine in E. coli K_{12}, B and W. Other amino acids did not have significant effect on the formation of this enzyme. Even under maximal repression of the enzyme by tyrosine, the cells were able to utilize this amino acid as the sole source of nitrogen and the activity of this enzyme was the first rate limiting factor for growth. It was concluded that normally the enzyme functions biosynthetically, but under special conditions it could catalyze a catabolic reaction in the deamination of tyrosine for nitrogen (Silbert et al., 1963).

The gene controlling this repression was later designated tyr R by Wallace and Pittard (1969). They postulated that tyr R codes for a protein aporepressor which, when combined with tyrosine, causes repression of DAHP synthetase (tyr), chorismate mutase T and its associated prephenate dehydrogenase activity, and

TABLE I

Transamination between α-keto acids and various amino acids catalyzed by extracts of E. coli.

(Rudman and Meister, 1953)

Transaminase	L-Amino Acid
A	aspartate, tyrosine, phenylalanine, tryptophan, (methionine, leucine)
B	leucine, isoleucine, valine (methionine, phenylalanine)
C	valine, alanine, aminobutyrate

transaminase A. Davidson and Pittard in 1971 postulated that the aporepressor can (i) combine with tyrosine to control the regulon consisting of the aro F tyr A operon and the structural gene for transaminase A and (ii) combine with phenylalanine and repress the synthesis of DAHP synthetase (Phe). The aporepressor must, therefore, have affinity for both tyrosine and phenylalanine.

E. Purpose of Present Work

The work in this Thesis was prompted by the observation referred to previously, namely, that transaminase A was repressible by only 70% by tyrosine. It was considered possible that the remaining 30% of unrepressible activity might represent an alternate form of transaminase A, not subject to repression. In the course of our studies it was found that tyrosine does not, in fact, repress transaminase A but another, new enzyme, aromatic-amino-acid amino-transferase.

II. MATERIALS AND METHODS

A. Materials:

Chemicals and their sources are listed below.

Eastman Kodak Co. (Rochester, N.Y.)

Acrylaminde, bis-acrylamide, tetramethyl ethylenediamine (TEMED), 2-mercaptoethanol.

Fisher Scientific Co. Ltd. (Montreal, Quebec)

Ampholytes (L.K.B.), Ammonium persulfate

Ethylene diamine-tetraacetic (EDTA), and all buffer salts.

Mandel Scientific Co. Ltd. (Montreal, Quebec).

Diethylaminoethyl-cellulose (DE-52)

Pharmacia Fine Chemicals AB (Montreal, Quebec).

Sephadex G-200 superfine, chymotrysinogen A, aldolase, ovalbumin, bovine serum albumin.

Pierce Chemical Co. (Rockford, Illinois)

Sodium dodecyl sulfate (SDS)

Schwartz/Mann (Orangeburg, N.Y.)

Urea (ultra pure), Ammonium sulfate (enzyme grade).

Sigma Chemical Co. (St. Louis, Missouri)

amino acids.

pyridoxal phosphate, dithiothreitol

α-ketoglutarate, nitroblue tetrazolium

nicotinamide adenine dinucleotide (NAD^+), NADH

sodium azide, phenazine methosulfate glutamate dehydrogenase, malate dehydrogenase, subtilisin, tautomerase, trizma, sucrose, dichlorophenolindophenol (DCIP).

B. Methods:

1. Enzyme Assays

Tyrosine: 2-oxoglutarate aminotransferase was assayed by the method of Diamondstone (1966). The reaction mixture contained in 3.2 ml the following: 0.2 M potassium phosphate buffer, pH 7.3, 6.0 mM L-tyrosine, 9.4 mM α-ketoglutarate, 38 μM pyridoxal phosphate and enzyme. The mixture was preincubated at 37° for 10 min and the reaction was started by adding α-ketoglutarate. After 10 min the reaction was stopped with 0.2 ml of 10 N NaOH. After 30 min at room temperature the optical density at 331 nm was read against a control to which NaOH had been added prior to α-ketoglutarate. A molar extinction coefficient of 19,000 M^{-1} cm^{-1} was used. Tyrosine aminotransferase in some kinetic experiments in the presence of aspartate was assayed by the enol borate-tautomerase method (Lin et al., 1958) because a substance absorbed strongly just below 331 nm in the Diamondstone assay.

Phenylalanine aminotransferase was assayed as the tyrosine aminotransferase except that tyrosine was replaced

by 6 mM phenylalanine and the optical density was read at 315 nm. A molar extinction coefficient of 17,500 M^{-1} cm^{-1} was used.

Tryptophan aminotransferase was assayed also as the tyrosine aminotransferase except that tyrosine was replaced by 6 mM L-tryptophan and the optical density was read at 335 nm. A molar extinction coefficient of 10,000 M^{-1} cm^{-1} was used.

Aspartate: 2-oxoglutarate aminotransferase (E.C. 2.6.1.1) was assayed by the method of Karmen (1955) by coupling with malate dehydrogenase at 25°C. The reaction mixture contained in 3.0 ml the following: 0.1 M potassium phosphate buffer, pH 7.6, 178 mM L-asparate, 6.4 mM α-ketoglutarate, 38 µm pyridoxal phospate, 0.24 mM NADH, 10 units malate dehydrogenase and enzyme. The reaction was started by adding the enzyme and was followed by the fall of optical density at 340 nm. A molar extension coefficient of 6.2×10^3 M^{-1} cm^{-1} was used.

Methionine aminotransferase was assayed by the method of Chesne and Pelmont (1973). The reaction mixture contained in 3.0 ml the following: 1 µ mole α-ketoglutarate, 40 µ moles methionine, 0.15 µ mole NAD^+, 0.08 µ mole pyridoxal phosphate, 0.08 µ mole phenazine methosulfate, 0.06 µ mole dichlorophenolindophenol, 100 µg glutamate dehydrogenase, 0.1 M potassium phosphate buffer and enzyme. The reaction

was started by adding α-ketoglutarate and the optical density was read at 600 nm. A molar extinction coefficient of 16,000 M^{-1} cm^{-1} was used.

In some experiments all enzyme activities were assayed by estimating the product (either glutamic acid or aspartic acid). 0.8 ml of 30% trichloroacetic acid was added to terminate the reaction and the product (in the supernatant fluid, after removing the precipitated protein by centrifugation if necessary) was estimated in a Beckman amino acid analyzer, Model B.

One enzyme unit is defined as that amount of enzyme which catalyzes the conversion of 1 μ mole of substrate to product per minute at the temperature of the assay. Enzyme specific activities are expressed as units per mg protein. Protein concentration was determined by the method of Lowry et al. (1951) or spectrophotometrically (Layne, 1957).

2. Growth of Microorganisms

E. coli B were grown in 100 ml of the minimal medium 56 (Monod et al. 1951) with 0.125% glucose in 300 ml nepheloflasks at 37° in a rotary shaker at 200 r.p.m. The logarithmically growing cells were harvested 4-5 hours after inoculation by centrifugation and washed twice with 0.9% NaCl.

Frozen Escherichia coli Crookes (H.T.C.C. No. 8379) were obtained from General Biochemicals. The organisms had

been grown at pH 6.5 to late log phase in a minimal medium with sodium succinate as the carbon source.

3. Preparation of Crude Extract

For each experiment approximately 100 g of E. coli Crookes were suspended in a Waring blender in about 1 liter of 0.2 M potassium phosphate buffer, pH 7.3 containing EDTA 1 mM, dithiothreitol 1 mM, α-ketoglutarate 2.0 mM and pyridoxal phosphate 0.2 mM. The suspension was sonicated with a Bronwill Biosonic III Ultrasonic disintegrator for 1 min using the standard 3/4" probe in volumes of 50 ml and was centrifuged for 30 min at 27,000 x g in a Sorvall RC-2 ultra-centrifuge. The clear supernatant fluid (crude extract) was used for further purification.

4. Enzyme Purification

All purification steps were carried out at 0-4° and all potassium phosphate buffers were at pH 7.0 and contained EDTA and dithiothreitol 1 mM, α-ketoglutarate 2.0 mM and pyridoxal phosphate 0.2 mM.

a) Ammonium Sulfate Fractionation

Crystalline ammonium sulfate (enzyme grade from Schwatz/Mann) was added slowly to the clear crude extract to 35% saturation (199 g per liter). After constant stirring for 1 hour, the mixture was centrifuged for 10 min at 27,000 x g

and the precipitate was removed and discarded. The resulting supernatant fraction was brought, without pH adjustment, to 70% saturation with ammonium sulfate (449 g per liter). The pH at this point was about 5.2. After stirring for 1 hour or more, the precipitate was collected by centrifugation (27,000 x g) and dissolved in a minimal amount of 0.02 M potassium phosphate buffer. Trace amount of insoluble material was removed by centrifugation.

b) Heat Treatment

The enzyme solution was diluted to a protein concentration of about 10 mg per ml with 0.02 M potassium phosphate buffer. The solution was brought rapidly to 55° in batches of 200-300 ml in a boiling water bath and maintained at this temperature in another water bath at 55° with constant stirring for 5 minutes after which it was quickly chilled to 0-4° in a 0.9% NaCl - ice water bath. The bulky precipitate was removed by centrifugation and discarded. Solid ammonium sulfate was added to the supernatant fluid to 70% saturation. The precipitate which formed was collected by centrifugation, dissolved in a minimal volume of 0.005 M potassium phosphate buffer, and the solution was centrifuged at 27,000 x g for 10 minutes to remove insoluble material. The clear preparation was then dialyzed for 16 hours against 4 liters of the above buffer. The solution was then centrifuged to remove trace precipitate

which was formed during the dialysis.

c) Calcium Phosphate Gel Fractionation

Calcuium phosphate gel was prepared according to the method of Swingle and Tiselius (1951). To 450 g pure sucrose in 2 l. of water 75 g CaO was added. The suspension was agitated periodically for several hours until most had dissolved, and was clarified. Conc. H_3PO_4 (about 18 ml) was added dropwise with continuous stirring over the course of an hour to 800 ml of the calcium-sucrose solution, chilled to about 5°, until the pH dropped to 9.5; after this the stirring was continued for 4 hours. The resulting precipitate of calcium phosphate was thoroughly washed with 200 l. of distilled water (20 l. each time). The gel concentration was kept at about 30 mg/ml, and the gel was stored at 4° for several months prior to use.

The enzyme solution was diluted with 0.005 M potassium phosphate buffer to a protein concentration of about 1%. A suspension of calcium phosphate gel was then added to give a gel:protein ratio of 3:1, by weight. After stirring for 10 min, the mixture was centrifuged at 5,000 x g for 15 minutes. Most of the enzyme activity remained in the supernatant fluid. The enzyme solution (supernatant fluid) was concentrated by ultrafiltration in a Diaflo apparatus (Amicon Corporation, with PM-10 membrane). During this procedure 200 ml of 0.005 M potassium

phosphate buffer, pH 7.0 were passed through the enzyme solution.

d) DEAE-Cellulose Column Chromatography

Wet microgranular DEAE-cellulose (Whatman DE-52) was equilibrated in ample 0.05 M potassium phosphate buffer pH 7.0 (10 times the volume of cellulose). This suspension was stirred and allowed to stand for 15 minutes. Fine particles were removed by sucking off the supernatant fluid. The process was repeated until the supernatant fluid reached the same pH as that of the buffer (pH 7.0). The column (2.7 x 70 cm) was packed and washed with 500 ml of 0.05 M potassium phosphate buffer. The concentrated enzyme solution obtained by calcium phosphate gel fractionation was then applied to the column. Elution was carried out with two successive linear sodium chloride gradients, the first (1000 ml) 0.1 - 0.5 M, and the second (500 ml) 0.5 - 1.0 M sodium chloride solution. Fractions of 5 ml each were collected and assayed for enzyme activity. Pooled fractions were concentrated in the Amicon ultrafiltration units. During this procedure 1 liter of 0.05 M potassium phosphate buffer, pH 7.0 were passed through the enzyme solution.

e) Isoelectric Focusing

Isoelectric focusing was performed according to the procedure described in the manufacturer's instruction mannual. Both

LKB 4102 (110 ml) and LKB 8102 (440 ml) electrofocusing columns were used. The narrow pH range used to achieve the highest possible resolution was obtained by an initial focusing of 6% carrier ampholytes, pH 4 - 6. A linear gradient of sucrose including ampholytes was formed manually in the electrofocusing column. The gradient was layered over the anode solution (56 ml distilled water, 48 g sucrose and 0.8 ml concentrated sulfuric acid for LKB 8102 column) and the cathode solution (1% sodium hydroxide) was layered over the gradient. Five to six watts of power output was maintained for the duration (6 days) of the electrofocusing procedure. After electrofocusing was completed, the column contents were drained and collected in 3 ml fractions. The pH of the fractions was determined immediately and subsequently the fractions in the 4.3 - 4.9 pH range were pooled. The pooled fractions were used to form the second gradient as described before. In this final focusing with the enzyme preparation (2.0 ml, 160 units) from the previous purification step (DEAE-cellulose column chromatography) the concentration of carrier ampholytes was about 1 - 1.5%. Electrofocusing was carried out for 5 days and a power load of 2 watts was maintained for that duration. After electrofocusing the column contents were drained and 2 ml fractions were collected. The pH of the fractions was determined immediately and the fractions were assayed for tyrosine aminotransferase. In one electrofocusing

experiment the fractions were assayed also for aminotransferase activities toward the L-aromatic amino acids, (tyrosine, phenylalanine tryptophane) L-aspartic acid and L-methionine. Fractions containing enzyme activity were pooled and then placed in an Amicon ultrafiltration unit (membrane PM-10) attached to a 2.5 liters reservior. The carried ampholytes were removed by passing 2-3 liters of 0.05 M potassium phosphate buffer, pH 7.0 through this pooled fractions. The enzyme solutions were then concentrated to a volume of 3-5 ml and kept at -20^{o} to -30^{o}. The concentrated fractions were used for further characterization studies.

5. Electrophoresis in Polyacrylamide Gel

Polyacrylamide disc gel electrophoresis was carried out at $1-2^{o}$ in the Tris-glycine buffer, pH 8.3, system as described by Davis (1964) except that the acrylamide monomer concentration was 10.5%. The gels were prepared in glass tubes 75 x 5 mm, and a current of 3 ma per gel was applied. Bromphenol Blue was used as the tracking dye. The dye ran off the gel at about 2½ hours, but runs were often extended to obtain better separation. At the completion of electrophoresis, after the power supply was turned off, the gel tubes were removed from the upper reservoir and the gels extruded from the tubes by rimming under water. The extruded gels were fixed with 12.5% trichloroacetic and for 30 minutes at 65^{o}, then stained with Coomassie Brilliant Blue solution (0.2% Commassie Blue in 45% ethanol and 10% acetic acid,

(w/v/v)) for 30 minutes at 65° and destained with ethanol: acetic acid:water (25:10:65 v/v/v), for two intervals of 30 minutes at 65°. The gels were then destained with several changes of 10% acetic acid at 65°. The destained gels were also kept in this solution (Bulletin, AN 32, Oretec Incorporated, 1970).

Aminotransferase activity was detected directly on the gel by the method of Ryan et al. (1972). After extrusion, the gels were incubated at 35° in the dark in a mixture containing the following in 60 ml of 0.2 M potassium phosphate buffer, pH 7.5: 60 mg α-ketoglutarate, 120 mg NAD^+, 2 mg phenazine methosulfate, 20 mg nitrobluetetrazolium, 4 mg pyridoxal phosphate, 40 mg NaN_3, 6 units of glutamate dehydrogenase and the desired amino acid at 6 mM, except aspartate and methionine which were present at 150 mM. Incubation was carried out for 20 - 30 minutes. The presence of aminotransferase was indicated by a purple band on a light violet background when stored in 10% acetic acid. The staining was entirely satisfactory for all the aromatic amino acids. The staining for aspartate and methionine were very poor and the resulting bands were very faint. Prolonged incubation with aspartate to 45 - 60 minutes improved the staining but it also resulted in much darker background.

The staining method for aminotransferase activities in gels is actually mainly applicable for aromatic aminotransferase activity. It is based on the reaction between phenazine metho-

sulfate and arylpyruvates, i.e., p-hydroxyphenylpyruvate, phenylpyruvate and indolepyruvate (the products of aromatic amino acid transamination) (Schepartz, 1969). Valeriote et al. (1969) used a similar method to stain tyrosine aminotransferase activity in gels. They used monoiodotyrosine and p-iodonitrotetrazolium instead of tyrosine and nitrobluetetrazolium in the absence of NAD^+, NaN_3 and glutamate dehydrogenase.

After completion of the experiments described in this Thesis Dr. Mavrides applied the method used by Rehfeld et al. (1972) to stain aspartate aminotransferase in gels. The staining mixture contains the substrates for the transaminase reaction and Fast Violet B salt which reacts with oxaloacetate to form a chromogenic compound (Brewbaker et al., 1968). Bright red bands appear at the position of enzyme activity.

In the repression experiment, tyrosine aminotransferase activity of the two enzymes in the gels was assayed by the Diamondstone method (1966) in gel slices corresponding to the activity bands as visualized in one gel by the staining technique. The gel slices were mashed in a 7-ml glass homogenizer with a glass rod and subsequently with the plunger, in 2.0 ml of assay buffer containing tyrosine and PLP. The gel suspension was transferred to a 25-ml Erlenmeyer flask and the homogenizer was rinsed with the remaining assay mixture (1.1 ml) and the rinsings were transferred to the flask. The reaction was

started as usual with α-KG and terminated after a 10 min incubation at 37° with 10 N NaOH. The assay mixture was centrifuged at 27,000 x g and the absorbance of the clear supernatant was read at 331 nm by scanning. The linearity of the reaction with time and enzyme concentration had been ascertained in preliminary experiments.

In studies concerned with the relative size and charges of the enzymes, gels were prepared at various acrylamide concentrations and a constant acrylamide to bis-acrylamide ratio of 30:1 (Hedrick and Smith, 1968).

The molecular weight of subunits was estimated by sodium dodecyl sulfate (SDS) gel electrophoresis as described by Weber and Osborn (1969). Samples and protein standards were incubated at 60° for 10 minutes in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS, 2.5% 2-mercaptoethanol and 2 M urea (ultra pure). The gels were electrophoresed at 3 ma per gel at room temperature and stained as described for Davis gels. The following protein standards were used: chymotrypsinogen A, aldolase (tetramer), ovalbumin and bovine serum albumin.

6. Molecular Weight Estimation

a) Gel Filtration

The molecular weights of the pure enzymes were estimated by gel filtratration in a calibrated column (1.5 x 100 cm) of Sephadex G-200 (Superfine) that had been previously equilibrated

in 0.05 M potassium phosphate buffer. The column was eluted with the same buffer at a flow rate of 4.0 ml/hour. Fractions (0.5 ml) were collected and assayed for aminotransferase activity. The following protein standards were used: Chymotrysinogen A, ovalbumin, bovine serum albumin and aldolase. The protein was monitored by absorption at 280 nm (Layne, 1957).

b) Sodium Dodecyl Sulfate (SDS) Acrylamide Gel Electrophoresis

The molecular weight of subunits was estimated by SDS acrylamide gel electrophoresis as described previously.

7. Amino Acid Composition

Samples of enzyme A (38 µg) and enzyme B (17 µg) were hydrolyzed with 6 N HCl solution and transferred to a heavy wall Pyrex glass tube. The solutions were frozen, the tubes constricted, evacuated for 15 minutes, sealed and then placed in an oven at 110° for 24 hours. After hydrolysis the tubes were opened and HCl was removed in a vacuum descicator and the hydrolyzate resuspended in 0.2 sodium-citrate buffer, pH 2.0 and applied to a cartridge. Analysis of the hydrolyzate was carried out on a TSM amino acid autoanalyzer equipped with an automatic integrator.

8. Heat Sensitivity Tests

The solutions of purified enzymes were diluted 10-fold with 0.2 M potassium phosphate buffer, pH 7.3 to a final volume of 4.0 ml and bovine serum albumin was added to a final concentration of 5 mg/ml. The solutions were placed in thin-walled 20 x 100 mm glass tubes supplied with small magnetic stirring bars and were immersed into a water bath at 100° with constant stirring until the temperature was raised to about 52°. This required about 20 seconds. The tubes were then transferred to a water bath at 55° and kept at this temperature for 10 minutes with constant stirring. Aliquots were withdrawn at specified time intervals, quickly chilled in ice and centrifuged at 27,000 x g for 10 minutes. The supernatant fractions were assayed for enzyme activity. An aliquot was withdrawn before heating and kept in ice throughout and served as the zero time control. Activities were expressed as percentage of the control.

9. Apoenzyme Formation

The purified enzyme solutions were allowed to form the pyridoxamine form which is believed to be more dissociable than the pyridoxal form (Guirard and Snell, 1964) in the presence of L-tyrosine. Purified enzymes A and B were diluted 20-fold and 40-fold, respectively, with 0.1 M potassium phosphate buffer, pH 7.0, supplemented with 1 mM EDTA, 1 mM dithiothreitol and 5 mM L-tyrosine to a volume of 8.0 ml, and were allowed to stand at 4° for four hours. They were then dialyzed for 19 hours against two changes of 2 liters each of the above buffer minus L-tyrosine.

In the absence of added pyridoxal phosphate enzyme A still possessed 56% of the activity obtained upon full activation with the cofactor, the corresponding figure being 9% for enzyme B. Full restoration of the original activity was observed in the presence of pyridoxal phosphate. The experiment was repeated replacing L-tyrosine with L-phenylalanine with no more success. Therefore, in kinetic experiments designed to calculate the Km and Vmax of pyridoxal phosphate the plots of the velocity (vertical axis) versus the apparent (added) pyridoxal phosphate concentration were extrapolated to zero velocity. The intercept of the curve with the horizontal concentration axis (to the left of the velocity axis) was set as zero pyridoxal phosphate concentration and the new real cofactor concentrations were written on the horizontal axis. Double reciprocal plots were then constructed to calculate the approximate Km and Vmax values for pyridoxal phosphate.

10. Kinetic Experiments

Double reciprocal plots according to Lineweaver and Burk (1934) were used for the calculation of kinetic constants. Since in transamination reactions more than one substrate is involved, the Km and Vmax values are apparent values at pH 7.3 and were calculated for each variable substrate while pyridoxal phosphate and α-ketoglutarate were at saturating and L-tyrosine at 6.0 mM concentrations. The amount of enzymes used for these experiments were: enzyme A, 1-2 µg for the aromatic activities and about 0.29 µg for the aspartate activity; enzyme B, 0.07 -

0.15 µg for the aromatic activities.

11. The Effect of pH on the Km

Km values were calculated by the method of Lineweaver and Burk (1934). The effect of pH on the Km values was studied by calculating Km values over a wide pH range. As discussed by Dixon and Webb (1964) the ionization constant of ionizing groups at the active site of the free enzyme (Ke), or in combination with the substrate (Kes) may be calculated as pKe (-logKe) or pKes (-logKes) values from plots of pKm versus the pH. Bends in the curves with the concave side downwards represent the ionizations of the free enzyme or the free substrate, whereas bends with the concave side upwards represent modified ionizations of the enzyme or substrate in the enzyme-substrate complex.

12. Proteolysis of Enzyme B

Enzyme B was incubated at 37° in 4.0 ml of 0.02 M Tris-HCl buffer pH 8.0, in the presence of bovine serum albumin (0.3 mg/ml) and 0.2 µg of subtilisin. In some experiments the amount of subtilisin was increased to 0.4 and 0.8 µg. Aliquots (0.5 ml) were withdrawn at 5 minutes intervals, quickly chilled in ice and subsequently assayed for tyrosine and aspartate aminotransferases activities. Aliquots were withdrawn after 5, 10, and 15 minutes incubation and were pooled and the pooled mixtures were subjected to acrylamide gel electrophoresis for 4 hours at 3 mA per gel. The gels were then stained for tyrosine aminotransferase activity.

Another proteolytic enzyme, trypsin, was also tried and the amounts of trypsin used were varied from 0.2 to 40 µg.

III RESULTS AND DISCUSSION

A. Purification of Tyrosine Aminotransferase Activity and its Separation into two Fractions by Isoelectric Focusing

Table II summarizes the purification procedure. Enzyme activity was monitored as tyrosine aminotransferase but the fractions collected in the isoelectric focusing experiment (with the 110 ml column) were assayed also for aspartate aminotransferase, tryptophan aminotransferase and phenylalanine aminotransferase. The fraction after the calcium phosphate gel step was dialyzed, concentrated and subjected to DEAE-cellulose chromatography. The elution pattern for this procedure is shown in Fig. 5. Six peaks were obtained but the first peak (peak I) constituted 99% of the total enzyme activity, the other five peaks representing only 1% of the total activity. These five peaks may represent other minor forms of aminotransferase of unknown significance and they were not investigated further. When the pooled fractions of peak I were assayed with different amino acids as substrates a specificity emerged which was in agreement with that of transaminase A as first reported by Rudman and Meister (1953) as shown in Table III.

Since staining for protein after polyacrylamide gel electrophoresis of peak I revealed a large number of bands (Fig. 6), it was decided to attempt a further fractionation of the activity

TABLE II

Summary of purification of tyrosine aminotransferase activity and its seperation into two fractions by isoelectric focusing

Purification Step	Volume (ml)	Protein (mg)	Enzyme Units	Specific Activity Units/ mg protein	% Recovery
Crude Extract	690	16560	1771	0.108	
Ammonium Sulfate (35%-75%)	130	5395	1888	0.35	107
Heat Treatment	80	3064	1685	0.55	95
Calcium Phosphate gel	11	607	1184	1.95	67
DEAE-cellulose (peak I)	9	86.4	726	8.4	41
Isoelectric Focusing of peak I	2	19.2	160	8.4	
Enzyme A	3.2	0.384	10	26	6.25*
Enzyme B	3.4	0.129	26.7	207	16.7*

*Recoveries in terms of the amount (160 units) placed in the 440 ml column. These recoveries are underestimates since only selected fractions from the focusing column were pooled and assayed.

Figure 5

DEAE-Cellulose Chromatography of Aminotransferase Activity

The enzyme after the calcium phosphate gel step was chromatographed on a DEAE-cellulose column (2.7 x 70 cm). The enzyme activity was eluted with two successive linear NaCl gradients and 5 ml fractions were collected. The column fractions were assayed as tyrosine aminotransferase (•). Protein (o) was determined by the Lowry method (1951). Peak I is plotted on a contracted activity scale (insert).

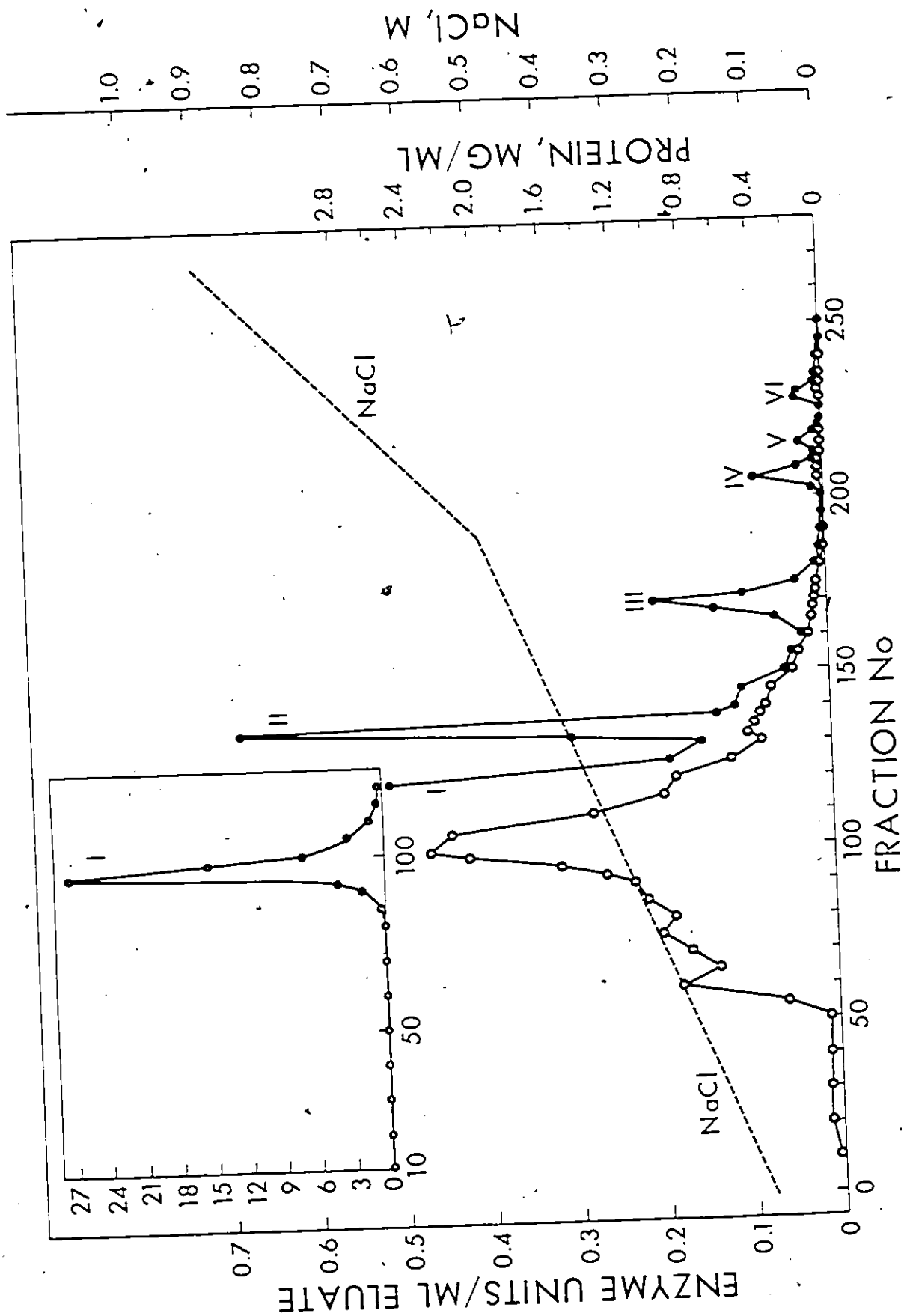

ENZYME UNITS/ML ELUATE
PROTEIN, MG/ML
NaCl, M
FRACTION No
NaCl
NaCl
I
II
III
IV
V
VI

Table III

Relative amino acid specificity of peak I with α-ketoglutarate or oxaloacetate as cosubstrate

Amino Acid	Relative activity with:	
	α-ketoglutarate	oxaloacetate
L-aspartic acid	4.35	-
L-glutamic acid	-	4.60
L-tyrosine	1.00	1.00
L-tryptophan	0.95	0.66
L-phenylalanine	0.86	0.64
L-methionine	0.21	0.28
L-valine	0.03	0.04
L-cysteine	0.04	0.01
L-leucine	0.03	0.00
L-alanine	0.03	0.04
L-isoleucine.	0.00	0.00

Amino acid concentration in assay mixtures was 6 mM. Keto acid concentration was 10 mM. The specific tyrosine aminotransferase activities were set equal to 1.0. Activities were determined by measuring glutamate and asparatate with the amino acid analyzer.

Figure 6

Polyacrylamide gel Electrophoresis of Peak I

The partially purified enzyme (peak I) by DEAE-cellulose chromatography was electrophoresed in 10.5% Davis gels (1964). 7.5 µg of protein was applied in the gel. The left gel wa stained for tyrosine aminotransferase and the right gel for protein.


TYR Protein

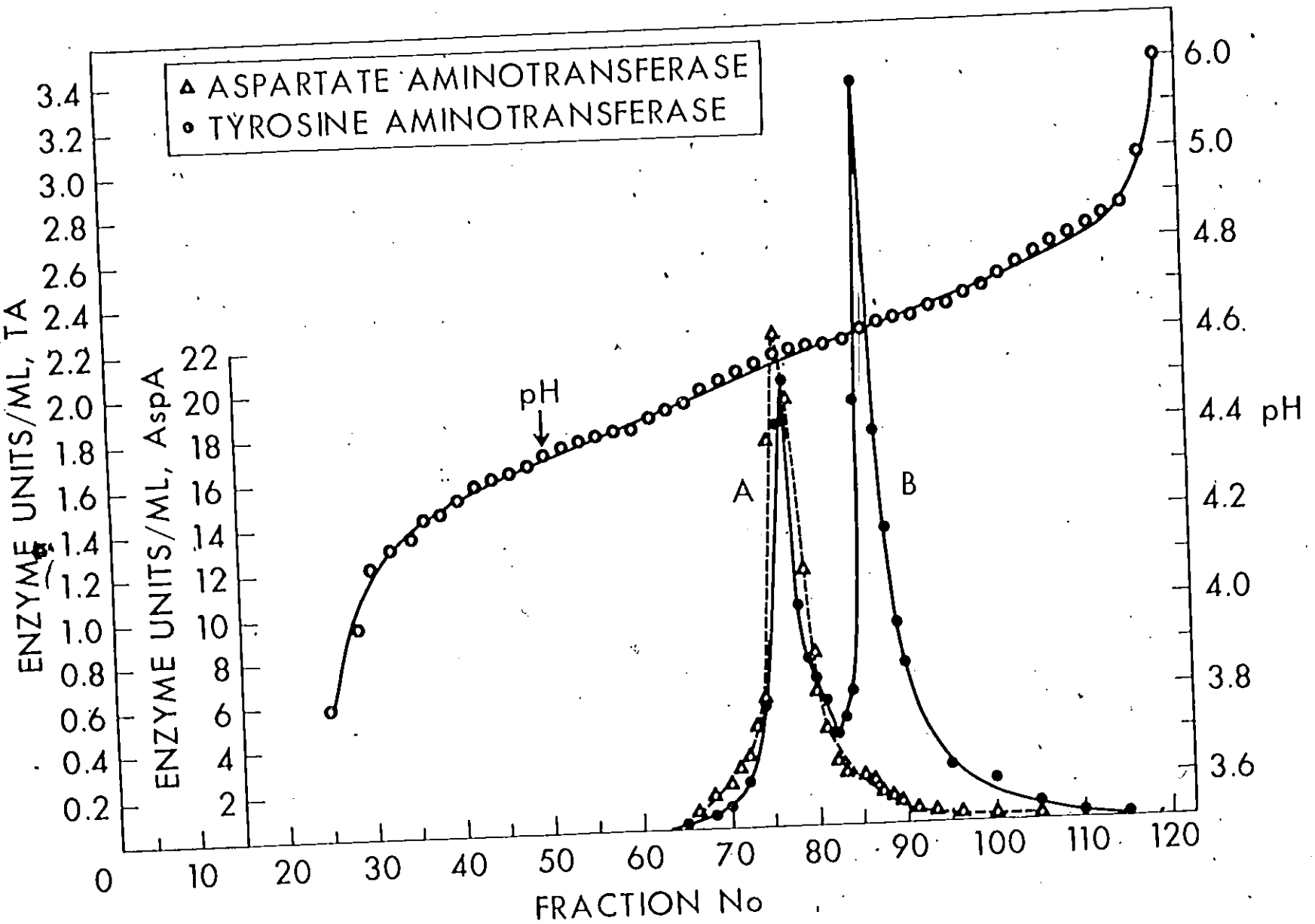
by isoelectric focusing. Isoelectric focusing is obtained by imposing a potential on an electrolyte system in which the pH steadily increases from the anode to the cathode. Provided that the pH gradient is sufficiently stable for the duration of an experiment, ampholytes, such as proteins and peptides, present in the electrolyte system will be repelled by both electrodes, and each ampholyte species will collect at the place in the gradient where the pH of the gradient is equal to the isoelectric point of that species. This focusing is caused by the electric field, thus the name isoelectric focusing.

Isoelectric focusing in the small (110 ml) column resulted in the purification and resolution of the tyrosine aminotransferase activity into two partially overlapping peaks (Fig. 7). Aspartate aminotransferase activity virtually coincided with peak A. Because of the overlap only small amounts of enzyme from either peak could be obtained without cross-contamination. In the large (440 ml) preparative column, which could be loaded with 5-6 times more enzyme units, superior resolution was obtained and sufficient amounts of enzymes were recovered for kinetic and other experiments. Fig. 8 shows the separation obtained in the large column. Isoelectric points of 4.54 and 4.61 were observed for peaks A and B, respectively. This profile was reproducible. The intermediate low activity fractions were rejected and only the fractions of the highest activity from each peak were pooled, dialyzed and concentrated by ultrafiltration and tested for protein and

Figure 7

Isoelectric focusing of Peak I from DE-52 column chromatography

Isoelectric focusing of an enzyme preparation (peak I, Fig. 5) obtained after the step of DEAE-cellulose column chromatorgraphy. 27 units of enzyme in 0.5 ml (total protein 7.5 mg) were applied on the column. The run lasted 96 hours with a voltage range of 300-490 V and a current range of 1.4-1.5 mA. 1.0 ml fractions were collected and assayed for the two activities. TA, tyrosine aminotransferase; AspA, aspartate aminotransferase.


ASPARTATE AMINOTRANSFERASE
TYROSINE AMINOTRANSFERASE
ENZYME UNITS/ML, TA
ENZYME UNITS/ML, AspA
pH
A
B
FRACTION No
0
10
20
30
40
50
60
70
80
90
100
110
120
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
2
4
6
8
10
12
14
16
18
20
22
3.6
3.8
4.0
4.2
4.4 pH
4.6
4.8
5.0
6.0

enzyme activity by gel electrophoresis in order to check the homogeneity of the purified enzymes.

There have been reports of artifacts formed during electrofocusing due to Ampholine-protein interaction (Illingworth, 1972). Such a possibility may be checked by refocusing of the peaks. If refocusing of the original peak yields again a single peak with the original pI it may be concluded that the original peak is not artifactual. As shown in Fig. 9, individual refocusing of enzyme A and B yielded single peaks with isoelectric points of 4.55 and 4.60 respectively, which coincided with the original pI of enzyme A and B (Fig. 8). Thus, the possibility of artifacts due to Ampholines was eliminated in this case.

B. Homogeneity of Final Preparation

The enzymes separated by electrofocusing (Fig. 8) were electrophoresed in 10.5% Davis gels (Figs. 10, 11, 12). Each of the two enzymes gave a single protein band and a single enzyme activity band when stained in the presence of L-tyrosine, L-phenylanine, and L-tryptophan. Therefore, by the criterion of gel electrophoresis, the two preparations, A and B, were obtained free of each other and foreign proteins. These preparations were used for the study of many enzyme properties including subunit structure, heat sensitivity, enzyme kinetics, amino acid composition, and the conversion of enzyme A by subtilisin.

C. Characterization of the Enzymes

Figure 8

Isoelectric focusing of Peak I on 440 ml column

Isoelectric focusing of an enzyme preparation (Peak I) obtained after the step of DEAE-cellulose column chromatography. Tyrosine aminotransferase, 160 units, was placed in the large 440 ml preparative column maintained at 2-3°. The experiment proceeded until the maximum power had reached 0.8 watts and had stabilized at that level for 24 hours. The average run lasted 111 hours. Two ml fractions were collected and assayed for tyrosine aminotransferase. Fractions 147 to 149 from Peak A and fractions 166 to 169 from Peak B were pooled, dialyzed, and concentrated as described under "Materials and Methods".

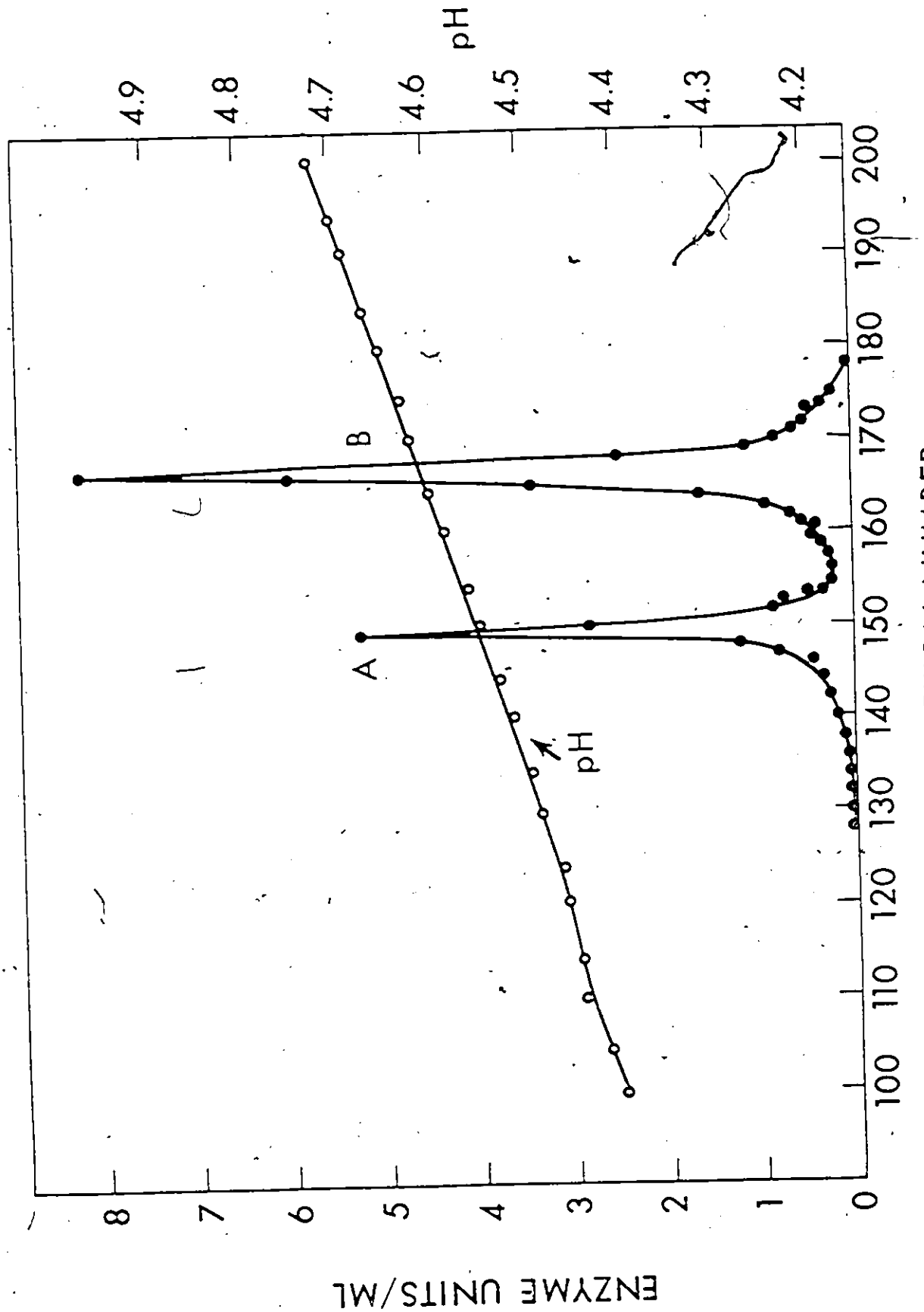

ENZYME UNITS/ML
FRACTION NUMBER
pH
A
B
pH
0
1
2
3
4
5
6
7
8
100
110
120
130
140
150
160
170
180
190
200
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9

Figure 9

Refocusing of enzymes A and B

0.8 and 1.65 units of enzymes A and B previously separated as in Fig. 7 were applied separately on a 110 ml isoelectric focusing column. Procedures and conditions were as described in "Materials and Methods".

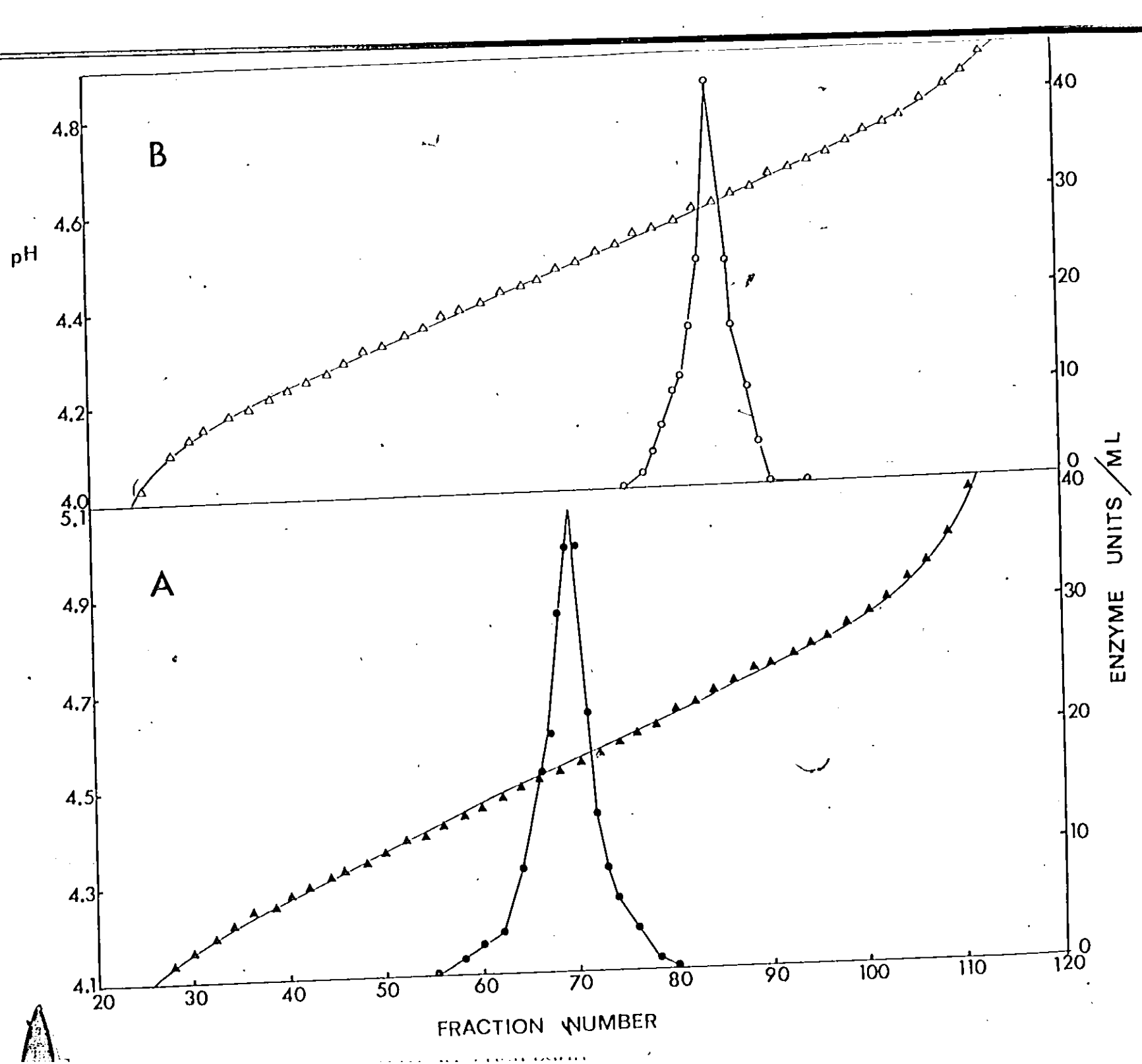

B
A
pH
4.8
4.6
4.4
4.2
4.0
5.1
4.9
4.7
4.5
4.3
4.1
40
30
20
10
0
ENZYME UNITS/ML
20
30
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

Figure 10

Polyacrylamide gel electrophoresis of enzymes A and B

Polyacrylamide gel electrophoresis (in 75 x 5 mm tubes) after isoelectric focusing, dialysis and concentration. A, enzyme A; B, enzyme B were electrophoresed in 10.5% Davis gels. All the gels were pre-run for 1 hour at 3 mA per gel. The three gels on the left were loaded with 0.96 µg of enzyme A and 0.29 µg of enzyme B, run for 2 hours at 3 mA per gel and stained for protein. The three gels on the right were loaded with 0.48 µg of enzyme A and 0.15 µg of enzyme B (0.24 µg of enzyme A in the mixture), and were run as the gels on the left but in a different experiment. They were stained for tyrosine aminotransferase activity.

PROTEIN TYROSINE

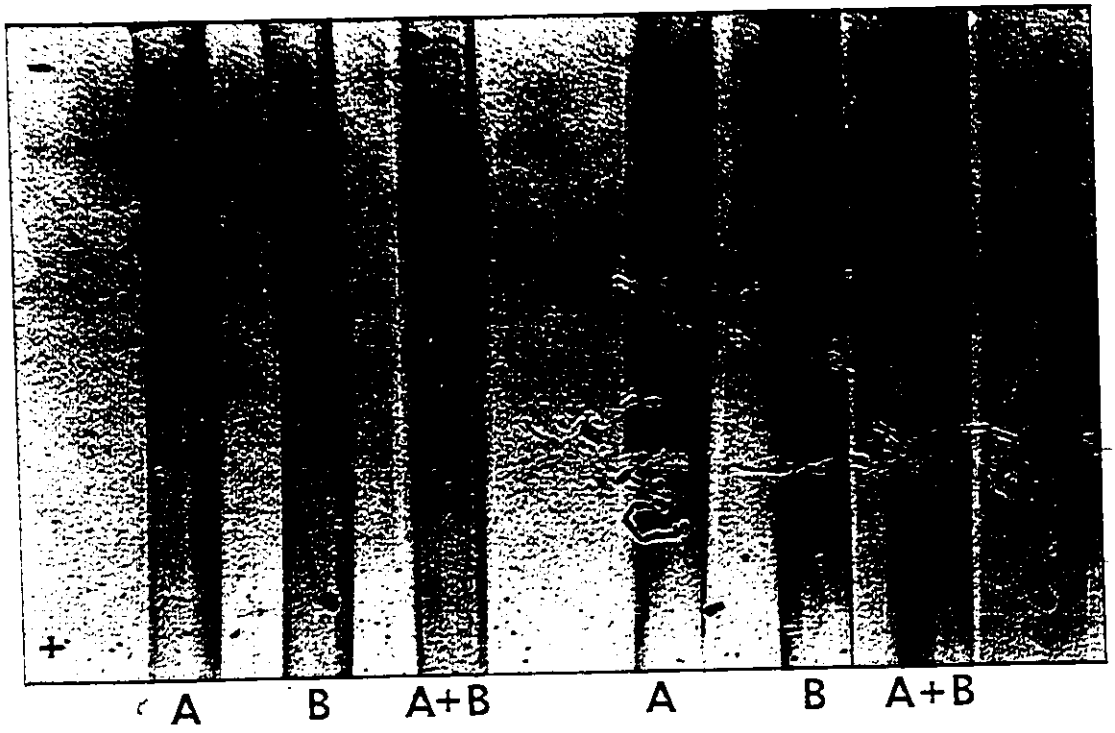


Figure 11

Polyacrylamide gel electrophoresis of enzymes A and B

Polyacrylamide gel electrophoresis (in 75 x 5 mm tubes) after isoelectric focusing, dialysis and concentration. Enzyme A and enzyme B were electrophoresed in 10.5% Davis gels. All the gels were pre-run for 1 hour at 3 mA per gel. 0.48 µg of enzyme A and 0.15 µg of enzyme B (0.24 µg of enzyme A in the mixture) were applied. They were stained for phenylalanine aminotransferase activity.

PHENYLALANINE

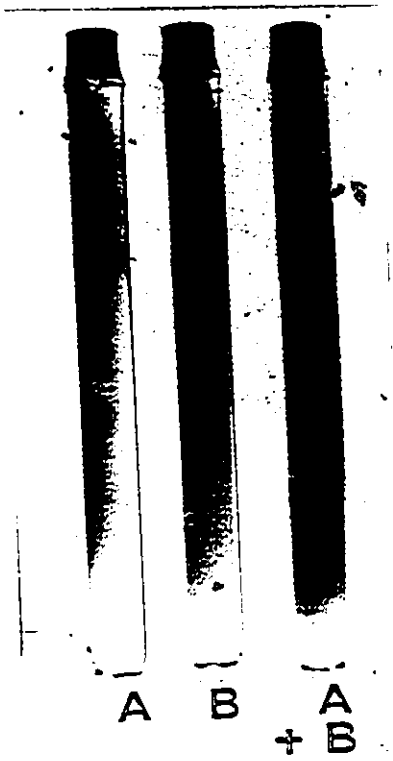


Figure 12

Polyacrylamide gel electrophoresis of enzymes A and B

Enzymes A and B were electrophoresed in 10.5% Davis gels. All gels were pre-run for 1 hour at 3 mA per gel. The concentration of the proteins applied to the gels were 0.48 µg and 0.15 µg of enzymes A and B respectively. (0.24 µg of enzyme A in the mixture). The gels were stained for tryptophan aminotransferase activity.

TRYPTOPHAN

1. Enzyme stability

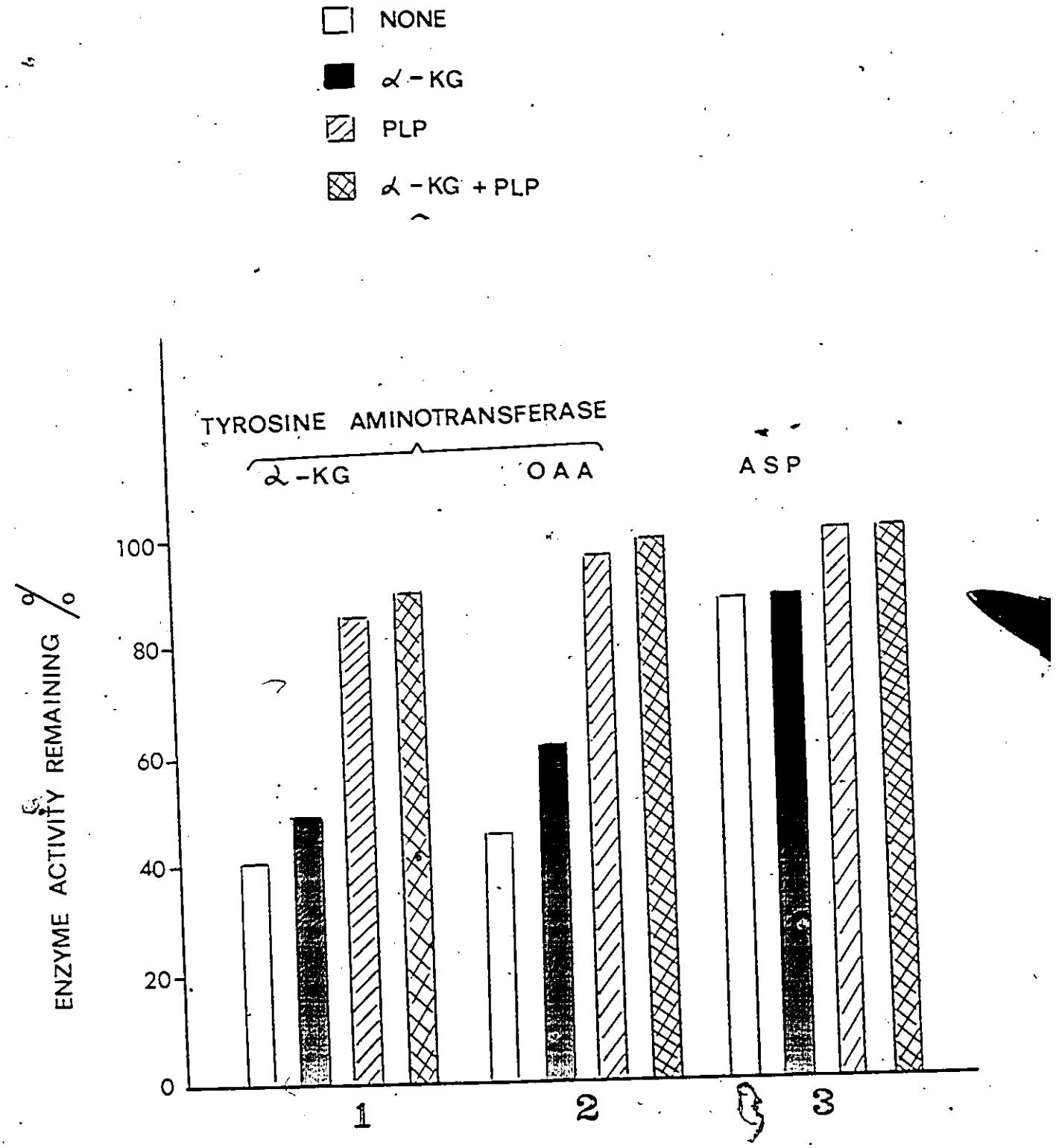
At all stages of the purification, aminotransferase activity was stable to freezing and thawing in the presence of 0.2 mM pyridoxal phosphate (PLP), 20 mM α-ketoglutarate (α-KG), 1 mM dithiothreitol (DTT), and 1 mM ethylenediamine tetra-acetate (EDTA). The purified enzymes A and B are stable even at very low protein concentration varying from 30 to 200 µg protein per ml, without loosing significant activity at -25° after several months.

As is the general practice, DTT and EDTA were used to prevent denaturation of enzyme during enzyme purification procedures. Pyridoxal phosphate and α-ketoglutarate protect tyrosine aminotransferase against heat inactivation. Fig. 13 shows that α-ketoglutarate when added to partially purified enzyme (peak I), affords slight protection of aminotransferase activity against heat inactivation and that pyridoxal phosphate gives much better protection (87% recovery of activity) than α-ketoglutarate. It was shown that the combination of PLP and α-KG gives slightly better protection than the former alone when the enzyme is heated at 55° for 5 minutes in 0.2 M potassium phosphate buffer, pH 7.3. When aspartate was used as the substrate the aminotransferase activity was about 90% stable and PLP alone protected 100%.

Figure 13

Protection of aminotransferase against heat inactivation

The partially purified enzyme (peak I from DEAE-cellulose column chromatography) was diluted to a protein concentration of 0.3 mg per ml. PLP and α-KG were at 0.2 mM and 2.0 mM respectively. The mixture was heated for 5 min at 55° and the enzyme was assayed as tyrosine aminotransferase activity by using α-KG and OAA as substrate (1 and 2) whereas 3 was assayed as aspartate aminotransferase with α-KG as cosubstrate.


NONE
α-KG
PLP
α-KG + PLP
TYROSINE AMINOTRANSFERASE
α-KG
OAA
ASP
ENZYME ACTIVITY REMAINING %
100
80
60
40
20
0
1
2
3

2. Substrate Specificities

0.3 ml (18 units) of partially purified enzyme (peak I) was electrofocused over a narrow pH range from 4.3 to 4.9. An isoelectric focusing experiment is illustrated in Fig. 14. Two peaks, enzyme A and enzyme B, were observed with isoelectric points of 4.54 and 4.60 respectively. When the fractions were assayed with the amino acids L-tyrosine, L-phenylalanine, and L-tryptophan (with α-ketoglutarate as cosubstrate), the peak activities with the three amino acids occurred in fraction 55 for enzyme A and fraction 61 for enzyme B (Fig. 14a). When the fractions were assayed for aspartate aminotransferase (Fig. 14b), the activity completely coincided with enzyme A. The conclusion for the identity of aspartate and aromatic aminotransferase activities in enzyme A was confirmed by gel electrophoresis of enzyme A. Staining the gel for protein, aspartate aminotransferase, and a mixture of aspartate and tyrosine aminotransferases resulted in a single band in each case (Fig. 15). In another isoelectric focusing experiment (not shown) the fractions were assayed for methionine aminotransferase also. Two peaks were observed for this activity also which completely coincided with the two peaks observed with the other activities. Thus enzyme A had the original specificity of peak I (Table III) and was transaminase A but enzyme B was a new enzyme specific for the aromatic amino acids only.

Figure 14

Isoelectric Focusing of Peak I from DE-52 column chromatography

Isoelectric focusing of an enzyme preparation (peak I, Fig. 5) obtained after the step on DEAE-cellulose column chromatography. Eighteen units of tyrosine aminotransferase were placed in the small 110 ml column. The run lasted 114 hours with a voltage range of 300-500 V and a current range of 1.10-1.30 mA. The maximum power reached 0.65 W and had stabilized for 20 hours prior to the collection of fractions. 0.8 ml fractions were collected and assayed for amino acid aminotransferase activities with tyrosine (● - ●), phenylalanine (o - o) and tryptophan (▲ - ▲) as substrates. The profile of aspartate aminotransferase (Δ - Δ) is shown in the right-hand panel together with that of tyrosine aminotransferase for easy comparison. The standard abbreviations of amino acids followed by the letter A indicate the respective aminotransferase.

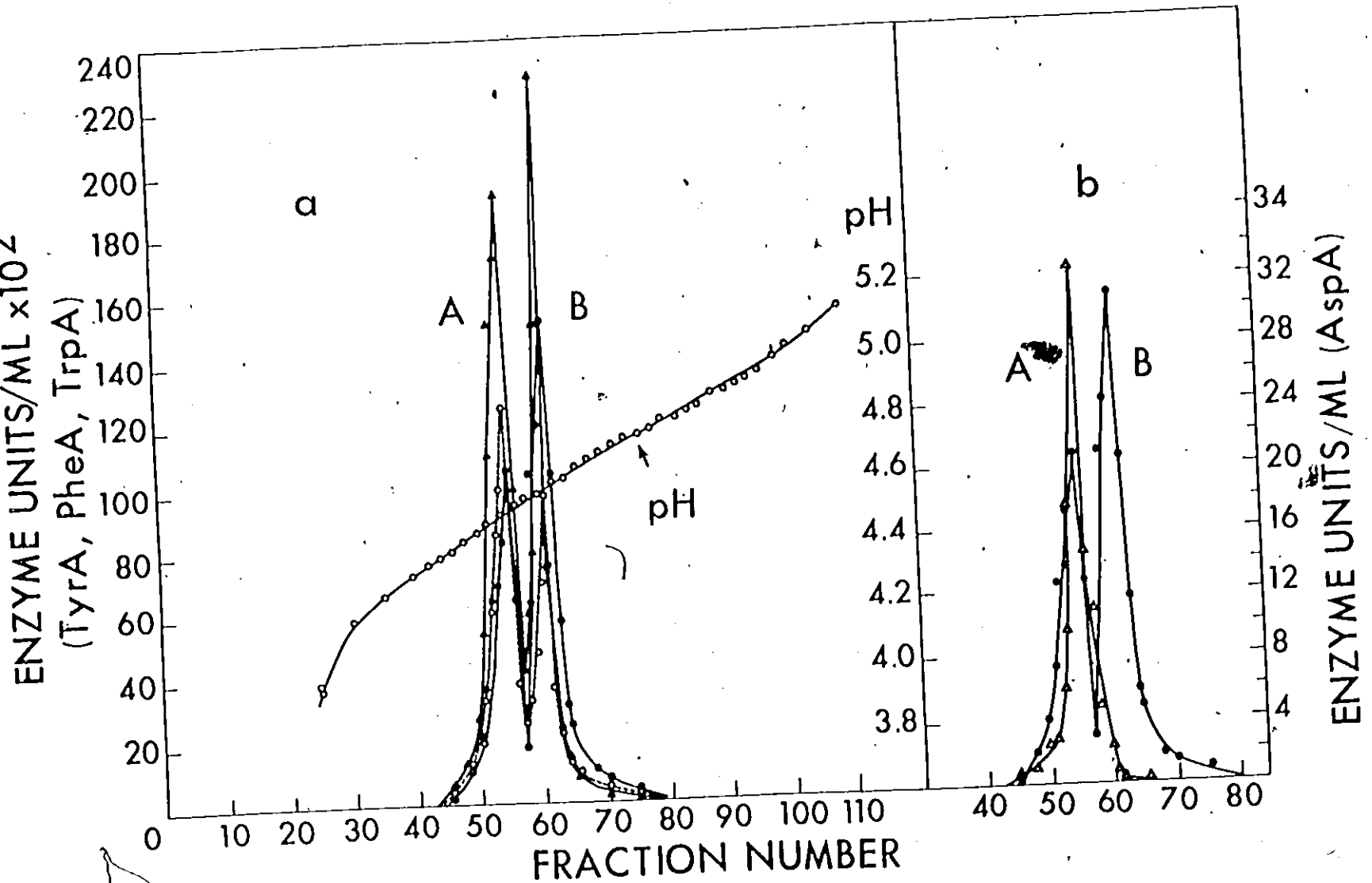
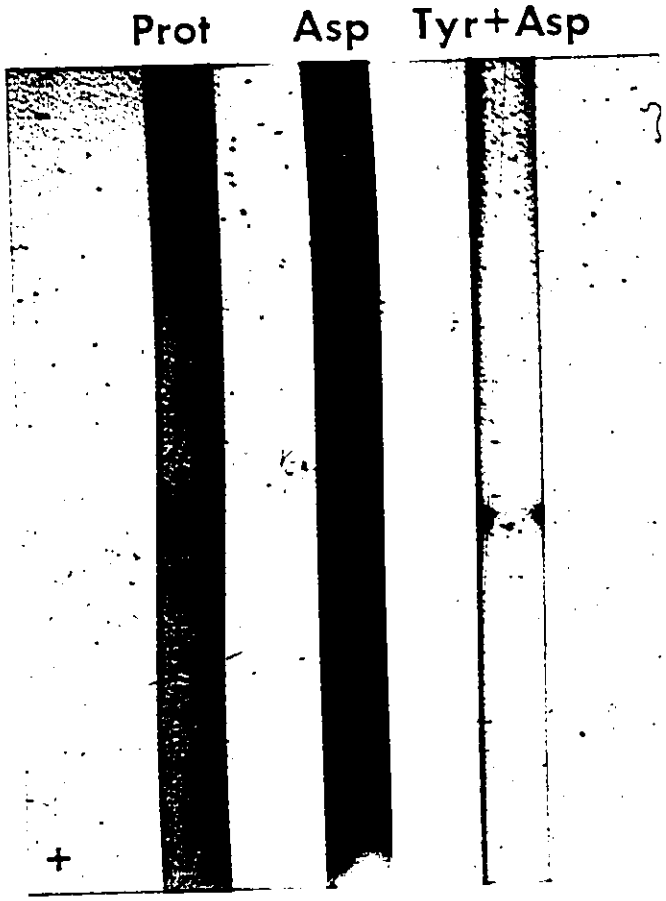

a
b
A
B
A
B
pH
pH
ENZYME UNITS/ML x10²
(TyrA, PheA, TrpA)
ENZYME UNITS/ML (AspA)
FRACTION NUMBER
0
10 20 30 40 50 60 70 80 90 100 110
40 50 60 70 80
20 40 60 80 100 120 140 160 180 200 220 240
4 8 12 16 20 24 28 32 34
3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2

Figure 15

Polyacrylamide gel electrophoresis of enzyme A

Polyacrylamide gel electrophoresis (in 100 mm x 5 mm tubes) after isoelectric focusing, dialysis, and concentration of enzyme A. The gels were pre-run for 1 hour and then were loaded with 0.48 µg (left gel) and 1.92 µg (middle and right gels) of enzyme A and run for 4.5 hours at 3 mA per gel. The left gel was stained for protein (Prot), the middle gel for aspartate aminotransferase (Asp), and the right gel for both tyrosine and aspartate aminotransferases (Tyr + Asp).


Prot
Asp
Tyr+Asp
+

3. Repression of enzyme B by tyrosine

An important and consistent visual observation was that only enzyme B stained less intensely from extracts of cells grown in the presence of tyrosine, whereas enzyme A was not affected. This effect is clear in Fig. 16. Densitometric tracings confirmed the visual observation (Figs. 17,18). This method however, could not quantify the effect because only the enzyme near the periphery of the gel would react and the intensity of staining did not, most probably, reflect initial velocity. Table IV shows the effect of tyrosine on enzymes A and B in cells grown in the presence of this amino acid. The effect was quantified as described in Materials and Methods and in the legend. Enzyme B was repressed by 90% whereas A was not affected.

The above results show that L-tyrosine represses the new enzyme B and not enzyme A (transaminase A) as had been reported by others (Silbert et al., 1963).

4. Heat Sensitivity

The time course of activity loss of the two enzymes toward their substrates (tryptophan was not included) at 55° is shown in Fig. 19. Enzyme B lost activity rapidly with tyrosine and phenylalanine as substrates so that after 10 min less than 10% of the original activity was left. Enzyme A, however, was relatively heat-stable, with 52% and 64% of the original activity remaining after 10 min with tyrosine and phenylalanine, respectively, as substrates. For each enzyme the initial rate of inactivation as well as the final activity loss (especially for enzyme A) was

Figure 16

Polyacrylamide gel

Electrophoresis of two Crude Extracts of E. coli B

Enzyme activity bands in acrylamide gel (Davis gel) electrophoresis of two crude extracts of E. coli B grown in the absence (left gel) and in the presence (right gel) of L-tyrosine (25 µg/ml). 56 µg of protein was applied on each gel. Electrophoresis was carried out for 4.5 hours at 3 mA per gel. The gels were stained for tyrosine aminotransferase acitivity.

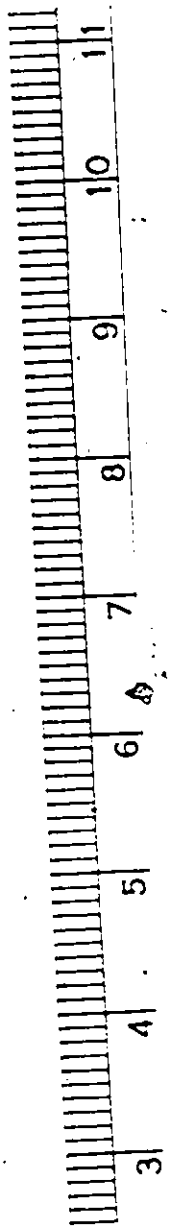

3
4
5
6
7
8
9
10
11

Figure 17

Densitometer tracings of tyrosine aminotransferase activity bands after acrylamide gel electrophoresis

The control gel from Figure 16 was scanned at 570 nm in a Gilford Spectrophotometer. The scanning rate was 2 inch/min. The full scale absorbance was 2.0 OD units.

CONTROL

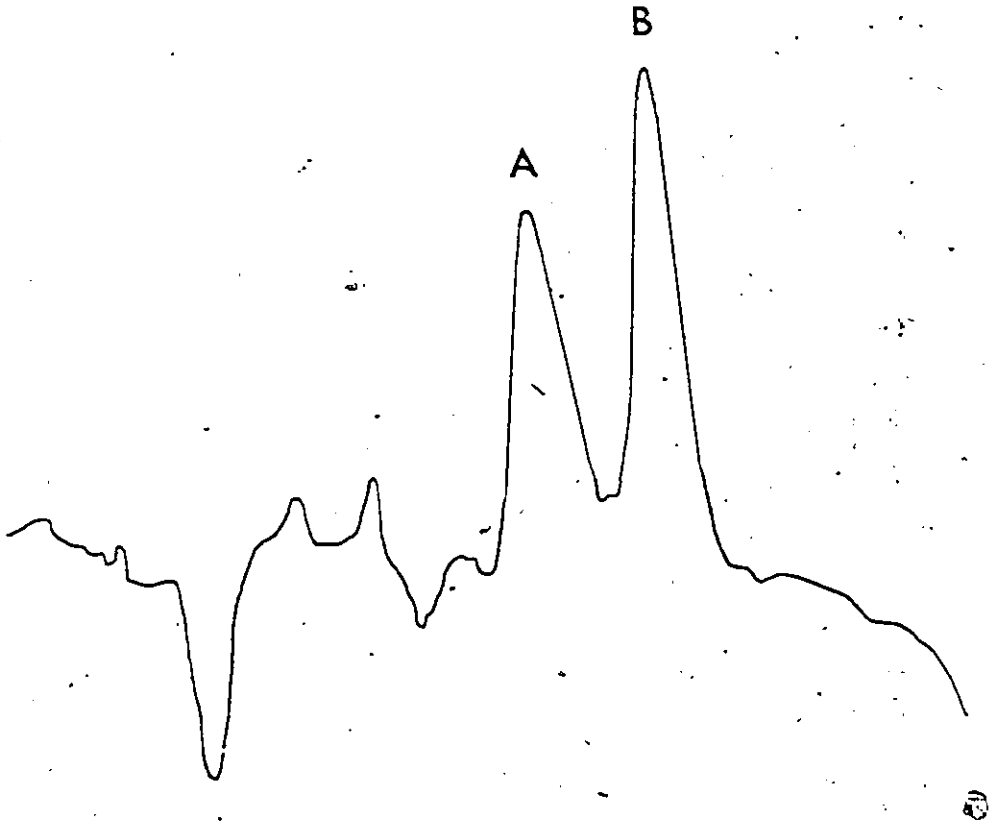


Figure 18

Densitometer Tracings of Tyrosine aminotransferase activity bands after acrylamide gel electrophoresis

The gel from Figure 16 (+ tyrosine) was scanned at 570 nm in a Gilford Spectrophotometer. The scanning rate was 2 inch/min. The full scale absorbance was 2.0 OD units.

+ TYROSINE

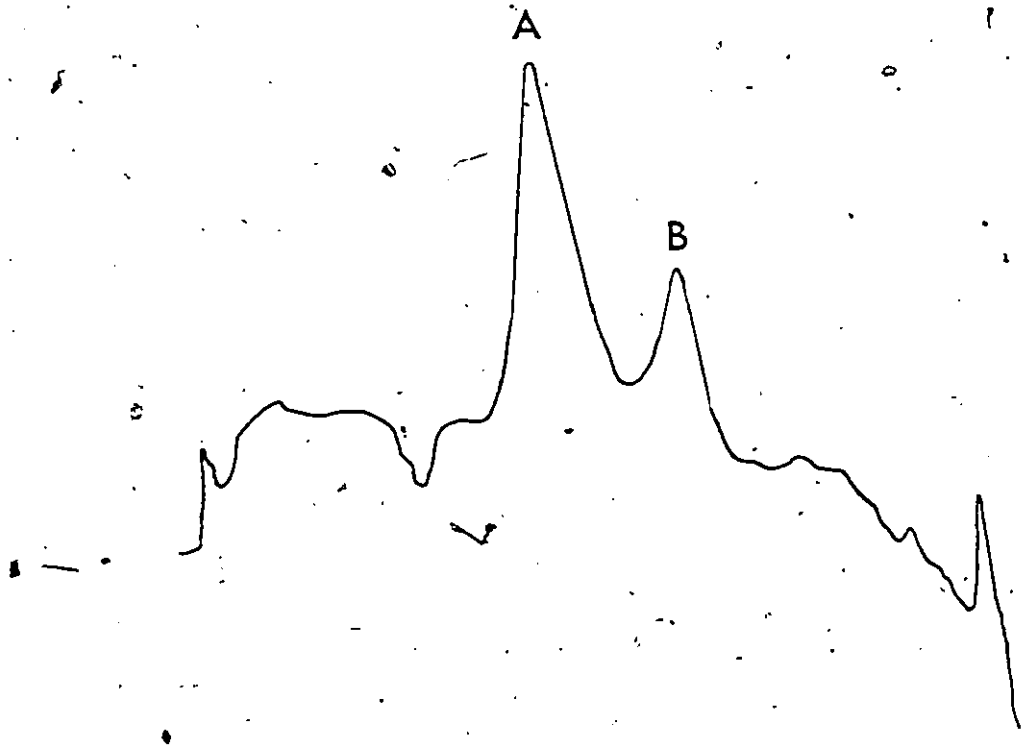


Table IV

Effect by L-tyrosine on enzymes A and B assayed as L-tyrosine aminotransferase

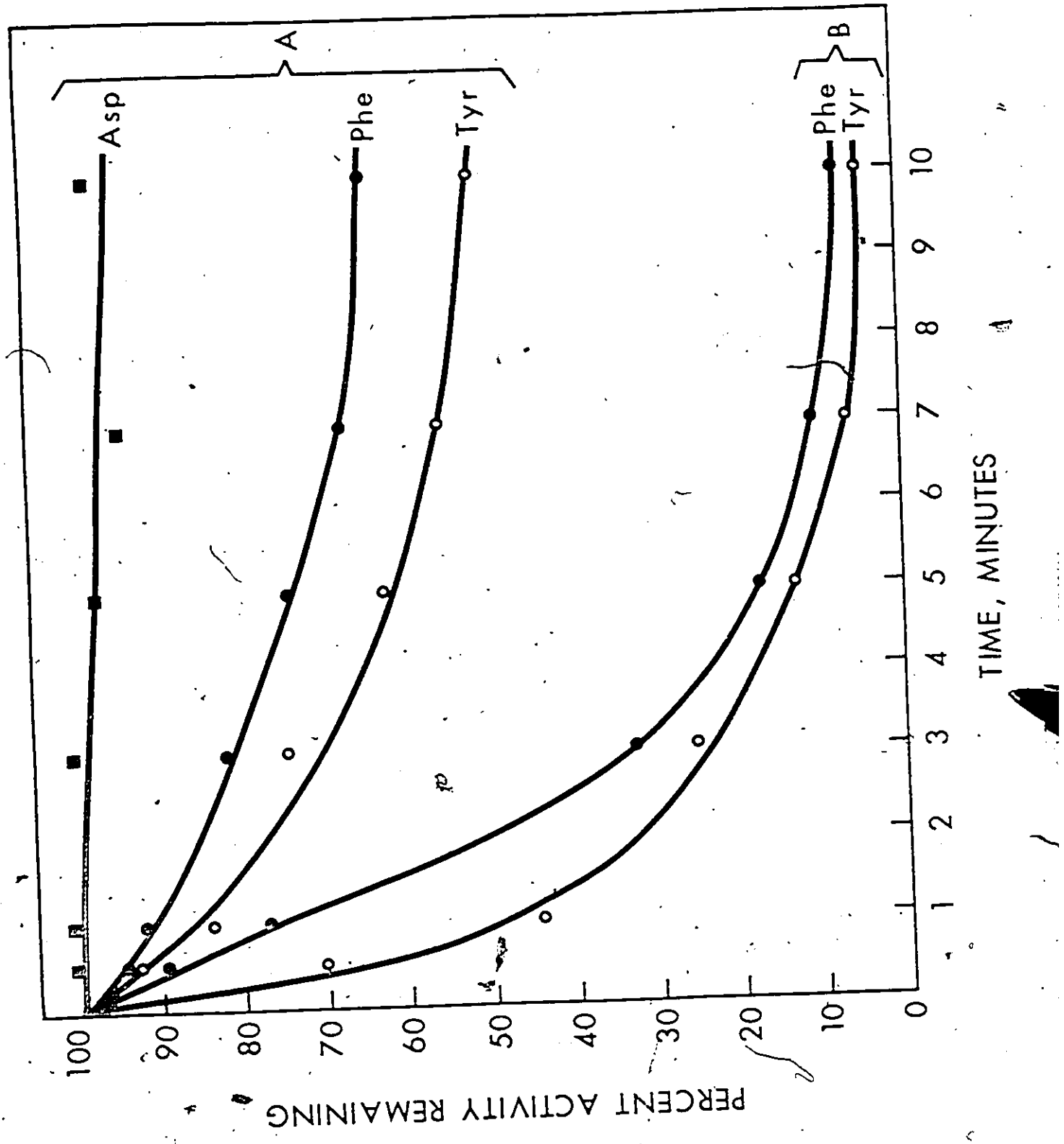
E. coli B were grown in the absence and presenceof L-tyrosine (25 µg/ml). Crude extracts (27,000 x g supernatant) corresponding to 432 µg protein (94.8 m units from the control and 27.6 m units from the tyrosine-supplemented culture in term of tyrosine aminotransferase) were applied on the gels. Electrophoresis was carried out for 4.5 hours at 3 mA gel. The slices from 5 gels corresponding to enzyme A were combined and treated as described in "Materials and Methods". The same procedure was carried out for 5 gels corresponding to enzyme B. They were then divided into 5 equal portions. One was added to the blank assay flask and the remaining 4 were assayed. The figures under "enzyme m units" represent average activities per gel but the individual values were very similar.

Growth Medium	Enzyme m units		percent repression by tyrosine			
	A	B	Crude Extract	A	B	A + B
Minimal	3.52	12.36	-	-	-	-
Minimal plus tyrosine	3.86	1.26	71	nil	90	68

Figure 19

Heat stability of the purified enzymes A and B at 55°

The purified enzyme solutions were diluted 10-fold with 0.2 M potassium phosphate buffer, pH 7.3, to a final volume of 4.0 ml. Bovine serum albumin was added to a final concentration of 5 mg/ml. After heating at 55° at various time intervals as indicated in the figure, enzyme A was assayed with aspartate (Asp), phenylalanine (Phe), and tyrosine (Tyr) and enzyme B was assayed with phenylalanine and tyrosine.


A
B
Asp
Phe
Tyr
Phe
Tyr
PERCENT ACTIVITY REMAINING
TIME, MINUTES
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10

more pronounced with tyrosine than with phenylalanine as substrate. More striking was the almost complete absence of inactivation of enzyme A with aspartate as substrate seen against its partial loss of activity with the two aromatic amino acids (Fig. 19). A heat sensitivity test such as this applied to a crude or semipurified preparation containing the two enzymes would yield a heat-stable aspartate aminotransferase, a more labile phenylalanine aminotransferase and an even more labile tyrosine aminotransferase and it would be misinterpreted to suggest the existence of one enzyme specific for aspartate and one enzyme (and possibly two) specific for the aromatic amino acids. Such a plot, derived from an experiment with a crude extract, was published by Collier and Kohlhaw (1972) in support of the non-identity of the two types of activity. The heat stability of enzyme A with aspartate as substrate does not in itself support the notion that enzyme A consists of different enzymic proteins. It simply reflects the existence of a single multispecific enzyme as was shown previously (Figs. 14, 16). Experiments addressed to this problem are described later in this thesis (p. 103).

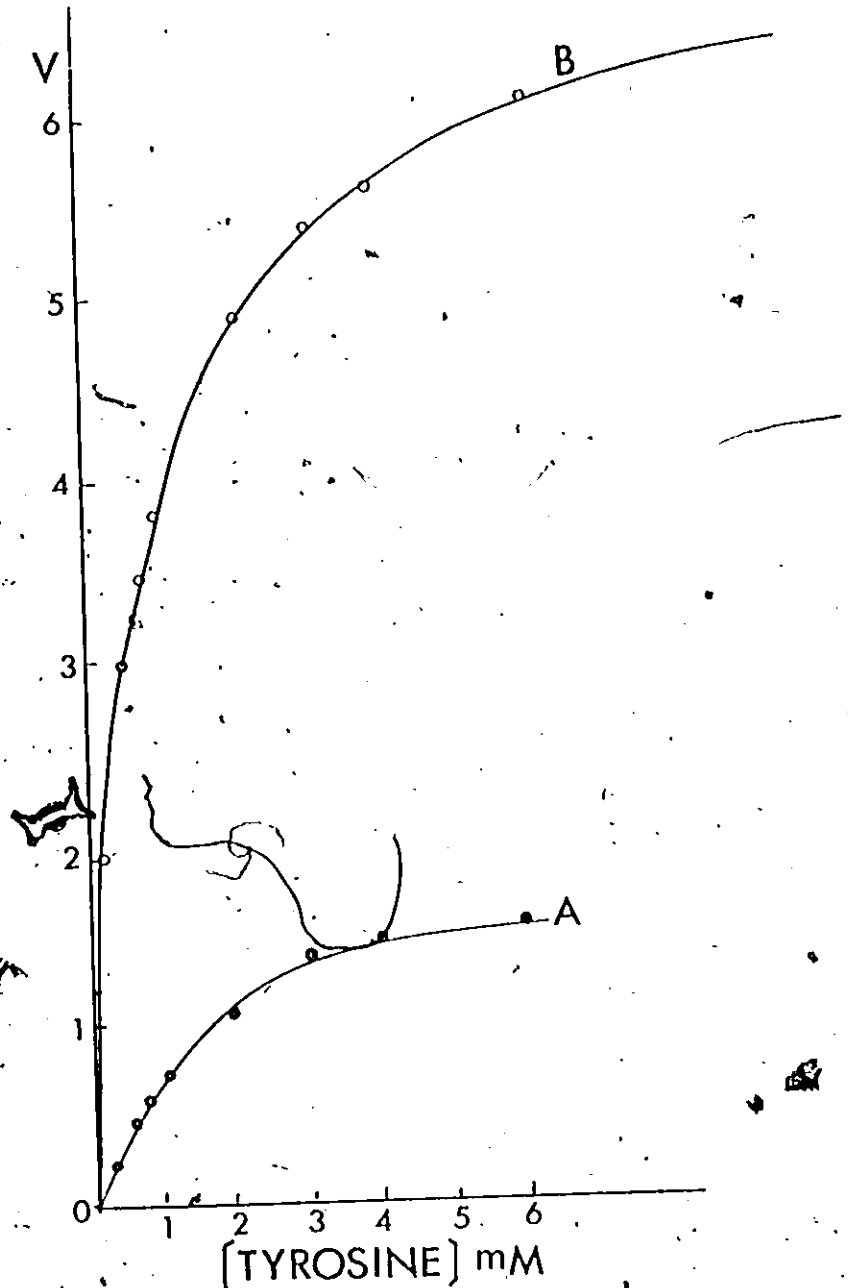
5. Kinetic Experiments

Figures 20-26 show the Michaelis-Menten plots and the corresponding Lineweaver-Burk plots for enzymes A and B with their substrates and PLP. Table V lists the various Km and Vmax values for enzymes A and B. The Vmax values clearly indicate that the major activity toward the aromatic amino acids resides in enzyme B, the B/A ratio for Vmax varying from about 14 to 25. In the presence

Figure 20

Michaelis-Menton and Lineweaver-Burk plots for Enzymes A and B

Effect of amino acid (tyrosine) concentrations on enzyme velocity and corresponding Lineweaver-Burk plots with enzyme A (●———●) and enzyme B (o———o) assayed as tyrosine amino-transferase. 0.015 units of enzymes A and B were used in this experiment.


V
B
A
0
1
2
3
4
5
6
[TYROSINE] mM

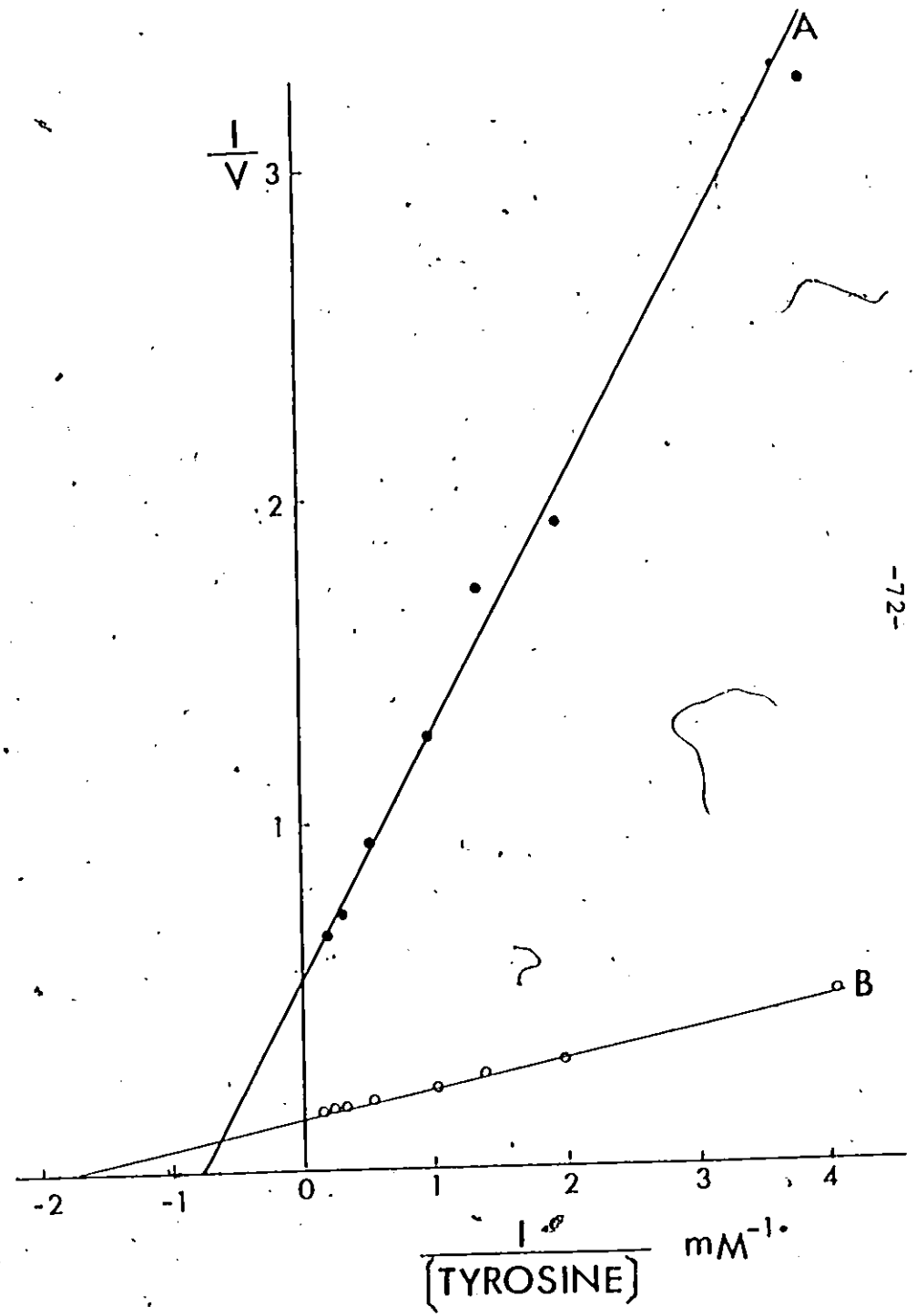

I/V
A
B
-2
-1
0
1
2
3
4
I / [TYROSINE] mM⁻¹

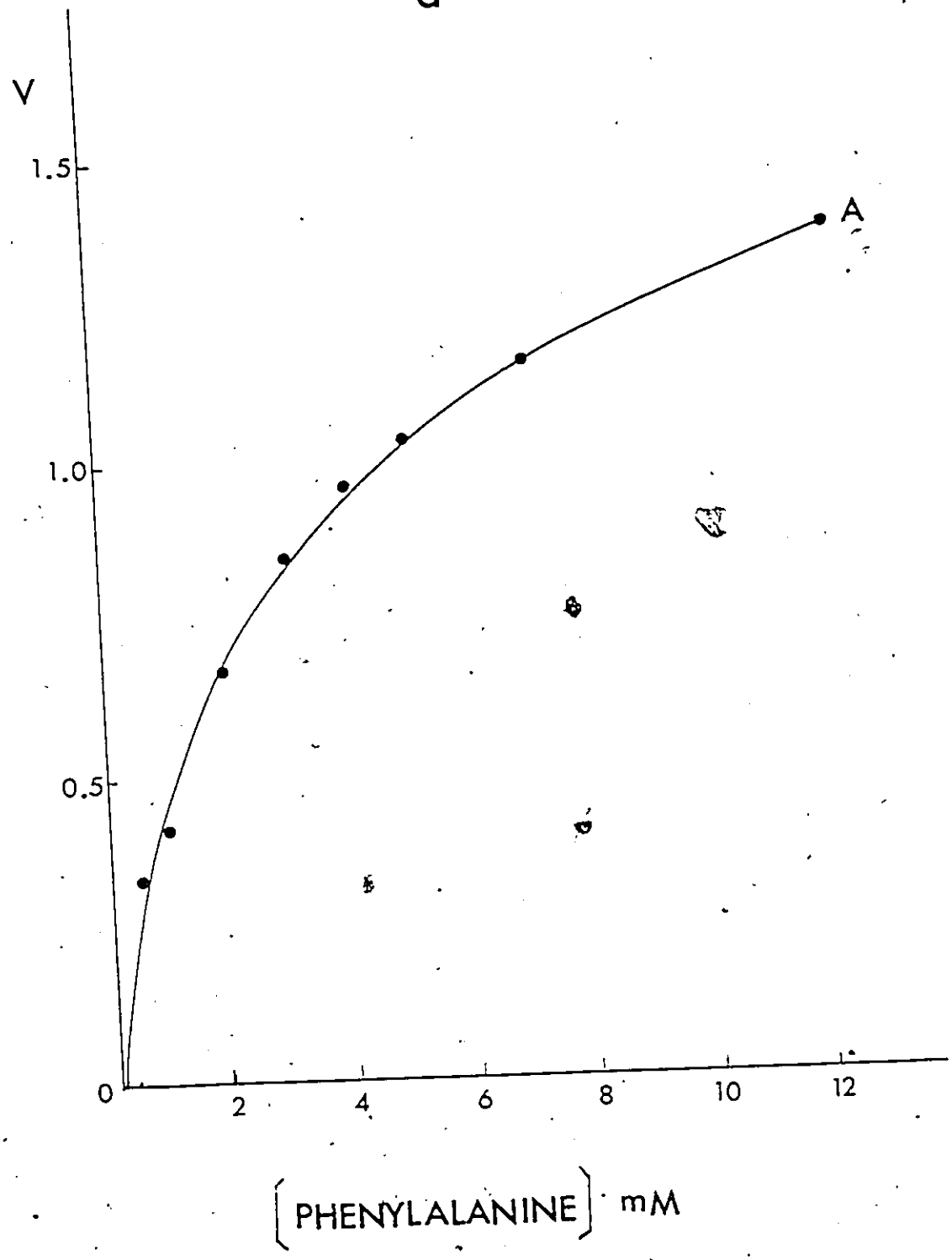
Figure 21

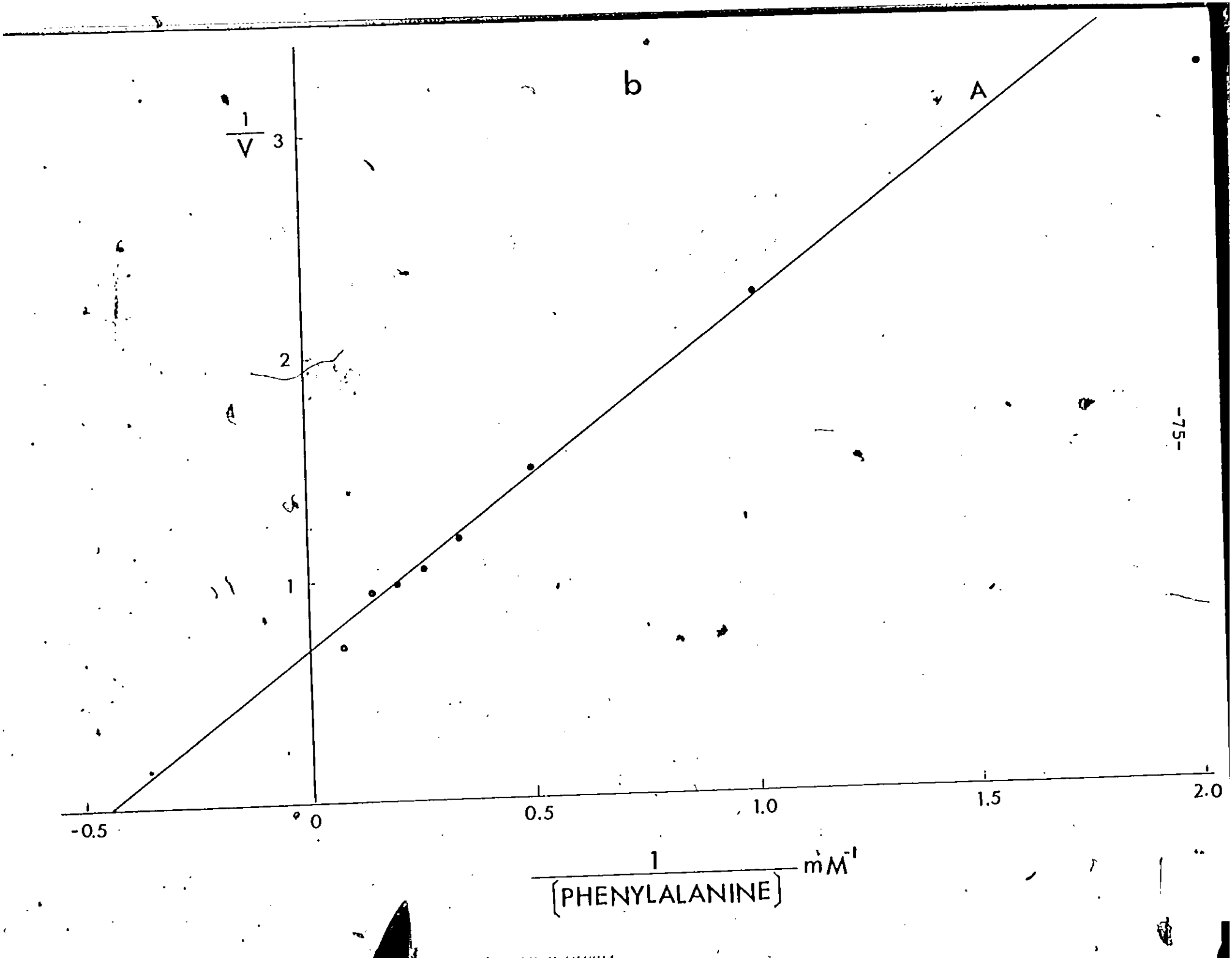
(a,b,c,d)

Michaelis-Menten and Lineweaver-Burk plots for enzymes A and B

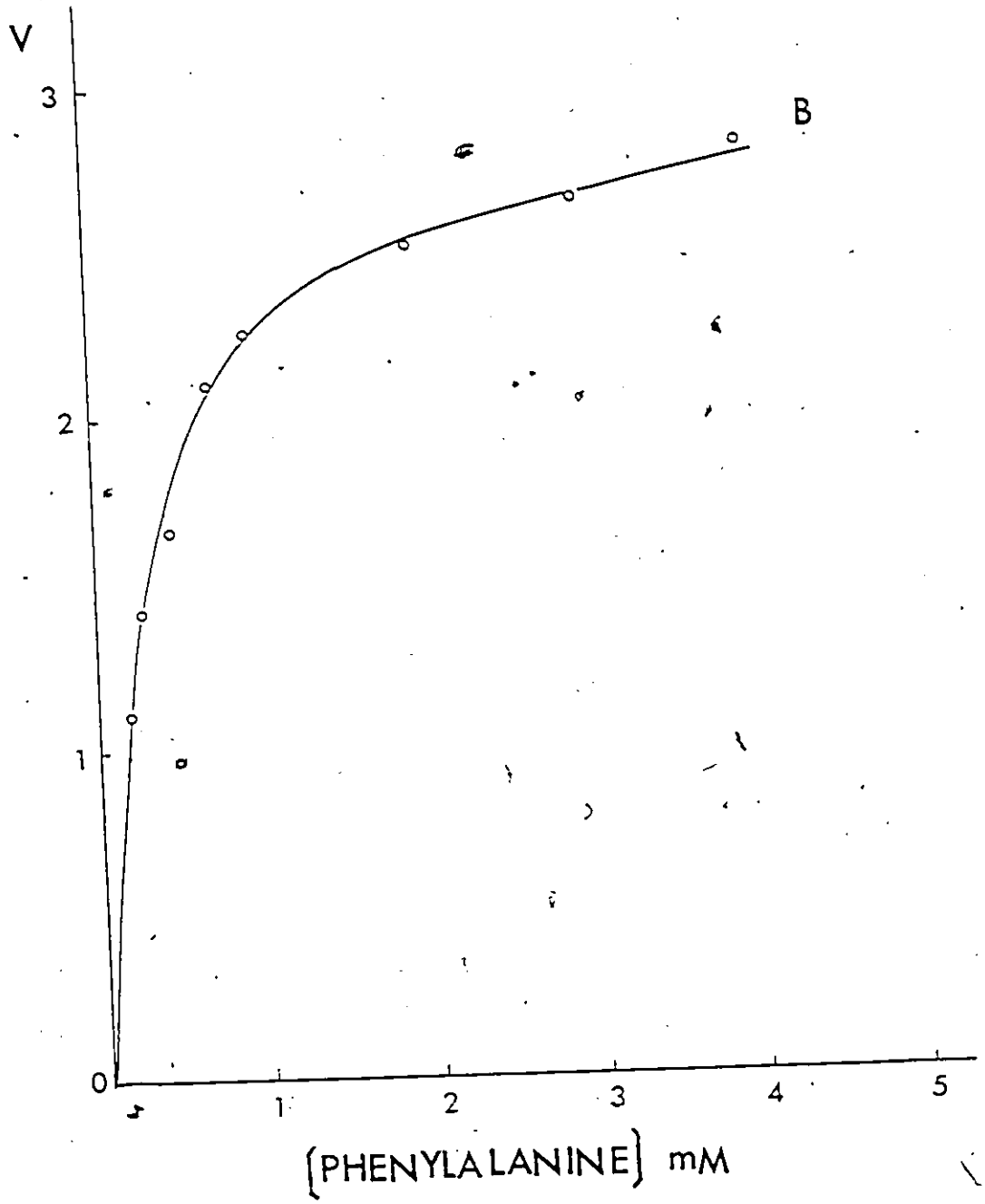
Effect of amino acid substrate (L-phenylalanine) on enzyme A (●————●) and B (o————o) assayed as phenylalanine amino-transferase.

a




b
A
1/V
3
2
1
-0.5
0
0.5
1.0
1.5
2.0
1 / [PHENYLALANINE] mM⁻¹

C



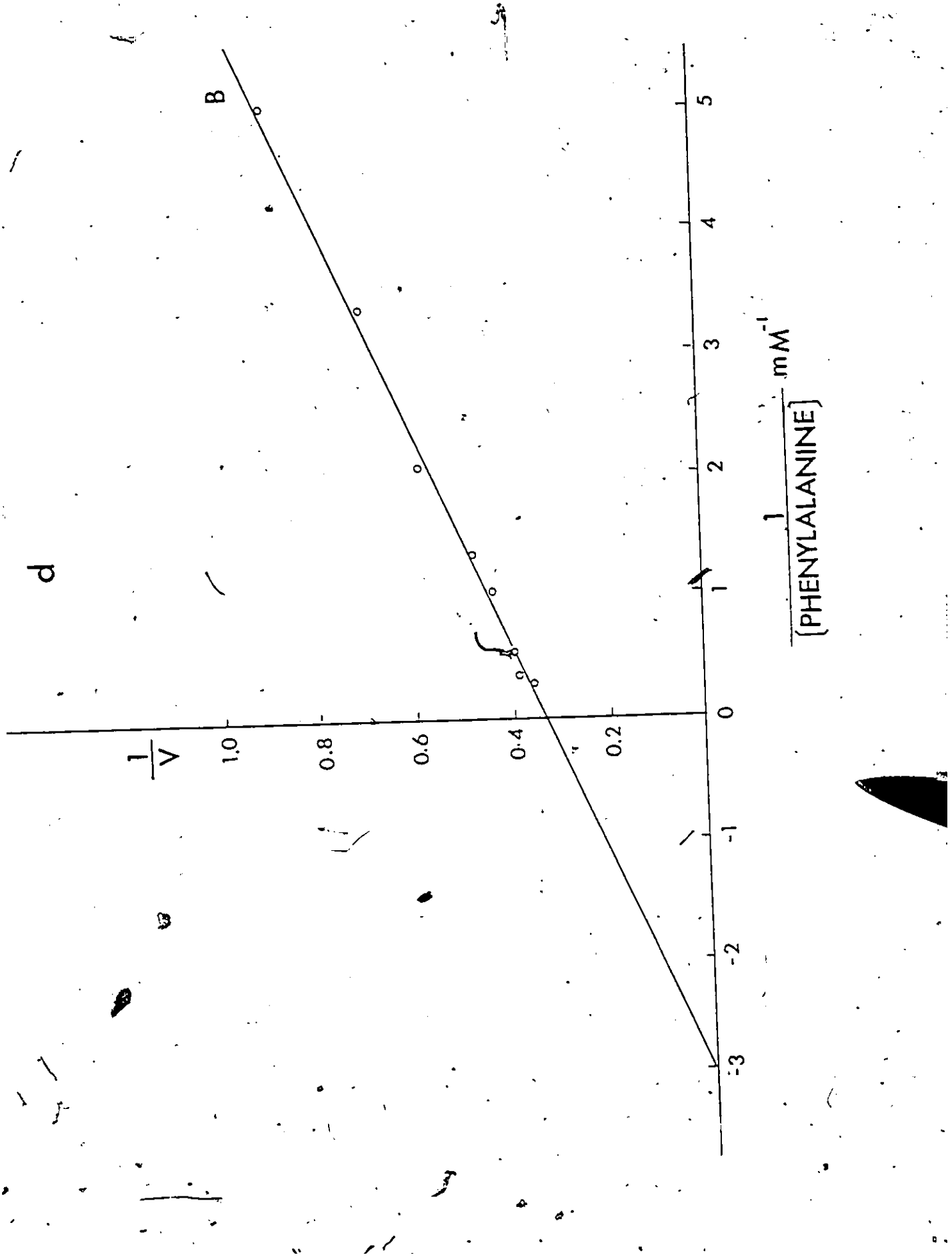

d
B
1/V
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
-1
-2
-3
1/[PHENYLALANINE] mM⁻¹

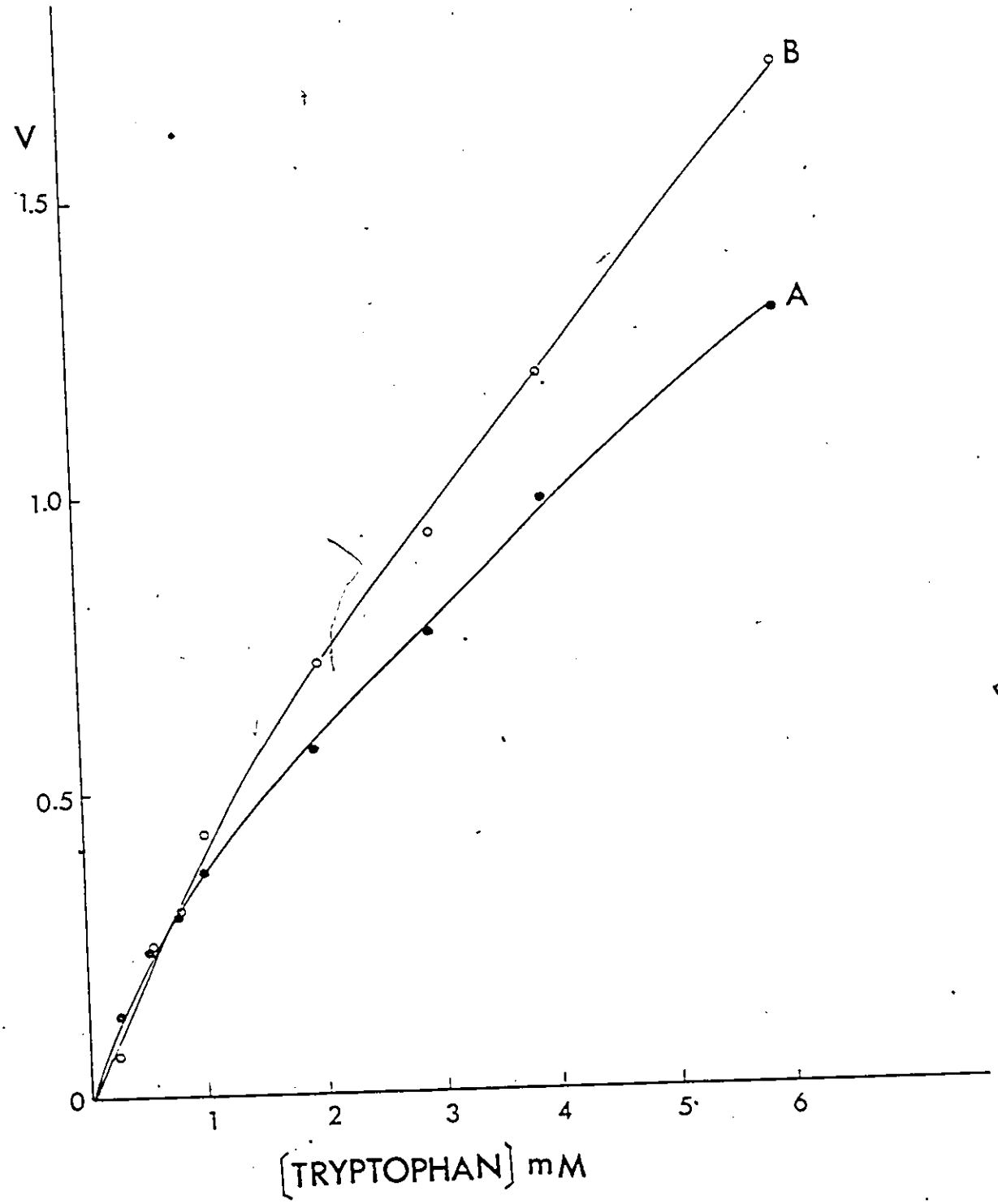
Figure 22

(a,b)

Michaelis-Menten and Lineweaver-Burk plots for enzymes A and B

Effect of substrate (tryptophan) on enzymes A (●————●) and B (o————o) assayed as tryptophan aminotransferase.

a



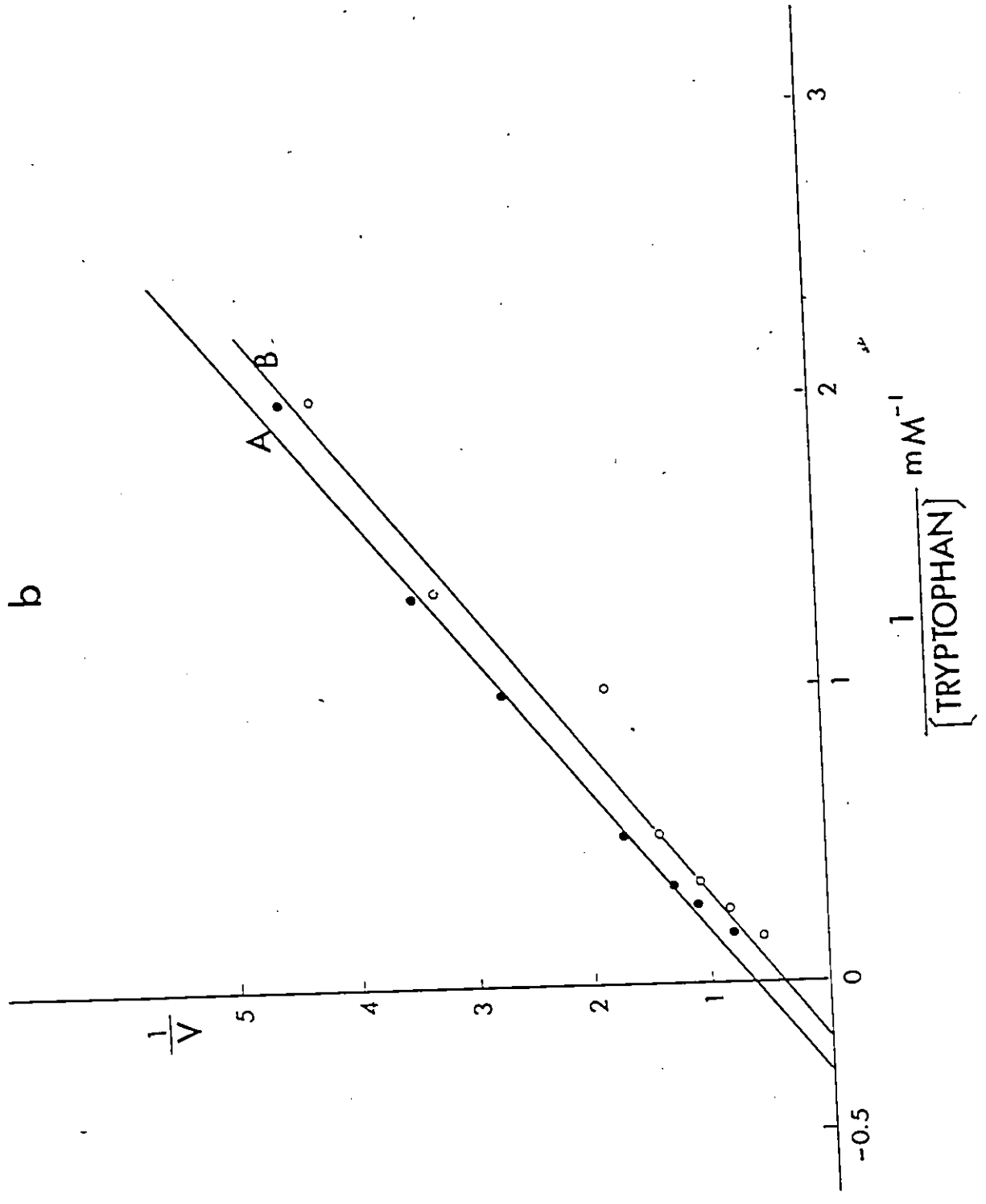

b
A
B
$\frac{1}{V}$
5
4
3
2
1
0
-0.5
1
2
3
$\frac{1}{[\text{TRYPTOPHAN}]}$ mM^{-1}

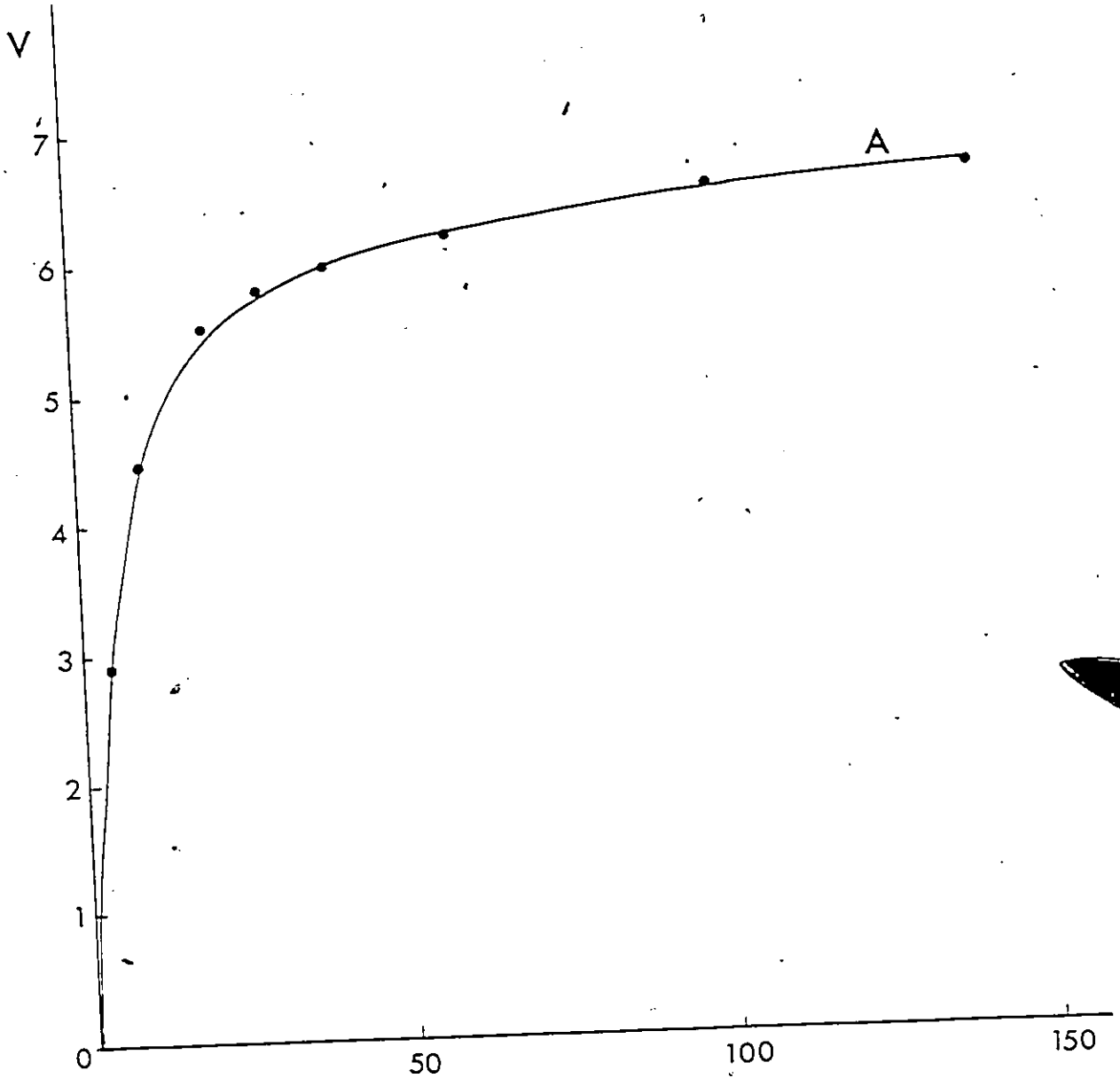
Figure 23

(a,b)

Michaelis-Menten and Lineweaver-Burk plot for Enzyme A

Effect of substrate (Aspartate) on enzyme A (●——●) assayed as aspartate aminotransferase.

a



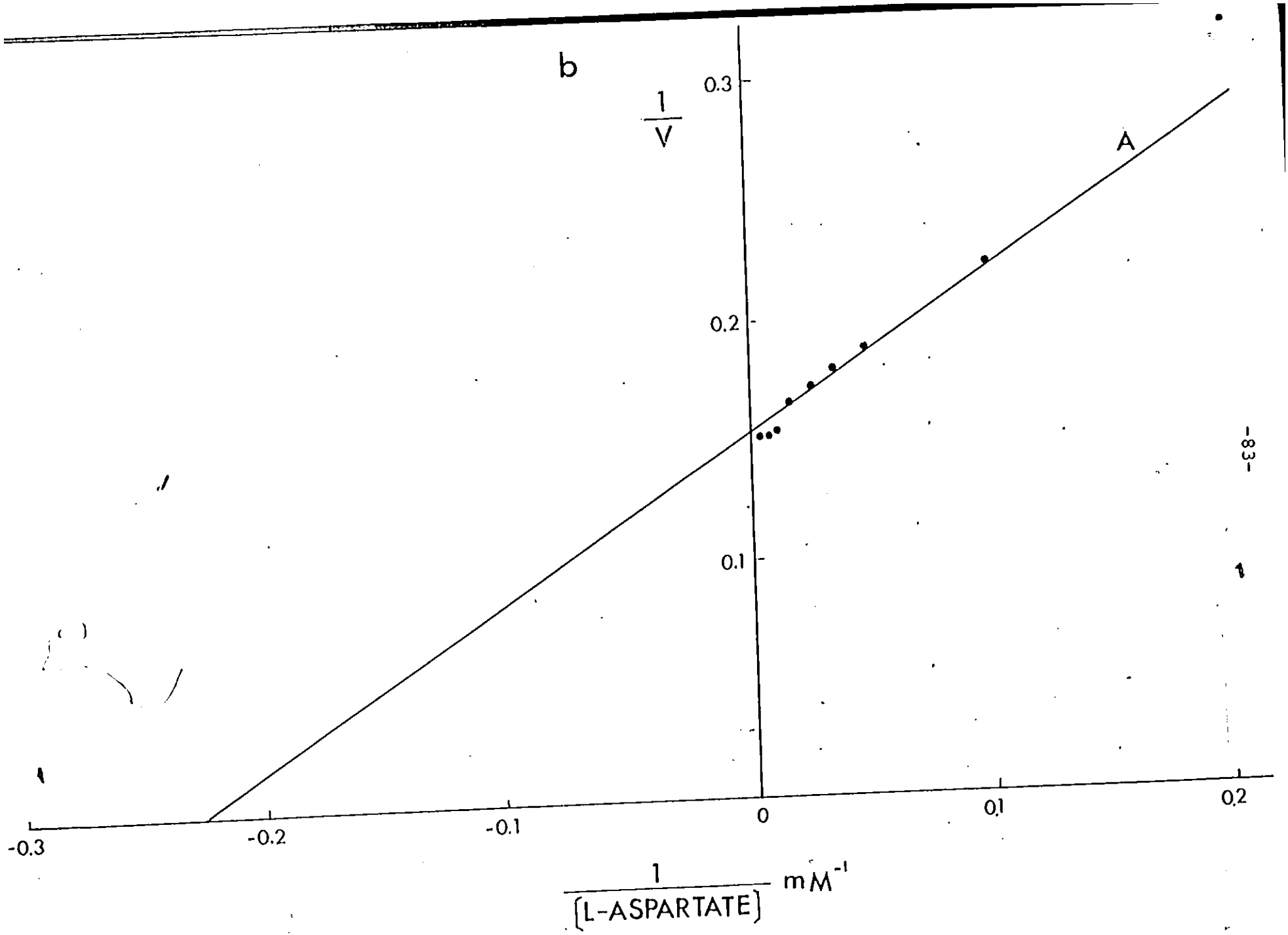

b
1/V
0.3
0.2
0.1
A
-0.3
-0.2
-0.1
0
0.1
0.2
1/[L-ASPARTATE] mM⁻¹

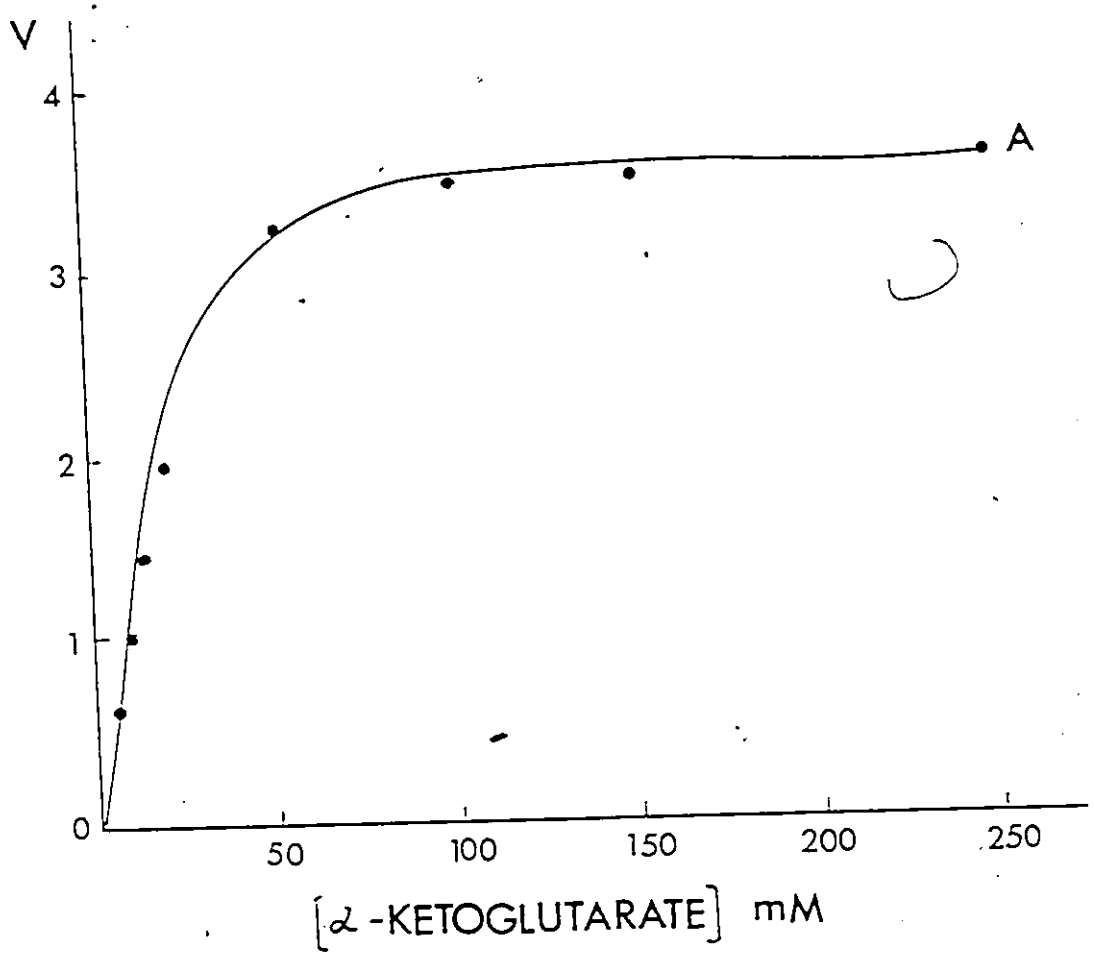
Figure 24

(a,b,c,d)

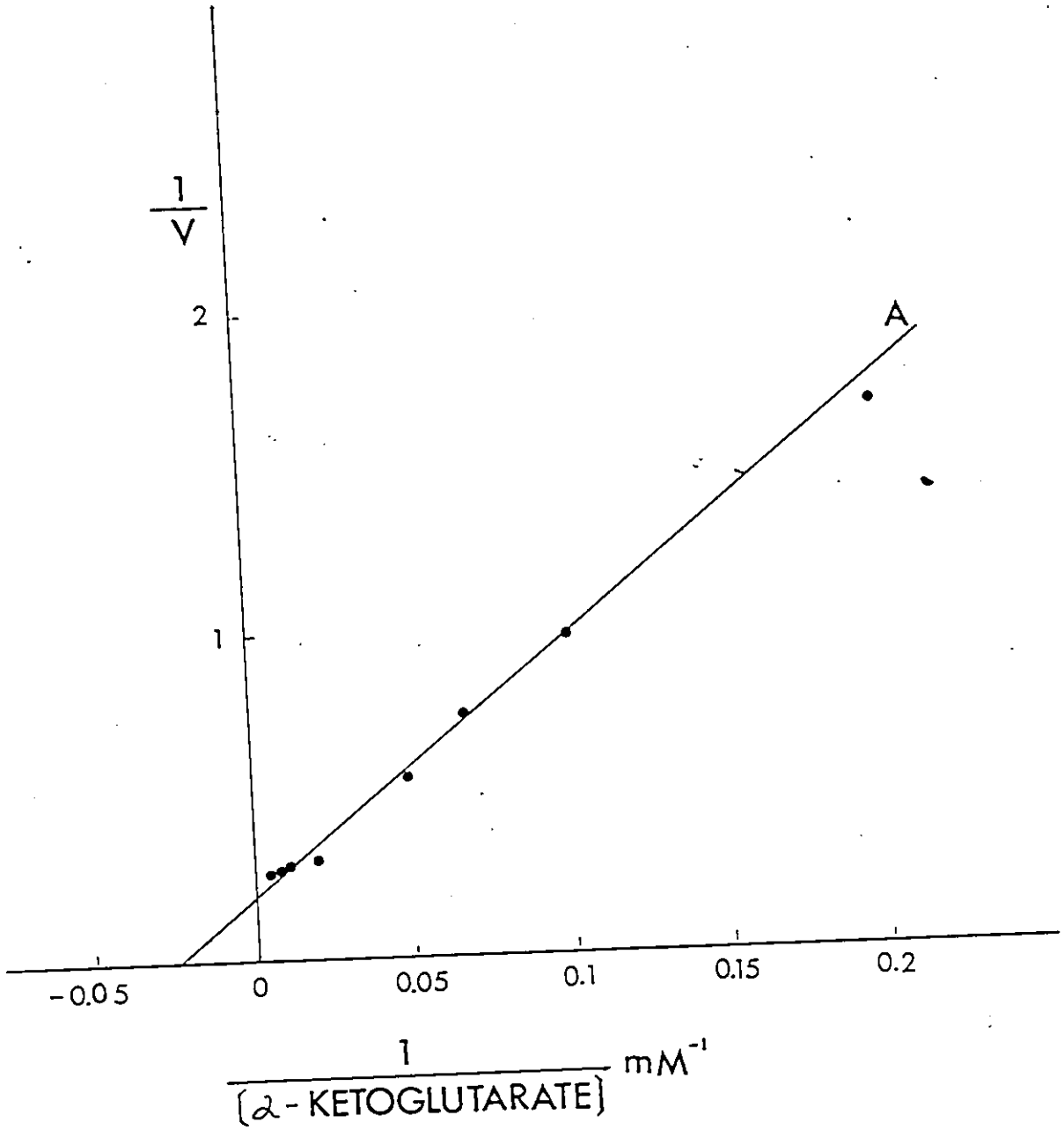
Michaelis-Menten and Lineweaver-Burk plots for enzymes A and B

Effect of substrate (α-ketoglutarate) on enzyme A (●————●) and B (o————o) assayed as tyrosine aminotransferase.

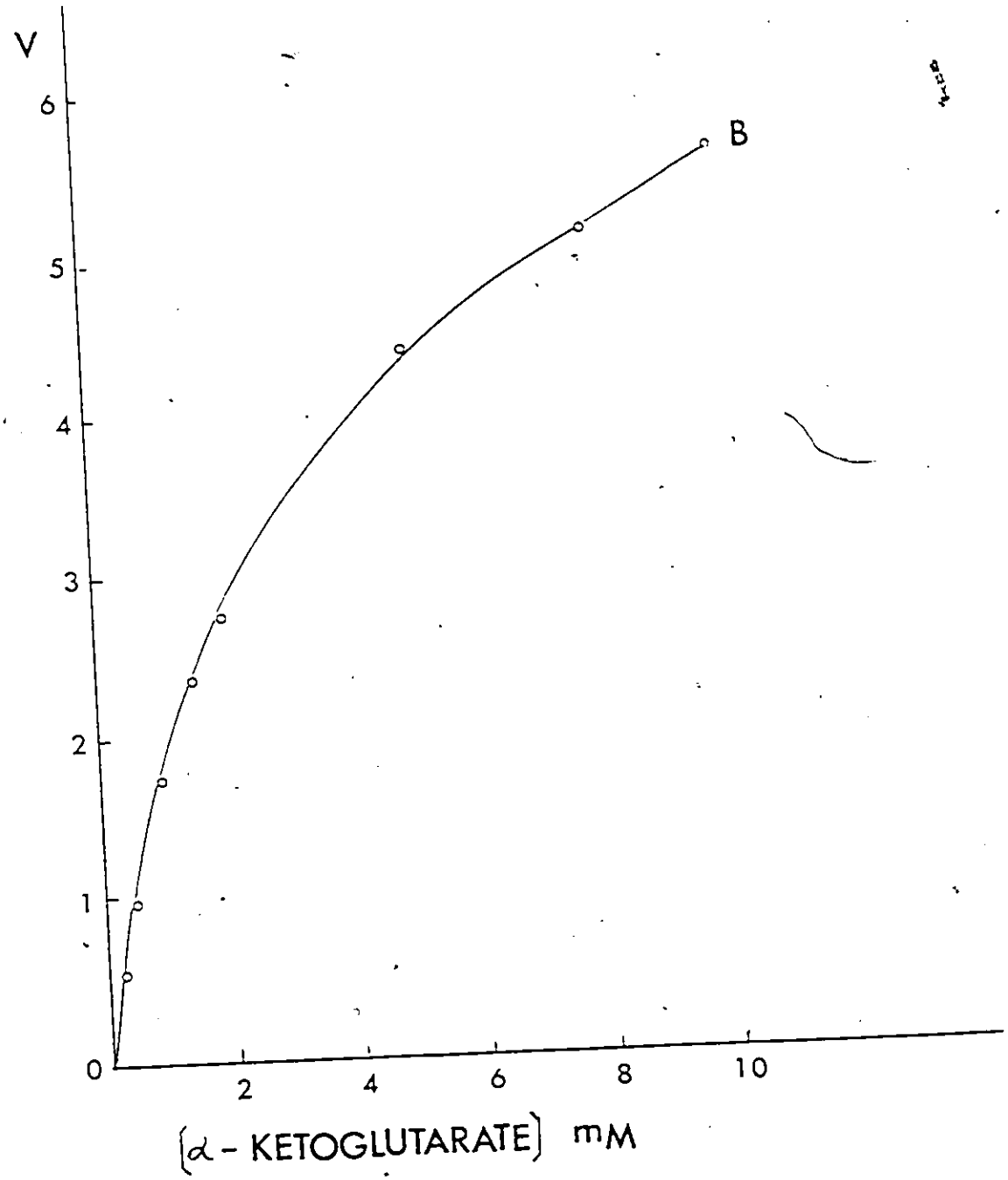
a



b



C



d

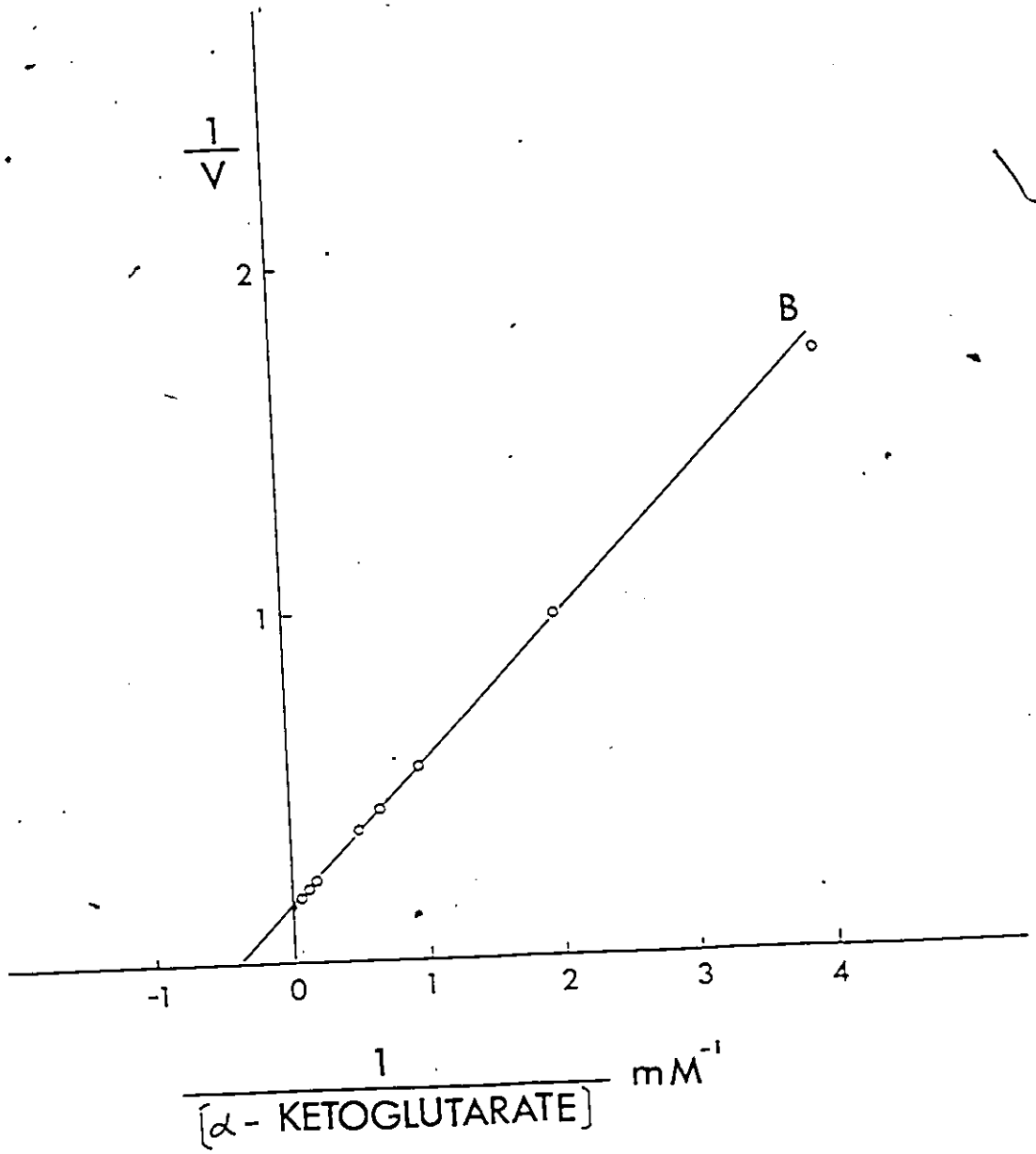
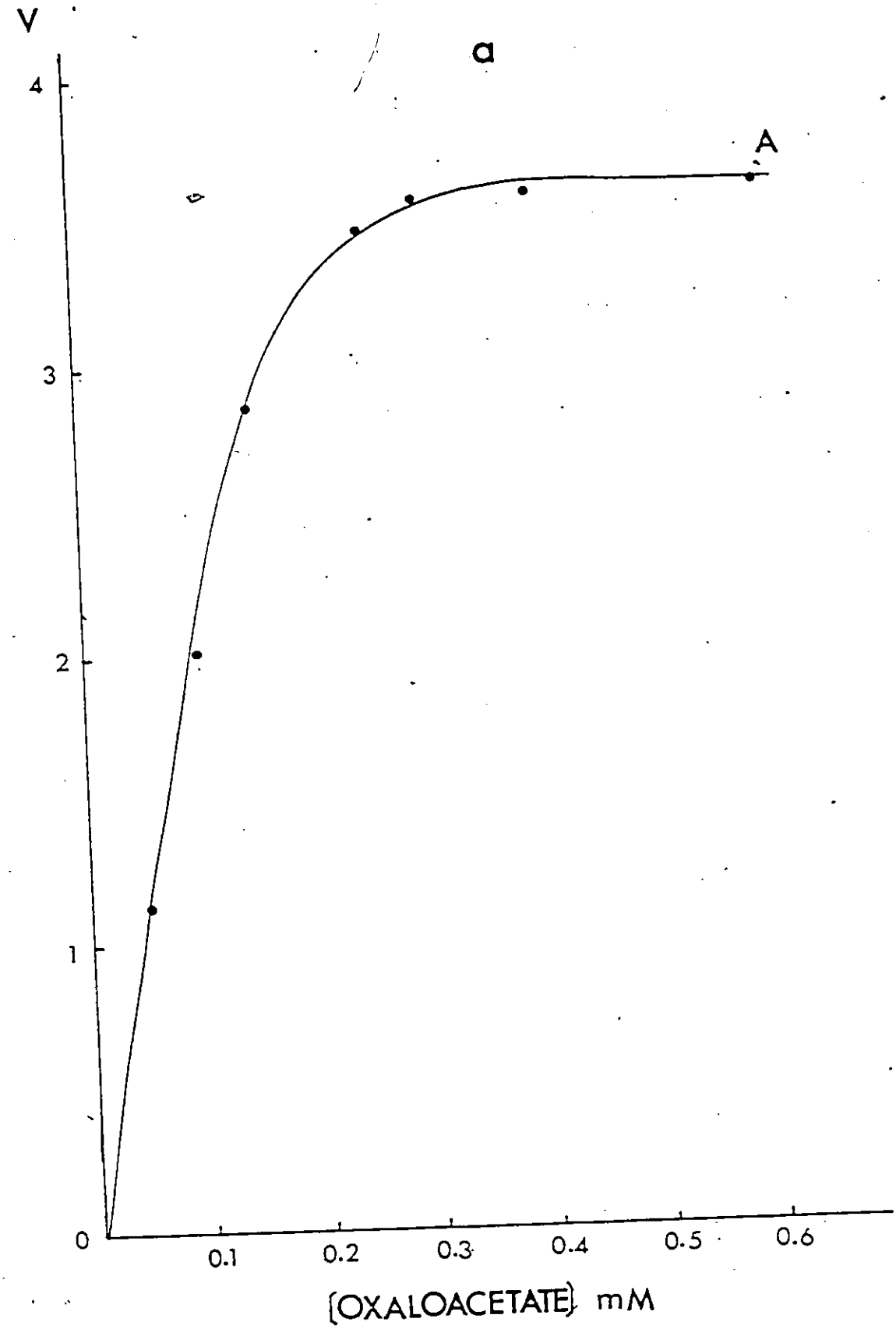


Figure 25

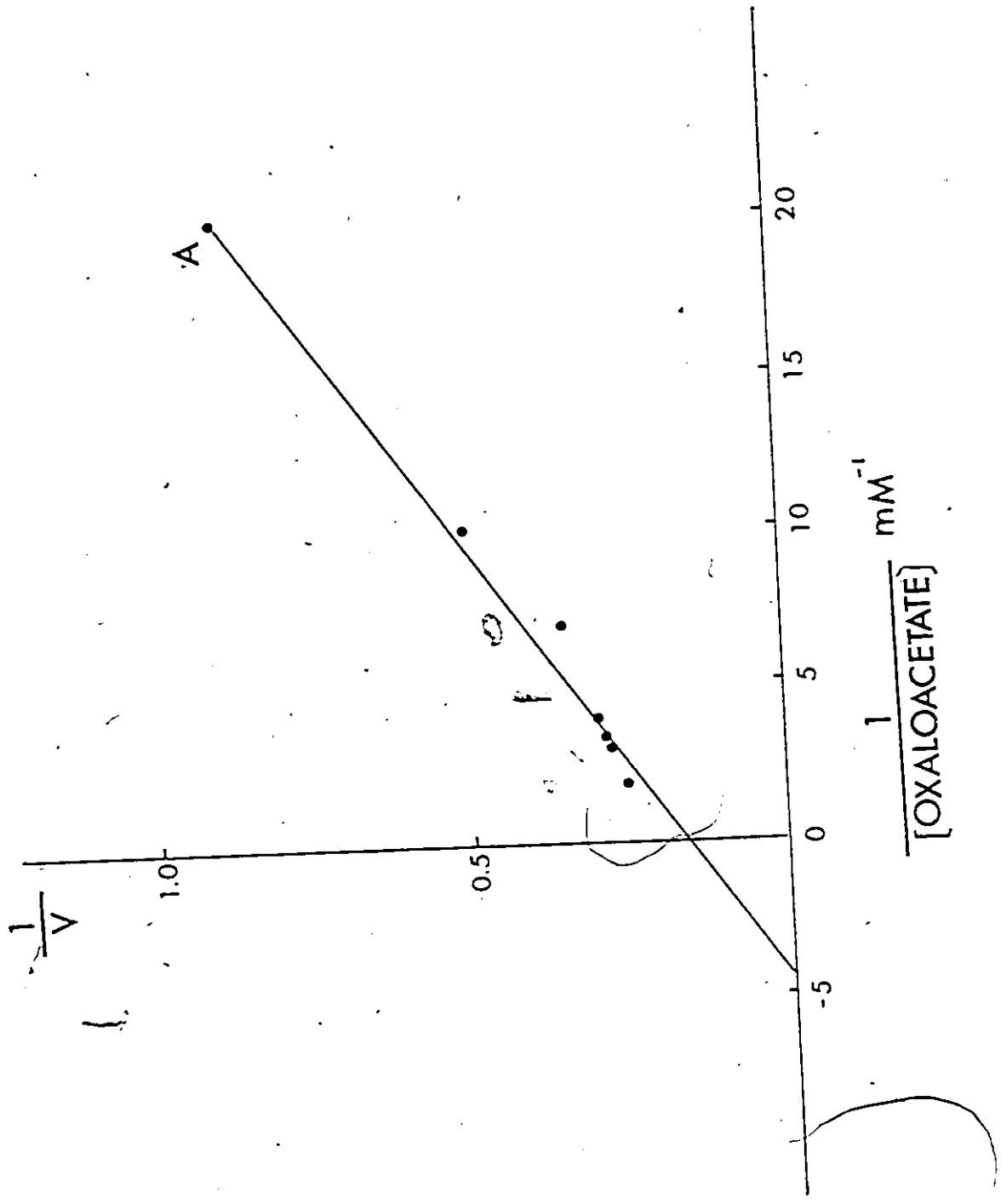
(a,b,c,d)

Michaelis-Menten and Lineweaver-Burk plots for enzymes A and B

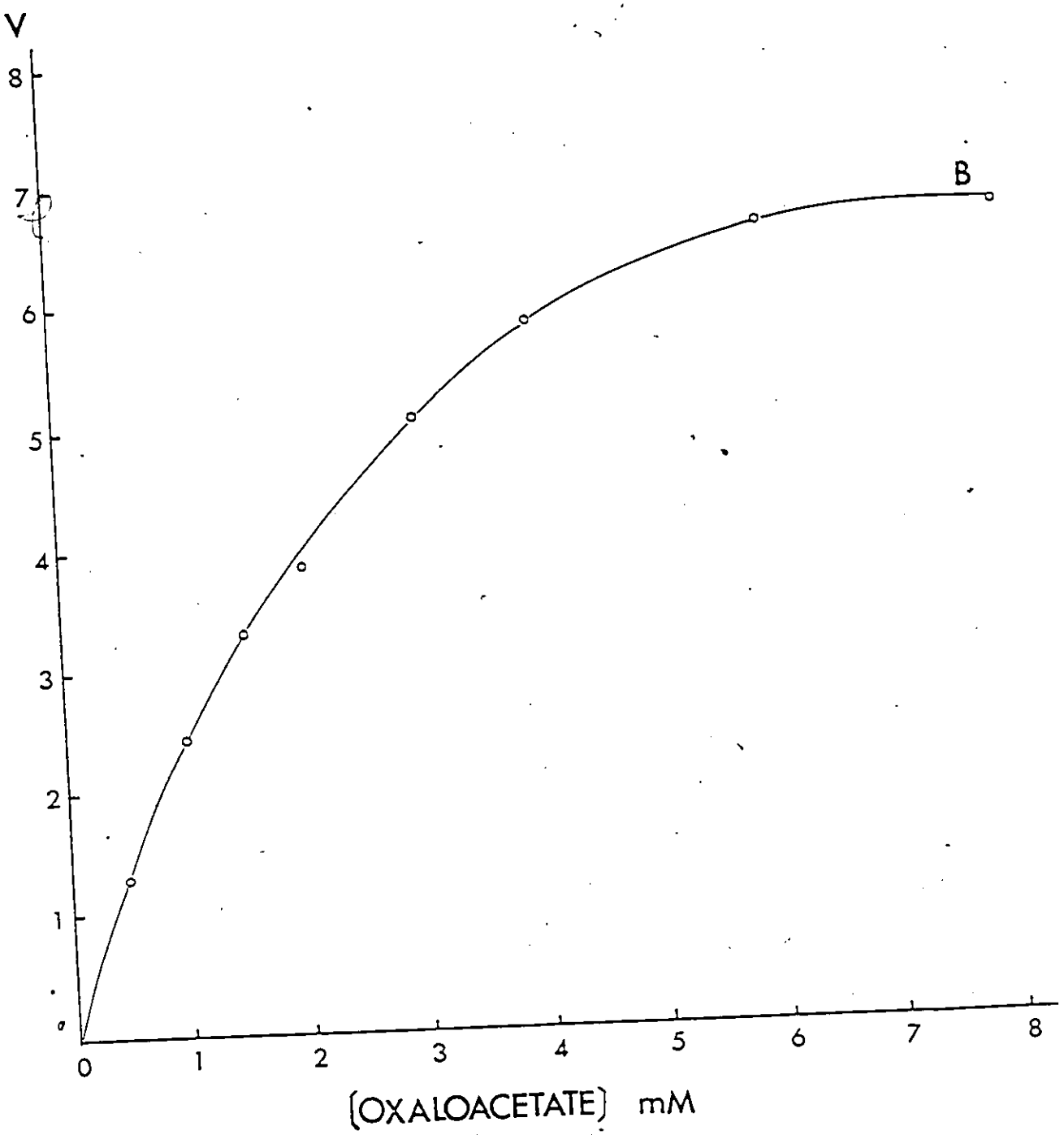
Effect of substrate (oxaloacetate) on enzymes A (●————●) and B (o————o) assayed as tyrosine aminotransferase.


V
a
4
3
2
1
0
A
0.1
0.2
0.3
0.4
0.5
0.6
[OXALOACETATE] mM

b



C



d

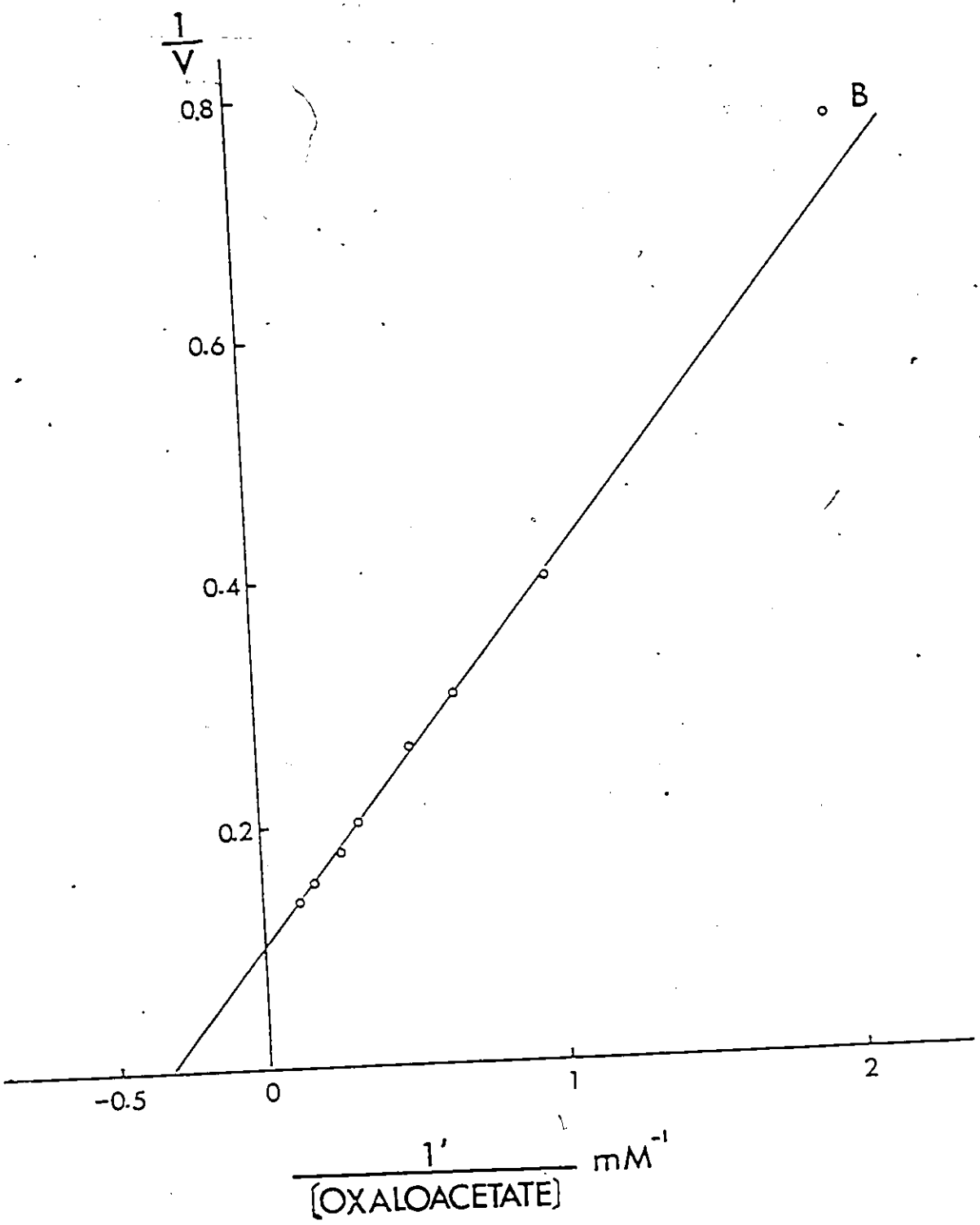


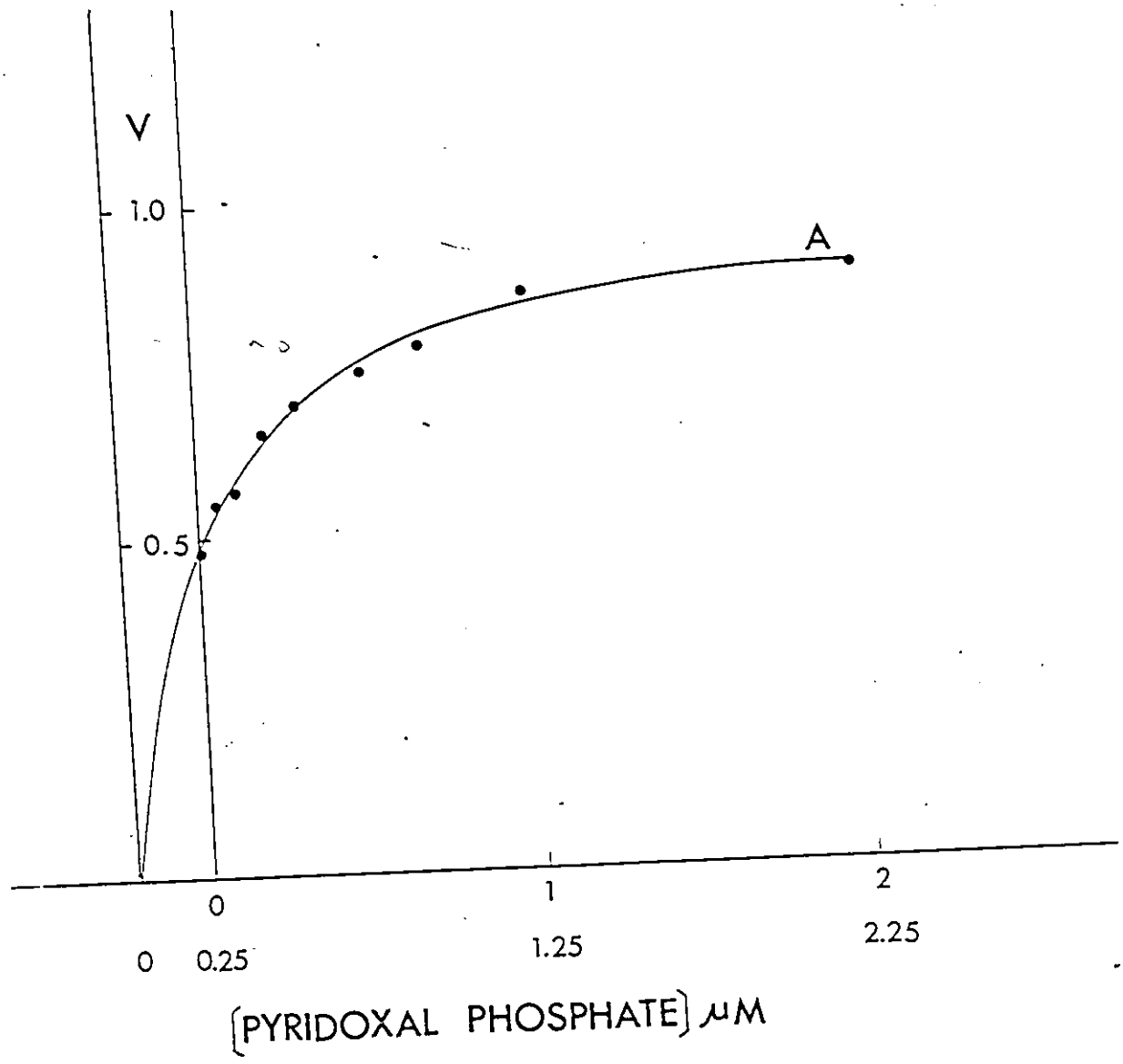
Figure 26

(a,b,c,d)

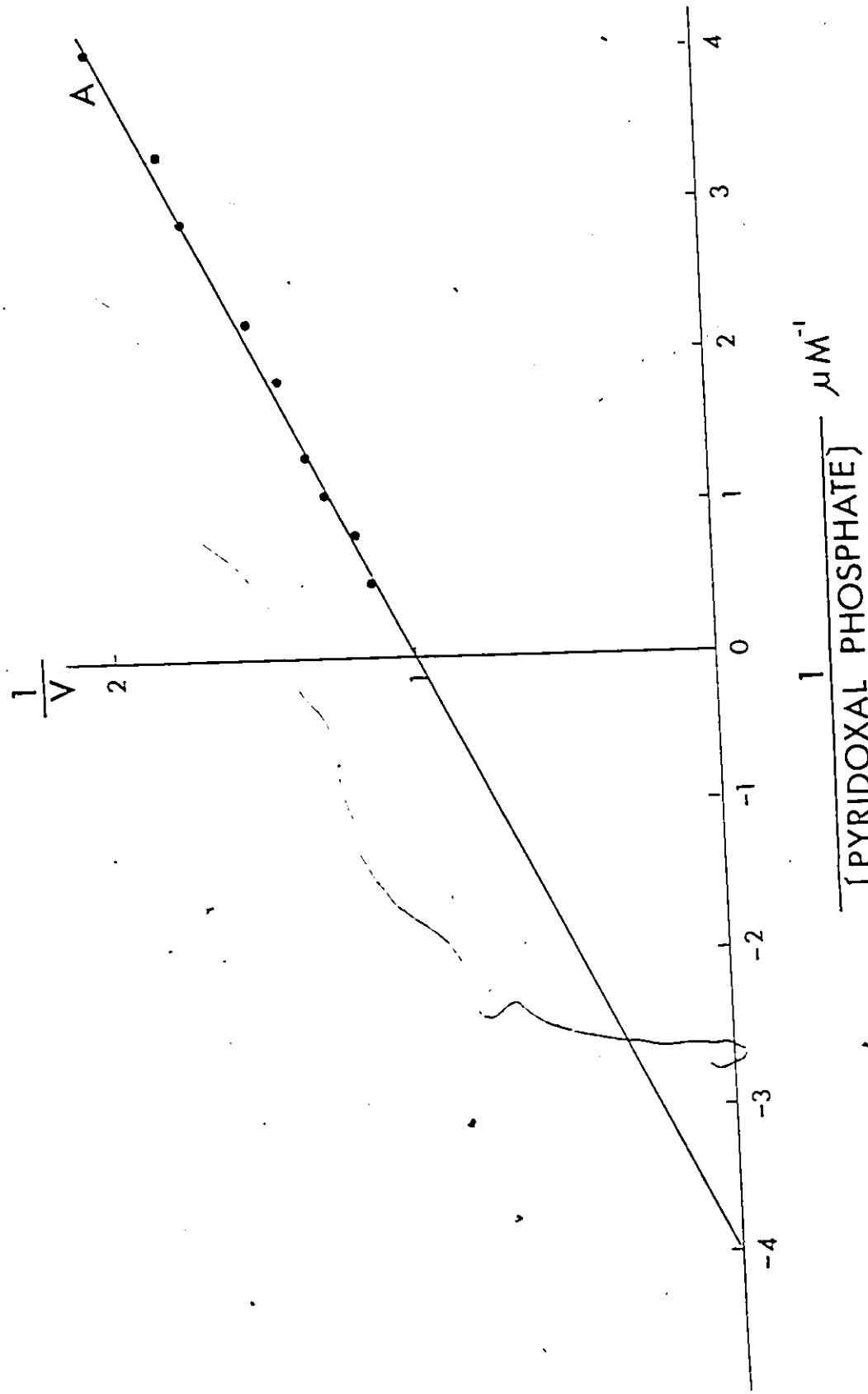
Michaelis-Menten and Lineweaver-Burk plots for Enzyme A and B

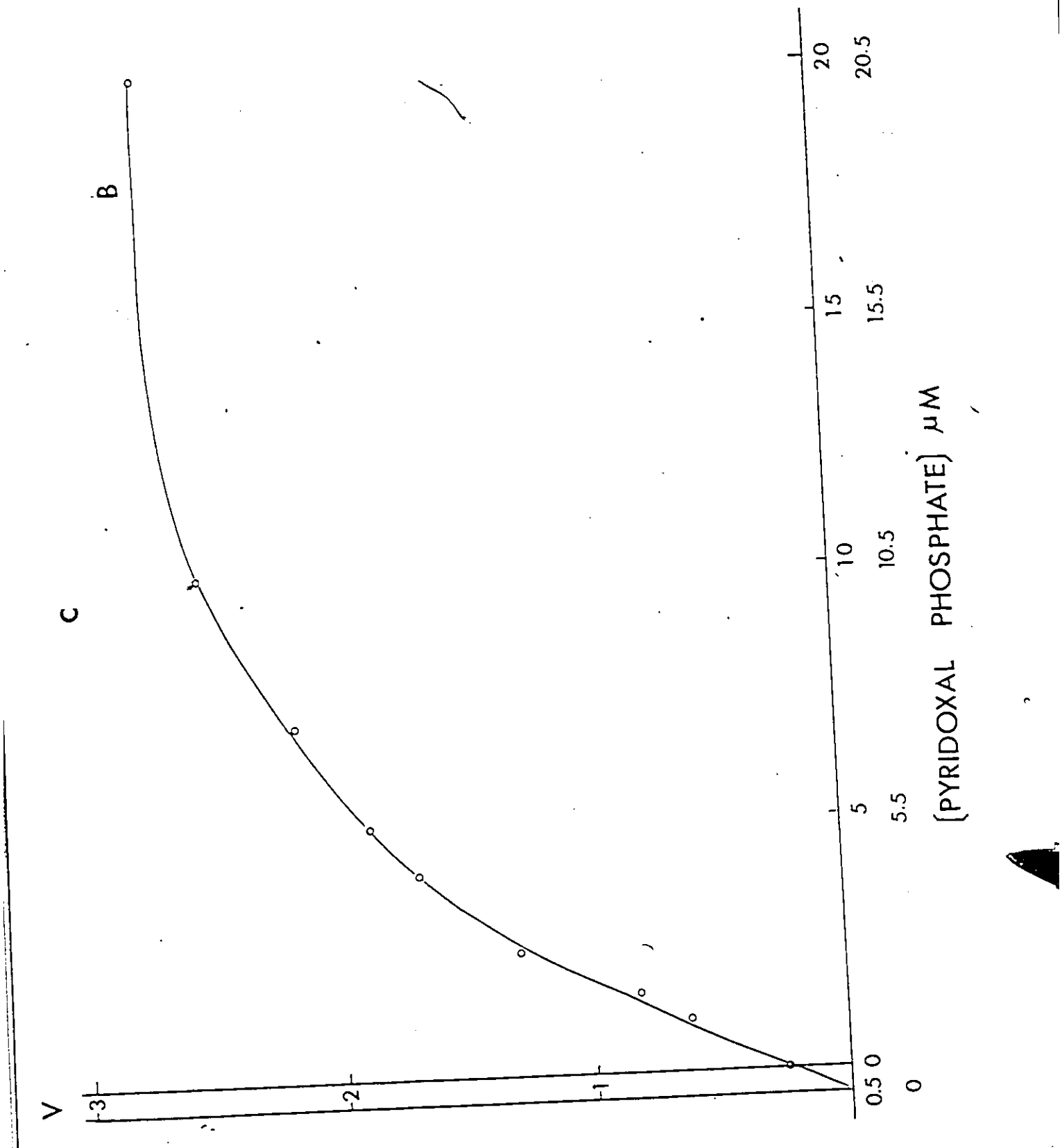
Effect of pyridoxal phosphate concentration on enzyme A (●——●) and enzyme B (o——o) assayed as tyrosine aminotransferase. The plots of the velocity (vertical axis) versus the apparent (added) pyridoxal phosphate concentration were extrapolated to zero velocity. The intercept of the curve with the horizontal concentration ax[illegible] (to the left of the velocity axis) was set as zero pyridoxal phosphate concentration and the new PLP concentrations were also written below the horizontal axis. Double reciprocal plots then were constructed to calculate the approximate Km and Vmax values for PLP. 0.015 units of enzymes A and B were used in this experiment.

a



b




V
3
2
1
0
0.5 0
5
5.5
10
10.5
15
15.5
20
20.5
[PYRIDOXAL PHOSPHATE] μM
B
C

d

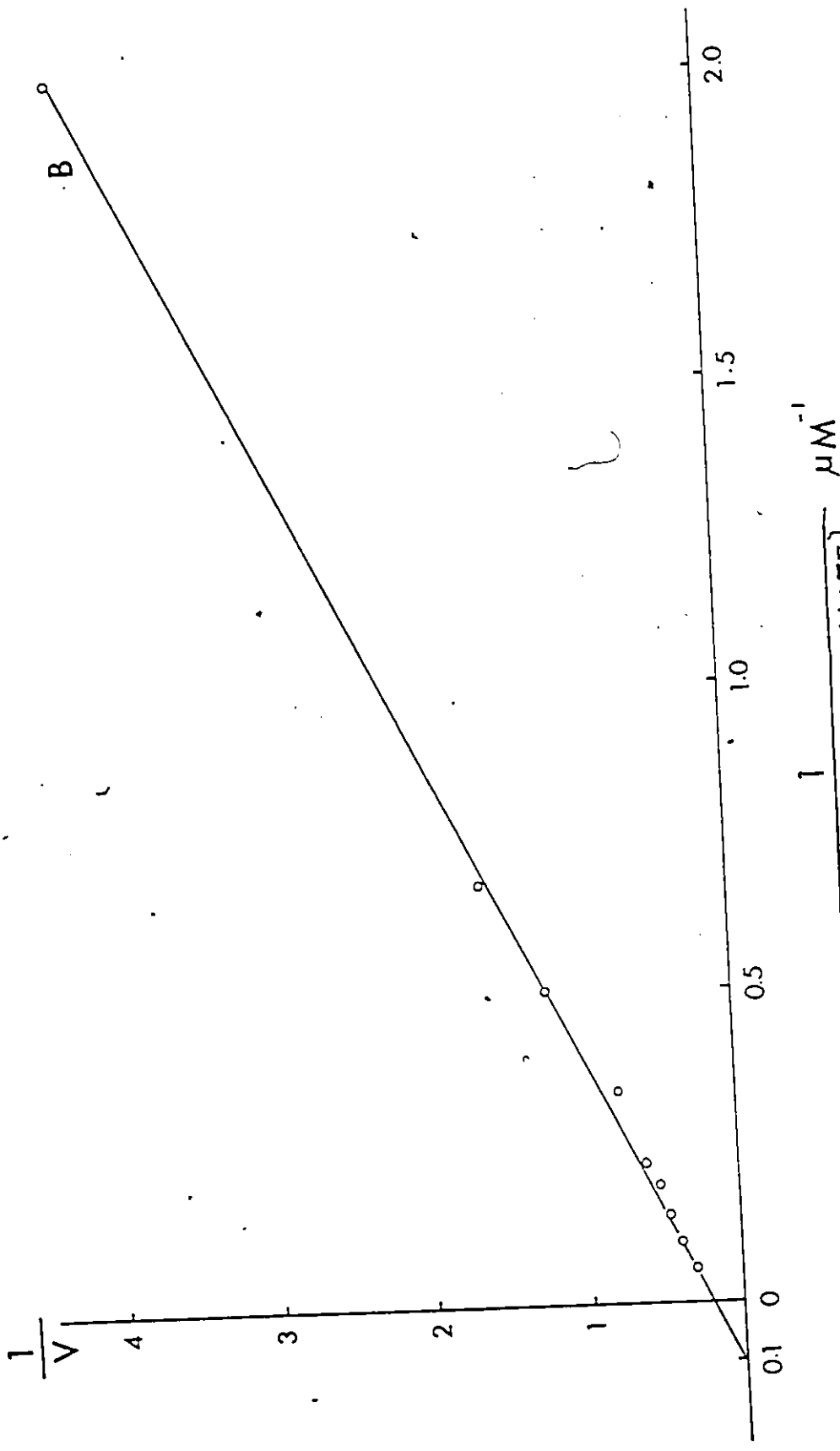


Table V

Km and Vmax values for enzymes A and B

The nmoles of each enzyme in the purified preparations were estimated from the known molecular weights and protein concentrations. The latter were calculated from the amino acid analyses.

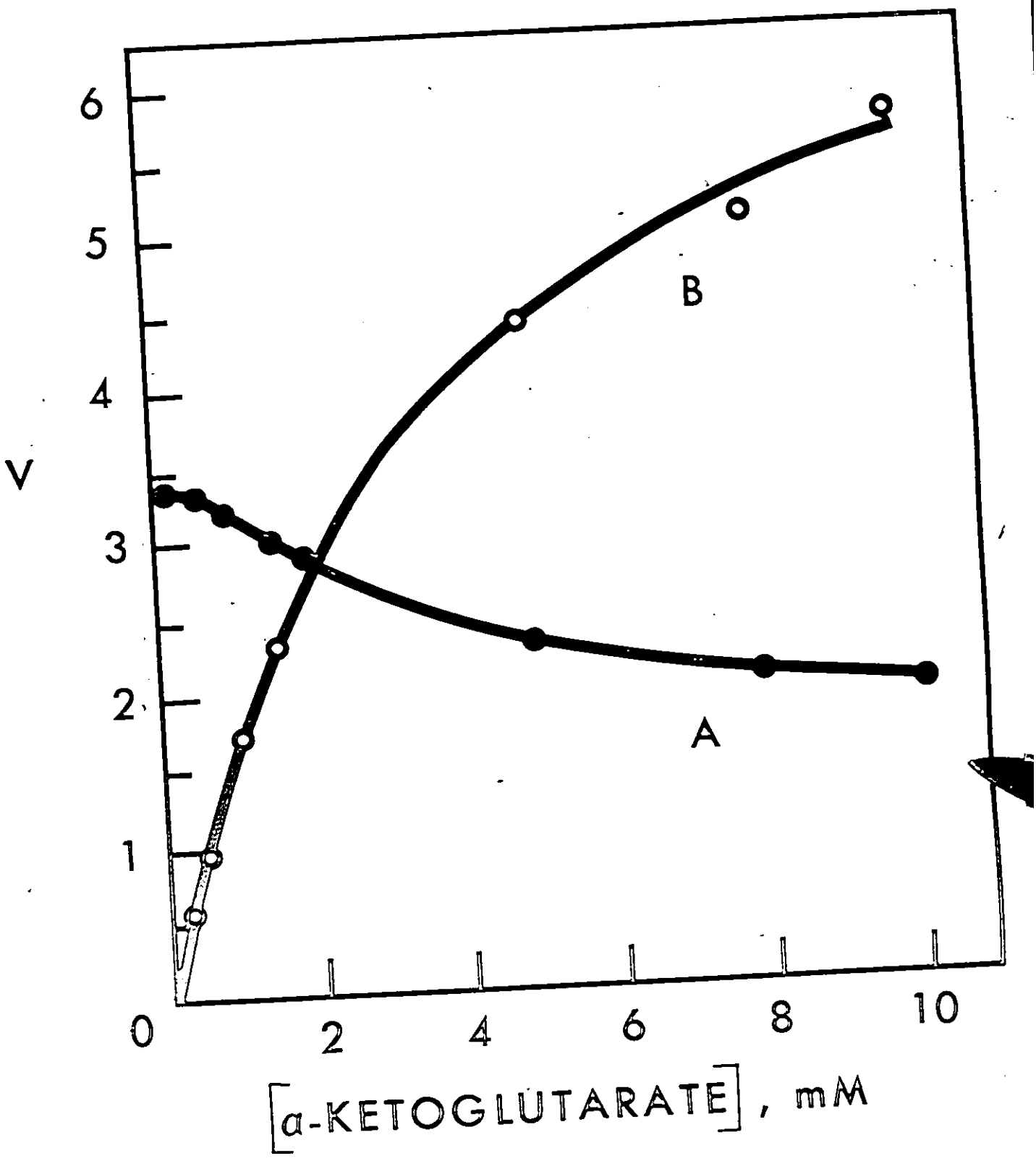
Substrate	Enzyme	Km	Vmax	B/A ratio for Vmax	B/A ratio for Km
		M	units/nmole		
L-tyrosine	A	1.43×10^{-3}	0.79	25.3	0.43
"	B	6.25×10^{-4}	20.00		
L-phenylalanine	A	2.17×10^{-3}	0.66	13.8	0.15
"	B	3.33×10^{-4}	9.10		
L-tryptophan	A	5.00×10^{-3}	0.85	17.7	2.00
"	B	10.0×10^{-3}	15.02		
L-aspartate	A	4.40×10^{-3}	3.14		
α-ketoglutarate	A	0.71×10^{-4}	3.28	5.1	35.2
"	B	2.50×10^{-3}	16.67		
oxaloacetate	A	3.70×10^{-4}	2.52	11.9	8.5
"	B	3.13×10^{-3}	30.03		
pyridoxal phosphate	A	2.50×10^{-7}	0.43	34.9	40.0
" "	B	1.00×10^{-5}	15.02		

of equimolar amounts of the two enzymes a maximum of only about 7% of the total Vmax toward phenylalanine and only a few percent toward tryptophan and tyrosine would be contributed by enzyme A. The major activity of enzyme A however, is toward aspartate as attested by the high Vmax for this amino acid in contrast to the much lower Vmax values for the aromatic amino acids (Table V). Since aspartate aminotransferase was assayed at 25° whereas the aromatic aminotransferases were assayed at 37°, the relative magnitude of Vmax for aspartate is actually underestimated in Table V by a factor of about 2 (if about 2.0 be considered the value of temperature coefficient Q_{10} in this instance). Thus, in in vitro assays of crude extracts the aspartate aminotransferase is contributed by enzyme A whereas the aromatic amino acid aminotransferase activity is for all practical purposes attributable to enzyme B. The Vmax values for the two keto acids and pyridoxal phosphate (in the presence of tyrosine as the amino acid substrate) were also higher with enzyme B than with enzyme A as indicated by their high B/A ratio. The Km values for α-ketoglutarate and pyridoxal phosphate were 35-fold and 40 fold higher with enzyme B than with enzyme A, respectively, indicating significantly higher affinities of the keto acid and the cofactor for enzyme A than for enzyme B. Fig. 27 shows in fact that enzyme A is inhibited by α-ketoglutarate at concentrations which do not yet saturate enzyme B.

Figure 27

Michaelis-Menten plot for enzymes A and B

Velocities (μ moles/min/ml) for tyrosine aminotransferase activities against the concentration of α-ketoglutarate with enzyme A (curve A) and enzyme B (curve B).


6
5
4
3
2
1
0
V
B
A
2
4
6
8
10
[α-KETOGLUTARATE], mM

6. Effect of pH on Km values

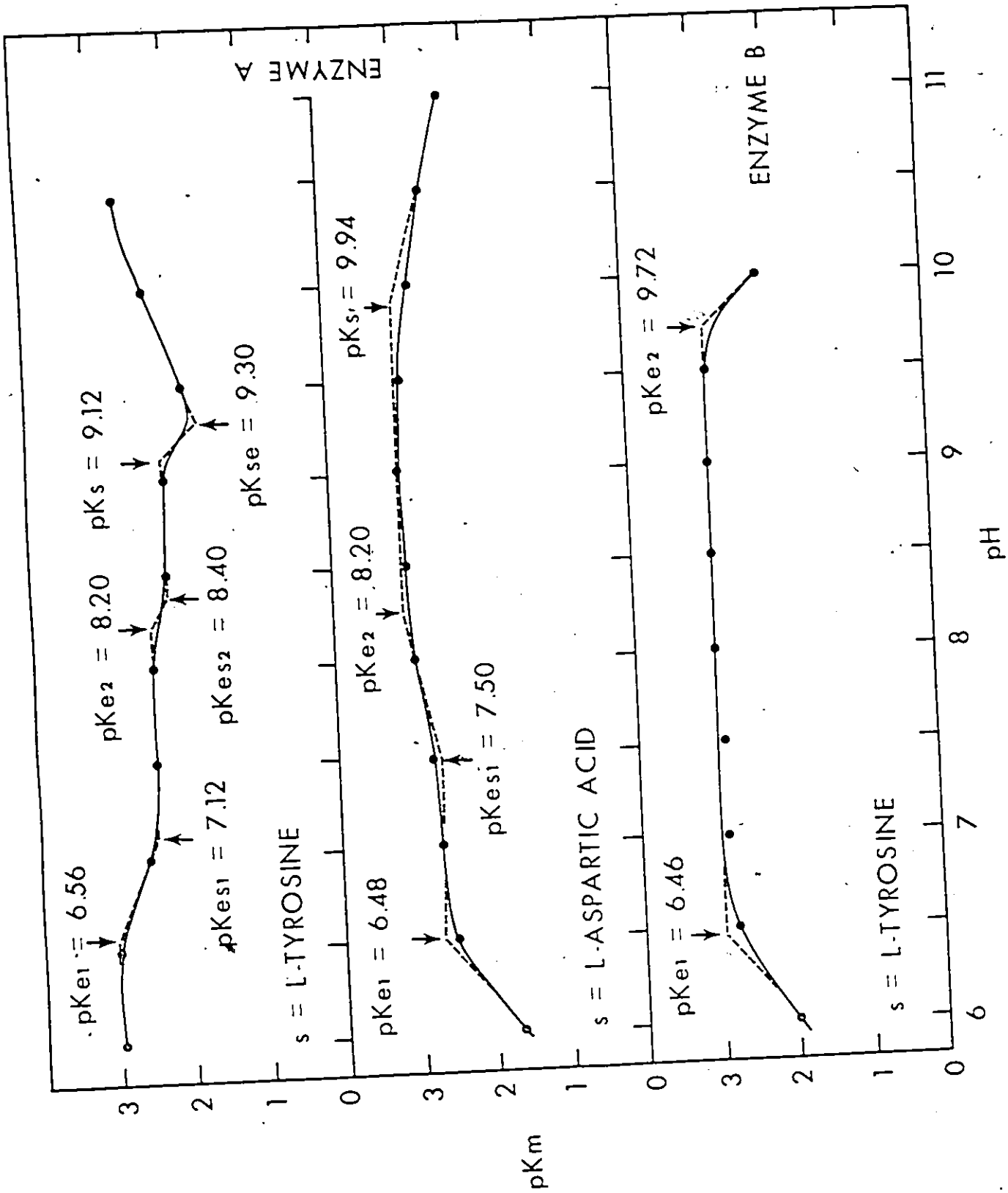
The following experiments were designed in an attempt to gain some insight into the cause for the different heat stabilities of enzyme A with aspartate and the aromatic amino acids as substrates (Fig. 19).

As discussed by Dixon and Webb (1964) the ionization constants of ionizing groups at the active site of the free enzyme (Ke), or in combination with the substrate (Kes) may be calculated as pKe (-logKe) or pKes (-logKes) values from plots of pKm versus the pH. Bends in the curves with the concave side downwards represent the ionizations of the free enzyme or the free substrate, whereas bends with the concave side upwards represent modified ionizations of the enzyme or substrate in the enzyme-substrate complex. As seen in Fig. 28 there appear to be two ionizing groups at the active site of the free enzyme A with the pK values, pKe_1 and pKe_2, of about 6.5 and 8.2, respectively, whether estimated in the presence of aspartate or tyrosine as substrates. This is kinetic evidence, in addition to the physical evidence (Figs 14, 15), that the activities toward the aliphatic and aromatic substrates reside in the same protein and that a single site is involved in the binding of both aspartate and tyrosine. With tyrosine as substrate the above pKe values are increased to the pKes values of 7.1 and 8.4, respectively. This indicates that the pKe values of both ionizing groups are affected by the binding of tyrosine. With

Figure 28

The effect of pH on the pKm

The effect of pH on the pKm (-log Km) of enzyme A with L-tyrosine and L-aspartate (two upper panels) and of enzyme B with L-tyrosine (bottom panel). K = ionization constant; pK = -log K; pKe refers to an ionizing group of the free enzyme; pKes to enzyme combined with substrate; pKs refers to an ionizing group of the free substrate; PKse refers to an ionizing group of the free substrate; PKse refers to an ionizing group of the free substrate in combination with the enzyme. The following buffers were used: pH 6.0-6.5, citrate-phosphate; pH 7.0-9.0, Tris. HCl; pH 9.5-11.0, glycine-NaOH.


ENZYME A
pKe1 = 6.56
pKe2 = 8.20
pKs = 9.12
pKes1 = 7.12
pKes2 = 8.40
pKse = 9.30
s = L-TYROSINE
pKe1 = 6.48
pKe2 = 8.20
pKs1 = 9.94
pKes1 = 7.50
s = L-ASPARTIC ACID
ENZYME B
pKe1 = 6.46
pKe2 = 9.72
s = L-TYROSINE
pKm
pH
0
1
2
3
6
7
8
9
10
11

aspartate as substrate a single pKes value of 7.5 appears, which may have resulted either from a decreased pKe2 or an increased pKe1. In either case, it appears that only one of the ionizing groups is affected by the binding of aspartate.

The effect of pH on the Km with enzyme B was also studied (Fig. 28). Two pKe values were revealed equal to about 6.5 and 9.7. The latter may in fact reflect denaturation of the enzyme. At any rate no other bends were present and their absence suggests that no modification of ionizing constant(s) occurred with tyrosine as substrate.

The above results suggest that the different heat sensitivities of enzyme A with its two substrates may be interpreted in terms of the optimal ionic environment at the active site required for the binding of tyrosine and aspartate. That ionic environment may change as a result of conformational changes caused by heat. The difference between the effects of binding of tyrosine and aspartate on the ionization constants at the active site may suggest different modes of binding for these two substrates, as might be expected for two so dissimilar molecules. Hence, it would be expected that the range of conformations, within which optimal binding is still possible, is different for the two substrates. The most stringent conformation, in terms of ionic environment at the active site, is that which is involved in the binding of tyrosine, so that heat may well affect the binding of

this substrate (observed as partial loss of catalytic activity) while the binding of aspartate is not affected. This seems to be a plausible explanation for the observed differential heat sensitivity of this homogeneous enzyme for aspartate and the aromatic amino acids. Subtle differences in the modes of binding of tyrosine and phenylalanine may also be responsible for the slightly higher heat lability of the enzyme in the presence of tyrosine than in the presence of phenylalanine as substrate (Fig. 19).

Other ionizing groups, unrelated to the ionizations of the free or combined enzyme, are as follows: With tyrosine as substrate, a third downward bend at pH 9.12 most likely represents the second ionization constant (9.11) of tyrosine (Handbook of Chemistry and Physics, 1969). It is followed by an upward bend at pH of 9.30 which represents the modified pKs of tyrosine once bound to the enzyme, and is indicated as pKse in Fig. 28. A similar change in the ionization constant of nitrocatechol sulfate by binding to arylsulfatase has been reported (Dodgson et al. 1955) and is quoted by Dixon and Webb (1964). With aspartate as substrate the last downward bend at pH 9.94 most likely represents, being so similar to it, the third ionization constant of 9.82 of free aspartic acid (Handbook of Chemistry and Physics, 1969).

The plots of Fig. 28 should be viewed as an attempt only

and not as a definitive answer to the problem raised by the heat stability experiments. The bends are caused by slight changes in the pKm values and the linear parts do not always approximate integral slopes of - 1 and + 1 as required by theory. Since no statistical tests were applied, it is not known whether the deviations from the theoretical requirements are due to experimental errors and the conclusions are essentially correct, of that the experimental points are essentially correct and the interpretation given above improper.

7. Inhibition Studies

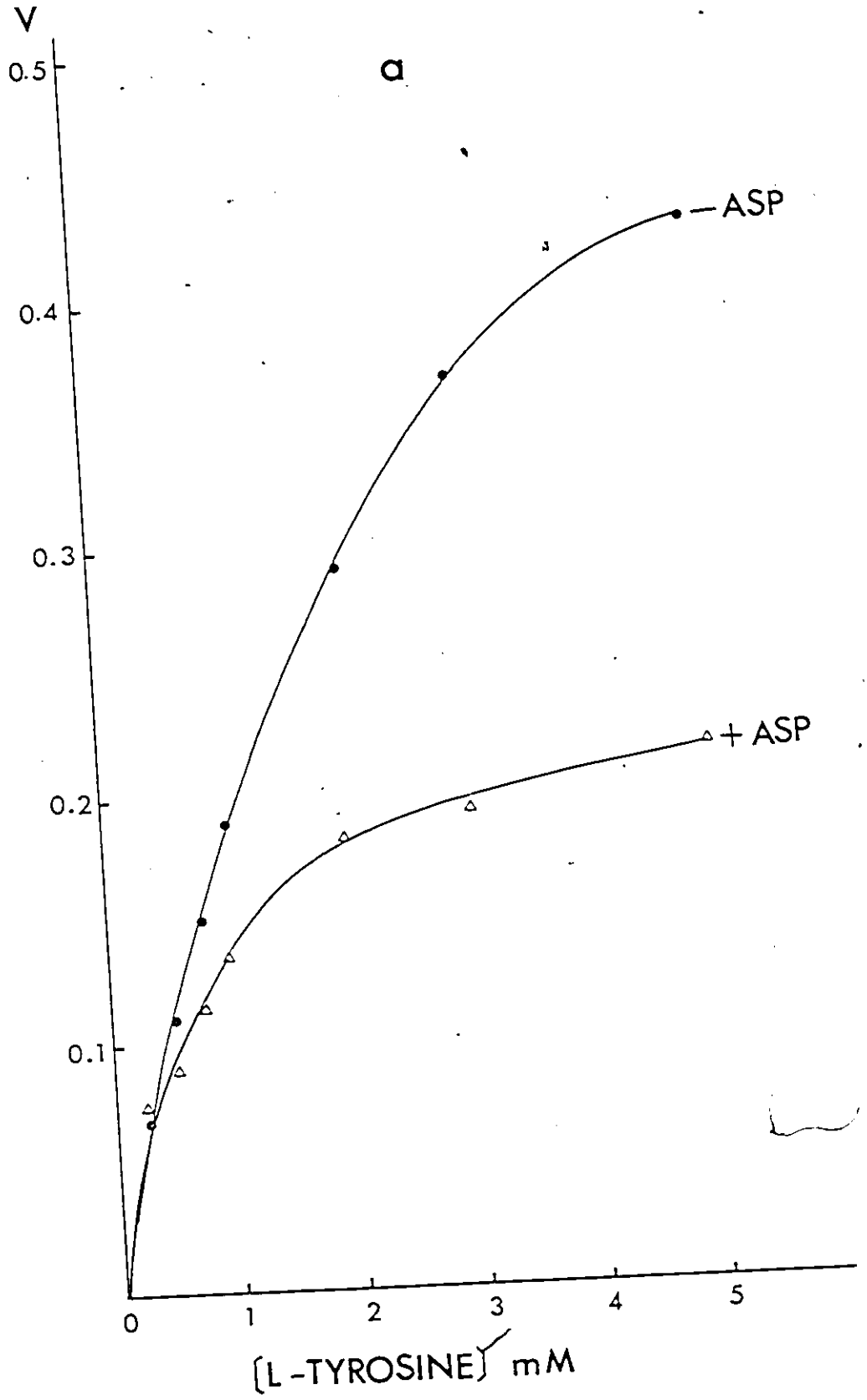
Because aspartate is the substrate of an enzyme (enzyme A) with significant activity toward the aromatic amino acids it was decided to study its possible role as an inhibitor when both enzyme A and enzyme B were assayed as aromatic aminotransferases. Figures 29-32 show the Michaelis-Menten and Lineweaver-Burk plots for the aspartate inhibition of enzymes A and B, with L-tyrosine (Figs. 29,30) and α-ketoglutarate (Figs. 31, 32) as the varied substrates. Table VI lists the types of inhibition by L-aspartate of both enzymes with respect to L-tyrosine and α-ketoglutarate. Aspartate is an uncompetitive inhibitor with respect to tyrosine (parallel lines were obtained in the double reciprocal plots) and a non-competitive inhibitor with respect to α-ketoglutarate, for enzyme A. In addition, the aromatic substrate phenylalanine (used because of its solubility, instead of

Figure 29

(a,b)

Inhibition by L-aspartate of enzyme A assayed as tyrosine aminotransferase

Michaelis-Menten and Lineweaver-Burk plots with tyrosine as the varied substrate and aspartate (3 mM) as the inhibitor. The control (-Asp) contained no aspartate. Tyrosine aminotransferase was assayed by the enol borate-tautomerase method (Lin et al., 1958).


V
a
0.5
0.4
0.3
0.2
0.1
— ASP
+ ASP
0
1
2
3
4
5
[L-TYROSINE] mM

b

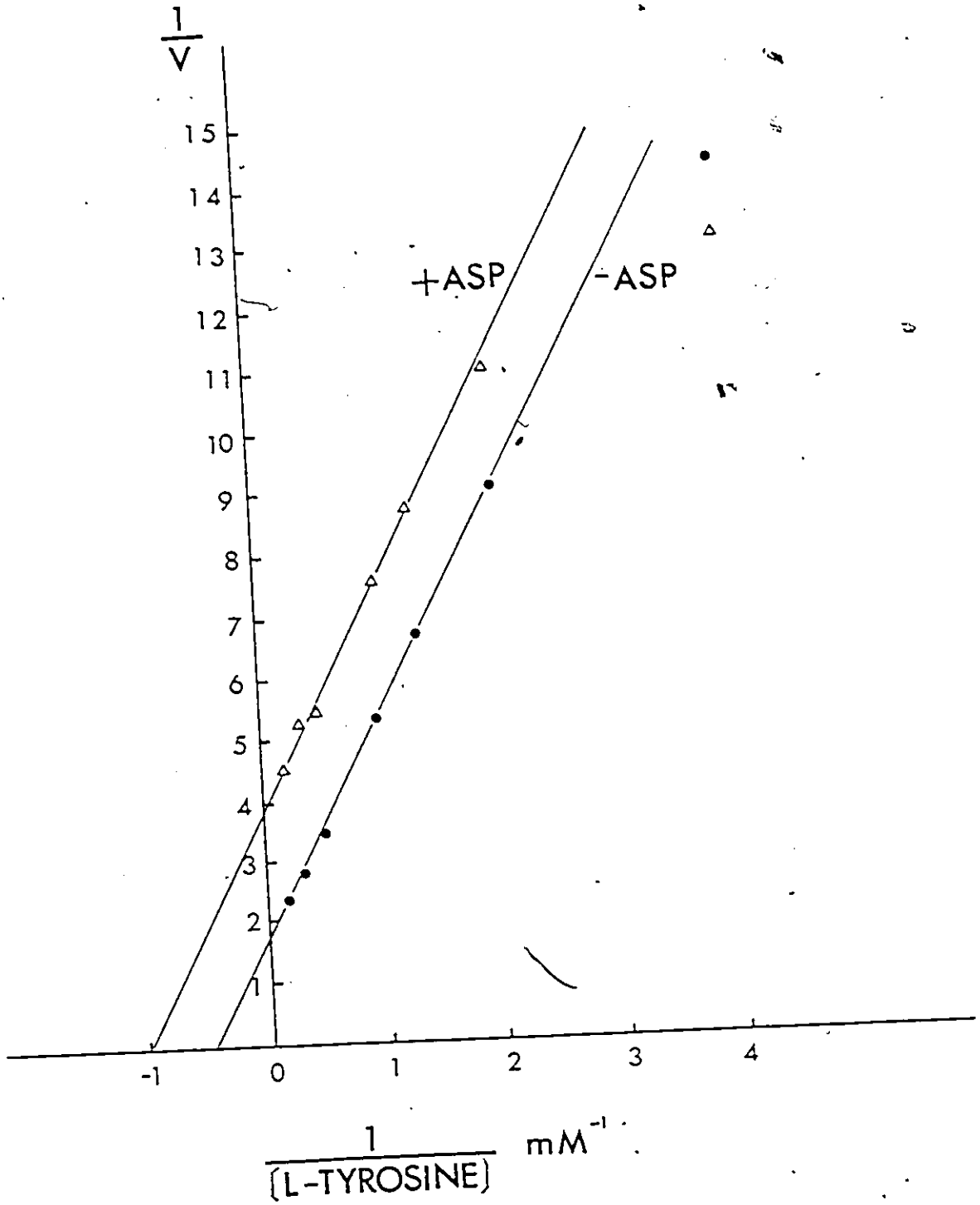


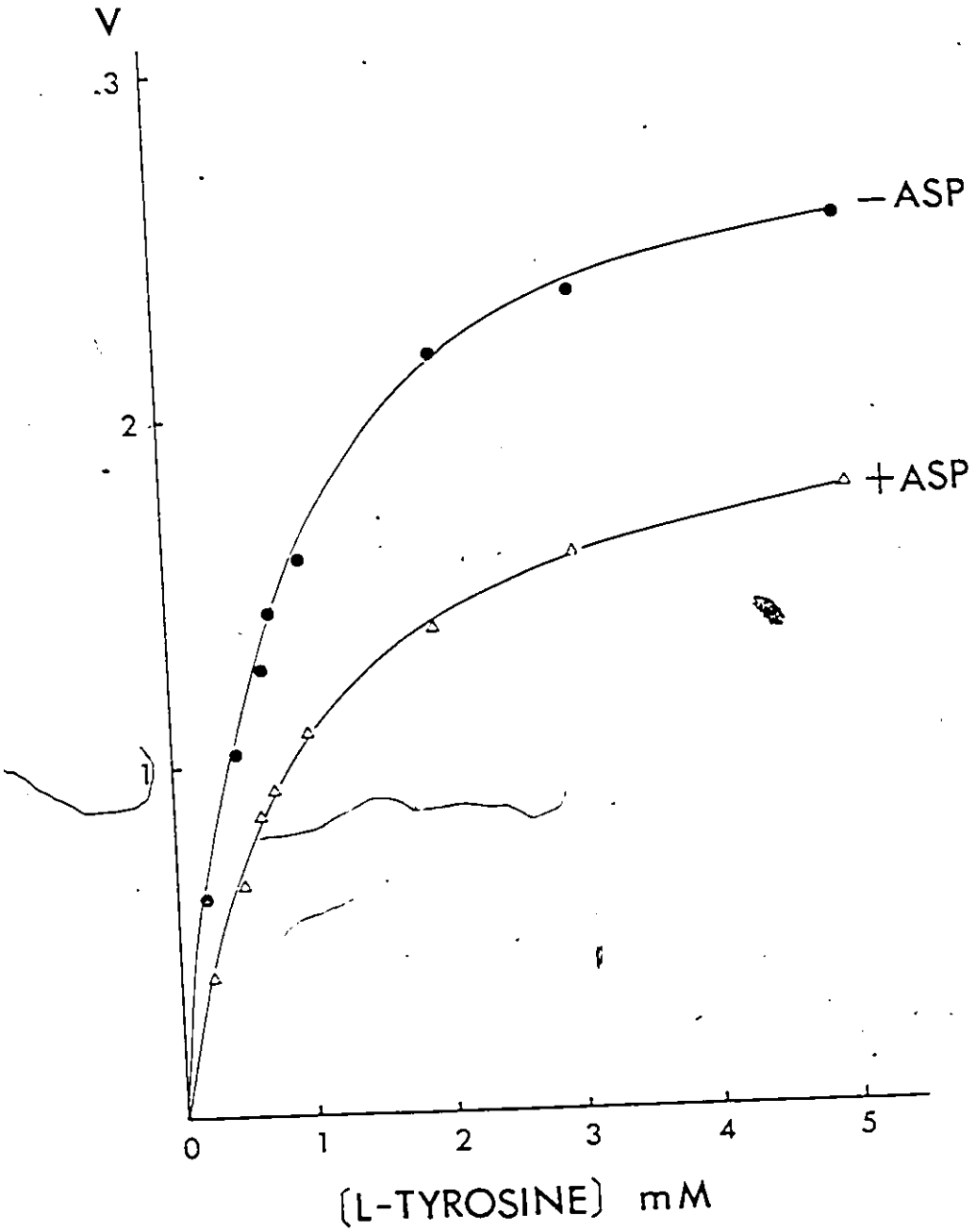
Figure 30

(a,b)

Inhibition by L-aspartate of enzyme B assayed as tyrosine aminotransferase

See Fig. 29 for conditions.

a



b

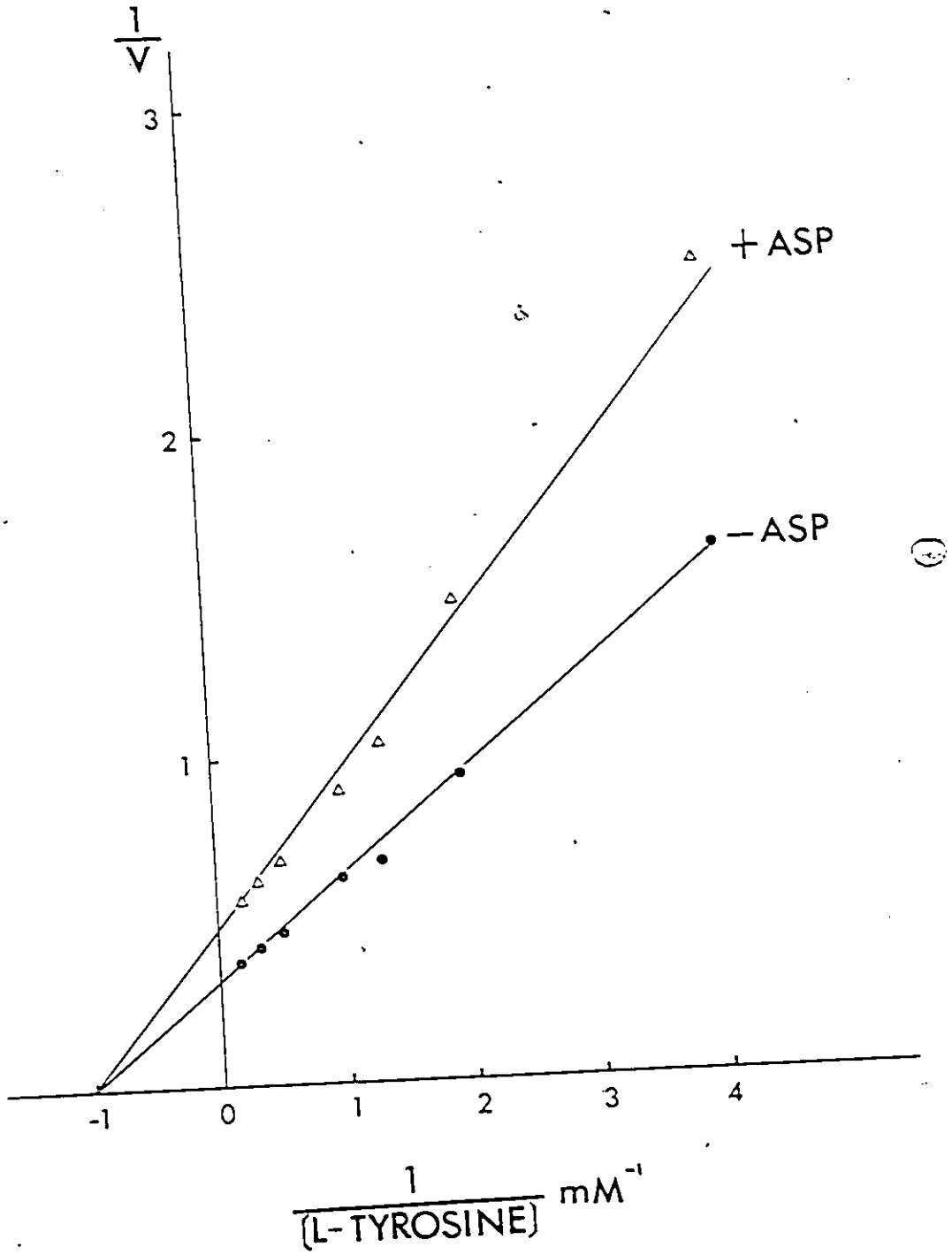


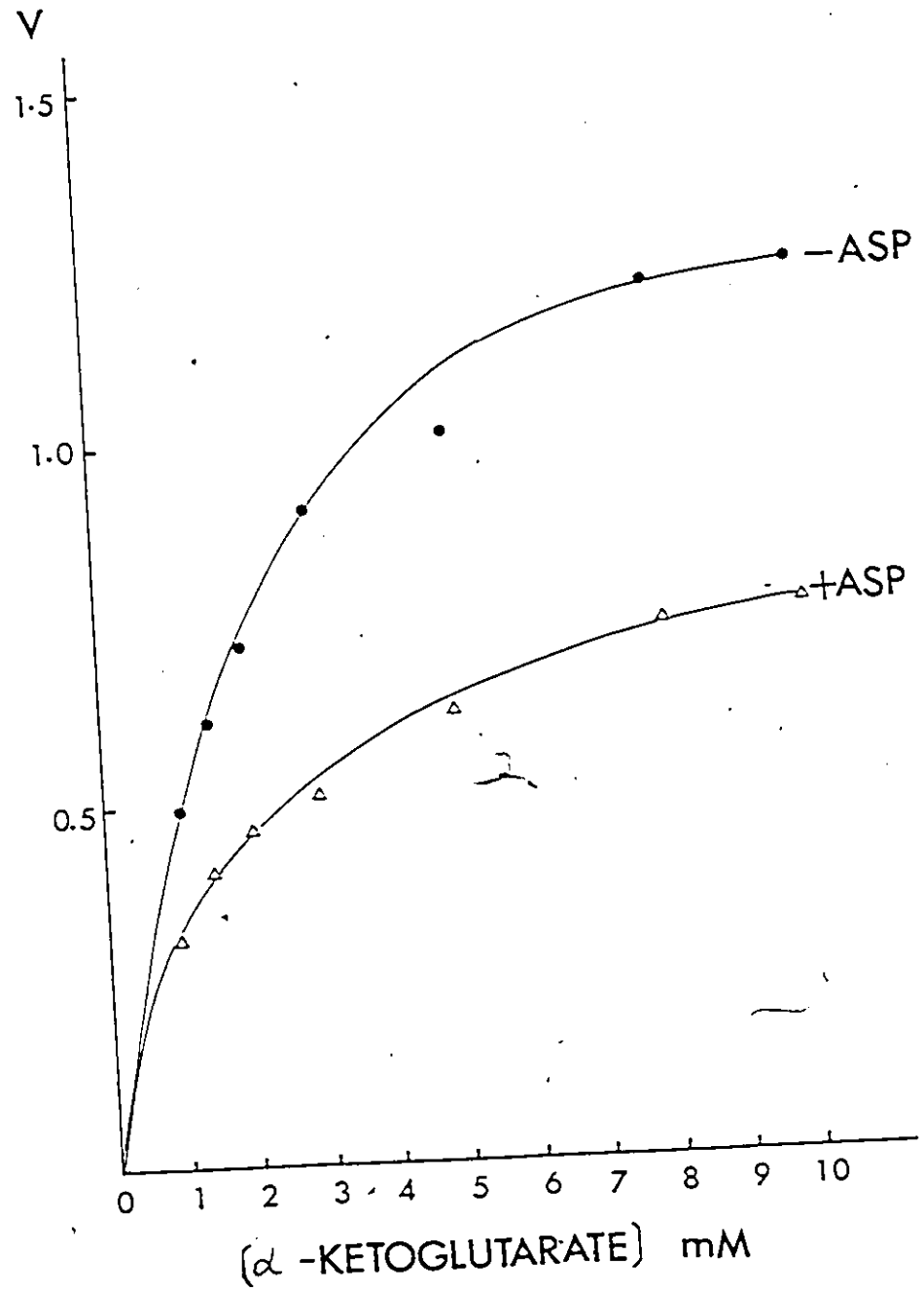
Figure 31

(a,b)

Inhibition by L-aspartate of enzyme A assayed as tyrosine aminotransferase

Michaelis-Menten and Lineweaver-Burk plots with α-ketoglutarate as the varied substrate and aspartate (3mM) as the inhibitor. The control (-Asp) contained no aspartate. Tyrosine aminotransferase was assayed by the enol borate-tautomerase method (Lin et al., 1958).

a



b

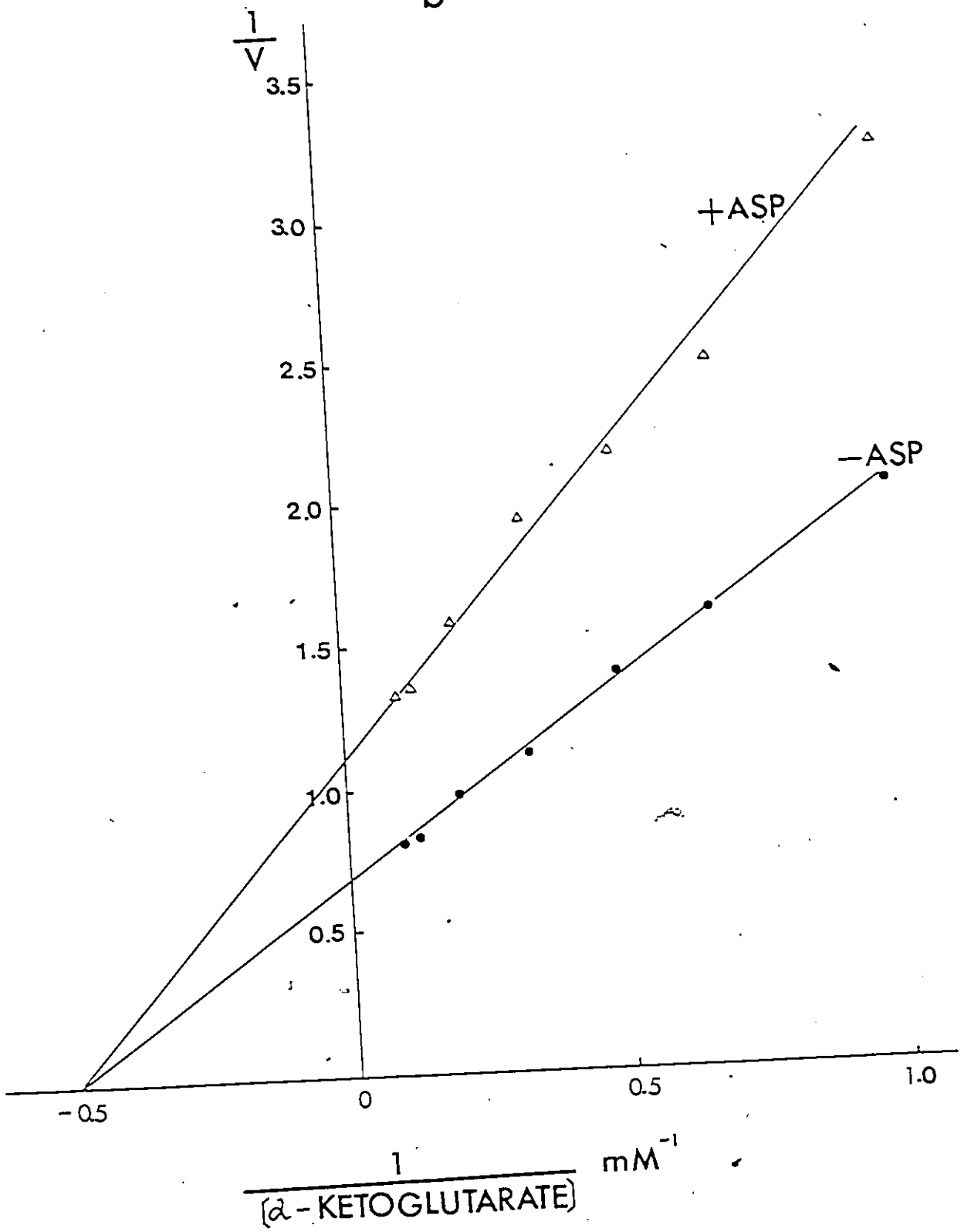


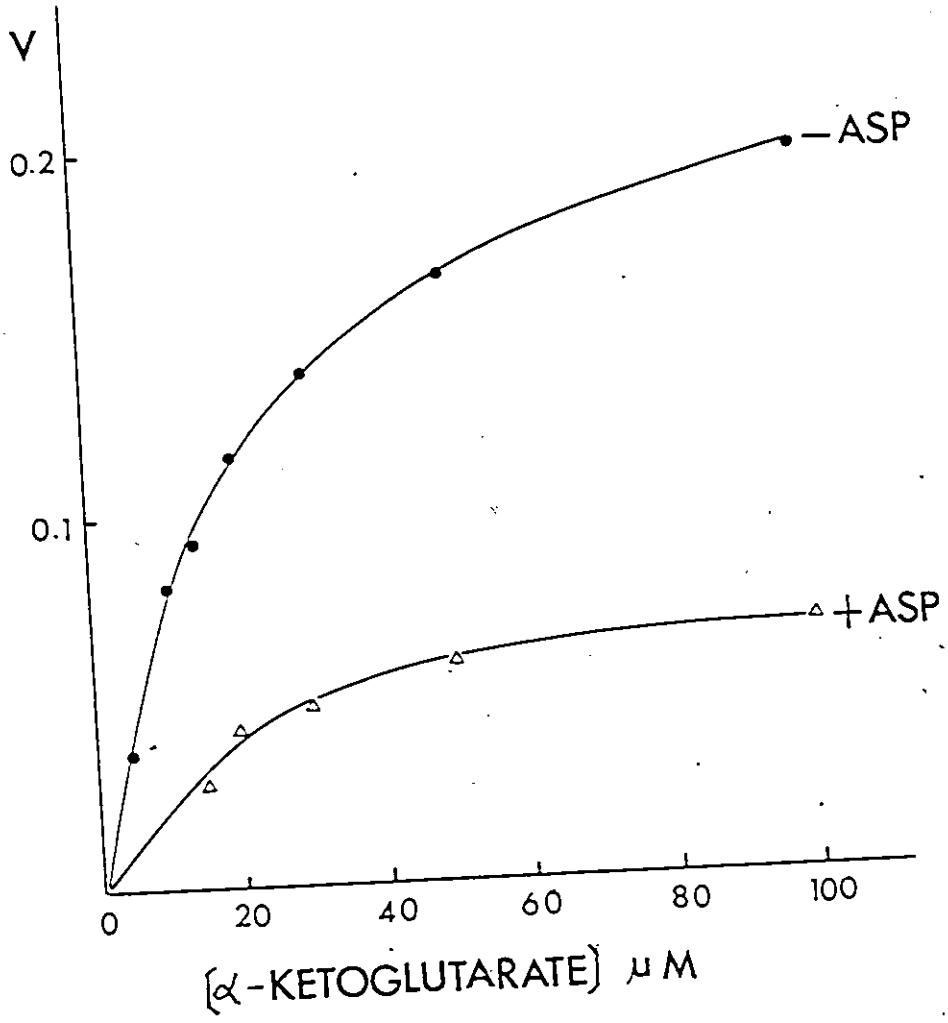
Figure 32

(a,b)

Inhibition by L-aspartate of enzyme B assayed as tyrosine aminotransferase

See Fig. 31 for conditions.

a



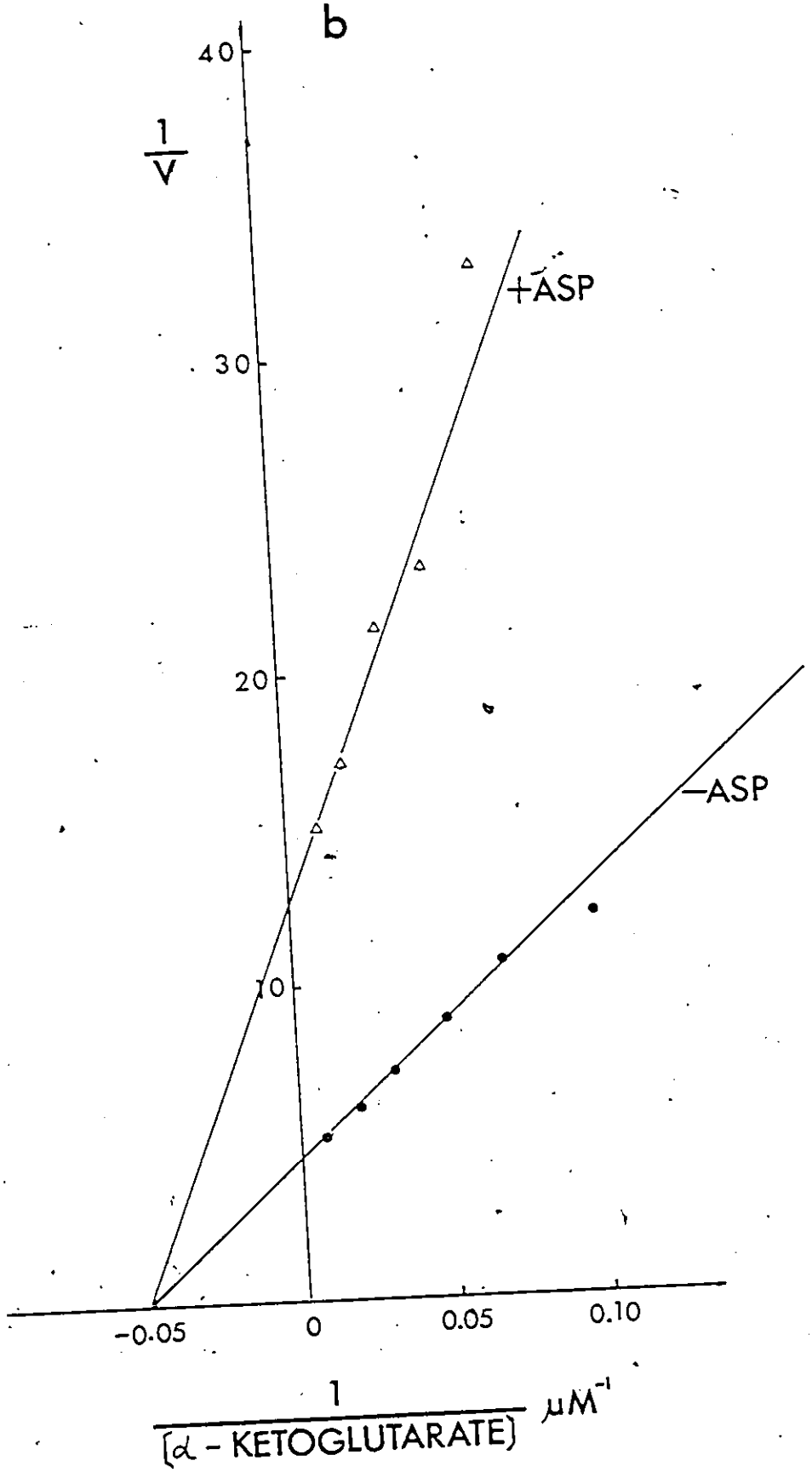

b
40
30
20
10
1/V
+ASP
−ASP
-0.05
0
0.05
0.10
1 / [α - KETOGLUTARATE] μM⁻¹

Table VI

Types of inhibition by L-aspartate and inhibition constants

Enzyme	Varied Substrate	Type of inhibition	K_i (M)
A	Tyrosine	uncompetitive	1.96×10^{-3}
	α-ketoglutarate	noncompetitive	1.67×10^{-3}
B	Tyrosine	noncompetitive	6.20×10^{-3}
	α-ketoglutarate	noncompetitive	4.86×10^{-3}

The double reciprocal plots according to Lineweaver and Burk were used and L-aspartate was present at a fixed 3 mM concentration. The enzyme activity was assayed as tyrosine aminotransferase.

tyrosine) is an uncompetitive inhibitor of enzyme A with aspartate as the varied substrate (Fig. 33). With enzyme B both inhibitions were of the non-competitive type (Table VI).

The significance of uncompetitive inhibition is generally obscure although it is thought by some to reflect binding of the inhibitor with the ES complex (Dixon and Webb, 1964). This, in fact, is supported by Fig. 29a where the inhibition by aspartate increases with increasing substrate (tyrosine) concentration. Thus it seems that in enzyme A one site (the active site) serves for the binding of both aspartate and tyrosine (different sites would generate noncompetitive inhibition) and this would be in agreement with the multiple substrate specificity of enzyme A. The presence of a single site for tyrosine and aspartate agrees with the same tentative conclusion derived from the effects of pH on Km (see previous section).

On the other hand, the noncompetitive inhibition of enzyme B by aspartate with tyrosine as the varied substrate (Table VI) underlines the difference between enzyme A and enzyme B with respect to aspartate binding: aspartate binds enzyme B at a site other than the active site and aspartate is not a proper substrate for enzyme B.

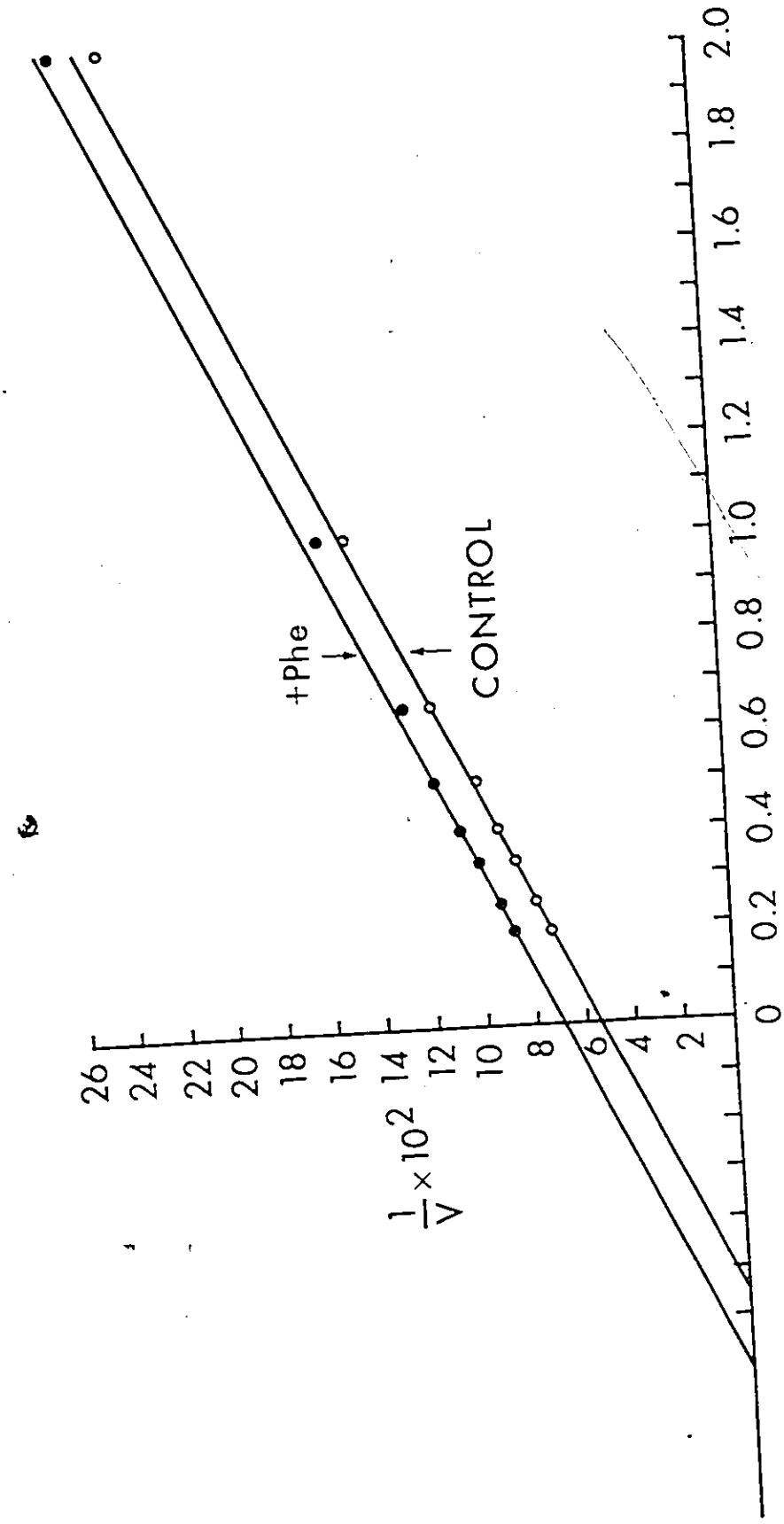
8. pH-Activity Curves

In general, enzymes are only active over a limited range of pH and in most cases a definite optimum pH is observed. The pH

Figure 33

Inhibition by phenylalanine of enzyme A assayed as aspartate aminotransferase

Lineweaver-Burk plots with aspartate as the varied substrate and phenylalanine (10mM) as the inhibitor.


+Phe
CONTROL
$\frac{1}{V} \times 10^2$
$\frac{1}{[S]}$, mM^{-1}
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2
4
6
8
10
12
14
16
18
20
22
24
26

optima of enzymes A and B with different substrates are shown in Fig. 34 which illustrates the dependence of activity of the two enzymes on pH. The optima for enzyme A with its four substrates vary from 8.0 (toward aspartate) to 9.0 (toward tyrosine) with 8.5 being the pH optimum toward phenylalanine and tryptophan (Fig. 34A). For enzyme B the pH optimum toward tyrosine drops to 8.0 with 7.5 and 8.0 being the optima toward phenylalanine and tryptophan (Fig. 34B). In the view of the physical evidence of homogeneity the differences in the pH optima of each enzyme with its substrates reflect the differences in the pK values of the substrates rather than the existence of different enzymes.

9. Molecular Weight Estimation and Subunit Structure Study

The molecular weights of enzymes A and B were estimated by gel filtration. When fraction I (from DEAE-cellulose column chromatography) was passed through a column of Sephadex G-200 it resolved into two peaks (Fig. 35). Their elution volumes corresponded to those obtained when pure enzymes A and B were individually or as a mixture passed through the same column. The molecular weights of the two enzymes were calculated by gel filtration in a calibrated column of Sephadex G-200 and found to be 82,000 and 88,000 for A and B, respectively (Fig. 36).

The subunit structure was determined by sodium dodecyl sulfate (SDS) gel electrophoresis. Each enzyme on acrylamide gel electrophoresis in the presence of SDS yielded a single protein

Figure 34

pH-activity curves for enzymes A and B

Dependence of activity on pH of the purified enzymes A (left) and B (right). The following buffers were used: pH 6.0-6.5, citrate-phosphate; pH 7.0-9.0 Tris. HCl; 9.5-11.0, glycine-NaOH. The enzymes were assayed with tyrosine (• - •), phenylalanine (Δ - Δ), tryptophan (o - o) and enzyme A in addition with aspartate (▲ - ▲). The standard abbreviations of amino acids, followed by the letter A indicate the respective aminotransferases.

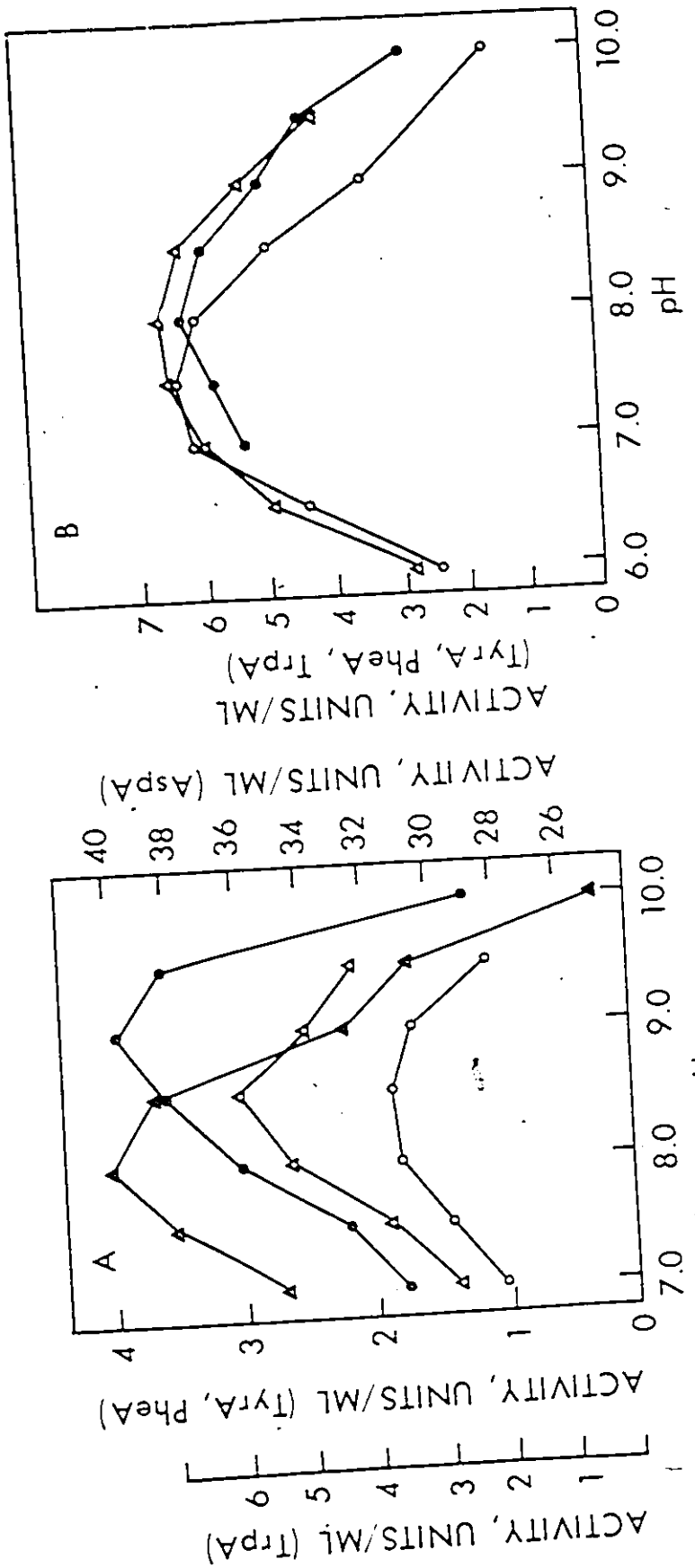

ACTIVITY, UNITS/ML (TrpA)
ACTIVITY, UNITS/ML (TyrA, PheA)
ACTIVITY, UNITS/ML (AspA)
pH
A
B
ACTIVITY, UNITS/ML
(TyrA, PheA, TrpA)
pH

Figure 35

Gel Filtration of Fraction I

Gel filtration of fraction I on Sephadex G-200 (fine grade) column (1.5 cm x 100 cm). Fractions 1-30 were about 1.5 ml each and the remaining fractions about 0.5 ml each. 150 µg of protein was applied on the column and elution was carried out with the buffer described in the "Materials and Methods".

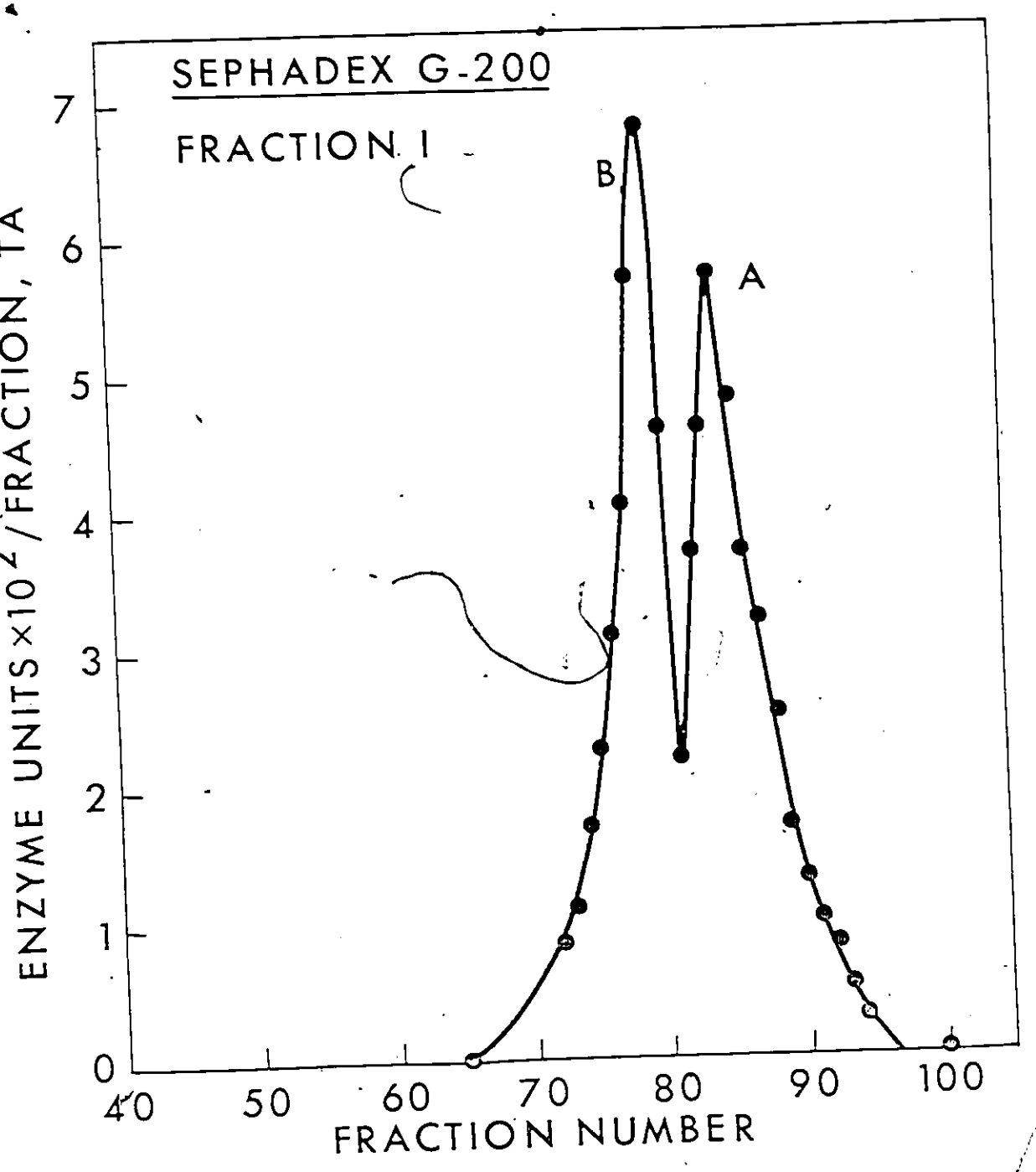
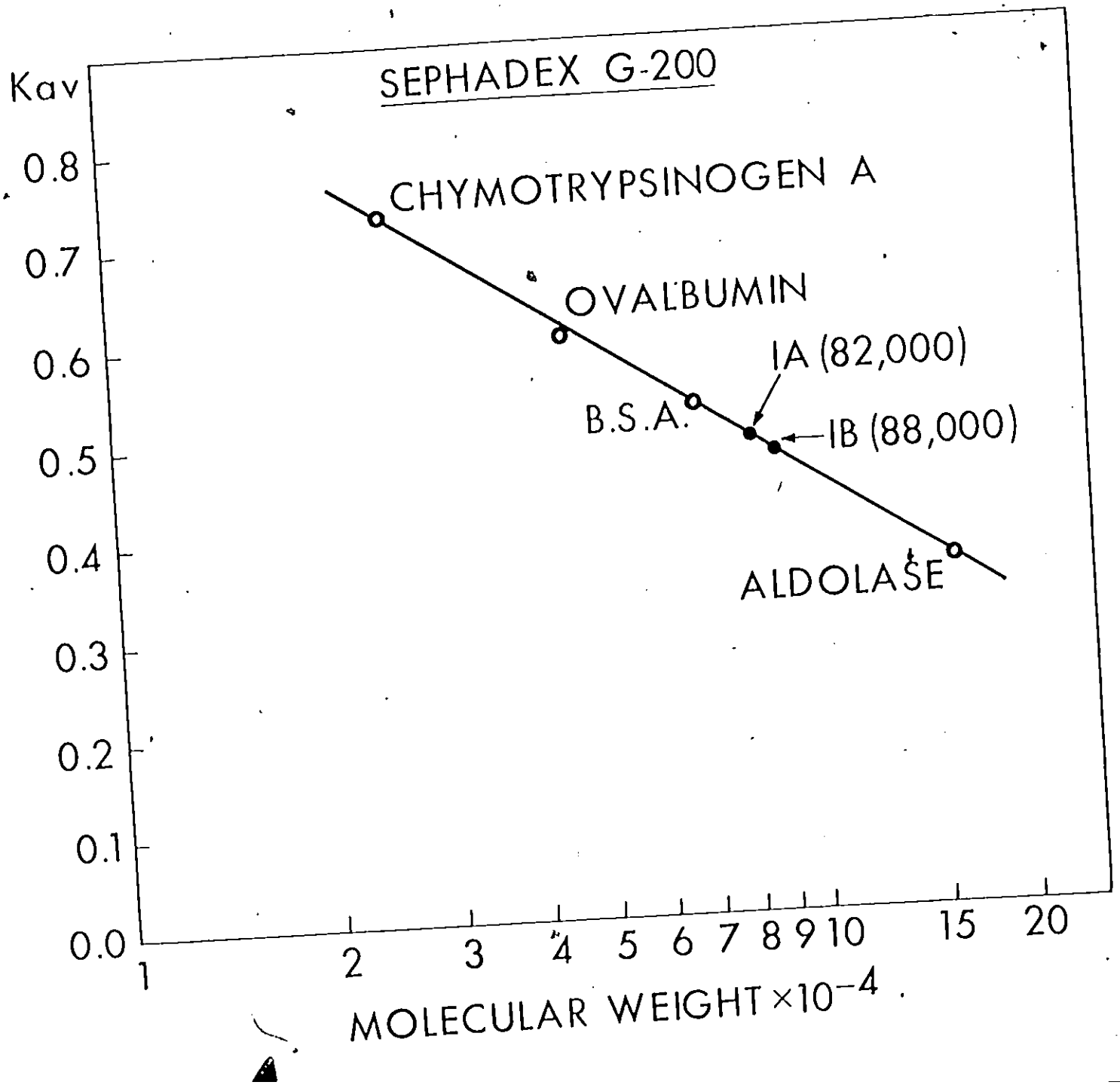

SEPHADEX G-200
FRACTION I
B
A
ENZYME UNITS ×10² / FRACTION, TA
0
1
2
3
4
5
6
7
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 36

Standard curve of molecular weight estimation

Estimation of the molecular weights of enzyme A and B by gel filtration in the column described in Fig. 35. The column was calibrated with four known molecular weight protein standards: chymotrypsinogen A, ovalbumin, B.S.A. (bovine serum albumin) and aldolase. The Kav value was calculated with the fromula: Kav = $\frac{Ve - Vo}{V_t - Vo}$ where Ve = elution volume, Vo = elution volume for Blue Dextran 2000, and V_t = total bed volume.


SEPHADEX G-200
Kav
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CHYMOTRYPSINOGEN A
OVALBUMIN
IA (82,000)
B.S.A.
IB (88,000)
ALDOLASE
1
2
3
4
5
6
7
8
9
10
15
20
MOLECULAR WEIGHT ×10⁻⁴

band which corresponded to a molecular weight of 42,000 to 45,000 (range of five experiments). Since the molecular weights of enzymes A and B were estimated to be about 82,000 and 88,000, respectively, each appears to consist of two subunits of equal size. In two experiments, one of which is shown in Fig. 37, a slightly higher subunit molecular weight for enzyme B (43,000) than for enzyme A (42,000) was calculated. The failure to calculate consistently a higher subunit molecular weight for enzyme B than for enzyme A is due to the short distance traveled by proteins of so similar size in the SDS gel (about 1.7 cm in 5 hours at 8 ma per gel).

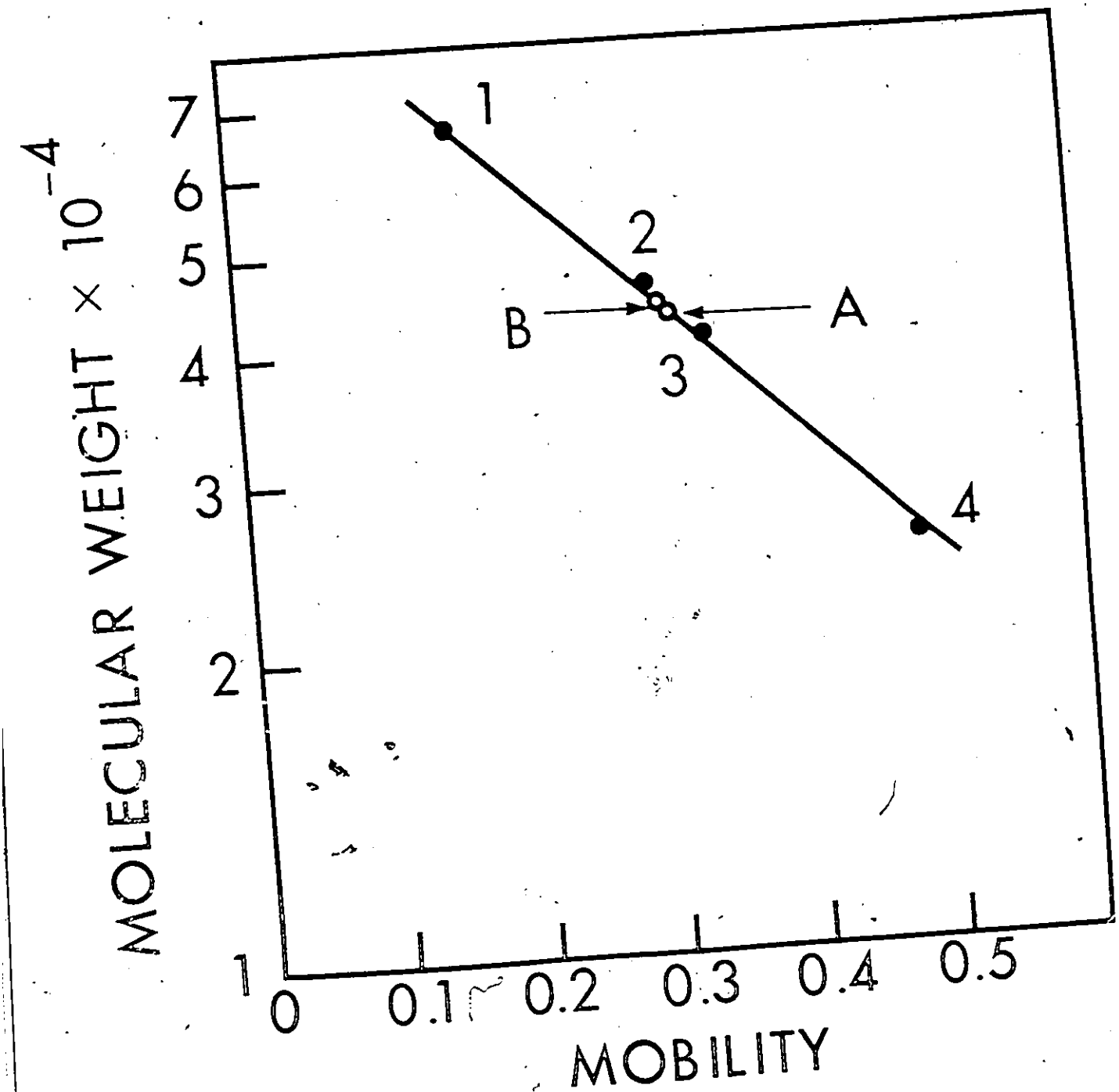
10. Electrophoresis at Various Gel Concentrations

The method of Hedrick and Smith (1968) affords a way to establish whether the separations observed on gel are due to difference in charge, size or both. When the log of protein mobility relative to the dye front is plotted versus acrylamide gel concentration, size isomeric proteins give a family of nonparallel lines extrapolating to a common point in the vicinity of 0% gel concentration while charge isomeric proteins give a parallel family of lines. Proteins differing in both charge and size give nonparallel lines intersecting at gel concentrations other than 0%. When enzymes A and B were applied separately on acrylamide gels at various concentrations (from 5 to 10%) single protein bands were observed and Fig. 38 shows two nonparallel

Figure 37

Molecular weight Estimation by S.D.S. acrylamide gel Electrophoresis

Estimation of the molecular weights of enzymes A and B by gel electrophoresis in the presence of sodium dodecyl sulfate (S.D.S.). The standard proteins used were: 1. bovine serum albumin; 2. ovalbumin; 3. aldolase; 4. chymotrypsimogen A.


MOLECULAR WEIGHT × 10^{-4}
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
MOBILITY
1
2
3
4
A
B

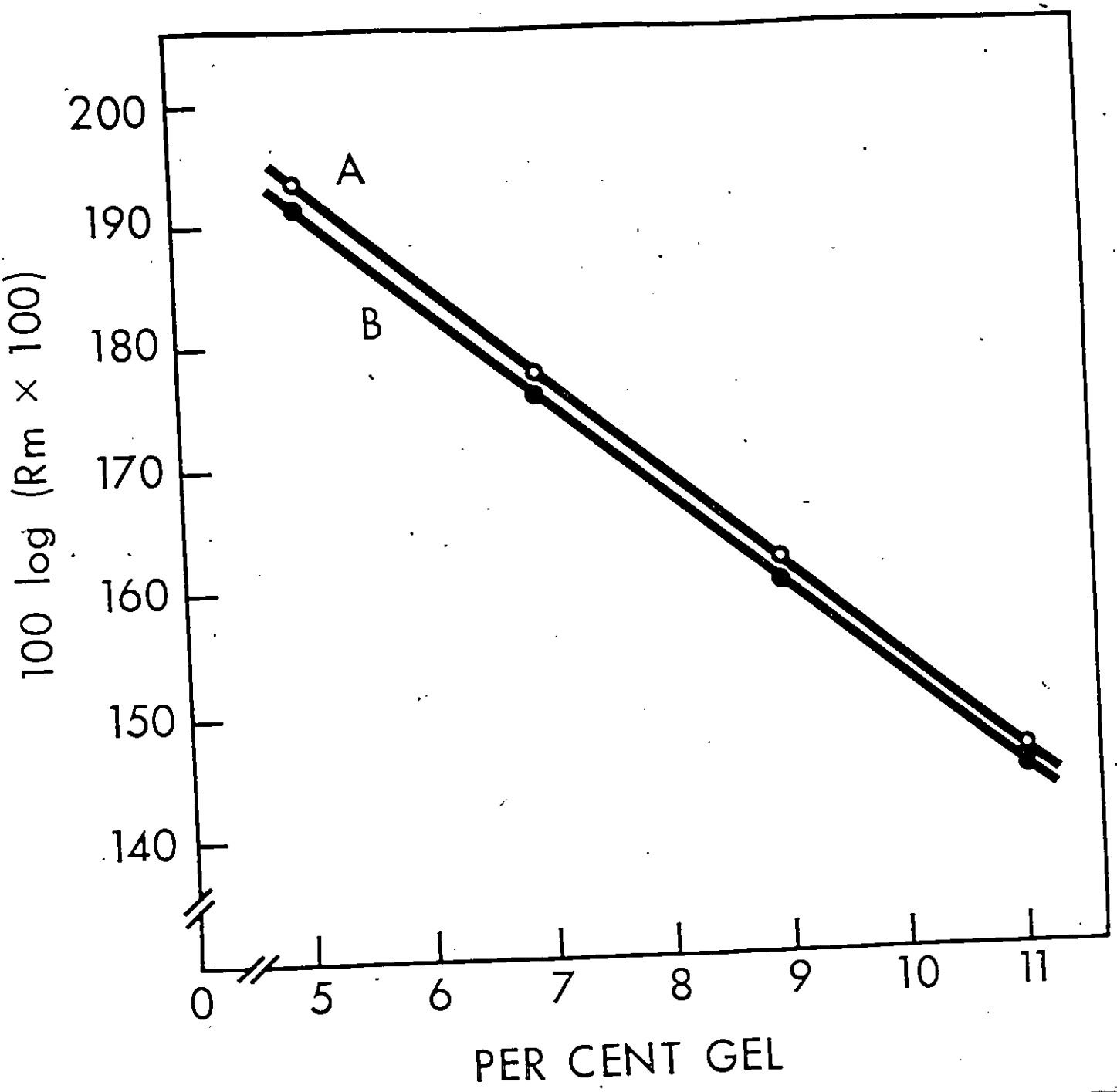
lines which on extrapolation intersect at about 18.5% acrylamide gel concentration. The nonparallel lines of Fig. 38 indicate that the separation of enzymes A and B is due to both charge and size differences. The charge difference made isoelectric separation possible and the size difference made separation by gel filtration possible.

11. Electrophoretic Behaviours of Enzymes A and B in Polyacrylamide Gels.

Enzyme A appears to be a slightly more acidic protein than enzyme B (Fig. 7, 8). Hence, it would be expected to migrate faster than enzyme B in the basic Davis gels toward the anode. Being of lower molecular weight than enzyme B, and presumably smaller, enzyme A would be expected to migrate faster in the gels for this reason too. However, it is enzyme B that displays a higher mobility (Fig. 10). The cause for this unexpected behaviour must probably be sought in the unreliability of isoelectric focusing as a means for determining true isoelectric points. It has been reported (Illingworth, 1972) that the isoelectric point of a single protein band in the presence of commercial carrier ampholytes might be in error due to their adsorption on to other polyelectrolytes such as proteins, which might also result in the formation of pseudoisoenzymes. The possibility of false isoelectric points may have materialized in this instance. It is possible that the removal of "sticky"

Figure 38

The effect of different acrylamide concentrations on the mobilities of the purified enzymes A and B [lines A and B, respectively]. The lines on extrapolation intersect at about 18.5% acrylamide concentration. Rm, relative mobility [Hedrick and Smith, 1968]. Each point is the average of triplicate samples.


200
190
180
170
160
150
140
100 log (Rm × 100)
A
B
0
5
6
7
8
9
10
11
PER CENT GEL

carrier ampholytes (Illingworth, 1972) from either one or both enzymes by ultrafiltration dialysis restored the original order in the relative magnitude of the isoelectric points of the native proteins and resulted in the mobilities observed in gel electrophoresis. The relative mobilities observed in gels (Fig. 10) may simply mean that the difference in the isoelectric points is the reverse of that suggested by isoelectric focusing (Figs. 7,8) and is sufficiently large to override the size difference, thus causing the larger enzyme B to migrate faster than the smaller enzyme A.

12. Amino Acid Composition

The amino acid composition of the two enzymes was looked into with the purpose of obtaining some insight into the chemical constitution of the two enzymes. Their similarity in size and especially in charge suggested that they might be similar proteins. The data of Table VII suggested similarities in the number of many amino acid residues. The significant differences in the number of amino acid residues appear to be limited to glutamic acid, glycine, alanine, valine and possibly to lysine and phenylalanine.

The possibility that enzyme A might be obtained by controlled proteolysis of enzyme B was examined in the experiments described below.

13. Conversion of Enzyme B to Enzyme A by Subtilisin Treatment

Incubation for 30 minutes of enzyme B at 37^{o} in the presence

Table VII

Amino acid composition of enzymes A and B

The figures indicate the nearest integral number of amino acid residues per molecule of enzyme. Approximately 40 µg of enzyme A and 18 µg of enzyme B were hydrolysed.

Amino acid	Enzyme A	Enzyme B	Difference (enzyme B - enzyme A)
Asp	86	83	-3
Thr	42	39	-3
Ser	37	40	+3
Glu	89	109	+20
Pro	33	39	+6
Gly	61	77	+16
Ala	87	101	+14
Val	50	63	+13
Met	15	14	-1
Ile	30	34	+4
Leu	74	71	-3
Tyr	12	14	+2
Phe	38	30	-8
His	12	14	+2
Lys	35	46	+11
Arg	43	37	-6

of subtilisin resulted in a nearly six-fold increase in its very low basal aspartate aminotransferase activity in the first 10 minutes and the activity declined only slightly thereafter (Fig. 39). The tyrosine aminotransferase activity declined rapidly in the first 10 minutes to about 50% of its zero time value and remained practically unchanged thereafter. Incubation in the absence of subtilisin (control) resulted in some loss of tyrosine aminotransferase activity, whereas the aspartate aminotransferase activity did not change, in agreement with the relative heat stabilities of these two activities (Fig. 19). These results suggested that enzyme A with its typical aspartate aminotransferase activity was the product of the controlled proteolysis of enzyme B by subtilisin. This was demonstrated by gel electrophoresis of subtilisin-treated enzyme B. Figure 40 (gel 2) shows clearly an activity band with the mobility of enzyme A. This band is absent from the zero time control (gel 1) and the subtilisin control (gel 4). Incubation with subtilisin concentrations double and quadruple that used in Fig. 39 resulted in the generation of much lower net aspartate aminotransferase activity (Fig. 41). This is probably due to more advanced proteolysis of enzyme B resulting in lower aspartate aminotransferase activity.

Another proteolytic enzyme, trypsin, was also tried without success, in concentrations varied fron 0.2 - 40 µg. Both tyrosine and aspartate aminotransferase activities were lower than that

Figure 39

Generation of Aspartate Aminotransferase activity from Enzyme B by limited proteolysis with subtilisin

Enzyme B (1.8 units) was incubated at 37°C in 4.0 ml of 0.2 M Tris. HCl buffer, pH 8.0, in the presence of bovine serum albumin (0.3 mg/ml) and 0.2 µg of subtilisin. Aliquots (0.5 ml) were withdrawn at the indicated times and assayed for enzyme activities. Control, in the absence of subtilisin; AspA, aspartate aminotransferase; TyrA, tyrosine aminotransferase.

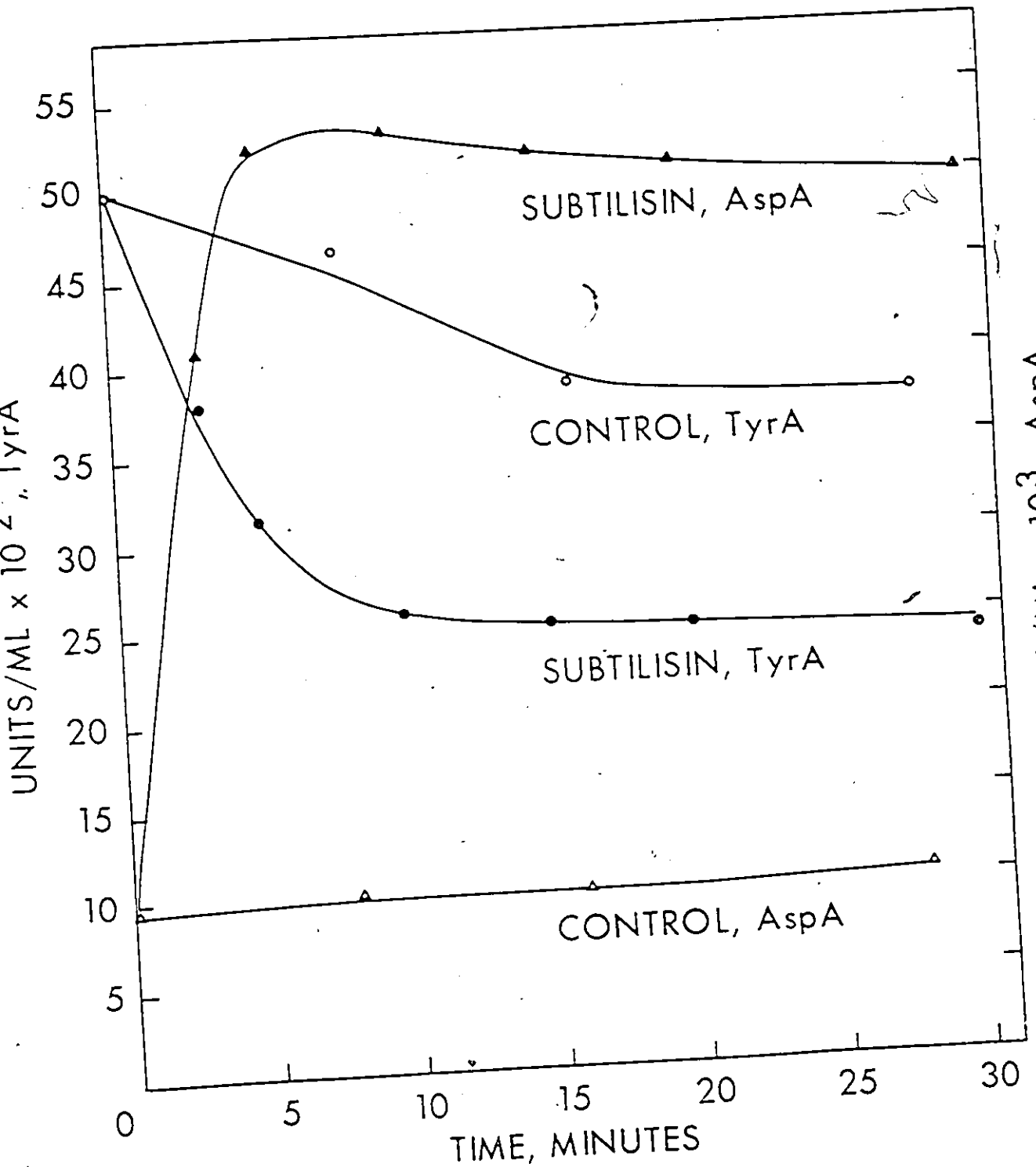

SUBTILISIN, AspA
CONTROL, TyrA
SUBTILISIN, TyrA
CONTROL, AspA
UNITS/ML x 10^2, TyrA
UNITS/ML x 10^3, AspA
TIME, MINUTES
0
5
10
15
20
25
30
35
40
45
50
55

Figure 40

Acrylamide gel Electrophoresis of Enzyme B After Proteolysis with Subtilisin

Generation of enzyme A (upper band) after limited proteolysis of enzyme B (lower band). Enzyme B was incubated in the presence of subtilisin exactly as described in legend of Fig. 38. Aliquots were withdrawn after 5, 10 and 15 min. of incubation were pooled and the pooled mixtures were subjected to acrylamide gel electrophoresis for 4 hours at 3 mA per gel. The gel then were stained for tyrosine aminotransferase activity. Gel 1, enzyme B (zero time); Gel 2, enzyme B after incubation with subtilisin; Gel 3, mixture of authentic enzyme A and B; Gel 4, enzyme B after incubation in the absence of subtilisin; Gel 5, enzyme B incubated in the presence of subtilisin plus authentic enzyme A. About 0.15 µg of original (before incubation) enzyme B, 1 µg of authentic enzyme A, and 0.3 µg of enzyme B were applied on the gels.

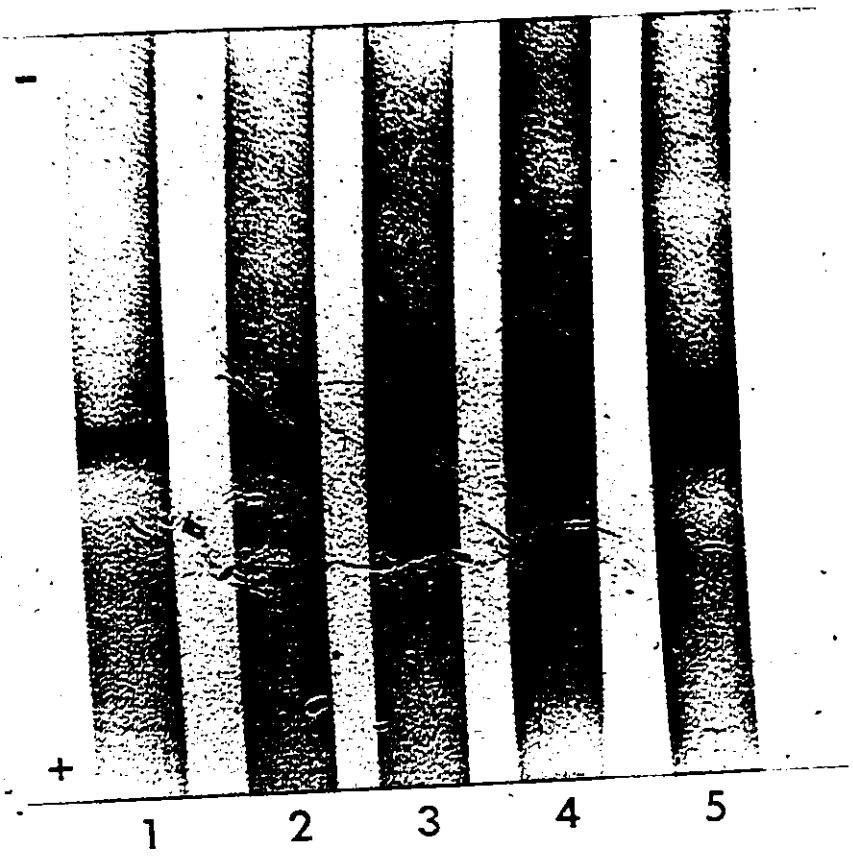
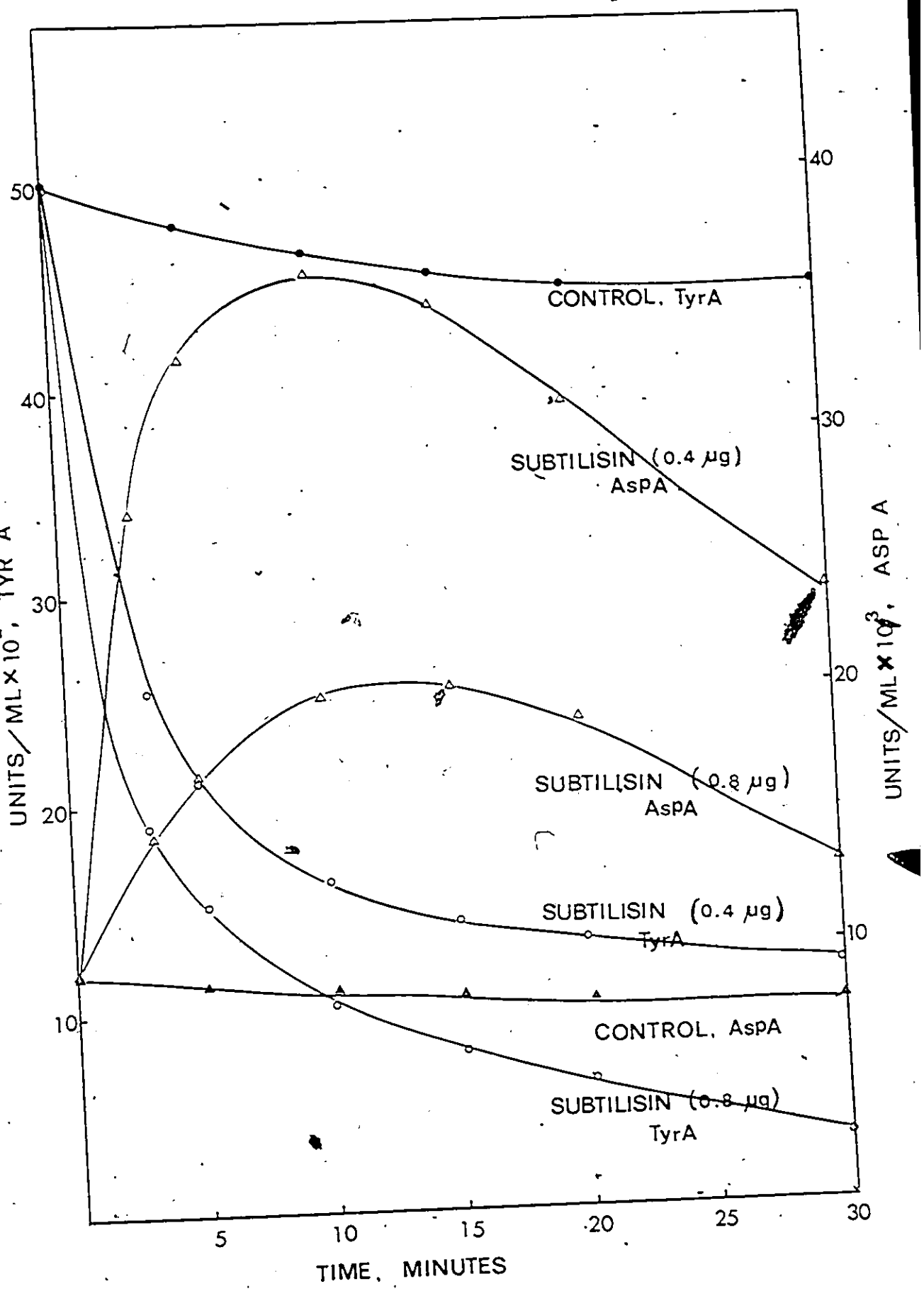

-
+
1
2
3
4
5

Figure 41

Proteolysis of Enzyme B at Different Concentrations of Subtilisin

Conditions were the same as in Figure 39 except that the amount of subtilisin was increased to 0.4 µg and 0.8 µg.


CONTROL, TyrA
SUBTILISIN (0.4 µg) AspA
SUBTILISIN (0.8 µg) AspA
SUBTILISIN (0.4 µg) TyrA
CONTROL, AspA
SUBTILISIN (0.8 µg) TyrA
UNITS/ML×10², TYR A
UNITS/ML×10³, ASP A
TIME, MINUTES
50
40
30
20
10
40
30
20
10
5
10
15
20
25
30

of the zero time control (not shown). Trypsin hydrolyzes peptide bonds formed from the carbonyl groups of lysine and arginine. On the other hand the specificity of subtilisin is very broad.

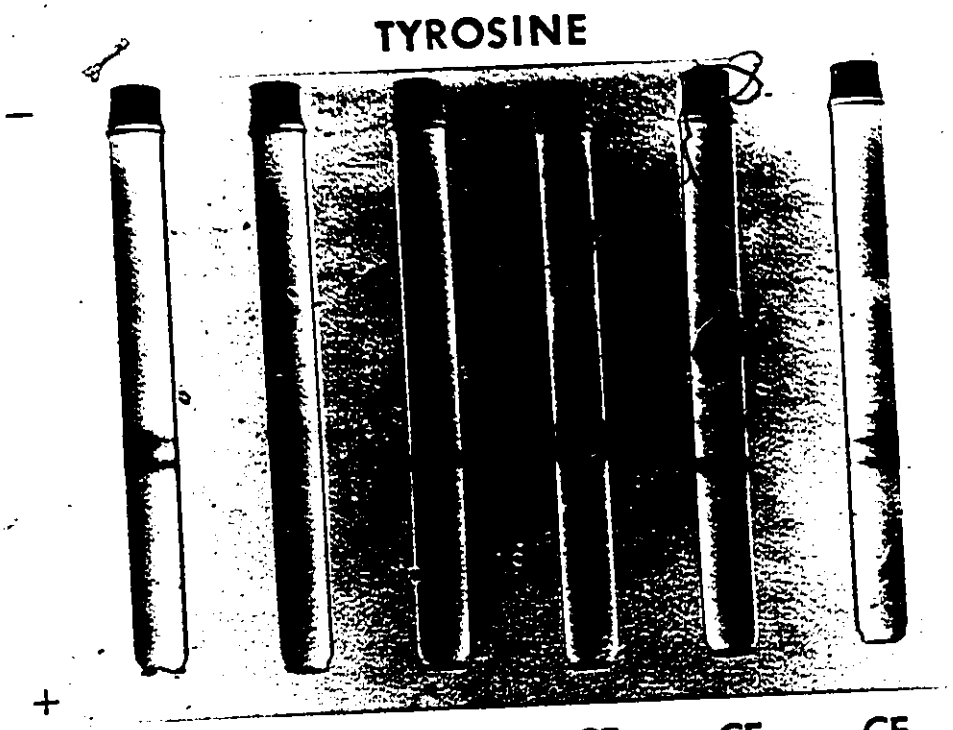
D. Detection of Enzymes in Fresh Crude Extracts of Other Strains

In addition to detecting the enzymes in the fresh crude extracts of E. coli B by polyacrylamide gel electrophoresis and the staining technique (Fig. 16) the enzymes were also detected in the fresh crude extracts of E. coli Crookes and the tyrosine auxotroph M 83-8 (not shown). Interestingly, the activity bands of the two strains corresponding to enzyme B migrated with different mobilities in the gels, the one from strain B being the faster. Electrophoresis of a mixed extract and staining for tyrosine aminotransferase activity resulted in three activity bands (Fig. 42). The activity bands corresponding to enzyme A had the same mobility from the two strains. Apparently, the larger protein (enzyme B) may be slightly different in different strains.

Figure 42

Polyacrylamide Gel Electrophoresis of Purified Enzymes A and B, and Crude Extracts of E. coli B and Crookes

Polyacrylamide gel electrophoresis (in 5 x 75 cm tubes) of fraction I (after DEAE-cellulose chromatography), purified enzymes A and B, crude extracts from E. coli Crookes [CE (Crookes)] and fresh E. coli B [CE (B)]. All the gels were pre-run for 1 hour at 3 mA per gel. Fraction I, 7.5 µg; enzyme A, 0.48 µg; CE (Crookes), 38 µg; CE (B), 100 µg. Stain for tyrosine aminotransferase activity.


TYROSINE
−
+
I
A
Crookes
B
CE
(Crookes)
CE
(B)
CE
(Crookes+B)

IV GENERAL DISCUSSION AND CONCLUSIONS

The present investigation demonstrated the separation of two apparently structurally similar aminotransferases from E. coli which were purified to homogeneity by the criterion of gel electrophoresis. These two enzymes were designated as enzymes A and B for convenience. Enzyme A is an aspartate aminotransferase (EC 2.6.1.1) better known as transaminase A (Rudman & Meister, 1953). Enzyme B is an aromatic-amino-acid aminotransferase and has been recently assigned the number EC 2.6.1.57 (Enzymes Nomenclature: Recommendations (1972) of the International Union of Pure and Applied Chemistry and International Union of Biochemistry. Supplement No. 1: Additions and Corrections (1975). Biochim. Biophys. Acta. 429 (1976), 1-45). Enzymes A and B showed the following differences in their properties:

a) Substrate specificity.-
Enzyme A is specific for aspartate and the aromatic amino acids and enzyme B for the aromatic amino acids only.

b) K_m and V_{max} values (Table V).

c) Heat sensitivity with different amino acids as substrates.-
With tyrosine and phenylalanine as substrates,

enzyme A is more heat-stable than enzyme B after 10 minutes at 55° and with aspartate as a substrate, enzyme A is heat-stable under the same conditions (Fig. 19).

d) Optima pH for the same substrate (Fig. 34).

e) Repression by tyrosine.-
When organisms were grown in the presence of L-tyrosine, enzyme B is selectively repressed whereas enzyme A is unaffected.

f) Difference in electrophoretic mobility.-
Enzyme B has a slightly higher electrophoretic mobility than A towards the anode.

g) Although the isoelectric points of enzymes A and B obtained from the isoelectric focusing experiment, may not represent their true isoelectric points (Results and Discussion), they still indicate a slight difference between them.

h) Difference in molecular weights of native protein and subunits.-
The two enzymes have similar but definitely different molecular weights, the latter being 82,000 and 88,000 for enzymes A and B, respectively. Each enzyme consists of equal subunits with the molecular weights of 42,000 to 45,000 for enzymes A and B respectively.

i) Amino acid composition.-

The enzymes show a broad similarity in amino acid composition.

The similarities in size and charge readily explain the failure of Rudman and Meister (1953) to distinguish between the two enzymes in their classic study on transamination in E. coli with the then available techniques.

The isoelectric focusing experiment (Fig. 14) indicates that enzyme A is specific for the aromatic amino acids and aspartate and enzyme B is specific for the aromatic amino acids only. From the results of the kinetic study (Table V), V_{max} values clearly indicate that the major activity of enzyme A is towards aspartate with minor but significant activities towards the aromatic amino acids. All aspartate aminotransferases so far studied show significant activity towards the aromatic amino acids (Shrawder and Martinez-Carrion, 1972; Miller and Litwack, 1971; Forest and Wightman, 1973). The major activity towards the aromatic amino acids resides in enzyme B.

Isoelectric focusing and acrylamide gel electrophoresis failed to resolve the activity of enzyme A towards aspartate from the activity towards the aromatic amino acids in enzyme A (Figures 14A, 15). With the latter method, a single protein band was obtained even when the acrylamide concentration was varied from 5 to 11%. Thus, the two kinds of activity could be

distinguished by neither size nor charge differences. Collier and Kohlhaw (1972) reported the nonidentity of aspartate and aromatic amino acid activities in transaminase A, a conclusion based on experiments with crude extracts. Our own experiments confirm the original specificity of transaminase A (aspartate aminotransferase) as given by Rudman and Meister (1953) but, in addition, demonstrated the presence of a distinct enzyme for the aromatic amino acids.

Enzyme B, formerly reported as form IB by Mavrides and Orr (1974) is an aromatic-amino-acid aminotransferase and its "dissection" from enzyme A is of some consequence regarding the actual aminotransferase involved in the regulation of tyrosine and phenylalanine biosynthesis. It has been reported (Silbert et al, 1963) that transaminase A of E. coli is specifically repressed by L-tyrosine and so is the new enzyme B (Table IV). The enzyme, assayed only as tyrosine and phenylalanine aminotransferases, was found in the crude extract to be heat-labile and to account for most of the activity towards the two aromatic amino acids (Silbert et al., 1963). The heat-lability and substrate specificity conform with the properties of enzyme B. It is clear that by assaying only for aromatic aminotransferase activities in the crude extract, the above-mentioned investigators (Silbert et al, 1963) were not observing the repression of transaminase A but of the new enzyme B. They were led to their conclusion because of

omission to assay for aspartate aminotransferase. Accordingly, references in the literature on the repression by tyrosine of transaminase A assayed as tyrosine aminotransferase (which is the usual practice) reflect the repression of enzyme B, not of transaminase A, which is not repressed by tyrosine (Collier and Kohlhaw 1972; Mavrides, unpublished results).

In the study of Silbert et al, (1963), evidence was presented purporting to show that there exists in E. coli a specific phenylalanine aminotransferase distinct from transaminase A with the following properties studied in crude extracts: 1) relatively heat-stable at 60° (25-30% loss of activity after 10 minutes); 2) non-repressible by tyrosine or any other amino acids; 3) it contributed about 20% of the total activity towards phenylalanine; 4) its resolution from the activity towards tyrosine could not be achieved by ammonium sulfate fractionation and DEAE-cellulose chromatography. These are properties of enzyme A which: 1) is relatively heat-stable with phenylalanine (30% loss of activity at 55°, Fig. 19); 2) is not repressed by tyrosine (Table IV); 3) contributes little to the total activity towards phenylalanine (Table V); and 4) was separated with some difficulty from the strictly aromatic activity of enzyme B, with techniques not used by or unavailable to the previous investigators (Silbert et al, 1963). It is conceivable that these investigators were describing properties of transaminase A itself against the background of the

unsuspected enzyme B and attributed them to a specific phenylalamine aminotransferase which has never been purified and charactrized. In polyacrylamide gel electrophoresis of crude extracts we have never detected a phenylalanine aminotransferase activity band in addition to the bands due to the two enzymes studied in this Thesis.

The conversion of enzyme B to enzyme A by controlled proteolysis in the presence of subtilisin is of interest. The proteolytic removal of polypeptide tract(s) appears to result in the generation of aspartate aminotransferase activity. It is unlikely that the process is of physiological significance. Since enzyme B is selectively repressed by L-tyrosine whereas enzyme A is unaffected, the two enzymes are most probably products of two genes different only in a short, probably terminal, nucleotide sequence. The enzymological implications of the conversion are of interest also. It clearly seems that cleavage of polypeptide(s) from enzyme B is sufficient to generate a new activity, aspartate aminotransferase, typical of enzyme A. In this respect, the conversion is reminiscent of the long known zymogen-enzyme relationships. To the best of our knowledge this is the first instance of an enzyme being converted to another distinct, physiologically occurring enzyme by controlled proteolysis.

Enzyme B has been included as a new enzyme with the recommended name aromatic-amino-acid aminotransferase (EC 2.6.1.57)

in Supplement No. 1 to the Enzyme List (Enzyme Nomenclature: Recommendations (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry. Supplement No. 1: Additions and Corrections (1975). Biochim., Biophys. Acta 429 (1976), 1-45).

REFERENCES

Abderhalden, E., amd Stix, W. (1923) Heppe-Seyler's Z. Physiol. Chem., 129, 143-156

Braunstein, A.E., and Kritzmann, M.G. (1937) Enzymologia, 2, 129-146.

Braunstein, A.E. (1939) Enzymologia, 7, 25-52.

Brewbaker, J.L., Upadhya, M.D., Mäkinen, Y., and Macdonald, T. (1968) Physiologia Plantarum, 21, 930-940.

Chesne, S., and Pelmont, J. (1973) Biochimie, 55, 237-244.

Cleland, W.W. (1963) Biochim. Biophys. Acta, 67, 188-196.

Cleland, W.W. (1963) Biochim. Biophys. Acta, 67, 104-137.

Cohen, P.P. (1939) Biochem. J., 33, 1478.

Cohen, P.P. (1940) J. Biol. Chem., 136, 565-584.

Collier, R.H., and Kohlhaw, G. (1972) J. Bacteriol., 112, 365-371.

Cotton, R., and Gibson, F. (1965) Biochim. Biophys. Acta, 100, 76-88.

Cotton, R., and Gibson, F. (1967) Biochim. Biophys. Acta, 147, 222-237.

Davis, B.J. (1964) Ann. N.Y. Acad. Sci., 121, 404-427.

Dayhoff, M.O., and Eck, R.V. (1968) Atlas of Protein Sequence and Structure. National Biochemical Research Foundation, Silver Spring, Md.

Dodgson, K.S., Spancer, B., and Williams, K. (1955) Biochem. J., 61, 374-380.

DeMoss, J.A. (1965) J. Biol. Chem., 240, 1231-1235.

Diamondstone, T.I. (1966) Anal. Biochem., 16, 395-401.

Dixon, M., and Webb, E.C. (1964) Enzymes, 2nd Ed., 135-145; 315-324, Academic Press, New York.

Enzyme Nomenclature: Recommendations (1972) of the International Union of Pure and Applied Chemistry and International Union of Biochemistry. Supplement 1: Correction and Addition (1975). Biochim. Biophys. Acta. 429 (1976), 1-45.

Feldman, L.I., and Gunsalus, I.C. (1950) J. Biol. Chem. 187, 821-830.

Florkin, M., and Stotz, E.H. (eds) (1964) Comprehensive Biochemistry, Elsevier Publishing Co., New York, 15, 138-199.

Florkin, M., and Stotz, E.H. (eds) (1973) Commission on Biochemical Nomenclature in "Comprehensive Biochemistry", 13, 23-25 Elsevier, Amsterdam.

Forest, J.C. and Wightman, F. (1973) Can. J. Biochem. 50, 813-829.

Gibson, M.I., and Gibson, F. (1962) Nature 195, 1173-1175.

Gibson, M.I., and Gibson, F. (1962) Biochim. Biophys. Acta. 65, 160-163.

Gibson, M.I., and Gibson, F. (1964) Biochem. J. 90, 248-256.

Gibson, F., and Pittard, J. (1968) Bacteria Review 32, 465-492.

Guirard, B.M., and Snell, E.E. (1964) in Comprehensive Biochemistry (Florkin, M., and Stotz, Z.H. eds) 15, 138-199, Elsevier, Amsterdam.

Handbook of Chemistry and Physics (1969) 49 edition, 703. The Chemical Rubber Publishing Co., Cleveland.

Hedrick, J.L., and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155-164.

Herbst, R.M., and Engel, L.L. (1934) J. Biol. Chem. 107, 505-512.

Illingworth, J.A. (1972) Biochem. J. 129, 1125-1130.

Im, S.W.K., Davidson, H., and Pittard, J. (1971) J. Bacteriol. 108, 400-409.

Ito, J., and Crawford, I. (1965) Genetics 52, 1302-1316.

Karmen, A. (1955) J. Clin. Invest. 34, 131-133.

Knoop, F. (1910) Z. Physiol. Chem. 67, 489-502.

Layne, E. (1957) Methods in Enzymology (Colowick, S.P., and Kaplan, N.O. eds) 3, 451-454, Acad. Press, New York.

Lin, E.C.C., Pitt, B.M., Civen, M., and Knox, W.E. (1968) J. Biol. Chem. 233, 668-673.

Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658-666.

Lingens, F., and Müller, G. (1967) Z. Naturforsch. 22, 991-993.

Litwack, G., and Cleland, W.W. (1968) Biochem. 7, 2072-2079.

Lowry, O.H., Rosenbrough, N.J., Fan, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Margolin, P. (1967) Metabolic Regulation V, 389-447.

Mavrides, C., and Orr, W. (1974) Biochim. Biophys. Acta. 336, 70-78.

Miller, J.E., and Litwack, G. (1971) J. Biol. Chem. 246, 3234-3240.

Monod, J., Cohen-Bazire, G., and Cohen, M. (1951) Biochim. Biophys. Acta 7, 585-599.

Needham, D.M. (1930) Biochem. J. 24, 208-227.

Orectec Incorporated, Bulletin AN32 (1970).

Pittard, J., and Gibson, F. (1970) Current Topics in Cellular Regulation, 2, 29-36.

Rehfeld, D.W., and Tolbert, N.E. (1972) J. Biol. Chem. 247, 4803-4811.

Rivera, A., and Srinivasan, P.R. (1962) Pro. Natl. Acad. Sci. U.S. 48, 864-867.

Rudman, D., and Meister, A. (1953) J. Biol. Chem. 200, 591-604.

Ryan, E., Bodley, F., and Fottrell, P.F. (1972) Phytochemistry 11, 957-963.

Schepartz, B. (1969) Analytical Biochem. 30, 443-448.

Shaw, C.R. and Prasad, R. (1970) Biochem. Genetics 4, 297-320.

Shrawder, E. and Matinez-Carrion, M. (1972) J. Biol. Chem. 247, 2486-2492.

Silbert, D.F., and Jorgensen, S.E., and Lin, E.C.C. (1963) Biochim. Biophys. Acta 73, 232-240.

Swingle, S.M., and Tiselius, A. (1951) Biochem. J. 48, 171-174.

Szent-Gyorgyi, A. (1936) Z. Phys. Chem. Frankfurt, 244, 105.

Szent-Gyorgyi, A. (1937) Z. Phys. Chem. Frankfurt, 245,118.

Umbarger, E., and Davis, B.D. (1963) The Bacteria 3, 168-251.

Valeriote, F.A., Auricchio, F., Tomkins, G.M., and Riley, D. (1969) J. Biol. Chem. 244, 3618-3624.

Wallace, B.J., and Pittard, J. (1969) J. Bacteriology 97, 1234-1241.

Weber, K., and Osborn, M. (1966) J. Biol. Chem. 244, 4406-4412.

Wood, J.L., and Cooley, S.L. (1954) Proc. Soc. Expt. Biol. Med. 85, 409-411.