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PURIFICATION AND CHARACTERIZATION OF THE "B" TRANSAMINASE OF PSEUDOMONAS AERUGINOSA

A DISSERTATION

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JOHN ELDON NORTON

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PURIFICATION AND CHARACTERIZATION OF THE "B"

TRANSAMINASE OF PSEUDOMONAS AERUGINOSA

APPROVED BY

John R. Sokatch

L. Vernon Scott

Florence C. Kelly

A. C. Kurtz

Morgan H. Bradford

DISSERTATION COMMITTEE

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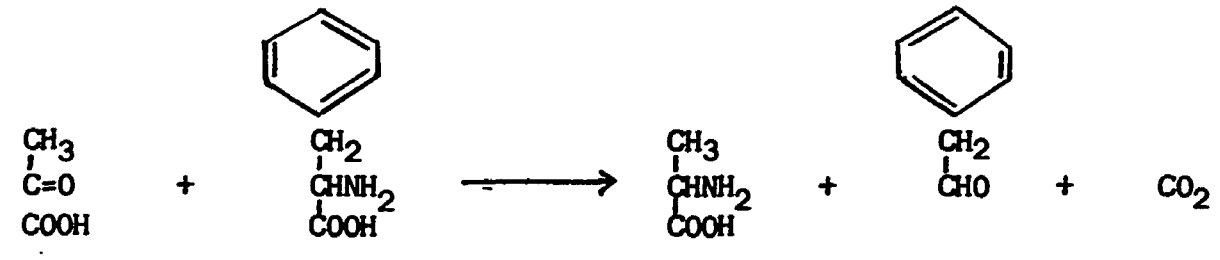
PURIFICATION AND CHARACTERIZATION OF THE "B" TRANSAMINASE OF *PSEUDOMONAS AERUGINOSA*

CHAPTER I

INTRODUCTION

Transamination is a chemical reaction in which amino groups of amino acids are transferred to the carbonyl carbon of 2-oxo (keto) acids. The reactions are usually reversible and without the liberation of ammonia during the reactions. Herbst and Engel (1934) first noticed transamination in boiling solutions of amino acid and 2-oxo acid mixtures. The donor amino acid was decarboxylated as part of the reaction:



Pyruvic acid + Phenylalanine → Alanine + Phenylacetaldehyde + CO_2

The amino acids Herbst and Engel used in their experiments were α-aminophenylacetic acid, glycine, phenylalanine, L-aspartic acid, and L-cystine. All of these amino acids could donate amino groups to pyruvate; α-methylaminophenylacetic acid and α-aminoisobutyric acid, which have no free

α-hydrogen atoms, could not participate in the non-enzymic transaminations. Phenylpyruvic acid was also found to be capable of transamination reactions with phenylalanine; the disappearance of phenylpyruvate was assayed. The amino acid end products of these reactions were racemic mixtures, as Herbst and Engel found by observing the gradual reduction of the original optical rotation. Addition of acid to the reaction mixtures had little effect on transamination; when an amount of base sufficient to titrate all the carboxyl groups of the reactants was added to the mixture, transamination ceased (Herbst and Engel, 1934).

Later, Braunstein and Kritzmann (1937) found that minces of pigeon breast muscle or rabbit skeletal muscle could catalyze the transamination of glutamate with pyruvate. The products were alanine and 2-oxoglutarate; the reaction was freely reversible; the yield of 2-oxoglutarate was improved when the mixtures were incubated anaerobically. They called the transfer reaction "umaminierung" and stated in very definite terms that this was the first time such a reaction had been noted in animal tissues: "Der in vorliegender Arbeit nachwiesene Prozess ist das erste Beispiel einer biochemischen Synthese von Aminosäuren durch intermoleculare Übertragung von Aminogruppen."

In 1940 Philip P. Cohen extended the investigation of transamination in animal tissues. He used chloramine-T to decarboxylate amino acids formed by transamination, and measured the liberated carbon dioxide by manometry; his assay depended on the fact that two moles of carbon dioxide were liberated from the aspartate which was formed; Cohen criticized the methods of Braunstein as being antiquated and inaccurate. Braunstein and Kritzmann had suggested that three types of transaminase

might be found in tissues, one to catalyze each of these reactions:

1) L-glutamate + oxaloacetate ⇌ 2-oxoglutarate + L-aspartate

2) L-glutamate + pyruvate ⇌ 2-oxoglutarate + L-alanine

3) L-asparate + pyruvate ⇌ oxaloacetate + L-alanine

Cohen did not believe that a separate enzyme existed for the third reaction. He suggested instead that aspartic acid and pyruvic acid could undergo transamination when trace amounts of glutamic acid were present. This, in effect, would be a coupling of reactions (1) and (2). He also found that pig heart muscle was a better source of transaminase than either pigeon breast muscle or rabbit skeletal muscle. For the first time an attempt was made to purify the transaminase and to see whether the reactions were due to one or several enzymes; he believed the reactions in pig heart were due to one enzyme. Cohen's work is difficult to compare with that of later workers because he did not convert the μliters of carbon dioxide liberated from newly formed aspartic acid into moles; he also failed to report the amount of protein which he had used in his experiments (Cohen, 1940_a). Cohen does, however, give some interesting data in another article in the same journal. He shows curves of glutamate and aspartate formation as a function of the incubation time. He also attempted to determine Michaelis constants for the substrates, but only showed values for K_m when the concentrations of 2-oxo acids and amino acids in the mixtures were initially identical. Calculated in this way, the formation of aspartate from glutamate and oxaloacetate has a K_m of 1.38×10^{-2} molar (for both substrates), which is a high concentration. The optimum pH for the glutamate-aspartate reaction was shown to be 7.5

and the best temperature was 40 C (Cohen, 1940_b).

The controversy about whether there were separate enzymes for the three known transaminative reactions was resolved by the experiments of Green, Leloir and Nocito (1945), who separated extracts of pig heart into two distinct fractions. They identified one enzyme as a glutamate-aspartate transaminase and the second as a glutamate-alanine transaminase. Green and his coworkers offered evidence which suggested the two enzymes had pyridoxal derivatives as cofactors.

More definitive evidence that a pyridoxal derivative was the cofactor in enzymic transamination reactions was obtained by Lichstein and his coworkers (1945_b). Enzyme extracts of Streptococcus faecalis grown in a medium deficient in pyridoxal exhibited reduced transaminative activity. Glutamate-aspartate transaminase activity could be entirely restored to the inactive extracts by adding pyridoxal phosphate or pyridoxal and adenosine triphosphate to the reaction mixtures.

In preliminary works, the synthesis of pyridoxal phosphate in aqueous solutions by treatment of pyridoxal with phosphorus trichloride was described by Gunsalus, Umbreit, Bellamy, and Foust (1945). They showed their synthetic product to be active as a transaminative coenzyme. Additional evidence that pyridoxal derivatives were cofactors in transamination was the fact that rats grown on a diet deficient in vitamin B_6 were shown to have low transaminative activity. The enzymic activity was increased when pyridoxamine was fed to these rats (Schlenk and Snell, 1945).

Also in 1945, Lichstein and Cohen showed that glutamate-asparate

transaminase was present in these [sic] bacteria:

Bacillus coli
Bacillus dysenteriae (shiga)
Bacillus typhosus
Bacillus proteus
Bacillus pyocyaneus
Azotobacter vinelandii
Staphylococcus aureus
Staphylococcus albus
Bacillus welchii
Streptococcus hemolyticus
Streptococcus viridans
Pneumococcus Type I

This report was the first to show that bacteria definitely possessed transaminase systems; Adler and his coworkers had briefly mentioned transamination by Escherichia coli extracts, but no quantitative data were given in their report (Adler, Hellström, Günther and von Euler, 1938).

Although Braunstein and Kritzmann (1938) stated that many amino acids, in addition to aspartate and alanine, could donate amino groups to 2-oxoglutarate, Cohen's criticism of their assay techniques made them less sure of their results. By 1950, the improved methods of amino acid separation and detection had made Cohen's position that there was only one transaminase less tenable; other investigators began writing of more than one or two transaminases (Broquist and Snell, 1949). Cohen published a paper which stated that animal tissues could, indeed, catalyze transamination between 2-oxoglutarate and a series of amino acids (Cammarata and Cohen, 1950). Pig heart muscle could use branched chain amino acids as transaminative substrates and other amino acids, such as the aromatic ones, were also transaminated by the extracts. Cohen now suggested that, probably, more than two enzymes were involved in transamination in living tissues.

Rudman and Meister found that extracts of E. coli could be

treated in such a way that three transaminative enzymes could be separated. One of these, which they called "B", was capable of catalyzing transamination between glutamate and the branched chain amino acids. Their "B" preparation was free of "A" or glutamate-aspartate transaminase, but its activity was less than 20 times that of the crude extract. The "B" transaminase was reported to be capable of catalyzing transamination, not only between 2-oxoglutarate and the branched chain amino acids, but also between those amino acids and their corresponding 2-oxo acids. It was shown that 2-oxo-3-methylvaleric acid could transaminate with both L-valine and L-leucine; 2-oxoisovaleric acid could serve as a transaminative partner with L-isoleucine and L-leucine. A mutant which was unable to make L-isoleucine was found to be capable of forming L-valine and L-leucine by transamination of their 2-oxo acids with L-alanine. Wild type E. coli extracts could also perform this type of transamination. This report showed that two types of transaminase ("A" and "B") could be extracted from E. coli, and it strongly suggested that the microorganism also had a third type of transaminative enzyme, one for alanine-valine reactions (Rudman and Meister, 1953).

That same year, Altenbern and Housewright (1953) published data showing that cell-free extracts of Brucella abortus contained transaminative enzyme(s) capable of transferring amino groups from the branched chain amino acids to 2-oxoglutarate and to pyruvate. They prepared three ammonium sulfate fractions from the extract which had varying amounts of leucine-glutamate, leucine-alanine, alanine-glutamate and aspartate-glutamate activities. The enzymes were more sharply separated after protamine sulfate treatment of the extract.

The transaminases with branched chain amino acid substrates which were studied during the next thirteen years were primarily of animal origin. Jenkins and Sizer (1956) described a transaminase prepared from chicken liver extracts. The enzyme had a substrate specificity similar to that of the E. coli "B" transaminase, but the highest activity was found when donor-recipient amino acid-2-oxo acid pairs both had branched chain configurations. Transamination between glutamate and the branched chain amino acids proceeded at a slower rate. This enzyme had a pH optimum greater than 8.0, and its activity was inhibited by heavy metal salts. The heavy metal inhibition could be reversed by adding glutathione, cysteine or versene to reaction mixtures. This, the authors said, was good evidence for the presence of thiol groups at the enzyme's active site (Jenkins and Sizer, 1956).

Later, Seecof and Wagner (1959_a) purified, by a factor of 20 times, an enzyme (or possibly two enzymes) with transaminative activity for the branched chain amino acids from extracts of the fungus Neurospora crassa. This preparation could catalyze transamination between 2-oxoglutarate, 2-oxoisovalerate or phenylpyruvate and the branched chain amino acids. Glutamate-aspartate and glutamate-alanine transaminase activity was removed during the purification procedure. The Neurospora extract was stimulated in its action by adding pyridoxal phosphate to the reaction mixtures. Therefore, the enzyme was probably partially resolved of its cofactor. The pH optima for isoleucine-phenylalanine and valine-phenylalanine transaminations were found to be near 8.0. The Michaelis constants, which these workers determined for various substrates, ranged from a low of 1.2×10^{-4} molar for L-phenylpyruvate to 1.1×10^{-2} molar

for L-valine. The K_m's found for 2-oxo acids were always lower than those for amino acids. No K_m's were reported for L-glutamate, but the authors wrote that "rates with glutamate could be measured only at high glutamate concentrations" (Seecof and Wagner, 1959_b).

The "B" transaminase from pig heart muscle was purified extensively by Taylor and Jenkins (1966). With their purification procedures, 45 pounds of pig hearts yield 30 mg of the transaminase. The purified enzyme had a specific activity of 50-53 μmoles of glutamate produced per min per mg of protein. Taylor and Jenkins estimated that their preparations were 90 per cent pure on the basis of ultracentrifugal data. They found that addition of mercaptoethanol to the reaction mixtures after the sixth purification step increased the activity of the enzyme by about three times, to its highest level. The molecular weight of the enzyme was approximately 75,000, and the molar ratio of the enzyme protein to pyridoxal was 1:1. The presence of pyridoxal phosphate on the enzyme as a cofactor was shown by partially resolving the protein of its prosthetic group by incubating the enzyme with leucine and pH 7.5 phosphate buffer. Addition of pyridoxal phosphate to reaction mixtures almost completely restored the loss in activity due to resolution of the cofactor. Furthermore, when Taylor and Jenkins (1966) reduced enzyme preparations with sodium borohydride and then hydrolyzed them, they could isolate epsilon-pyridoxyllysine by descending paper chromatography of the hydrolysates. This suggests that the pyridoxal was bound to the lysine by a Schiff-base linkage.

The "B" transaminase of Salmonella typhimurium was purified by Coleman and Armstrong (1967) until the specific activity of the final

preparation was approximately 400 times greater than that of the crude extract (Coleman and Armstrong, 1967).

Searches of the scientific literature have failed to reveal any more recent definitive work on bacterial "B" transaminases. The interest of the author in this enzyme began while he was attempting to show oxidation of D- and L-isomers of valine by cell-free extracts of P. aeruginosa. The D-isomer was found to be oxidized by an enzyme specific for D-amino acids (Norton and Sokatch, 1966; Marshall and Sokatch, 1968). The L-isomer of valine was deaminated by the mechanism of transamination (Norton and Sokatch, 1966). Earlier, Feldman and Gunsalus (1950_a and 1950_b), found that a close relative of P. aeruginosa, Pseudomonas fluorescens, was capable of transaminating branched chain amino acids. They used the fairly new technique of paper chromatography and quantitation by visual comparison of ninhydrin stained amino acid spots with standards. Glutamate production was also assayed with glutamate decarboxylase by monometry.

Since the early 1950's, several new methods of protein separation have come into common usage. An example is the technique of chromatography of protein extracts on weak ion exchangers such as DEAE cellulose (diethylaminoethyl cellulose) and cellulose phosphate. Other aids to enzyme purification which have been developed in the past decade are molecular seiving materials such as the Sephadexes (Pharmacia, Uppsala, Sweden). These cross-linked dextrans were described first by Swedish workers (Porath and Flodin, 1959; Lindner, Emlqvist and Porath, 1959).

Because the bacterial transaminases had received so little attention, and more powerful tools, such as the chromatographic materials

just mentioned, had become available, it seemed reasonable to attempt to purify the "B" transaminase of P. aeruginosa. With the development of purification techniques for this enzyme, a study of its interaction with specific inhibitors in vitro could be made; the metabolic importance of the enzyme makes it a tempting target for chemotherapeutic agents. There is a need to study the mode of action of as many enzymes as possible so that man may one day construct his own organic catalysts. Production of a specific catalyst without the necessity of growing organisms and extracting an enzyme, which might be stable for only a short time, would be a great advance in industrial chemistry.

The objectives of this investigation were (1) to establish a procedure for purifying the "B" transaminase of P. aeruginosa and (2) to characterize the enzyme with respect to some of its chemical and physical properties.

CHAPTER II

MATERIALS AND METHODS

Cultural Methods

Microorganisms Used

Pseudomonas aeruginosa, from the stock culture collection of the Department of Microbiology of the University of Oklahoma Medical Center, was used in these and earlier studies. Throughout this study, the organism was maintained on agar slants of a chemically defined medium which contained DL-valine as its sole carbon source. Stock cultures were incubated at 37 C for 24 hrs after inoculation; the slants were stored in a refrigerator at a temperature of 4 C until they were needed. Transfers were made at 3 to 4 week intervals.

Stock Culture Medium

This chemically defined medium consisted of these ingredients:

DL-valine	5 gm
K_2HPO_4	3 gm
$(NH_4)_2SO_4$	1 gm
Salts "C"	5 ml
Agar	20 gm
Distilled water	1000 ml

Salts "C" (Roberts and Snell, 1946) was composed of these compounds:

$MgSO_4 \cdot 7H_2O$	10.0 gm
NaCl	0.5 gm
$FeSO_4 \cdot 7H_2O$	0.5 gm
$MnSO_4 \cdot 4H_2O$	2.0 gm
Distilled water	250 ml

The complete medium was heated to boiling to dissolve the agar and it was dispensed in 5 ml quantities into tubes. After the medium was autoclaved for 15 min at 121 C, it was cooled in a slanted position.

Large-scale Growth of Bacteria for Transaminase Preparation

The complex defined growth medium consisted of these inorganic ingredients:

Salts "C"	65.00 ml
$(NH_4)_2SO_4$	13.00 gm
KH_2PO_4	15.60 gm
K_2HPO_4	94.25 gm
Distilled water	11,450 ml

The medium contained these amino acids:

L-aspartic acid	6.50 gm
L-glutamic acid	3.25 gm
Glycine	1.95 gm
DL-alanine	1.95 gm
Distilled water	250 ml

The amino acid suspension was titrated to pH 7.5 with 1 M KOH. The neutralized amino acids were then completely soluble.

The inorganic salts solution and the amino acids solution were mixed to form a basal medium. The basal medium was prepared for autoclaving by dividing it into three portions of 50, 850 and 10,800 ml.

The energy source for the growth medium was made up for autoclaving as six % glucose; this solution was made by dissolving 78.3 gm of glucose in 1,305 ml of distilled water. The glucose solution was also divided into three portions of 5, 100 and 1200 ml.

The basal medium was autoclaved for 15 min at 121 C in sparger-equipped growth flasks in the case of the 50 and 850 ml portions; the large portion was autoclaved for 90 min in a carboy. The glucose solutions were autoclaved separately for 15 min. After the solutions were sterilized, the 50 ml of basal medium and the 5 ml of glucose solution were combined while still hot; the 850 ml of basal medium and the 100 ml of glucose solution were also mixed together. The largest portions of basal medium and glucose solution were mixed only at the time of inoculation. These mixtures could be stored for one week at refrigerator temperatures.

The smallest container of complete medium was inoculated with a suspension of bacteria obtained by washing the growth from a valine agar slant with 1 ml of the complete growth medium. The inoculated culture was aerated with an aquarium pump and incubated at 37 C. After 8 hours of incubation, the 55 ml of growing bacteria were added to the 950 ml flask of growth medium and the culture was incubated another 8.5 hr at 37 C with aeration.

The large portions of basal medium and glucose solution, prewarmed to 37 C, were placed in a 13 liter Bio-Kulture assembly

(Fermentation Design, Fogelsville, Pennsylvania) and were inoculated with the 950 ml of growing culture. The fermenter was set to maintain a temperature of 37 C, the agitator set at 400 revolutions per min, and 3.5 liters of air were delivered per min from two aquarium pumps. Bacterial growth was monitored by taking samples and measuring the turbidity of 1:5 dilutions of the medium at a wavelength of 660 millimicrons with a B & L spectronic 20 spectrophotometer.

The bacteria were harvested at the peak of their growth in a Szent-Gyorgyi and Blum continuous flow attachment to the Servall RC-2 centrifuge. The yield of wet cells was approximately 10 gm per liter. The harvested bacteria were stored in centrifuge tubes at -20 C until needed.

Preparation of the "B" Transaminase

Cell-free extracts of P. aeruginosa were made by suspending 150 gm of frozen bacteria in 200 ml of 0.05 M potassium phosphate buffer, pH 6.5. Suspension of the bacteria was aided by adding 40 mg of type DN-25 bovine pancreatic deoxyribonuclease (Sigma Chemical Company). After the slurry was stirred at room temperature for approximately 20 min, the bacteria were disrupted by subjecting them to sonic oscillation in a Model DF101 Raytheon Sonic Oscillator for 20 min at 100 watts and 10 kilocycles. The oscillator chamber was cooled with water at the temperature of melting ice. The suspension was divided into five portions for sonic oscillation.

Cellular debris was removed from the extract by centrifugation at 12,000 x g for 15 min. The extract was kept below 10 C during this and subsequent steps in the purification except where otherwise indicated.

Portions of each fraction (usually 1 ml) were frozen for assay procedures at the completion of the purification.

Sufficient 0.4 M, pH 6.5, potassium 2-oxoglutarate was added to the extract to give a final concentration of 0.005 M. Pyridoxal-5'-phosphate was added to the mixture to a final concentration of 50 μg per ml of solution. The mixture was heated to 62 C in a 95 C water bath and the fluid was transferred to a 62 C water bath, where it was kept for 15 min. The turbid fluid was chilled in an ice-salt bath and denatured proteins were removed by centrifuging for one hr at 37,000 x g. The pellet was washed by resuspending it in 75 ml of 0.05 M, pH 6.5, potassium phosphate buffer and centrifuging the suspension for one hr at 37,000 x g. The two supernatant solutions were combined and were heated to 75 C in a 95 C water bath. The extract was transferred to a 75 C water bath, where it was held for 5 min, before it was cooled in an ice-salt bath. Denatured proteins were removed by centrifuging the extract for one hr at 37,000 x g.

A one per cent solution of protamine sulfate (Salmine, grade II, Sigma Chemical Co., Inc.) equal to 0.8 of the extract volume was added to the fluid. The suspension formed by the reaction was stirred for 10 minutes and the particulate material was removed by centrifugation for 45 min at 37,000 x g. The pellet was discarded.

Solid ammonium sulfate was added to the extract until it was 35 per cent saturated with the salt. This calculation, adapted from data by Brenner-Holzach and Staehelin (1953), was used to determine the amount of ammonium sulfate to be added:

$$\frac{(1.77)(\text{ml of solution})(\text{sat. 2-Sat. 1})}{3.54\text{-Sat. 2}}$$

In practice, the formula was simplified by multiplying the volume of solution by 0.194 to find the gm of ammonium sulfate required for this step. The protein-salt solution was stirred for 10 min and insoluble proteins were removed by centrifuging the fluid for 30 min at 37,000 x g. The supernatant fluid was made 60% saturated with ammonium sulfate by adding the number of gm of salt equal to the volume of solution times the factor 0.15, which was also obtained from the formula previously mentioned.

The insoluble proteins were sedimented by centrifuging the fluid for 30 min at 22,000 x g. This pellet was dissolved in 35 ml of 0.05 M, pH 8.5, potassium phosphate buffer. The sample which was saved for assay was diluted 1:10 with the same buffer.

The concentrated enzyme solution was placed on a 10 x 22 cm column of G-200 Sephadex (Pharmacia, Uppsala, Sweden) which had been equilibrated with 0.05 M, pH 8.5, potassium phosphate buffer. The proteins were eluted with 2.5 liters of the same buffer and fractions of approximately 13 ml were collected. The flow rate was maintained at 90 ml per hr by using a Marriotte flask as a buffer reservoir and a pressure-head of approximately 10 cm, according to Pharmacia technical directives.

The active fractions, located by checking every fifth tube with a qualitative assay described on page 18, were pooled. Usually the active fraction was eluted between tubes 60 and 100. The pooled active fractions were usually about 370 ml in volume. The enzyme pool was concentrated by placing it in a dialysis bag which it filled to one-third capacity. The dialysis bag was folded loosely in a large funnel and enough sucrose was poured into the funnel to cover the bag. A wetted marble in the lower end of the funnel formed a sugar clot which prevented

loss of the dry sucrose. The funnel was placed stem down in an Ehrlenmeyer flask and the assembly was kept in a refrigerator until the extract was sufficiently concentrated, usually about 6 hr or overnight.

The concentrated enzyme solution was placed on a 1.3 x 47 cm DEAE (diethylaminoethyl) cellulose column which had been equilibrated with pH 9.2, 0.05 M dibasic potassium phosphate solution. The proteins were eluted with a linear gradient between 500 ml of 0.05 M dibasic potassium phosphate and 500 ml of 0.2 M, pH 7.5, potassium phosphate buffer. Fractions of approximately 10 ml were collected and every fifth tube from 60 to 100 was assayed qualitatively for activity. Those fractions containing transaminase were pooled. The volume of the pooled enzyme solution was usually approximately 250 ml.

The extract was concentrated to about 30 ml by dialyzing it against sucrose by the method described previously. The concentrated enzyme solution was titrated to pH 6.0 with 0.15 M phosphoric acid, and distilled water was added to the fluid to dilute it 1:5. After titration and dilution, the enzyme solution was reconcentrated by sucrose dialysis to about 30 ml. This solution was diluted 1:3 with distilled water to give a final concentration of 0.01 M potassium phosphate and a pH of 6.0. The enzyme solution was reconcentrated by sucrose dialysis to about 20 ml.

The concentrated protein solution was placed on a 1 x 45 cm cellulose phosphate column which had been equilibrated with 0.01 M, pH 6.0, potassium phosphate buffer. The proteins were eluted with a linear gradient between 200 ml of 0.01 M, pH 6.0, potassium phosphate buffer and 200 ml of 0.01 M dibasic potassium phosphate solution. Protein in the 5 ml fractions was located by measuring the absorption of the fluid at a

wavelength of 280 millimicrons.

Enzymic Methods

Qualitative Assay

The active enzyme fractions from column chromatography were located by mixing 0.1 ml of the fraction with 100 μmoles of pH 7.5 potassium phosphate buffer, 5 μmoles of pH 7.5 2-O-oglutarate and 20 μmoles of L-norvaline in a total volume of 0.4 ml. The mixtures were incubated for 15 min at 37 C in a water bath shaker, and the reactions were stopped by adding 0.5 ml of 1.0 M perchloric acid. The acid was neutralized by adding 0.5 ml of 1.0 M potassium hydroxide to the mixtures. The mixtures were shaken to insure complete reaction of the acid and base to insoluble potassium perchlorate.

Amino acids in the reaction mixtures were separated by rapid paper chromatography. Ten μliters of each mixture were spotted on chromatographic paper and the chromatogram was developed for 30 min with the methanol, pyridine and water solvent described by Redfield (1953). The paper was dried, dipped in Heilmann's ninhydrin reagent, and dried with a hair-dryer until the red amino acid spots appeared (Heilmann, Barrolier and Watzke, 1957). The active fractions were those whose reaction mixtures showed two amino acid spots: glutamate and norvaline.

Quantitative Enzyme Assays

Each reaction mixture contained the following ingredients in a volume of 1 ml:

pH 7.5 potassium phosphate buffer	100 μmoles
pyridoxal-5'-phosphate	50 μg

enzyme protein	0.2 units (approx.)
pH 7.5 L-norvaline	60 μmoles
pH 7.5 2-oxoglutarate	5 μmoles

The reactions were started by adding L-norvaline. One enzyme unit was defined as that amount which catalyzed the formation of one μmole of glutamate per min under standard conditions, and specific activity was defined as units per mg protein.

The mixtures were incubated for 15 min at 37 C in a water bath shaker and 0.5 ml of 1.0 M perchloric acid was added to stop the reaction. The mixtures were centrifuged for 15 min at 12,000 x g, if necessary, to remove precipitated proteins. After the fluid had been decanted from the protein pellets the acid was neutralized by adding 0.5 ml of 1 M potassium hydroxide. When the potassium perchlorate had finished precipitating, 20 μliters of each reaction mixture were spotted on chromatographic paper. An identical tube which contained 5 μmoles of L-glutamate and no enzyme was used as a standard. The chromatograms were developed in the same way and with the same reagents as in the qualitative assay. For quantitative assay, however, chromatograms were placed in a closed jar over concentrated sulfuric acid after the first color appeared. The jar and chromatogram were incubated at 37 C for 30 min to one hr to develop maximum color. The glutamate spots were excised, and the color was eluted in a mixture of 2.5 ml of absolute methanol and 0.1 ml of 2 N acetic acid. The acetic acid aided color extraction and prevented interference from phosphate salts which form blue compounds with the ninhydrin reagent. The tubes were shaken to extract the red color and the optical density was read against a methanol-acetic acid blank at a wavelength of 510 mμ in a

Beckman model DU spectrophotometer.

Protein was estimated by the colorimetric method of Lowry, et al. (1951).

The assay system used in determining the pH optima for L-norvaline, L-leucine, L-valine and L-isoleucine differed somewhat from the standard assay. The reaction mixtures were composed of: 100 μmoles of potassium phosphate of varying pH, 2 μg of protein, 30 μmoles of pH 7.0 2-oxoglutarate, 50 μg of pyridoxal-5'-phosphate, and 30 μmoles of the amino acid used. The reactions were initiated by adding the amino acid solution, which brought the total volume of the mixtures to 1 ml. The pH of each mixture was measured soon after the reactions were begun and again before they were terminated by adding 0.5 ml of 1.0 M perchloric acid. From this point, mixtures were chromatographed as if they were standard assays, described on page 19.

The reaction mixtures in the substrate specificity studies were also modifications of the standard assay. The mixtures for these determinations were composed of: 100 μmoles of pH 7.5 potassium phosphate buffer, 50 μg of pyridoxal-5'-phosphate, 15 μmoles per assay, respectively, of each of the 13 amino acids (the amino acids were in 0.05 M solutions. Very insoluble amino acids, such as tyrosine or tryptophan were made up as 0.05 M suspensions which were mixed thoroughly before adding them to the mixtures.), 1 μg of the enzyme and 15 μmoles of pH 7.5 2-oxoglutarate, which was added to start the reaction. The total volume of the mixture was 1.0 ml. The incubation time for these assays was 20 min. Except for the modifications described, the mixtures were spotted, chromatographed and quantitated in the usual way.

The amount of substrate required to obtain one half of the maximal velocity of the reaction (K_m) was determined first for 2-oxoglutarate by adding 60 μmoles of L-norvaline, 100 μmoles of pH 7.5 potassium phosphate buffer with 50 μg of pyridoxal-5'-phosphate, 1.0 μg of enzyme and varying amounts of 2-oxoglutarate from 0.5 to 5.0 μmoles in a total reaction volume of 1.0 ml. Incubation was for 15 min at 37 C; the reactions were terminated in the usual way with perchloric acid and 20 μliters of each neutralized reaction mixture were spotted on the paper. The K_m's of the amino acids were determined in similar fashion, with the amount of 2-oxoglutarate used in the assays held constant at 5 μmoles and the concentration of the amino acids varied. The reactions were started by adding the component which was held constant.

The kinetic formula,

$$V = \frac{V_{max}}{1 + \frac{K_m}{[S]}} ,$$

is derived from the data of Michaelis and Menten (1913). If it is inverted and multiplied through by [S], it assumes the form:

$$\frac{[S]}{V} \quad \frac{[S]}{V_{max}} \quad \frac{K_m}{V_{max}} .$$

In this equation, [S] is the concentration of substrate, V_{max} is the maximum reaction velocity, and K_m is the substrate concentration (moles per liter), at which the enzymic reaction proceeds at one-half its maximal rate. When S/V is plotted as a function of [S], a straight line is obtained. The intercept of the line with the S/V axis is $1/V_{max}$ and its slope is K_m/V_{max}. The K_m is obtained by multiplying the value of the slope by V_{max} (Dawes, 1963; White, Handler, and Smith, 1968).

The specific activity was also plotted as a function of the

substrate concentration. This was to show the substrate concentration at which the reaction changes from first order, with the rate and the substrate concentration related, to zero order, where the substrate concentration and enzymic activity are independent.

Physico-chemical Methods

The molecular weight of the "B" transaminase of P. aeruginosa was determined by filtration of the protein through standardized columns of G-200 Sephadex (Whitaker, 1963). Standard protein samples were eluted from 2.5 x 35 cm columns and mid-points of their elution volumes were plotted, in ml, against the logarithm of their molecular weights. Elution of the proteins was followed by recording the absorption of the emerging buffer at a wavelength of 215 mμ. The standard proteins used were bovine pancreatic trypsin, ovalbumin, bovine serum albumin and human gamma globulin. The "B" transaminase was concentrated during purification in an Amicon ultrafilter with nitrogen as a source of pressure for this determination.

The pyridoxal content of the protein was determined by the method of Wada and Snell (1961), with the total volume of the assay reduced to 1.0 ml. The sample was first dialyzed for six days against four changes of 0.02 M ammonium bicarbonate buffer to eliminate sucrose and nonvolatile salts. The sample was then lyophilized. The dried protein was weighed and hydrolyzed for 6 hrs in 0.055 N HCl in an autoclave at 121 C according to the method of Rabinowitz and Snell (1947). The sample was dried again and dissolved in distilled water. The assay mixtures and standard tubes were incubated at 60 C for 20 min and then for 10 min at room temperature to insure the complete reaction of all pyridoxal-5'-phosphate in

the sample. A solution of crystalline pyridoxal-5'-phosphate (Sigma Chemical Co., Inc.) was used as a standard.

CHAPTER III

EXPERIMENTAL RESULTS

Proportionality of Enzyme Concentration to Rate of Transamination Reaction

The activity of the "B" transaminase of P. aeruginosa proved to be linear with respect to the protein concentration in the reaction mixture, up to a protein concentration of 2.0 μg per ml (Fig. 1). The proportionality of catalyst to end product was not constant above this range, and therefore, no more than 2.0 μg of the purified enzyme was used in an assay. No more than 0.2 units of enzyme was used in any assay.

Localization and Assays of Enzyme Fractions

Protein was eluted from the final cellulose phosphate chromatographic column within the first twenty of the 5 ml fractions. The eluted protein was located by recording the absorbance of each fraction and constructing a curve from the optical density values. The peak shown in Figure 2 is of protein emerging from a cellulose phosphate column which had been equilibrated at pH 6.0 with 0.01 M potassium phosphate. When the entire emerging peak was pooled, the protein had several minor contaminants. This is shown in Fig. 3, which is a reproduction of a photograph of disc electropherograms from each of the steps of a typical enzyme purification. The protein from the last purification step is the

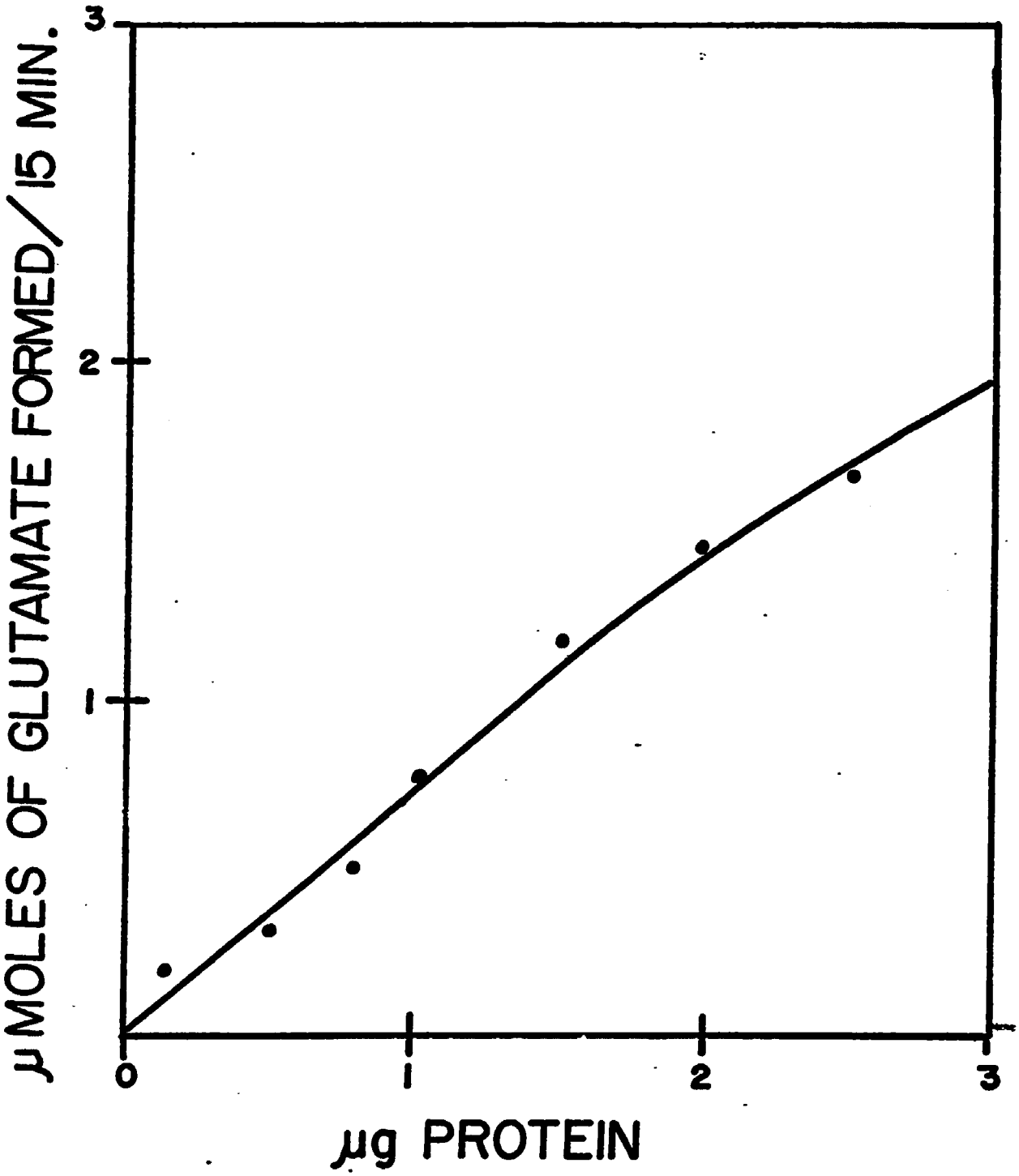


Fig. 1.--Proportionality of the concentration of purified transaminase to the production of L-glutamate in the standard assay system.

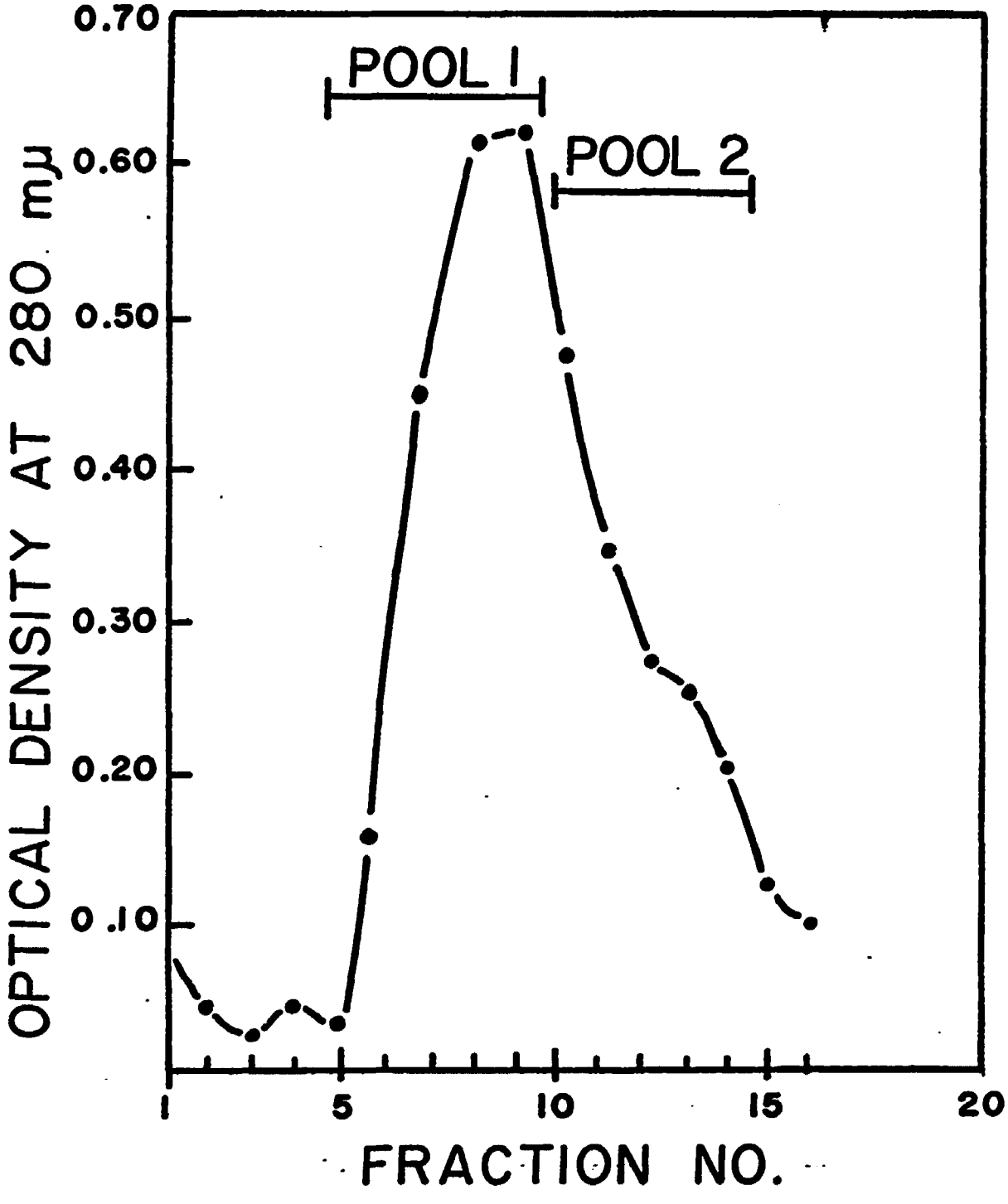


Fig. 2.--Elution profile of the "B" transaminase from a cellulose phosphate column which had been equilibrated at pH 6.0.

Fig. 3.--Disc electropherograms of fractions made from extracts of P. aeruginosa during purification of the "B" transaminase. The gel on the left contains the crude extract; progressive purification is seen from the left to the right. The last gel on the right is the total protein eluted from cellulose phosphate.

one at the right of the figure. In practice, the proteins emerging from a pH 6.0 cellulose phosphate column were divided into two pools, as is indicated in Fig. 2. The data from this purification are summarized in Table 1. The fractions were assayed simultaneously to conserve time and materials. In this instance, the cellulose phosphate column was equilibrated with pH 6.0 potassium phosphate buffer; later during the study when a purer enzyme preparation was needed for the estimation of the pyridoxal content of the protein, the cellulose phosphate column was equilibrated with 0.01 M pH 5.5 potassium phosphate buffer. The enzyme was then eluted in a slightly different peak, as is shown in Fig. 4. The electropherograms of the proteins contained in the three pools from this peak are shown in Fig. 5. Pools 1 and 2 were combined for enzyme characterization studies. The improvement in the enzyme purification obtained by decreasing the pH of the cellulose phosphate column to 5.5 and dividing the eluted protein into pools may be seen by comparing the electropherograms from pools 1 and 2 from the pH 5.5 preparation (shown in Fig. 5) with the last electropherogram on the right in Fig. 3, which was made from a pool of the entire protein peak eluted from a cellulose phosphate column at pH 6.0.

pH Optima of the "B" Transaminase

The specific activity of the enzyme at different pH's and with four different substrates is shown in Fig. 6. Each of the four amino acid substrates in these assays had different pH optima, and two of the activity curves exhibit bimodality. When L-isoleucine or L-norvaline were used as substrates for the enzyme, the resulting activity curves are bell-shaped. The pH curves obtained when L-leucine and L-valine were used

TABLE 1

RESUME OF PURIFICATION DATA

Fraction	Total Protein (mgs)	Total Units	Specific Activity	Purification
Crude Extract	24,500	2,890	0.118	---
Heated Extract	2,240	1,390	0.621	5.3
Protamined Extract	1,920	1,400	0.727	6.2
.35-.60 $(NH_4)_2SO_4$	756	1,380	1.83	15.2
G-200 Sephadex pool	116	1,220	10.5	89.0
DEAE Cellulose pool	27.2	888	32.7	277
Cellulose Phosphate pool 1	5.8	290	50.0	424
Cellulose Phosphate pool 2	4.0	172	43.0	364

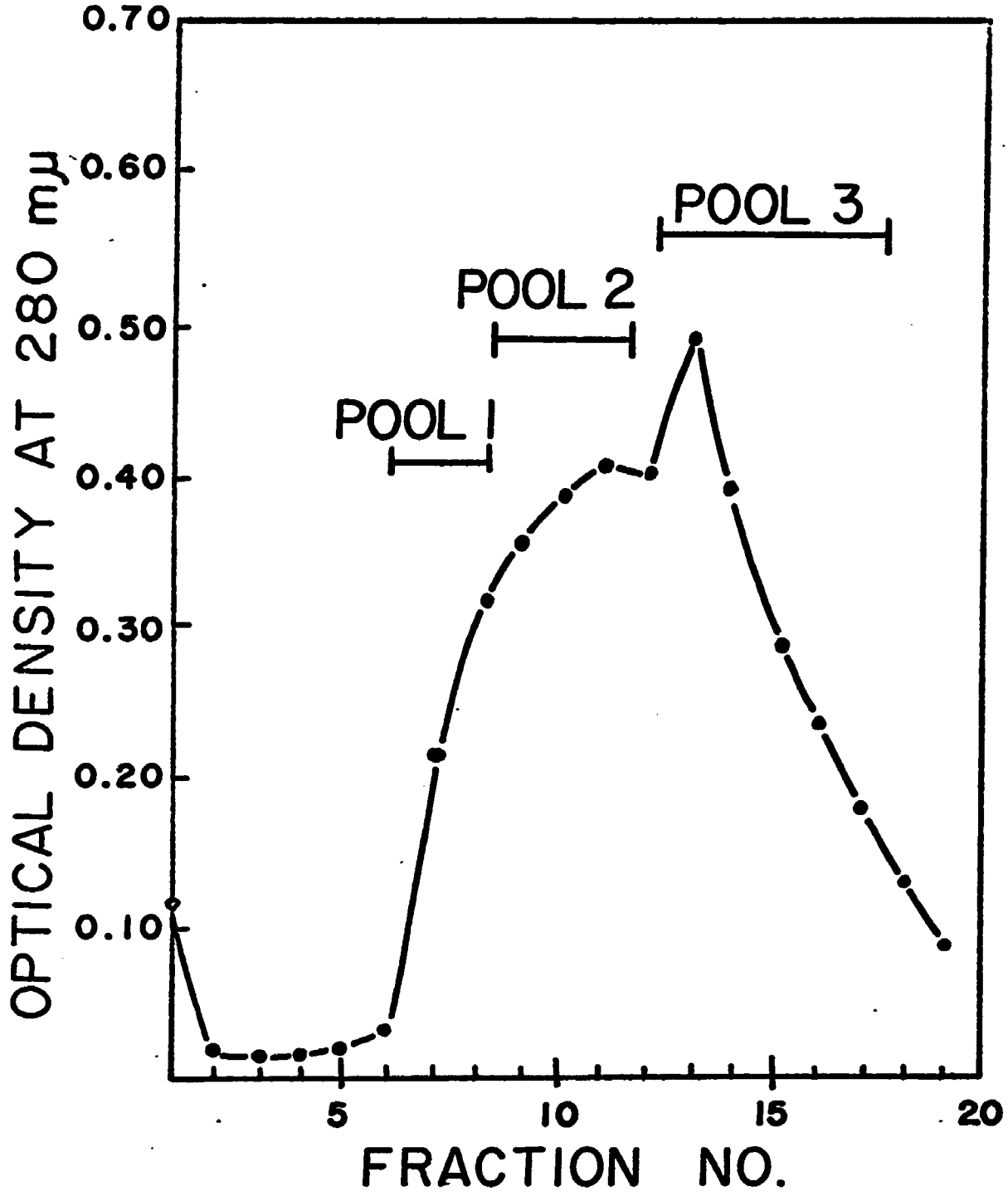


Fig. 4 --Elution profile of the "B" transaminase from a cellulose phosphate column which had been equilibrated at pH 5.5.

Fig. 5.--Disc electropherograms of the three protein pools from the eluate of a cellulose phosphate column at pH 5.5.

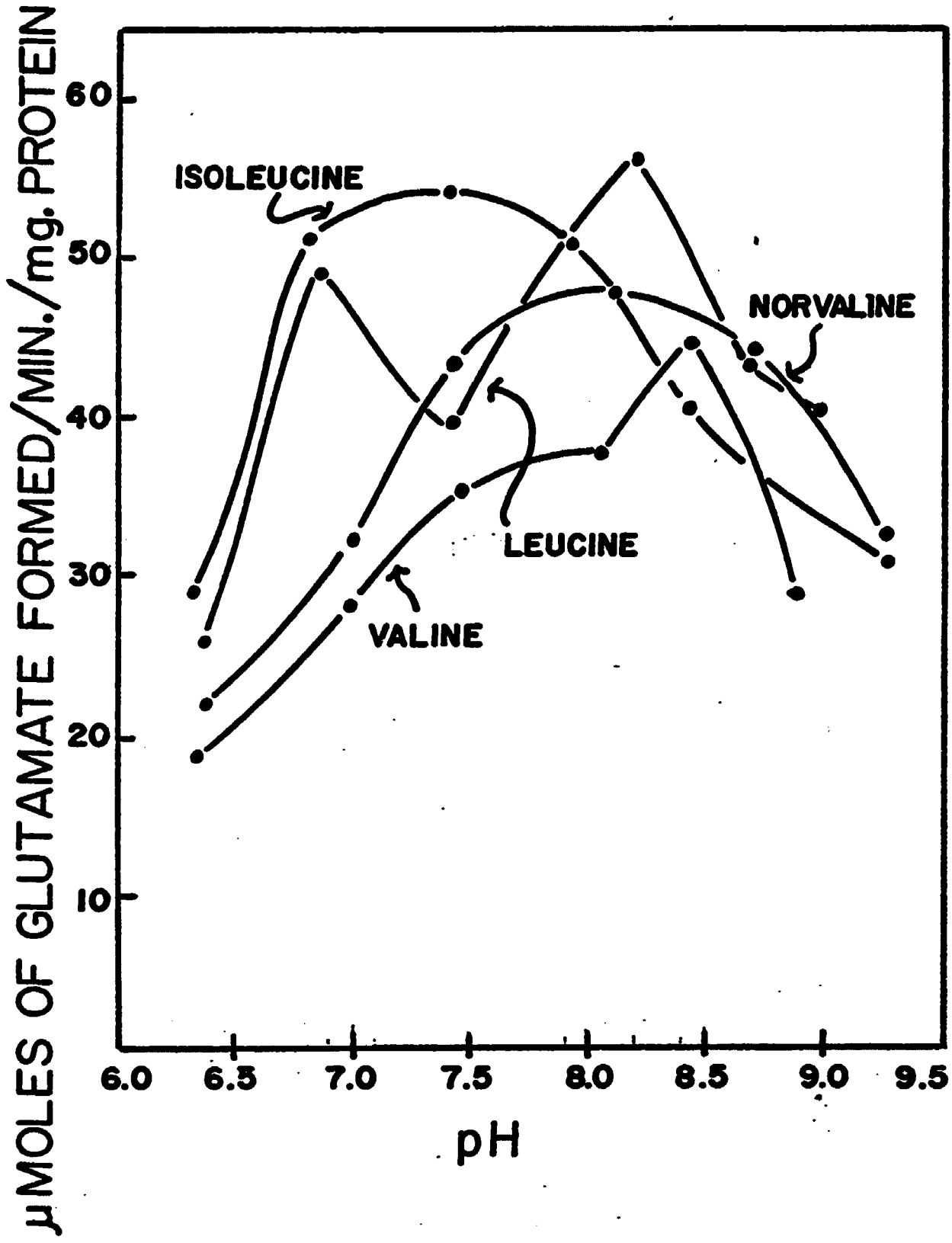


Fig. 6.--The relation of the hydrogen ion concentration to the activity of the "B" transaminase for four of its substrates.

as substrates for the "B" transaminase are biomodal.

Substrate Specificity of the "B" Transaminase

The "B" transaminase of P. aeruginosa utilized, as substrate, 6 of the 13 amino acids used in this assay as may be seen in Table 2. The L-amino acids isoleucine, leucine, methionine, norvaline, phenylalanine and valine are substrates for the enzyme; under the conditions of this assay the activity was greatest when leucine was the substrate and lowest when phenylalanine was the amino acid used.

Michaelis-Menten Constants of Several Enzyme Substrates

Arbitrarily, the Michaelis-Menten constants determined in this study are listed in Table 3 in order of increasing magnitude. The lowest K_m was that of 2-oxovalerate, shown plotted in Figure 7. The other 6 substrates for which constants have been determined are represented in Figures 8 through 13. The curve of S/V is linear in every K_m plot.

Determination of the Molecular Weight of the Enzyme

The mid-points of the elution peaks of the "B" transaminase were plotted on a graph which had as its abcissa the logarithm of the molecular weights of the proteins used as standards. The ordinate of the graph was the number of ml of eluant from the column. Figure 14 shows a curve constructed by plotting the molecular weights and elution volumes of bovine pancreatic trypsin, ovalbumin, bovine serum albumin and human gamma globulin. The point shown for the "B" transaminase at a position on the curve corresponding to a molecular weight of 130,000 is an average of five determinations on two different columns. The extremes of variation obtained

TABLE 2

SUBSTRATE SPECIFICITY OF THE "B" TRANSAMINASE OF PSEUDOMONAS AERUGINOSA

Amino acids	Specific activity
L-alanine	0
L-aspartic acid	0
Glycine	0
L-isoleucine	80
L-leucine	90
L-methionine	27
L-norvaline	38
L-phenylalanine	20
L-serine	0
L-threonine	0
*L-tryptophan	0
*L-tyrosine	0
L-valine	42

*These two amino acids were added to the reactions as suspensions.

TABLE 3

MICHAELIS-MENTEN CONSTANTS OF SEVERAL ENZYME SUBSTRATES

Substrate	K_m
2-oxovalerate	2.6×10^{-4} M
2-oxoglutarate	4.0×10^{-4} M
L-leucine	1.0×10^{-3} M
L-isoleucine	1.2×10^{-3} M
L-norvaline	3.0×10^{-3} M
L-valine	3.6×10^{-3} M
L-glutamate	1.8×10^{-2} M

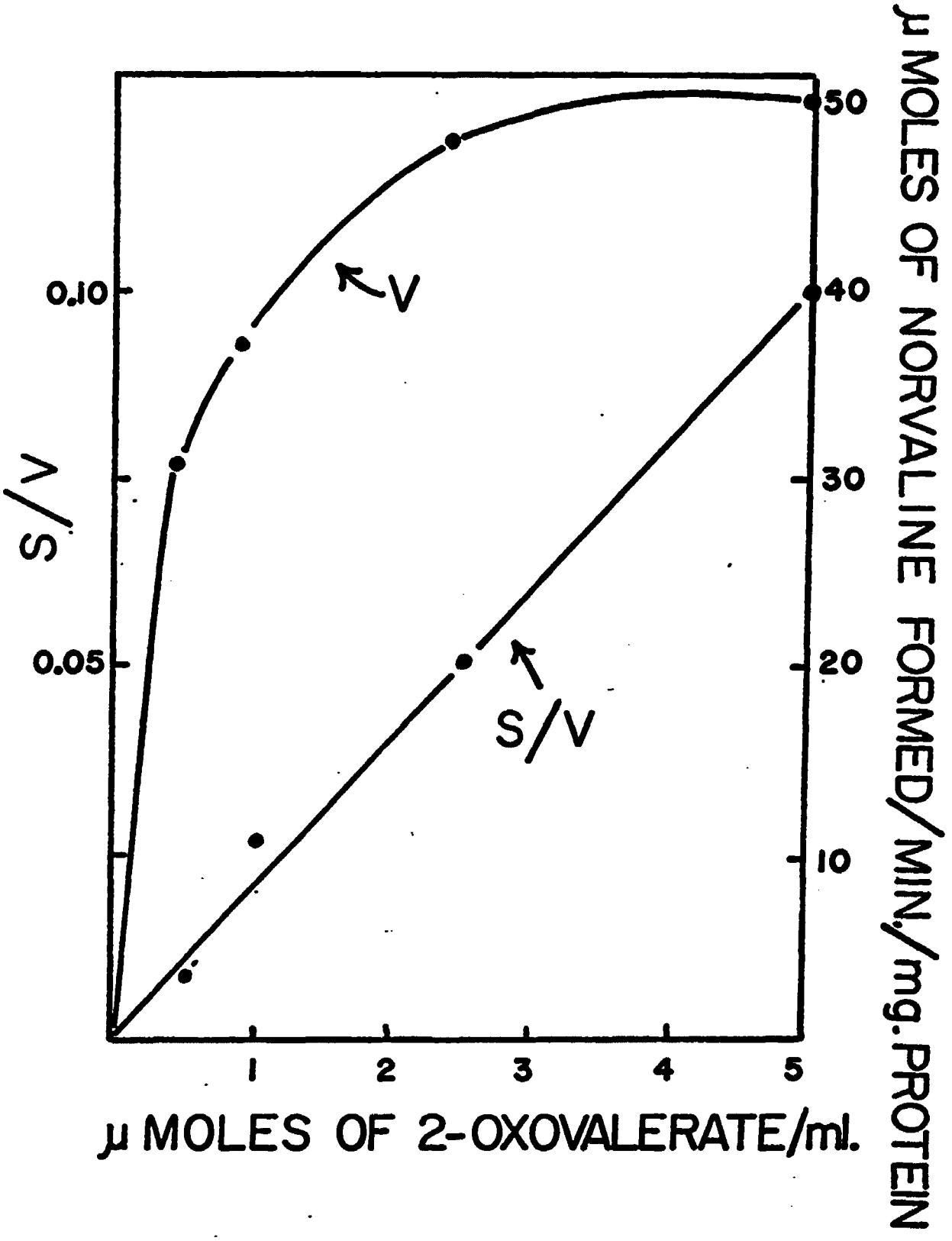


Fig. 7.--The velocity of the transamination reaction as a function of the concentration of 2-oxovalerate.

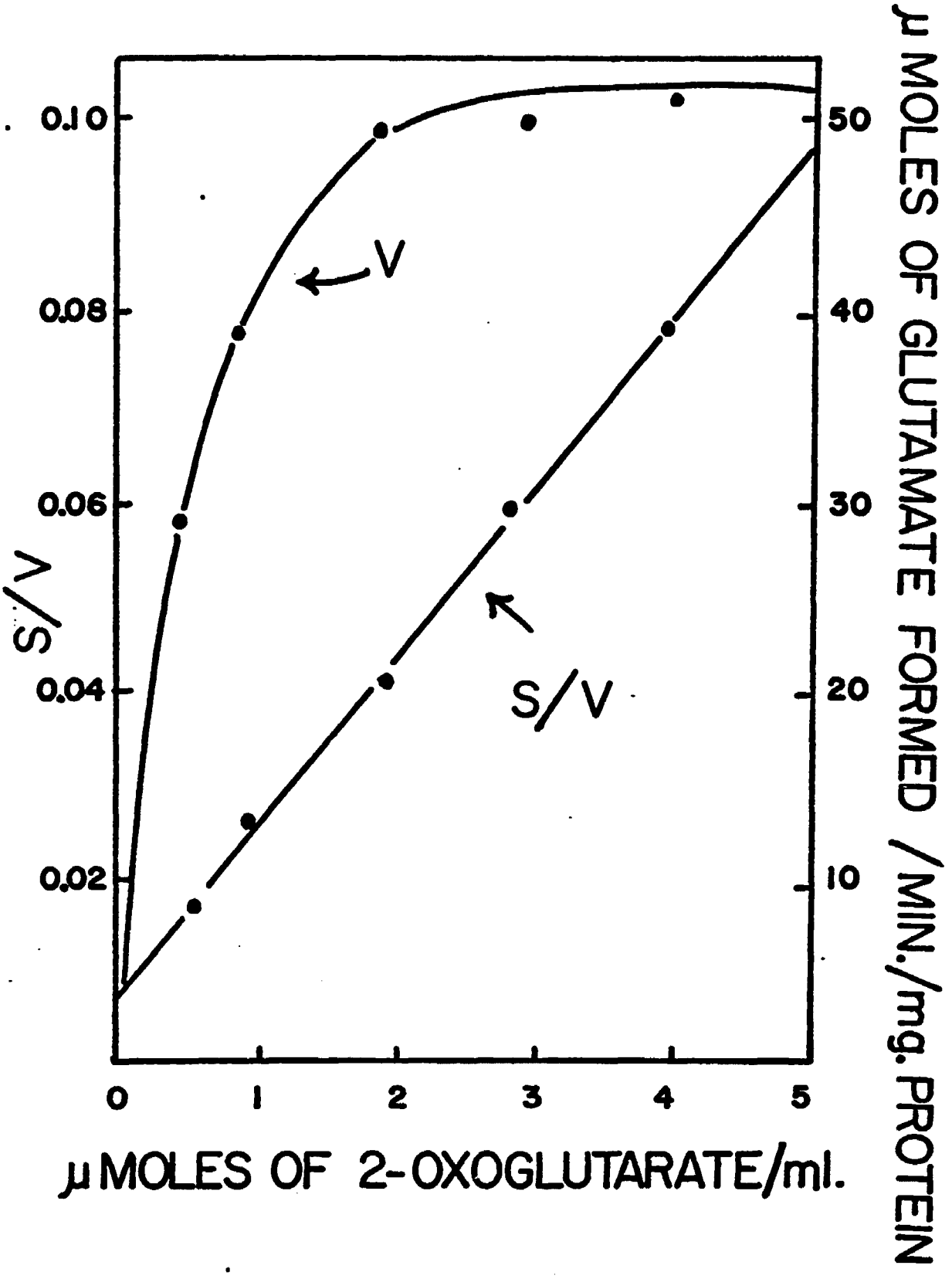


Fig. 8.--The velocity of the transamination reaction as a function of the concentration of 2-oxoglutarate.

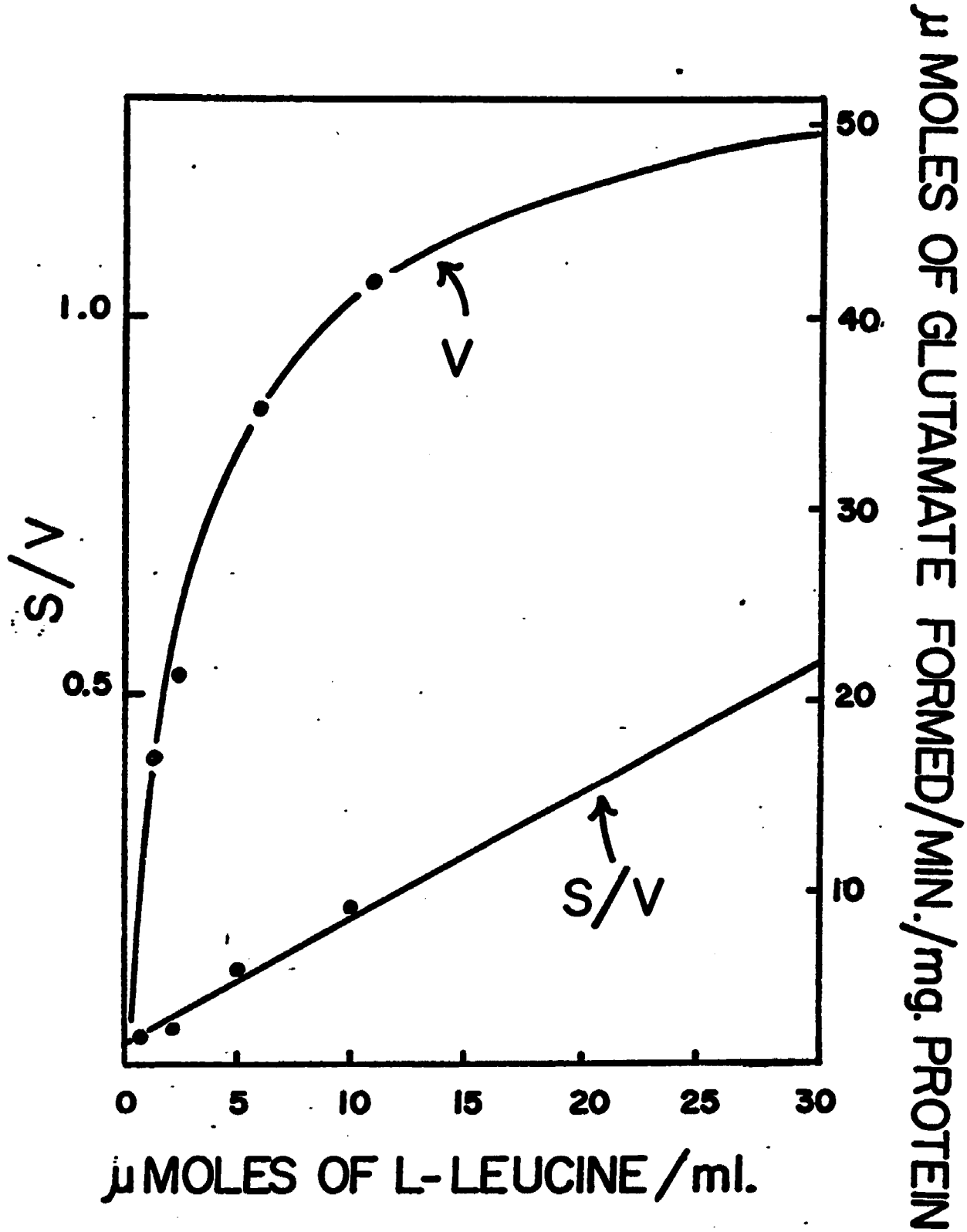


Fig. 9.--The velocity of the transamination reaction as a function of the concentration of L-leucine.

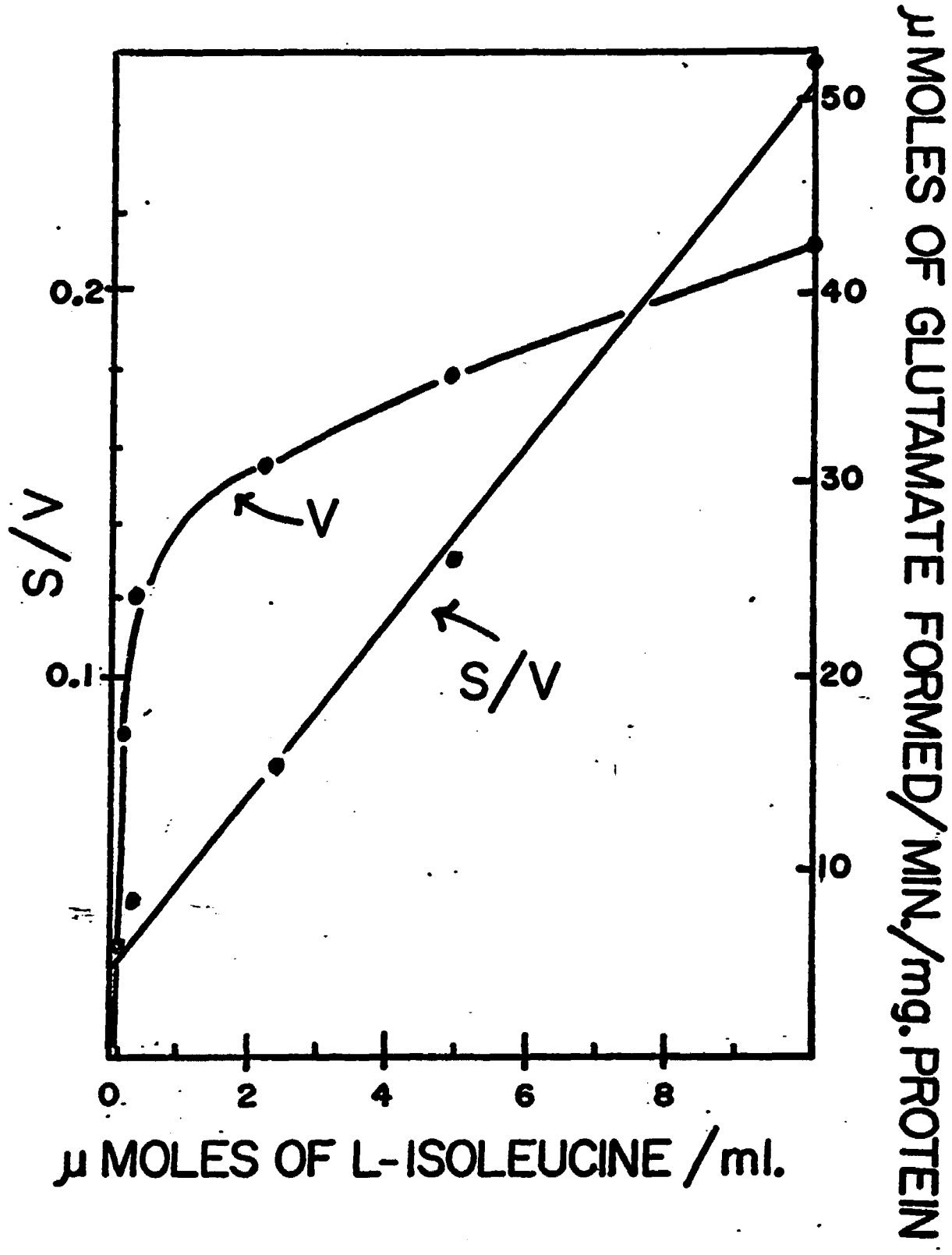


Fig. 10.--The velocity of the transamination reaction as a function of the concentration of L-isoleucine.

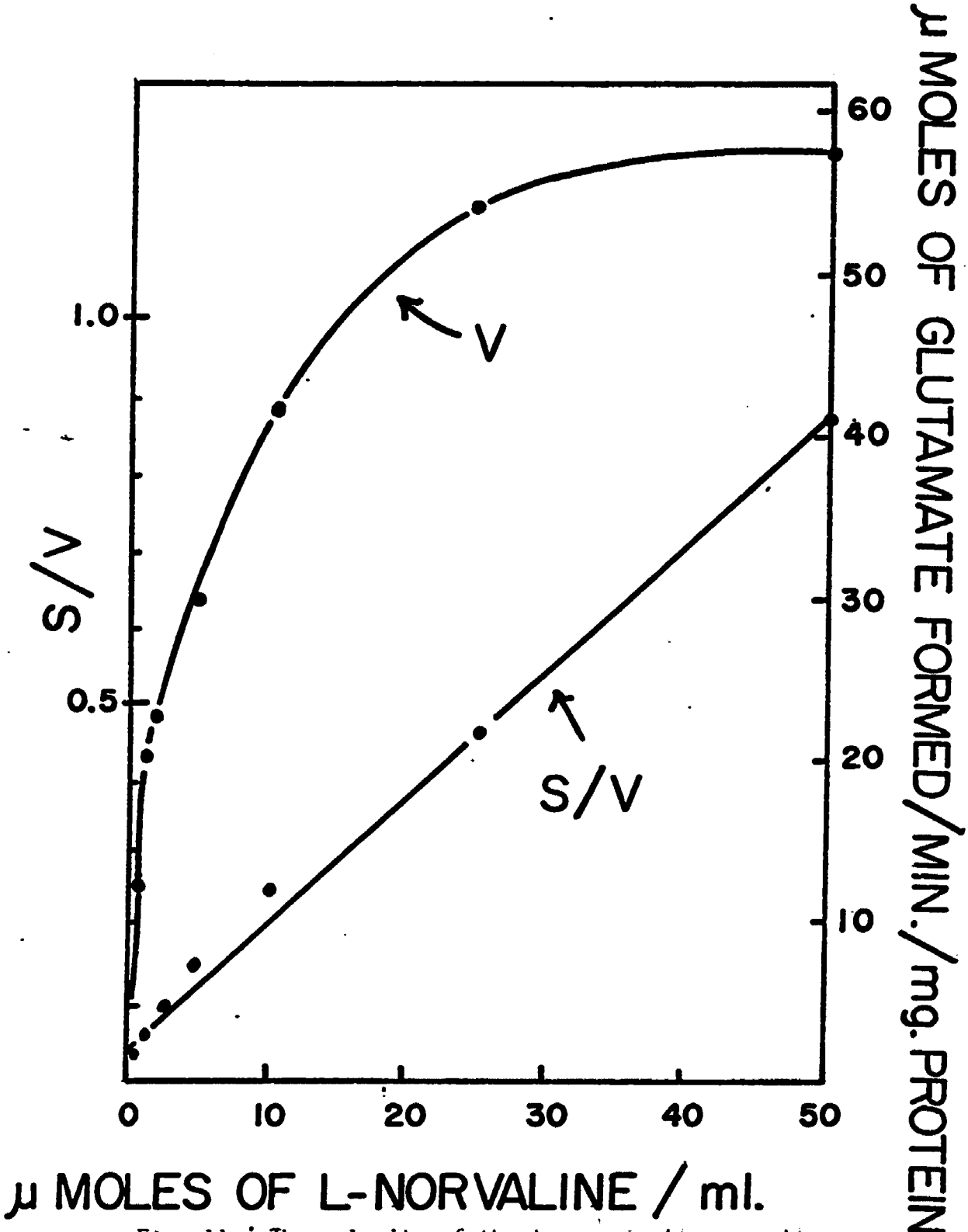


Fig. 11.--The velocity of the transamination reaction as a function of the concentration of L-norvaline.

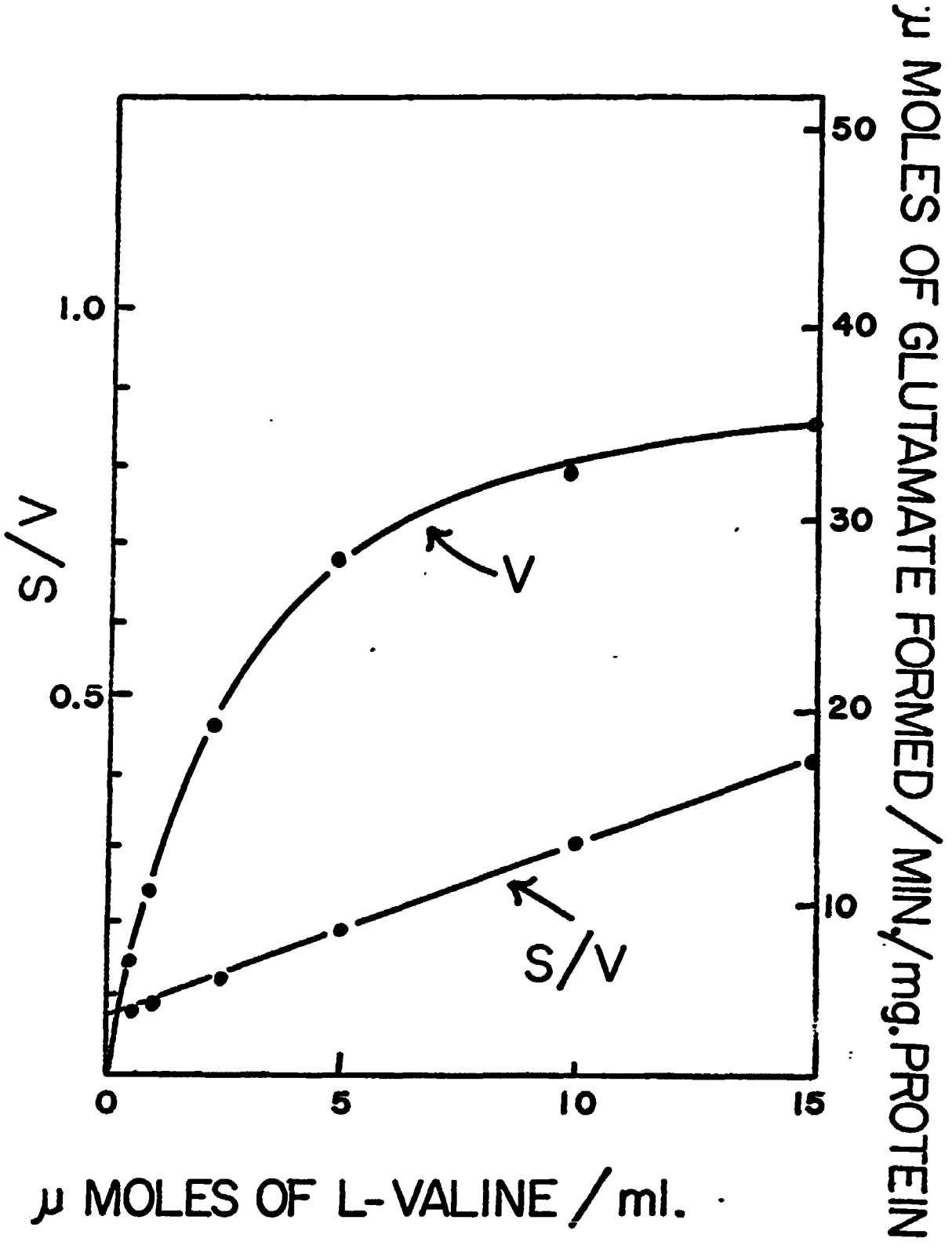


Fig. 12.--The velocity of the transamination reaction as a function of the concentration of L-valine.

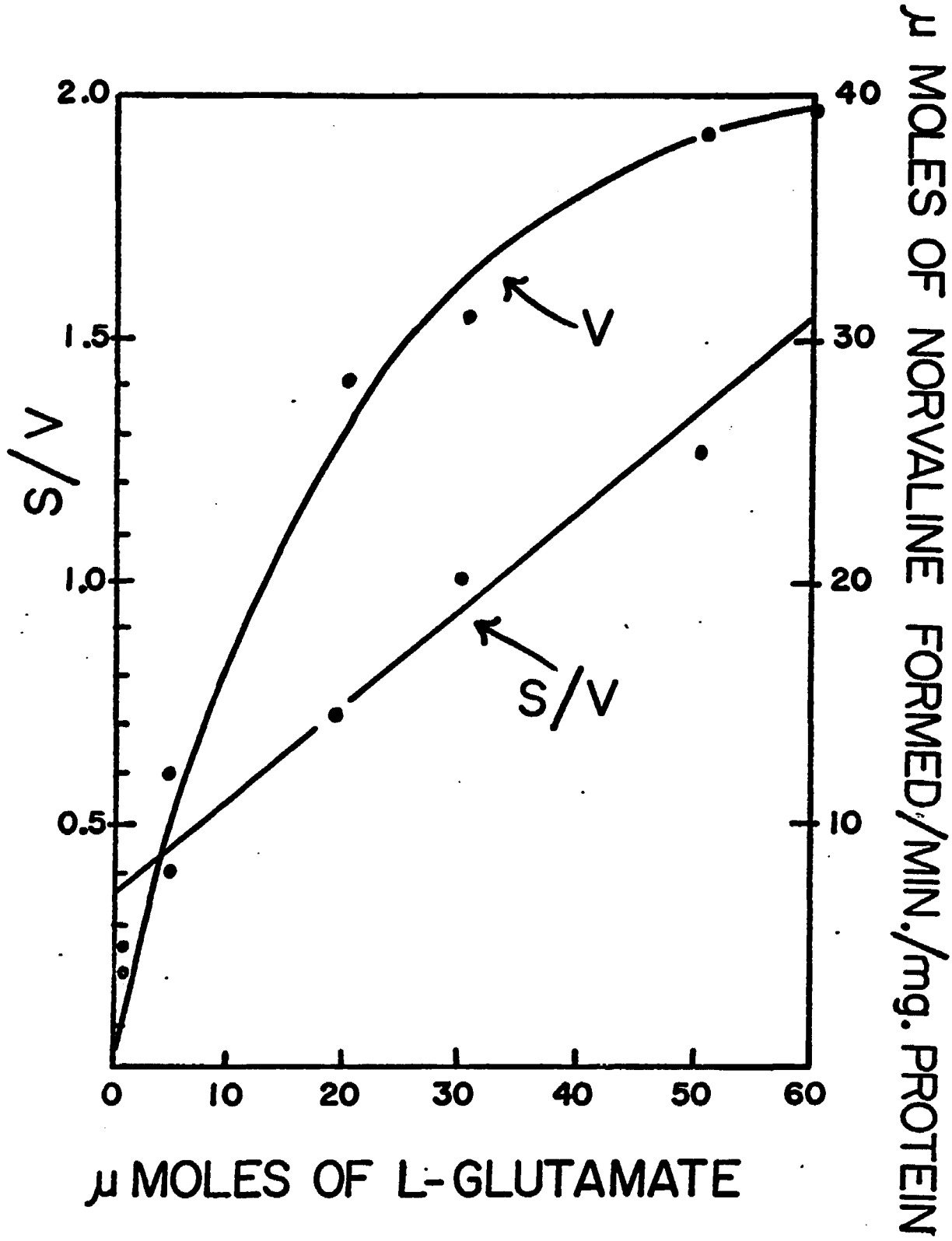


Fig. 13.--The velocity of the transamination reaction as a function of the concentration of L-glutamate.

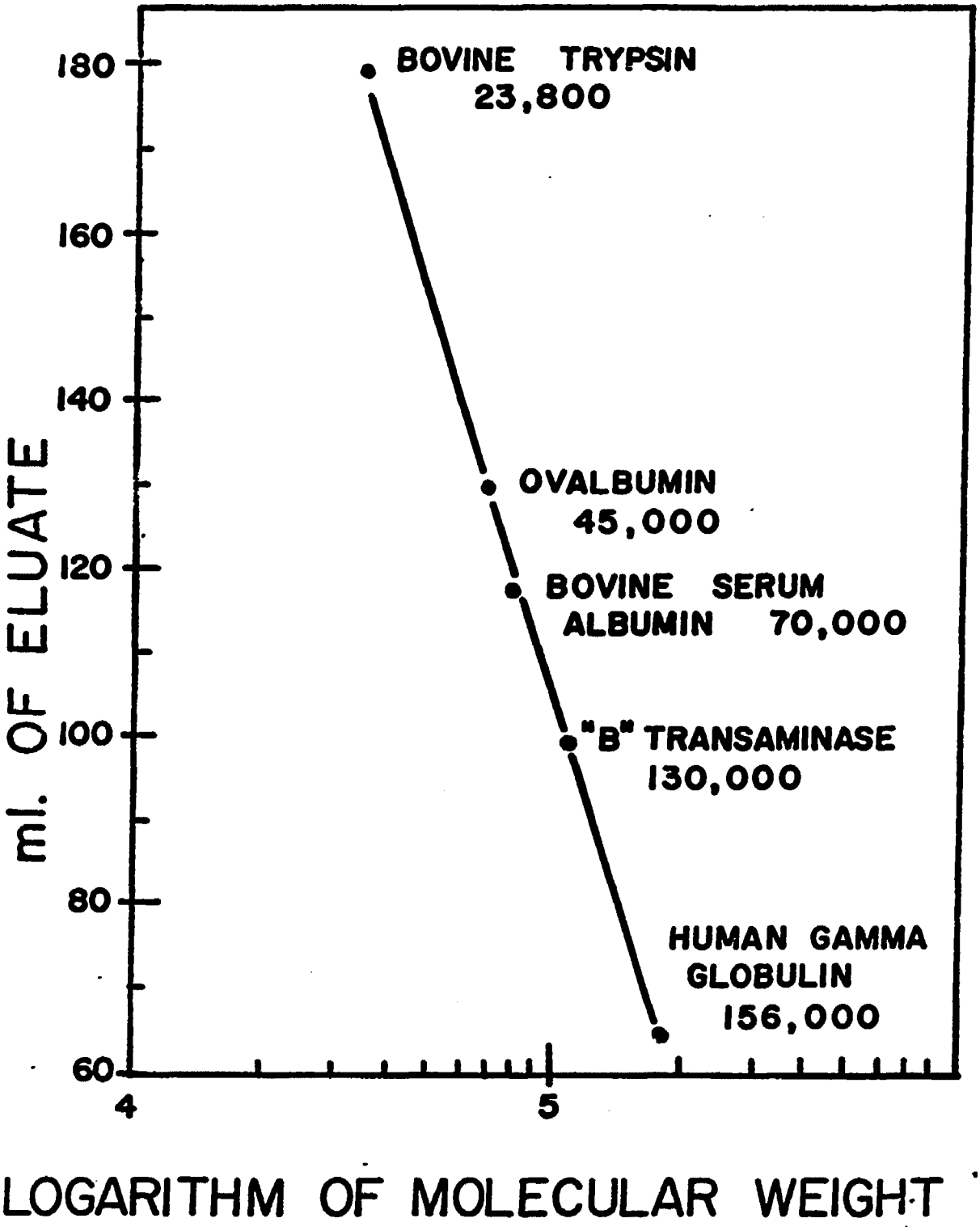


Fig. 14.--The curve obtained by plotting the mid-points of the elution volumes of standard proteins from a G-200 Sephadex column. The molecular weight of the "B" transaminase was equal to the anti-logarithm of the value found on the abcissa of the graph.

in the determinations were 127,400 and 134,900 gm per mole, or minus 2.0 and plus 3.8 percent.

Estimation of the Number of Molecules of Pyridoxal per Mole of Enzyme

The pyridoxal content of the enzyme protein was determined by the method of Wada and Snell (1961). The amounts of pyridoxal found in two separate determinations were 2.05 and 2.4 moles per mole of the enzyme protein, based on a molecular weight of 130,000 for the enzyme. One of the determinations gave a false high content of pyridoxal due to trace contaminants of sucrose in the protein which was not removed by the dialysis.

The absorption of the pyridoxal-phenylhydrazone in the assay at a wavelength of 410 mμ could be partially obscured by furfural derivatives. Furfural may be formed from sugars in hot acid solutions. The absorption curve of furfural was found to have values at a wavelength of 410 mμ 2.6 times as great as at 500 mμ; absorption curves of hydrolyzed sucrose were identical to the furfural curve. Pyridoxal-phenylhydrazone did not absorb light at a wavelength of 500 mμ. In the one case in which it was needed, the following corrections were made to account for sugar in the protein sample:

(O. D. of sample at 500 mμ)(2.6) = O. D. of furfural derivative at 410 mμ

O. D. found at 410 mμ-(O. D. at 500 mμ)(2.6) = O. D. due to pyridoxalphenylhydrazone

The corrected optical density at 410 mμ was used to calculate the pyridoxal content from a standard pyridoxal curve.

The weight of sugar which had been contaminating the protein was estimated directly from a curve of the O. D. at 500 mμ of furfural derivatives made by hydrolyzing sucrose under the same conditions as the protein. The curve was made by plotting μg sucrose versus O. D. at 500 mμ.

The pyridoxal to protein protein ratios were calculated in this way:

gm of hydrolysate used	moles of pyridoxal found
1.74×10^{-4}	3.0×10^{-9}
3.48×10^{-4}	5.4×10^{-9}
5.22×10^{-4}	8.0×10^{-9}
1.04×10^{-3}	1.64×10^{-8}

$$\frac{1.04 \times 10^{-3}}{1.64 \times 10^{-8}} = 6.34 \times 10^{4} \text{ gm/mole}$$

$$\frac{130,000}{63,400} = 2.05 \text{ moles of pyridoxal per mole of enzyme protein}$$

An average of the two values determined is 2.23 moles of pyridoxal per mole of protein.

CHAPTER IV

DISCUSSION

The "B" transaminase of Pseudomonas aeruginosa was not inducible in the usual sense of the word. The bacteria did have more of the enzyme if they were grown on glucose rather than on valine, but the total units were increased by only about two fold per gm of wet cells. When P. aeruginosa was grown in a medium with glucose as a sole carbon source, the yield of bacteria was about 7 gm of wet cells per liter. The medium described in Chapter II increases the yield of bacteria to 10 gm per liter and the specific activity from 0.07 to 0.12.

The "B" transaminase of P. aeruginosa, like the pig heart leucine aminotransferase of Taylor and Jenkins (1966), was most stable at pH 6.5. The purification obtained by heat treating the crude extract was best at that pH. It was necessary to add both pyridoxal-5'-phosphate and 2-oxoglutarate to the crude extract before the heat treatment to protect the transaminase from inactivation.

The effect of adding 2-oxoglutarate to the crude enzyme is not known with certainty. The leucine aminotransferase of pig heart is also stabilized by adding 2-oxoglutarate to a concentration of 0.005 M before the heat treatment, but apparently pyridoxal-5'-phosphate is not required as it is when the extracts of P. aeruginosa are heated. Both animal and

bacterial enzymes must have 2-oxoglutarate in the cathodic (negative) buffer chambers during electrophoresis to prevent what Taylor and Jenkins (1966) call "smearing" in the case of the animal enzyme and the formation of a double band in the case of the bacterial enzyme. This suggests that the polymeric form of these proteins may be unstable and easily converted to a monomeric form by mechanical or thermal stresses if the substrate is not present. Taylor and Jenkins (1966) believe that their enzyme is not a polymeric protein, but they made their determinations of molecular weight in 0.1 M mercaptoethanol, which could cause its dissociation into subunits.

Although the removal of nucleic acids from the heated bacterial extract with protamine sulfate did not increase its specific activity substantially, it was necessary to insure the reproducible fractioning of the protein with ammonium sulfate; without this step subsequent increases in activity were not the same from batch to batch. The transaminase was also separated more easily from nonactive proteins by Sephadex chromatography if nucleic acids had been removed from the extract. Altenbern and Housewright (1953) reported that the transaminases of Brucella abortus were more cleanly separated by ammonium sulfate precipitations after protamine sulfate treatment of their cell-free extracts.

The use of sucrose dialysis to concentrate the enzyme following G-200 Sephadex and DEAE cellulose chromatography has both advantages and disadvantages. The "B" transaminase of P. aeruginosa normally becomes unstable as it is purified. The sucrose which penetrates the dialysis bag stabilizes the enzyme; ordinarily a purified extract is active for about three to four days, but after sucrose concentration an extract still has

usable activity after 12 days to two weeks of storage at 0 C. Sucrose in an enzyme extract can be disadvantageous if inhibition studies are to be attempted; the aldehydic part of the molecule may react with carbonyl reagents. If a determination of pyridoxal is to be made by chemical methods, the sucrose must first be completely removed by dialysis; the hydrolysis of the protein may make furfural of the sucrose and interfere with the assay.

The highest specific activity of the "B" transaminase obtained was about the same as that of the leucine aminotransferase of pig heart. The pig heart enzyme has a specific activity of approximately 53 μmoles of glutamate formed per mg of protein used (Taylor and Jenkins, 1966). The "B" transaminase of P. aeruginosa may have specific activity as high as 60 to 70 μmoles of glutamate formed per min per mg of protein.

The substrate specificity of the "B" transaminase of P. aeruginosa was found to be virtually the same as that of the E. coli enzyme, but it is not identical to that of the branched chain aminotransferase of Neurospora crassa (Rudman and Meister, 1953; Seecof and Wagner, 1959$_a$). The "B" transaminase of P. aeruginosa is also similar in its substrate specificity to the branched chain amino acid transaminase of B abortus (Altenbern and Housewright, 1953). Most of the reports in the scientific literature show lists of specific activities, defined in varying terms, for several substrates which have been determined at one pH. The pH optima for four substrates presented in this report differed within a range from pH 7.0 to pH 8.5. It is clear that the specific activity of the enzyme from P. aeruginosa with L-norvaline as substrate should not be compared, in absolute terms, to its activity with L-isoleucine or L-valine

as a substrate at the same pH. A list of substrates on which a "B" transaminase is active should be considered just that, and no conclusions about which is the "best" or "poorest" substrate for the enzyme should be drawn from such data.

In cases where K_m's have been determined at different hydrogen ion concentrations, it has been possible to draw some conclusions as to which amino acids in the active site of an enzyme are responsible for the binding of the substrate to the protein (Dixon, 1953). If $\log_{10} K_m$ (pK_m) is plotted against pH, curves are produced which, it is claimed, show the number and charge of substrate or enzyme groups which have been titrated at a particular pH. When the slope of such curves is 0, no charges have been neutralized, a slope of +1 indicates change of +1, and a slope of -1 shows that the group titrated has changed in charge by -1. At the time his article setting forth this principle was published, Dixon stated that the effects of pH on K_m's had been determined for only seven enzymes, urease, phosphatase, arginase, saccharase, fumarase, cholinesterase and xanthine oxidase (Dixon, 1953). Later, Dodgson, Spencer and Williams (1955) used Dixon's method to determine pK_m's for the arylsulphatase of Alkaligenes metacaligenes. They obtained a curve, with nitrocatechol sulfate as substrate, which had two distinct peaks, one between pH 6.0 and 7.0 and the other between pH 7.5 and 10.0. The authors found that nitrocatechol had a pK at pH 6.5, which corresponded to the peak between 6.0 and 7.0. The peak between pH 7.5 and 10.0 they attributed to binding sites on the enzyme. Other substrates which they used had no titratable groups and the second pK_m was absent from their plots. In an earlier study, Dodgson and his coworkers found that the arylsulphatase had two

pH optima, depending upon the substrate used (Dodgson et al., 1954). The enzyme showed optimal activity at pH 7.8 when nitrocatechol sulfate was the substrate; when p-nitrophenyl sulfate and p-acetylphenyl sulfate were the substrates, the enzyme's optimal pH was 8.7.

The multiple pH optima of the "B" transaminase of P. aeruginosa are perhaps due to titratable binding sites on the enzyme's active site. If the peaks of highest activity are due to decreases in the K_m for a particular substrate, then perhaps binding sites on the enzyme may have pK's near 6.8, 7.5 and 8.3. The peaks are probably not due to the titration of substrate groups, because the pK's of all four amino acid substrates tested are very similar as is seen in these data by Edsall (1943):

Amino Acid	pK_1	pK_2
L-isoleucine	2.36	9.68
L-leucine	2.36	9.60
L-norvaline	2.36	9.72
L-valine	2.32	9.62

The variations seen in the pH optima curves must be due to titratable groups on the enzyme which interact with the amino acids according to their different spatial configurations. Since the amino acids are aliphatic, the binding site may have a benzene or imidazole ring structure capable of water-exclusion (hydrophobic) bonding with the amino acids.

The extreme difference in the binding constants ($1/K_m$) for 2-oxoglutarate and L-glutamate which were found for this enzyme are very interesting. It appears that the enzyme is poised to produce L-glutamate from 2-oxoglutarate. The binding constant for 2-oxoglutarate, 1/0.0004, was 44.9 times as great as the constant for L-glutamate, 1/0.018. The

disparity between the binding constants for 2-oxovalerate and L-norvaline was less, i.e., 1/0.00025 for 2-oxovalerate versus 1/0.003 for L-norvaline. The 2-oxo acid was bound to the enzyme 12 times more tightly than was the amino acid. A comparison of the available K_m's for three "B" transaminases has been made in Table 4. The K_m for glutamate of the chicken liver "B" transaminase is also high (Jenkins, 1957).

No other investigators, with the exception of Taylor and Jenkins (1966), have reported the molecular weight of a "B" transaminase. The animal enzyme, with a molecular weight of 75,000, is a little more than half as large as the enzyme from P. aeruginosa. Pig heart glutamate-aspartate transaminase has been known for some time to have a molecular weight of approximately 110,000 and a pyridoxal content of 2 moles per mole of protein (Jenkins, Yphantis and Sizer, 1959). Polyanovskii and Ivanov (1964) showed that the glutamate-aspartate transaminase of pig heart could dissociate into monomers at low protein concentrations, and that this dissociation increased the activity. Since Taylor and Jenkins (1966) also found increases in activity when the leucine aminotransferase was assayed in the presence of mercaptoethanol, it may be that this enzyme, like the glutamate-aspartate transaminase, is a dimer. This characteristic would make it like the P. aeruginosa enzyme with its molecular weight of 130,000 and two moles of cofactor per mole of protein.

The reason that the catalytic activity of the "B" transaminase from P. aeruginosa is not proportional to the enzyme concentration at levels above 2.5 μg per ml may be because the active sites of the enzyme are in some way "masked" by the protein molecules themselves. This theory could be checked by assays similar to those of Polyanovskii and Ivanov

TABLE 4

A COMPARISON OF K_m'S OF THREE "B" TRANSAMINASES

Substrates	*Pseudomonas aeruginosa* pH 7.5	*Neurospora crassa*[a] pH 8.0	Chicken[b] liver pH 7.0
2-Oxovalerate	2.6×10^{-4} M	-	-
2-Oxoglutarate	4.0×10^{-4} M	2.4×10^{-3} M	7.9×10^{-4} M
2-Oxoisocaproate	-	-	1.0×10^{-4} M
2-Oxo-3-methyl-valerate	-	1.0×10^{-3} M	-
2-Oxoisovalerate	-	1.5×10^{-3} M	-
Phenylpyruvate	-	1.2×10^{-4} M	-
L-Glutamate	1.8×10^{-2} M	high	6.7×10^{-2} M
L-Isoleucine	1.2×10^{-3} M	5.9×10^{-3} M	-
L-Leucine	1.0×10^{-3} M	-	1.7×10^{-3} M
L-Norvaline	3.0×10^{-3} M	-	-
L-Phenylalanine	-	1.3×10^{-3} M	-
L-Valine	3.6×10^{-3} M	2.4×10^{-3} M	-

[a]Seecof and Wagner, 1959.

[b]Jenkins, 1957.

(1964), but, unfortunately the type of equipment they used is not frequently employed in this country. The device which these Russian investigators used was a monochromator which projects, into the sample cuvette, light of a wavelength absorbed maximally by pyridoxal phosphate (approximately 340 mμ). At a right angle to the incoming light, a photomultiplier attached to another monochromator senses and amplifies the signal generated by fluorescent light of a longer wavelength (approximately 430 mμ) given off by excitation of the pyridoxal. An attachment to the device measures changes in the polarization of the fluorescent light.

Leucine aminotransferase of pig heart may be partially resolved of pyridoxal-5'-phosphate by incubating the enzyme with 0.05 M L-leucine in 1.0 M pH 7.5 potassium phosphate buffer. The lost activity may be restored by adding the cofactor to the reaction mixtures. When L-norvaline was used instead of L-leucine in a similar attempt to resolve the "B" transaminase of its cofactor, the enzyme remained active, even in the presence of 0.2 M L-norvaline. The bacterial enzyme may have its cofactor bound by linkages other than a Schiff-base between pyridoxal phosphate and the ε-amino group of lysine. Perhaps the phosphate is bound to an additional amino group near the active site by ionic forces, or it may be more tightly held by an ester linkage with the hydroxyl of a serine or threonine residue. If the amino acid sequence of the active site of the bacterial "B" transaminase is similar to the pig heart glutamate-aspartate transaminase, such an additional binding of the pyridoxal phosphate to the protein might be possible. Hughes, Jenkins and Fisher (1962) reduced the pig heart enzyme with sodium borohydride and hydrolyzed it with chymotrypsin. A determination of the sequence of the peptide which was

bound to pyridoxal phosphate showed it to have this structure:

N-Ser-Thr-Glu-Asp-Gly-Ala-Val-Ileu-ε-pyridoxyllys-Lys-Gly-Ser-Asp-Phe-C

The chief difficulty involved in chemical characterization of the active site of an enzyme, such as the "B" transaminase of P. aeruginosa, is the large quantity of starting material necessary. The total amount of the "B" transaminase present in 150 gm of wet bacterial cells was approximately 50 mg. Losses during the purification of the enzyme make it difficult to prepare even four or five mg of the protein. These problems may be relatively minor, however, now that a basic procedure for the purification has been established. It is possible that in the future much more will be known about the structure and mode of action of this enzyme.

CHAPTER V

SUMMARY

The enzyme which catalyzes the transamination reactions between the branched chain amino acids and 2-oxoglutarate in extracts of Pseudomonas aeruginosa was purified and partially characterized in this study. The purification methods included a two-step heat treatment, removal of nucleic acids with protamine sulfate, and separation of the "B" transaminase from other proteins with ammonium sulfate precipitation between 35 and 60 per cent of saturation. The extract was then further purified by molecular seiving with G-200 Sephadex and by column chromatography on ion exchangers, first on DEAE cellulose, then on cellulose phosphate.

The state of purity of the enzyme was checked by the use of acrylamide disc electrophoresis. This technique was useful when choosing the fractions of a chromatographic eluate which were suitable for pooling.

The molecular weight of the "B" transaminase was estimated tc be 130,000 gm per mole by placing it on a standardized G-200 Sephadex column and recording the mid-point of the elution volume for the protein. The elution volume was recorded on a graph made by plotting the elution volumes of bovine pancreatic trypsin, ovalbumin, bovine serum albumin and human gamma globulin against the logarithm of the molecular weight of these proteins.

"B" transaminase from P. aeruginosa catalyzed transamination between the L-isomers of isoleucine, leucine, methionine, norvaline, phenylalanine and valine and 2-oxoglutarate. The reactions were freely reversible. The optimal pH for enzyme activity was found to be 7.5 for isoleucine, 8.0 for norvaline and about 8.5 for valine. The enzyme had dual pH optima for leucine at 6.8 and 8.0. Assays were made routinely with L-norvaline and 2-oxoglutarate at pH 7.5.

The Michaelis-Menten constants were determined for seven of the potential substrates at pH 7.5. The 2-oxo acids used had K_m's in the 10^{-4} M range, amino acid substrates had K_m's of 1.0 to 3.6 x 10^{-3} M; L-glutamate had a K_m of 1.8 x 10^{-2} M. The enzyme seems poised for amino acid synthesis, especially glutamate production.

The pyridoxal content of the enzyme was found to be 2 moles per mole of protein. This determination was made chemically, with phenylhydrazine. The bacterial enzyme resisted resolution of its cofactor when procedures found successful with the leucine aminotransferase of pig heart were used.

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