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TITLE OF THESIS. Studies on the Pathway of Glutamic Acid Fermentation by Pepto.coccus aerogenes

UNIVERSITY. Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED. Ph.D.

YEAR THIS DEGREE GRANTED. 1971

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NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

STUDIES ON THE PATHWAY OF GLUTAMIC ACID FERMENTATION BY PEPTOCOCCUS AEROGENES

by

© Wendy M. Johnson

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Pathway of Glutamic Acid Fermentation by Peptococcus aerogenes" submitted by Wendy M. Johnson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date. Nov. 30, 1970

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External Examiner

ABSTRACT

It has been well established that there are major differences between aerobic and anaerobic modes of glutamate metabolism. Furthermore, all anaerobic pathways from glutamate are not identical since, on the basis of labelling patterns in the products of fermentation, the pathway in Peptococcus aerogenes has been shown to differ from the well-documented mesaconic acid pathway in Clostridium tetanomorphum.

In the present investigation, active cell-free extracts of P. aerogenes metabolized glutamic acid to α-hydroxyglutaric acid. Factors affecting the formation of this intermediate were studied by following the conversion of glutamic acid labelled with ^{14}C in the one or five positions. The production of α-hydroxyglutaric acid from glutamic acid by cell-free extracts was NAD-dependent. Labelled α-hydroxyglutaric acid produced by NAD-supplemented extracts was purified by anion exchange chromatography and identified by several methods including paper and thin-layer chromatography, mass spectrometry, and infrared spectroscopy.

The pathway from glutamate to α-hydroxyglutarate was shown to proceed via α-ketoglutarate. Moreover, two NAD-dependent enzymes involved in glutamate metabolism were isolated from cell-free extracts of P. aerogenes. One enzyme, glutamic dehydrogenase, was shown to oxidatively deaminate glutamate to α-ketoglutarate in the presence of NAD. The other enzyme, α-ketoglutarate reductase, reduced the α-keto acid to α-hydroxyglutarate and, in so doing, appeared to oxidize the reduced cofactor generated by the deamination of glutamate.

The two NAD-dependent enzymes involved in glutamate degradation were subjected to purification procedures, sedimentation and kinetic analyses, and general enzyme characterization. The results suggested that glutamate dehydrogenase, an enzyme not frequently implicated in anaerobic glutamate metabolism, was a predominating protein in extracts of P. aerogenes. The product of glutamate deamination, α-ketoglutarate, was in turn reduced by the α-ketoglutarate reductase. Kinetic data showed that the equilibrium of the second reaction favoured the direction of keto acid reduction, thereby producing α-hydroxyglutarate.

The metabolism of glutamate to α-hydroxyglutarate was correlated to radiotracer experiments monitoring glutamate fermentation by washed cells of P. aerogenes. In addition, hypotheses have been postulated for pathway intermediates beyond α-hydroxyglutarate to account for carbon distribution in the products and energy metabolism in the microorganism.

ACKNOWLEDGEMENTS

The author wishes to express appreciation to the National Research Council of Canada and the University of Alberta for providing financial assistance during the course of her graduate program.

The author also expresses specific thanks to Dr. D. W. S. Westlake for his generous assistance, optimism, and patience in supervising this project. In addition, appreciation is extended to Drs. W. E. Razzell and J. N. Campbell for their advice during specific phases of the work and to Dr. M. A. Pickard for his part in providing an enjoyable research atmosphere. Drs. R. A. Locock and R. T. Coutts are acknowledged for their assistance in obtaining and interpreting infrared spectra. The author also expresses gratitude to Mr. J. Kinnear for performing and programming the ultracentrifugal analysis and to Mrs. Colleen Dmytriw for her excellent photographic talents and technical assistance.

The author expresses special thanks to her husband, Ken, for his encouragement and interest during the course of these studies.

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ABBREVIATIONS

ATP	-	Adenosine triphosphate
°C	-	degrees Centigrade
cm	-	centimeter
DEAE	-	diethylaminoethyl
ε	-	extinction coefficient
E	-	extinction
EDTA	-	ethylenediaminetetraacetate
E.U.	-	enzyme unit
FAD	-	flavine adenine dinucleotide
FMN	-	flavine mononucleotide
g	-	gram
x g	-	gravity (centrifugal force)
hr	-	hour
°K	-	degrees Kelvin
K_{av}	-	distribution coefficient between liquid phase and gel phase
l	-	liter
M	-	molar
mA	-	millampere
mc	-	millicurie
m/e	-	mass to charge ratio
meq	-	milliequivalent
mg	-	milligram
ml	-	milliliter

mM	-	millimolar
mm	-	millimeter
M.W.	-	molecular weight
μc	-	microcurie
μg	-	microgram (10^{-6} g)
μl	-	microliter (10^{-6} l)
μmoles	-	micromoles (10^{-6} moles)
N	-	normal
NAD	-	nicotinamide-adenine dinucleotide
NADH	-	reduced nicotinamide-adenine dinucleotide
NADP	-	Nicotinamide-adenine dinucleotide phosphate
NADPH	-	reduced nicotinamide-adenine dinucleotide phosphate
nc	-	nanocurie
nmoles	-	nanomoles (10^{-9} moles)
O.D.	-	optical density
PCA	-	perchloric acid
psi	-	pounds per square inch
rpm	-	revolutions per minute
S.A.	-	specific activity
S_{obs}	-	observed sedimentation rate
TCA	-	trichloroacetic acid
TCS	-	trypticase soy
tris	-	tris(hydroxymethyl)aminomethane

V_e	-	elution volume
V_o	-	void volume
V_t	-	total volume
v/v	-	volume per volume
veronal	-	N,N-diethylbarbituric acid
w/v	-	weight per volume
w/w	-	weight per weight

LIST OF OFFICIAL ENZYME NOMENCLATURE

TRIVIAL NAME	E. C. NUMBER	SYSTEMATIC NAME
Catalase	1.11.1.6	H_2O_2:H_2O_2 oxidoreductase
Glutamic dehydrogenase	1.4.1.2	L-glutamate:NAD oxido-reductase (deaminating)
Histidase	4.3.1.3	L-histidine ammonia lyase
α-Ketoglutarate decarboxylase	4.1.1.1	2-oxo-acid carboxy-lyase
α-Ketoglutarate reductase (α-Hydroxyglutarate dehydrogenase)	1.6.99.3	Reduced NAD:(acceptor) oxidoreductase
Lysozyme	3.2.1.17	General Class: N-acetyl-muramide glycanohydrolase
L-Malate dehydrogenase	1.1.1.37	L-malate:NAD oxidoreductase
Alcohol dehydrogenase	1.1.1.1	alcohol:NAD oxidoreductase

INTRODUCTION

Several species of anaerobic or facultatively anaerobic bacteria have been shown to possess the ability to ferment single amino acids (Barker, 1961). These bacteria are generally grouped into one of two major classes, species of the anaerobic spore-forming rods Clostridium or the anaerobic cocci Micrococcus (Peptococcus) or Diplococcus. Glutamic acid is readily fermented by representatives of both major groups, specifically, Clostridium tetanomorphum (Woods, 1937) and Peptococcus (Micrococcus) aerogenes (Whiteley, 1957a).

Early observations indicated that a fundamental difference existed between aerobic and fermentative pathways of glutamate metabolism. The end-products of glutamate fermentation include acetate, butyrate, carbon dioxide, ammonia, and traces of hydrogen (Barker, 1937). Quantification of the products of glutamate fermentation by C. tetanomorphum (Wachsman and Barker, 1955a), Clostridium tetani (Pickett, 1943), and P. aerogenes (Whiteley, 1957a) illustrated that the yield of carbon dioxide and ammonia was consistently one mole per mole of glutamic acid fermented. Were glutamate deaminated to α-ketoglutarate and degraded to acetate and carbon dioxide through the tricarboxylic acid cycle (Hoare, 1963), the yield of carbon dioxide would be three moles per mole of glutamate fermented.

The pathway of glutamate decomposition has been well documented in C. tetanomorphum (Barker, 1961). In this organism, the branched-chain pathway from glutamate is known as the "mesaconic acid" pathway,

a term coined after the identification of mesaconate as a key intermediate (Wachsman, 1956). Several specialized enzymes found in this pathway convert glutamate to common metabolic intermediates. The first enzyme is glutamate mutase which rearranges glutamic acid to the branched-chain compound threo-3-methyl-L-aspartate (Barker et al., 1964). The second enzyme, β-methylaspartase, deaminates the product of the first reaction producing mesaconic acid (Barker et al., 1959). The next two enzymes in this metabolic sequence are known as mesaconase and citramalate lyase. Mesaconase hydrates mesaconic acid to L-(+)-citramalate (Blair and Barker, 1965) and the lyase cleaves citramalate to acetate and pyruvate, common metabolic intermediates (Barker, 1967). Studies using glutamic acid specifically labelled with carbon-14 (Wachsman and Barker, 1955a) indicated that carbon atoms 1 and 2 were primarily converted to acetate whereas carbon atoms 3 and 4 appeared in butyrate by the condensation of two-carbon fragments. The carbon dioxide was specifically derived from the carbon 5 carboxyl group of glutamate. It was suggested that the two-carbon fragment produced from carbons 3 and 4 must possess an activated group so that it may be distinguished from the free acetate released from carbons 1 and 2.

The pathway of glutamate degradation has not been so extensively investigated in other glutamate fermenting bacteria as in C. tetanomorphum. Solely on the basis of low levels of carbon dioxide production, one might presume that the mesaconic acid pathway functions in other anaerobes such as C. tetani (Pickett, 1943), P. aerogenes (Whiteley, 1957a), and Clostridium saccharobutyricum (Cohen et al., 1948), all of which readily ferment glutamate but produce only one mole of carbon

dioxide per mole of glutamate degraded. However, neither cells nor crude extracts of P. aerogenes which actively metabolized glutamic acid were able to degrade intermediates of the mesaconic acid pathway such as β-methylaspartate, DL-citramalate, or mesaconate (Whiteley, 1957a). These observations stimulated interest in the possibility of a unique pathway of glutamate fermentation by P. aerogenes.

Evidence for an alternate pathway of glutamate degradation in P. aerogenes was obtained by radioactive tracer experiments which established the fate of individual carbon atoms during the fermentation of specifically labelled glutamate (Horler et al., 1966a). The tracer experiments indicated that some of the butyric acid was derived from the first four intact carbon atoms of glutamate, not only from carbons 3 and 4 as in C. tetanomorphum. In addition, radioactive acetate was formed to the same extent from carbons 3 and 4 as from carbons 1 and 2. These observations were sufficient to indicate a distinct difference in the mode of glutamate fermentation by P. aerogenes from that by C. tetanomorphum.

Fractionation of reaction mixtures in which specifically labelled glutamic acid was fermented by cell suspensions of P. aerogenes resulted in the isolation of a radioactive, non-volatile, ether-soluble fraction (Horler et al., 1966b). The compound was isolated from reaction supernatants by steam distillation, ether extraction, and ion exchange chromatography. The radioactive product was identified by chemical analysis as glutaconic acid, the α:β-unsaturated dicarboxylic organic acid analogue of glutamic acid. Early chemical studies (Linstead, 1941) indicated that glutaconic acid reacted as a symmetrical compound.

These observations were probably valid since equilibrium frequently exists between α,β and β,γ isomers of α,β-unsaturated carbonyl compounds (Noller, 1965). In order to account for the specific release of carbon dioxide from the terminal carboxyl group of glutaconic acid, a mechanism was suggested for differentiating the two carboxyl groups of glutaconic acid. A system analogous to that found in the metabolism of glutaric acid by Pseudomonas fluorescens (Numa et al., 1964) was considered since the primary carboxyl group is involved as the Coenzyme A derivative leaving the terminal carboxyl group free for decarboxylation.

There has been some debate whether histidine fermentation by P. aerogenes could be considered the equivalent of glutamate fermentation. As documented by Barker, histidine, like glutamate, is readily fermented by species of both Clostridium (Wachsman and Barker, 1955a) and Micrococcus (Whiteley, 1957a). The pathway of histidine fermentation by these organisms appears to be similar to those used by aerobes such as P. fluorescens (Tabor, 1955) or Aerobacter aerogenes (Magasanik, 1955; Revel, 1958). Both C. tetanomorphum (Magasanik, 1955) and P. aerogenes (Whiteley, 1957a) have been shown to deaminate histidine to urocanic acid by action of the enzyme histidase. Whereas subsequent degradation of urocanate through α-formiminoglutamate by C. tetanomorphum resulted in the production of formamide and glutamate (Wachsman and Barker, 1955b), Whiteley found that P. aerogenes did not produce any formamide and only small quantities of formate (Whiteley, 1957a). The glutamate produced from histidine by C. tetanomorphum is only a transient intermediate since it is readily decomposed by cells or extracts of the organism,

presumably through the same pathway as glutamic acid is fermented. These observations suggested that glutamate and histidine fermentation are equivalent processes in C. tetanomorphum with formamide and ammonia as the additional end products. When Whiteley directly compared glutamate and histidine fermentation by P. aerogenes (Whiteley, 1957a), no glutamate, formamide, or equivalent amounts of formate could be detected during histidine metabolism. Moreover, quantification of the end products showed higher yields of acetate and lactate and lower yields of butyrate from histidine than from glutamate, indicating that histidine and glutamate were not decomposed by P. aerogenes through equivalent pathways.

The fermentation of histidine-α-^{14}C by P. aerogenes to acetate, butyrate, and formate has been documented (McConnell et al., 1967). Data gathered by these radiotracer studies indicated that histidine degradation does proceed via glutamate through the urocanic acid pathway. This conclusion was based on the distribution of carbon-14 in butyric acid, the accumulation of glutamic acid under sodium-deficient conditions, and the production of formate in addition to radioactive glutaconic acid. The accumulation of glutamate under conditions of sodium deficiency was consistent with observations of sodium stimulated glutamate utilization in P. aerogenes (Westlake et al., 1967).

This investigation was undertaken to determine the fate of glutamic acid when fermented by P. aerogenes ATCC 14963. Initially, experimentation was performed using washed cell suspensions in order to establish the distribution of fermentation products under variable extracellular conditions. Further analysis involved identification of

key metabolic intermediates and study of the enzymatic reactions during glutamate degradation by crude, particle-free extracts of P. aerogenes.

MATERIALS AND METHODS

I. Source of Chemicals and Biochemicals

All chemicals employed were obtained from commercial sources and were of reagent grade. The sources of enzymes, coenzymes, and radioactive compounds are specified in the text. Several radiochemicals which were required during experimentation were not commercially available. Procedures for the production and purification of these compounds are described in the text.

II. Cultural Conditions

Peptococcus aerogenes ATCC 14963, previously classified as Micrococcus aerogenes (Bergey, 1957), was obtained from the American Type Culture Collection. The culture was maintained in a lyophilized state at -20°C.

Cells of P. aerogenes were either grown in the "glutamate medium" as described by Whiteley (Whiteley, 1957a) or in the same medium supplemented with histidine (McConnell et al., 1967). Medium of the following composition was prepared in distilled water:

Difco Bacto-Peptone..... 2% (w/v)

Difco Yeast-Extract..... 1% (w/v)

Sodium glutamate (Fisher)..... 0.4% (w/v)

Difco Sodium thioglycollate... 0.1% (w/v)

Under histidine supplemented conditions, L-histidine (Sigma) was substituted for sodium glutamate at 0.4% (w/v). The medium was dispensed, autoclaved, and cooled quickly in an ice bath to ensure low oxygen tension. The medium was inoculated at 2% (v/v) and incubated at 37°C

under standing conditions.

Periodically the culture was checked for purity by determining the Gram reaction and fermentation pattern as previously described (Foubert and Douglas, 1948). Basal fermentation medium was prepared as follows and dispensed at 6 ml per tube prior to autoclaving:

Bacto-Peptone..... 2% (w/v)

Yeast-Extract..... 0.2% (w/v)

Sodium thioglycollate.. 0.1% (w/v)

0.2% Methylene Blue.... 0.1% (v/v)

One of the following sugar solutions was added at a concentration of 1 ml per tube: 6% glucose, 6% sodium pyruvate, 6% sodium malate, or 60% sodium lactate. Cultures were tested for indole production when grown in 1% Difco tryptone or 1% Trypticase Soy Broth. All tubes were inoculated with 0.1 ml of an 18 hour culture and incubated at 37°C for 24 hours.

Stock cultures were also examined periodically for aerobic contaminants by streaking samples of an 18 hour culture on plates containing plate count agar, eosin methylene blue agar, or phenol-red mannitol agar. Plates were prepared in duplicate and incubated aerobically at 30°C and 37°C for 24 hours.

Several anaerobes in addition to P. aerogenes ATCC 14963 were tested for their ability to utilize glutamic acid or histidine. These organisms were Streptococcus faecalis var. liquefaciens NCIB 7432, Peptostreptococcus spp. NCIB 8927, Staphylococcus aerogenes P.I. 1987, Pediococcus saccharolyticus ATCC 14953, and Fusobacterium polymorphum ATCC 10953. Since sodium has been shown to stimulate the rapid utiliza-

tion of glutamate by P. aerogenes (Westlake et al., 1967), duplicate tests were performed on all of the organisms in the presence of sodium. The anaerobes were grown, harvested, washed three times in 0.1 M potassium phosphate buffer, pH 7.0, adjusted to 10 O.D. units per ml, and tested for their ability to utilize glutamate or histidine at a concentration of 5×10^{-3} M. Sodium sulphate was added to duplicate reaction mixtures to a final concentration of 1×10^{-2} M to test for increased utilization in the presence of sodium.

III. General Analytical Techniques

The Lowry technique was employed for estimating the protein content of cell-free preparations using crystalline bovine serum albumin (Sigma) for standard reference (Lowry et al., 1951). The protein content of partially purified samples containing relatively low quantities of nucleic acids was determined from the extinction of diluted protein solutions at 260 nm and 280 nm (Warburg and Christian, 1941).

The Kjeldahl nitrogen method was employed for the microdetermination of ammonium and protein nitrogen (Ballentine, 1957). Hydrogen peroxide served as oxidant and nitrogen was determined using a ninhydrin assay. Samples containing ammonium sulphate were digested for preparation of standard curves. Alternatively, the Conway Diffusion Assay was employed for the determination of ammonia (Conway, 1962). Samples were titrated in the presence of Tashiro's indicator as described by Conway.

Free amino acids were estimated quantitatively by the ninhydrin method (Moore and Stein, 1948). Standard curves prepared using L-leucine exhibited a linear relationship to an optical density of 0.50.

Alpha keto acids were determined as their 2,4-dinitrophenylhydrazone derivatives. A modification of the documented technique (Friedemann, 1943) was used which permitted quantification using excess 2,4-dinitrophenylhydrazine reagent without organic solvent extraction.

The acetic anhydride-pyridine method was employed for the estimation of glutaconic acid (Spencer and Lowenstein, 1967). Whereas citrate and various other compounds also yield colored complexes in the reaction, the complex with glutaconate has a totally different absorption spectrum with a maximum at 480 nm.

Purified product intermediates were tested for unsaturation by Baeyer's test (Vogel, 1966). Cold 1% $KMnO_4$ was added a drop at a time and immediate bleaching indicated the presence of a double bond.

Carboxylic acids were determined quantitatively by titration of the free acids when suspended in distilled water. Salts were converted to the free acids by passage through excess Dowex-50 (H^+) prior to titration. The acids were titrated with standardized NaOH to the end point of a mixed indicator containing 5 parts 0.02% ethanolic bromcresol green and 1 part 0.02% methyl red (acid colour, red; alkaline colour, green). Total milliequivalents or milligrams of acid were calculated from the volume of NaOH required for titration and the normality of the NaOH (Newman, 1965).

When necessary, protein was precipitated by trichloroacetic acid (TCA), perchloric acid (PCA), or ethanol. A final concentration of 5% (w/v) TCA ensured complete protein precipitation. The precipitate was removed by centrifugation at 4°C for 10 minutes at 18,000 x g. A final

concentration of 5% (w/v) PCA followed by centrifugation as above removed all protein. Excess PCA was precipitated as the potassium salt by neutralizing the supernatant with KOH and cooling to 0°C before removing the $KClO_4$ precipitate by centrifugation. Addition of ethanol to a final concentration of 80% (v/v) followed by heat treatment, cooling, and centrifugation of the precipitate was also sufficient to remove protein.

IV. Radioactive Tracer Experiments

A. Methods for the Determination of Radioactivity

Quantitative absorption of radioactive carbon dioxide and homogeneous scintillation counting was achieved by the use of a phenethylamine (Packard) scintillator solution (Woeller, 1961). Radioactive carbon dioxide released from enclosed systems was trapped directly in scintillation vials containing 10 ml of phenethylamine scintillation fluid per vial. The vials were changed at appropriate intervals and counted for a minimum of 4 minutes per vial using a Nuclear Chicago liquid scintillation counter, Model Mark-I. Phenethylamine and other organic bases are the most satisfactory agents for absorption provided that volatile materials from the scintillation fluid do not interfere with the metabolic processes (Duncombe and Rising, 1969). In order to prevent interference from phenethylamine, the trapping vials for $^{14}CO_2$ were situated at a distance and the nitrogen flow was adjusted such that the positive pressure of nitrogen in the reaction vessel was sufficient to produce continuous bubbling of nitrogen-$^{14}CO_2$ through the scintillation fluid.

Radioactive compounds chromatographed on strips of Whatman No. 1 paper 4.0 cm in width were located by means of a Nuclear Chicago Actigraph III, Model 1002. Comparative peak areas determined by triangulation were used as a function of enzymatic conversion of radiochemicals from substrate to products.

Specific radioactive areas on chromatograms were removed, placed on planchets, and estimated in a Nuclear Chicago planchet counter. Alternatively, radioactive spots were eluted into water, concentrated, and suspended in a dioxane-based scintillator (Bray, 1960) prior to quantification in the Nuclear Chicago scintillation counter. Aqueous samples of amino, keto, or hydroxy acids were also dissolved in Bray's solution for quantitative estimation. Radiochemicals prepared in this manner were counted for a minimum of 4 minutes per vial. The scintillators and napthalene were obtained from Nuclear Enterprises Ltd.

Specifically labelled glutamic acids were obtained from New England Nuclear Corp. DL-Glutamic acid-1-^{14}C (S.A. = 2.03 mc/mM) and DL-Glutamic acid-5-^{14}C (S.A. = 2.66 mc/mM) were stored at -20°C in 0.1 N HCl. The radiochemical purity was tested periodically as described for paper chromatography of amino acids. Sodium-2-ketoglutarate-5-^{14}C (S.A. = 17.1 mc/nM) was obtained from Amersham-Searle and stored under nitrogen at -20°C. Radioactive solutions of α-ketoglutarate were tested frequently for radiochemical purity as described for paper chromatography of free α-keto acids.

B. Preparation of Specific Radiochemicals

Radiochemicals not readily available from commercial sources were prepared from related radioactive compounds and purified prior to use.

L-glutamic acid-5-^{14}C was prepared from DL-glutamic acid-5-^{14}C by the addition of nonradioactive L-glutamic acid carrier to the labelled DL-material and recrystallization of the L-isomer from ethanol-water mixtures. The product was recrystallized several times to constant specific activity (70 ncuries/mg). The radioactive L-isomer prepared by this procedure was used as substrate for conversion to $^{14}CO_2$ by washed cell suspensions of P. aerogenes.

A partially purified preparation of α-ketoglutarate reductase (E.C. 1.6.99.3) from P. aerogenes was employed for the preparation of α-hydroxyglutarate-5-^{14}C from sodium-2-ketoglutarate-5-^{14}C. The enzyme used for this conversion (S.A. = 27.12 E.U./mg) was prepared by ammonium sulphate fractionation of crude extracts followed by gel filtration on Sephadex G-200. The following components were added in a final volume of 5.0 ml:

α-ketoglutarate reductase in 0.05 M tris-HCl buffer, pH 8.8 (S.A. = 27.12 E.U./mg)..... 1 mg

Tris-HCl buffer, pH 8.8..... 250 μmoles

NADH (Sigma)..... 1.25 μmoles

Sodium-2-ketoglutarate-5-^{14}C (S.A. = 17.1 mc/mM) ..... 25 μcuries.

The mixture was incubated at 37° C for 1 hour and ethanol was added to a final concentration of 75-80% (v/v) to precipitate the protein. The

radioactive preparation was purified by ion exchange chromatography on Dowex-1-X8 (Baker-analyzed reagent). The labelled product was tested for radiochemical purity by co-chromatography with unlabelled α-hydroxyglutarate and stored in solution as the sodium salt at -20°C.

Alternatively, α-hydroxyglutarate-5-^{14}C was synthesized chemically from α-ketoglutarate-5-^{14}C by reduction with sodium borohydride (Juni, 1966). To a neutralized preparation of α-ketoglutarate-5-^{14}C (not more than 1.5 μmoles) was added 0.5 ml of 1.0 M $NaBH_4$. The solution was mixed, incubated at 30°C for 30 minutes, and 1.0 ml of 1.0 N H_2SO_4 added to destroy excess borohydride. After incubation for 10 minutes at 30°C, the solution was mixed vigorously for 30 seconds prior to the addition of 0.5 ml of 2.5 N NaOH for neutralization. The product was purified as before by ion exchange chromatography on Dowex-1. Both products were tested and compared as substrates for a partially purified preparation of α-hydroxyglutarate dehydrogenase.

V. Methods Involved in Product Accumulation Studies

Glass reaction vessels were designed such that the system was closed except for two outlets and a central tube extending to within a few millimeters of the bottom of the vessel. Rubber tubing or serum caps were attached to the outlets and highly purified nitrogen bubbled through the reaction mixture. This experimental technique permitted preincubation of cells, extracts, or substrates and rapid addition of the last component of the reaction through the third outlet. Dow Corning Antifoam A Spray was used when necessary to control excess foaming.

Cell-free extracts of P. aerogenes were prepared which, in the

presence of NAD, catalyzed the conversion of labelled glutamic acid to a major radioactive product that accumulated in the reaction mixture. Cells were grown for 10 hours at 37°C, harvested at 4°C by centrifugation for 15 minutes at 12,000 x g (Sorval, Model RC-2B), and washed twice in cold 0.05 M potassium phosphate buffer, pH 6.7. The washed cells were resuspended in the same buffer at a concentration of 250 mg wet weight per ml of buffer and treated with crystalline lysozyme (Sigma, E.C. 3.2.1.17). A solution containing 1 mg/ml lysozyme and 0.165% $MgCl_2$ in 0.05 M potassium phosphate buffer, pH 6.7, was added to the cell suspension to yield a final concentration of 100 µg/ml lysozyme (Nierlich et al., 1965), and the mixture was incubated for 20 minutes at 37°C. Cells were removed by centrifugation at 4°C for 20 minutes at 30,000 x g, resuspended in 0.1 M potassium phosphate buffer, pH 6.5, containing 0.01 M mercaptoethanol, and subjected to sonic disruption for 3 minutes at 0°C in a Bronwill Biosonik III set at maximum intensity. The particulate matter was removed by centrifugation at 4°C for 1 hour at 40,000 x g and the pellet discarded. Cell-free extracts prepared by this procedure were assayed for protein content by the method of Lowry.

Radioactive glutamic acid was separated from the products of enzymatic degradation by continuous ether extraction for 18 hours under acidic conditions (Neish, 1952). Preparations were acidified with 5 N HCl prior to extraction. This procedure was found to separate amino acids, which remained in the aqueous phase, from the non-amino, organic acids, which were extracted into the ether phase. The ether was evaporated to dryness and the residue dissolved in a known volume of water.

The ether-soluble fraction containing the accumulated reaction

product was purified by anion exchange chromatography. Dowex-1-X8(Cl^-) 200-400 mesh (Baker-analyzed reagent) was converted to the acetate or formate form by ion exchange to the base form followed by neutralization with the appropriate acid of the salt. The resin was washed with water to remove impurities and acid, neutralized to pH 6.8, and stored as a slurry. A column 3 x 15 cm was poured and the radioactive sample, pH 6.8-7.2, applied in as small a volume as possible. The column was developed using a gradient to 2.5 N acetic acid, followed by a gradient to 6.0 N formic acid as previously described (Newman, 1965). Fractions were collected as desired and the formic or acetic acid evaporated using a Buchler Rotary Evapo-Mix.

VI. Techniques for Product Identification

A. Chromatography

Preliminary analysis of reaction mixtures as well as tentative identification of purified products was achieved by descending paper chromatography on Whatman No. 1 paper. All ratios indicate solvent composition on a volume basis unless otherwise specified.

The following solvents were employed for the separation of glutamic acid, histidine, and closely related amino acids:

n-butanol:acetic acid:water (120:30:50) (Smith, 1960)

n-butanol:pyridine:water (1:1:1) (Morrison, 1953)

ethanol:ammonia (0.88):water (180:10:10) (Smith, 1960).

Amino acids were located by spraying with 0.25% (w/v) ninhydrin in acetone (Toennies, 1951) and heating at 100°C for 5 minutes. Under these conditions, glutamic acid is revealed as a purple spot and

histidine as a red-gray spot. A polychromatic technique comprised of a ninhydrin-cupric nitrate indicator was also used to differentiate individual amino acids on paper chromatograms (Moffat, 1959).

The separation and identification of α-keto acids such as α-keto-glutarate and pyruvate was achieved in the following solvents:

water-saturated n-butanol:formic acid (95:5) (Magasanik, 1950)

n-butanol:water:propionic acid (10:7:5) (Wieland, 1949)

toluene:acetic acid:water (100:5:60) (Liberman et al., 1951).

The third solvent required separation of the layers and the addition of 4.0 ml of acetic acid to the toluene layer. Keto acids were localized by spraying with 0.4% 2,4-dinitrophenylhydrazine in 2 N HCl (Mehlitz, 1963). The yellow colour of hydrazones turned to a red-brown colour after spraying with 10% NaOH or 10% Na_2CO_3 (Meigh, 1952).

The best separations of possible carboxylic acid intermediates were obtained in the following solvents:

n-butanol:acetic acid:water (120:30:50) (Smith, 1960)

ethanol:ammonia (0.88):water (80:5:15) (Cheftel, 1952)

ether:acetic acid:water (13:3:1) (Denison, 1952)

toluene:acetic acid:water (100:5:60) (Liberman, 1951).

Indicator sprays were employed for the location of acid spots. After development in strongly acidic chromatographic solvents, chromatograms were dried for 1 hour at 60°C then at room temperature for 2 to 3 hours. The chromatograms were then sprayed with a 0.04% bromophenol blue solution in 95% ethanol adjusted to pH 5.0 with NaOH (Bryant and Overall, 1953). Much better contrast was obtained when a few drops of NH_4OH were added just before spraying. After development in alkaline solvents,

chromatograms were sprayed with one of two indicator solutions. The first indicator, 0.04% aqueous chlorophenol red adjusted to pH 4.7 (Brown, 1951), was applied after the chromatograms were heated to 95°C for 5 minutes. Alternatively, a mixture containing 50 mg phenol red in 20 ml 95% ethanol plus 2.0 ml of N NaOH when diluted to 100 ml with water was sprayed on chromatograms to reveal acidic areas (Buffa et al., 1951).

Several specific spray reagents were utilized for locating organic acids such as α-hydroxyglutarate. The aniline-xylose reagent (Smith, 1960) or the Altman reagent of p-dimethylaminobenzaldehyde and acetic anhydride in acetone (Smith, 1960; Nordmann, 1955) was used extensively. The Altman reagent was particularly sensitive for glutaconic acid which produced an intense pink colour after heating the chromatogram for 2 to 3 minutes at 130°C. Keto and organic acids were also detected by a 0.1% (w/v) ethanolic solution of 2,6-dichlorophenolindophenol (sodium salt) followed by brief heating (Passera, 1964).

Thin-layer chromatography was also found to be useful for the separation of mixtures of carboxylic acids or the 2,4-dinitrophenylhydrazone derivatives of α-keto acids. Thin-layer plates of Silica Gel H (Stahl's) were prepared on glass plates 10 x 10 cm or 20 x 20 cm. All plates were activated by heating at 100°C for 1 hour prior to the use of diisopropyl ether:formic acid:water (90:7:3) (Knappe, 1965), or n-propanol:ammonium hydroxide (70:30) (Passera, 1964), solvent systems which resulted in good separation of carboxylic acid mixtures. The separation of 2,4-dinitrophenylhydrazone derivatives was best achieved in n-butanol:ethanol:0.5 N ammonia (70:10:20) (El Hawary et al., 1953)

or isoamyl alcohol: 0.25 N NH_4OH (95.2:4.8) (Dancis et al., 1963).

Low voltage paper electrophoresis was utilized for the separation and identification of glutaconic and glutaric acids. Electrophoresis was performed on 4 x 41 cm LKB filter paper strips in an LKB Electrophoresis apparatus at room temperature. The buffer for electrophoresis contained pyridine:acetic acid:water (5:3.4:90) at a final pH of 5.0 (Numa et al., 1964). The strips were run at 8 mA per strip for the desired length of time, dried, and sprayed with specific detection reagents mentioned previously.

B. Infrared Spectroscopy

The reaction product which was purified by anion exchange chromatography was analyzed by infrared spectroscopy. Samples of the purified product were taken to dryness in vacuo in preparation for analysis on a Beckman IR-10 performed by the Spectroscopy Laboratory, Faculty of Pharmacy, University of Alberta, or the Spectroscopy Laboratory, Department of Chemistry, University of Alberta. Pressed discs were formed by mixing the solid substance 0.5-1.0% (w/w) in pure, crystalline potassium bromide and compressing the mixture under high pressure at room temperature. Occasionally, samples were prepared for infrared analysis as a film on NaCl prisms. The infrared spectra were interpreted by reference to previously documented correlation charts and group absorption frequency tables (Cross, 1964). After tentative identification, unknown samples were tested in parallel with standard acids and the spectra compared directly.

C. Mass Spectrometry

Mass spectrometry confirmed the identity of the purified product.

Samples were taken to dryness in vacuo prior to analysis by the Spectroscopy Laboratory, Department of Chemistry, University of Alberta. The mass spectroscopic analysis was performed according to the following specifications: direct probe, source temperature of 150°C at 8 EM and 1.8 kV. The mass spectra were compared to standard spectra (Budzikiewicz et al., 1967) on the basis of m/e values and relative abundance.

VII. Isolation and Purification of Enzymes

P. aerogenes was grown and harvested as previously described under product accumulation studies. The cells were washed twice in cold 0.05 M tris-HCl buffer, pH 8.8, prior to breakage by rupture in the Hughes' Press or sonic disruption. Washed cells were formed into plugs and frozen before treatment in the Hughes' Press. Otherwise, washed cells were resuspended in the same buffer at a concentration of 250 mg wet weight per ml in preparation for sonic disruption in a Bronwill Biosonik III for 5 minutes at maximum intensity. Particle-free extracts were obtained by centrifugation of the ruptured cells as previously described.

Ammonium sulphate fractionations of cell-free extracts were performed at 0°C by the slow addition of solid $(NH_4)_2SO_4$ (Mann, ultra pure grade) with continuous stirring. Once the desired weight was added (Green and Hughes, 1955), the mixture was stirred for an additional 15 minutes in an ice bath.

Ion exchange chromatography on cellulose was performed using DEAE-cellulose (Sigma, medium mesh; 0.89 meq/g capacity). The exchanger was suspended in distilled water and charged by successive washings with NaOH, water, ethanol, water, versene, water, NaOH, and water (Peterson,

1962). The cellulose was then suspended in 0.5 M tris-HCl buffer, pH 8.8, and stored at 4°C until required. Columns were equilibrated with 0.05 M tris-HCl buffer, pH 8.8, in preparation for sample application. Protein was eluted from DEAE-cellulose by means of a NaCl gradient in 0.05 M tris-HCl buffer, pH 8.8.

Ion exchange chromatography on Sephadex was performed using DEAE-Sephadex A-50 (Pharmacia) which was allowed to swell in distilled water and washed repeatedly with 0.5 N NaOH. Excess NaOH was removed by rinsing with distilled water. The exchanger was then treated with 0.5 N HCl, rinsed with water, and equilibrated with 0.05 M tris-HCl buffer, pH 8.8, prior to use in a K 25/45 jacketed Sephadex column at 4°C. The column was subjected to downward flow gradient elution with increasing concentrations of tris-HCl buffer, pH 8.8.

Sephadex G-200 (Pharmacia) was employed for enzyme fractionations by gel filtration. Sephadex G-200 was swollen for 72 hours at room temperature before equilibration in 0.05 M tris-HCl buffer, pH 8.8. A column of G-200 was prepared in a K 25/100 jacketed Sephadex column at 4°C using a 10 to 12 cm operating pressure throughout the pouring procedure. The void volume, V_o , was determined by applying 1 ml of Blue Dextran 2000 (1 mg/ml) and monitoring at 620 nm. The flow rate did not exceed 12 ml/hour and in all cases the eluant buffer was 0.05 M tris-HCl pH 8.8.

Adsorption chromatography was performed on hydroxylapatite prepared from 0.5 M $CaCl_2$ and 0.5 M Na_2HPO_4 (Levin, 1962) and suspended in 0.005 M sodium phosphate buffer, pH 6.8. Protein was adsorbed from 0.005 M buffer and elution followed a gradient increase in buffer

concentration to 0.4 M at constant pH. Protein samples were dialyzed against 0.005 M sodium phosphate buffer, pH 6.8, at 4°C prior to application to the column and against 0.05 M tris-HCl buffer, pH 8.8, subsequent to pooling of the active fractions.

VIII. Enzyme Assays

Glutamic dehydrogenase activity (E.C. 1.4.1.2) was assayed by measuring the formation of reduced NAD at 340 nm. The assay mixture contained 40 μmoles tris-HCl buffer, pH 8.8, 10 μmoles L-(+)-glutamic acid, 1.0 μmole β-NAD (Calbiochem) and enzyme in a final volume of 1.0 ml. Initial velocity results were converted into International enzyme units as described by Cooper (Cooper et al., 1958). Hence, one enzyme unit (E.U.) is the amount of enzyme which converts 1 μmole of substrate per minute at 25°C under saturating conditions of substrate concentration. The "specific activity" of the enzyme was the activity of a milligram of the enzyme (i.e., E.U./mg protein). The extinction coefficient (ε) and factors for the conversion of O.D. (E) to concentration of reduced NAD at 340 nm were as follows (Bergmeyer, 1963):

$$\varepsilon(cm^2/mole) = 6.2 \times 10^6 \quad \text{(Horecker, 1948)}$$

$$\mu moles\ NADH/ml = E \times 0.1613$$

$$\mu g\ NADH/ml = E \times 107.3.$$

Reported values for enzymic activities were corrected for control levels of reduction observed in the absence of added glutamate.

The activity of α-hydroxyglutarate dehydrogenase (α-ketoglutarate reductase) (E.C. 1.6.99.3) could be assayed either as a function of α-keto acid reduction or of α-hydroxy acid oxidation. When measured by

the formation of reduced NAD, activity was followed by increase in absorbance at 340 nm. The assay mixtures contained 40 μmoles of tris-HCl buffer, pH 8.8, 5 μmoles of D-α-hydroxyglutarate (Sigma, sodium salt), 1.0 mole of β-NAD, and enzyme in a final volume of 1.0 ml. When DL-α-hydroxyglutarate (Sigma, hemi-zinc salt) was utilized as substrate, the final concentration in the reaction mixture was 10 μmoles/ml. A suspension of zinc α-hydroxyglutarate was converted to the sodium salt by batch treatment with Dowex-50-X8 (Na^+). Resin was added until no insoluble material remained, then removed by centrifugation or filtration. The supernatant was adjusted to pH 8.8 and diluted for use as substrate. Enzyme activity was expressed in terms of International units, i.e., either as μmoles of α-ketoglutarate produced or as μmoles of NAD reduced/minute at 25°C under conditions of saturating substrate concentration. Enzyme activities were corrected for control levels of NAD reduction in the absence of α-hydroxyglutarate. Enzyme units and specific activities were calculated as described for glutamate dehydrogenase. Enzyme activity could also be determined as a function of α-keto glutarate reduction in the presence of reduced NAD. Initial velocities were determined by absorbancy changes at 340 nm in an assay mixture containing 40 μmoles tris-HCl buffer, pH 8.8, 1 μmole α-ketoglutarate (sodium salt), 0.25 μmoles NADH (Sigma) and enzyme in a final volume of 1.0 ml. Concentrations of α-ketoglutarate and NADH were chosen to permit maximum velocity and avoid substrate inhibition. Enzyme activity was recorded in International units as previously described.

The assay employed for the quantification of histidase activity (E.C. 4.3.1.3) in histidine grown cells of P. aerogenes has been

documented in detail (Tabor and Mehler, 1955). The assay is spectrophotometric, based on the appearance of urocanic acid which absorbs at 277 nm. One enzyme unit was defined as the amount of enzyme which causes an increase in O.D. at 277 nm of 0.001/minute at 25°C. Specific activity was expressed as units/mg protein.

The assay for L-malate dehydrogenase activity (E.C. 1.1.1.37) was identical to that described by Yoshida for the oxidation of L-malate at pH 8.8 (Yoshida, 1969). Enzyme activity was expressed in International units (i.e., 1 μmole/minute at 25°C) and specific activity as units/mg protein.

The α-ketoglutarate dehydrogenase complex was assayed by the ferricyanide reduction assay originally described for the pyruvate decarboxylase component (E.C. 4.1.1.1) of the pyruvate dehydrogenase complex (Reed and Willams, 1966). The application of this assay for the determination of the α-ketoglutarate dehydrogenase complex has been previously documented (Reed and Mukherjee, 1969). One unit was the amount of enzyme required to produce 2 μmoles of ferrocyanide per hour at 30°C. Specific activity was expressed as units/mg of protein.

IX. Determination of Purity and Enzyme Characterization

Molecular weight approximations were obtained by means of gel filtration on Sephadex G-200 according to the procedure of Andrews (Andrews, 1965). G-200 was prepared as previously described under enzyme purification procedures. A Sephadex column (K 25/45) equipped with flow adaptors was filled with gel to a depth of 36 cm (i.e., 2.5 x 36 cm) and equilibrated in 0.05 M tris-HCl buffer, pH 8.8, under an

operating pressure of 10 cm. The flow rate was adjusted to 5 ml/hour and the V_o and V_t determined using Blue Dextran 2000 and sucrose respectively. Standard proteins were prepared in buffer in a sample volume of 1.0 ml, eluted with 0.05 M tris-HCl buffer, pH 8.8, and collected in 1.0 ml fractions. Standard proteins, amounts, and assay conditions are summarized in Table I.

Elution volumes were determined for each protein and expressed in terms of the partition coefficient, K_{av} ,

$$K_{av} = \frac{Ve - V_o}{V_t - V_o}$$

where V_e is the elution volume, V_o is the void volume, and V_t is the total volume of the gel bed. A selectivity curve was constructed for G-200 by plotting K_{av} versus log molecular weight for the standard proteins. After determining the K_{av} values for purified glutamic acid dehydrogenase and α-ketoglutarate reductase, their molecular weights were estimated from the selectivity curve.

Sedimentation analyses on purified enzyme preparations of glutamic dehydrogenase and α-ketoglutarate reductase were performed using a Beckman Model E Analytical Centrifuge employing Schlieren optics. Protein concentration was determined by the method of Lowry. During sedimentation velocity experiments, the time at which the rotor reached the desired speed was designated time zero.

Purified glutamic dehydrogenase was dialyzed at 4°C in 2 litres of 0.05 M tris-HCl buffer, pH 8.8, for 24 hours prior to protein estimation. The protein concentration was then adjusted to 4 mg/ml before sedimentation velocity analysis according to the following

TABLE I

STANDARD COMPOUNDS, AMOUNTS, AND ASSAY CONDITIONS FOR MOLECULAR WEIGHT APPROXIMATIONS ON SEPHADEX G-200

COMPOUND	AMOUNT	ASSAY
Blue Dextran 2000 (Pharmacia)	1.0 mg	E_{620} (Andrews, 1965)
Sucrose	3.0 mg	Anthrone (Trevelyan, 1952)
Cytochrome c	2.0 mg	E_{412} (Edsall, 1953)
Ovalbumin	3.0 mg	E_{280}
Serum Albumin Monomer (Bovine)	5.0 mg	E_{280}
Serum Albumin Dimer (Bovine)	0.5 mg (approx.)	E_{220} (Andrews, 1965)
Yeast Alcohol Dehydrogenase	2.0 mg	NAD reduction (Racker, 1955)
Catalase (Horse)	0.1 mg	E_{230} (Chance, 1955)

specifications:

Cell type: 4° single sector, Kel-F 12mm centerpiece

Speed: 60,000 rpm

Temperature: 5°C

Length of Run: 80 minutes

Photo interval: 8 minutes

Phase Plate Angle: 60° .

Purified α-ketoglutarate reductase was dialyzed as described for glutamate dehydrogenase and the protein concentration adjusted to 4 mg/ml. The speed, temperature, and phase plate angle were identical to those presented above. However, the sedimentation cell had a 4° synthetic boundary and a 12 mm centerpiece, the duration of the run was 40 minutes, and photographs were taken at 4 minute intervals.

Conventional sedimentation equilibrium experiments were performed in preference to the classical sedimentation and diffusion method. Thermodynamic equilibrium was established such that the solute was distributed throughout the centrifuge cell and the solute concentration at the meniscus was not zero. The time required for equilibrium to be attained was 98 hours for glutamic dehydrogenase and 54 hours for α-hydroxyglutarate dehydrogenase. Both enzymes were suspended in 0.05 M tris-HCl buffer at concentrations of 2,4, and 6 mg protein/ml. Separate equilibrium experiments were performed for each protein concentration in a charcoal-epon interference cell with a 12 mm centerpiece. Sedimentation equilibrium data were obtained at 5°C using Rayleigh interference optics with an equilibrium rotor speed of 4,840 rpm for glutamic dehydrogenase and 11,000 rpm for α-hydroxyglutarate.

Polyacrylamide gel electrophoresis of crude and purified protein preparations was performed in the classical manner (Davis, 1964). Polyacrylamide gels containing 7% (w/v) polyacrylamide were prepared with tris plus glycine buffer in 6 x 64 mm glass tubes. Gels prepared in this manner were designed to stack at pH 8.9 and run at pH 9.5, providing maximum resolution of proteins from 30,000 to 300,000 molecular weight. The polymerized gels were subjected to electrophoresis in a Canalco Electrophoresis Apparatus at 4 mA/column for 50 minutes or until the riboflavin migrated to the end of the small pore gel. After electrophoresis and removal of the gels from the glass columns, enzymes were located in situ by a system similar to that used to locate multiple forms of D-(-)-3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30) in several species of Rhizobium (Fottrell, 1969). The gels were immersed in solutions containing the following reagents and incubated for 20 minutes at 37°C for development:

Tris-HCl buffer, pH 8.8..... 50 µmoles/ml

NAD..... 1 mg/ml

Phenazine methosulphate (Sigma)..... 25 µg/ml

Nitro-blue tetrazolium (Sigma).....250 µg/ml

Sodium glutamate (Fisher).....16.9 mg/ml (0.10 M)

OR Sodium D-α-hydroxyglutarate (Sigma).... 9.6 mg/ml (0.05M).

The enzyme location solution for glutamic dehydrogenase contained sodium glutamate whereas solutions for α-hydroxyglutarate dehydrogenase activity contained sodium D-α-hydroxyglutarate. After development of the gels, the location reagent was removed and the gels were fixed in 7% (w/v) acetic acid for permanent storage.

For detection of non-specific proteins, gels were simultaneously fixed and stained in 7% aqueous acetic acid containing 1% (w/v) Buffalo Black for 2 hours at room temperature. The gels were rapidly destained in a Canalco Rapid Destaining Apparatus for 15 minutes in two changes of 7% acetic acid.

The quantity of protein required for the most effective location of enzyme or protein bands was found to vary, depending on the purity of the protein preparation. Satisfactory resolution of crude extracts was achieved using 15 μg of protein for the in situ assay as well as the protein stain. The in situ enzyme assays for gels containing purified preparations of glutamic dehydrogenase required only 3 μg of protein whereas those containing purified α-hydroxyglutarate dehydrogenase required 20 μg of protein. For the non-specific protein stain, 15-20 μg of the purified enzyme preparations was found to produce discrete, well-defined bands of protein.

Enzyme characterization experiments were performed on purified preparations of glutamic dehydrogenase and α-hydroxyglutarate dehydrogenase in order to establish basic properties of the enzymes including such aspects as kinetic parameters, stability, and molecular constants. Details of the characterization experiments are presented in the text of the experimental section or in the legends to the appropriate figures of tables.

EXPERIMENTAL

I. Cultural Characteristics

P. aerogenes grew well on medium supplemented with either glutamate or histidine, producing gas and reaching an optical density of 2.0 at 600 nm after 12 hours growth at 37°C. Microscopic examination of 18 hour cultures revealed Gram-positive cocci occurring singly, in pairs, tetrads, or irregular clumps. Pure cultures of P. aerogenes did not ferment glucose, lactate, pyruvate, or malate when these compounds were supplemented to the basal fermentation medium, although growth occurred in all cases. This fermentation pattern agrees with that previously documented for P. aerogenes (Foubert and Douglas, 1948). Moreover, pure cultures were also found to produce indole when grown in 1% TCS broth (Bergey, 1957). Although several anaerobes were tested for their ability to utilize glutamic acid and histidine in the absence and presence of sodium as described in Section II of Materials and Methods, the only positive results were obtained with P. aerogenes (ATCC 14963). This organism fermented both glutamic acid and histidine, confirming earlier observations of histidine degradation (McConnell et al., 1967) and sodium-stimulated glutamate utilization (Westlake et al., 1967).

II. Fermentation of Radioactive Glutamic Acid by Washed Cells

The effect of sodium on the utilization of glutamic acid was determined using washed cells of P. aerogenes. Cells were grown for 10 hr on glutamate medium, washed three times in 0.1 M potassium phosphate buffer, pH 7.0, and added to reaction mixtures containing buffer and

substrates as outlined in the legend to Figure 1. Sodium sulphate was added at 8.8×10^{-3} M when required and the release of $^{14}CO_2$ from L-glutamic acid-5-^{14}C was followed as a function of time at 37°C. The results in Figure 1 indicate that sodium does stimulate glutamic acid utilization by whole washed cells of P. aerogenes. In the presence of Na_2SO_4, 72% of the ^{14}C-glutamic acid was degraded to $^{14}CO_2$ in 4 hr as compared to 5% in the absence of Na_2SO_4. These results compare favorably with those for sodium-stimulated glutamate utilization cited previously (Westlake et al., 1967).

The effect of growth conditions on glutamic acid utilization by washed cells was also investigated. Cells were grown for 10 hr on medium supplemented with glutamate or histidine and washed as before. Reaction mixtures containing glutamic acid-5-^{14}C as substrate were incubated at 37°C and $^{14}CO_2$ release was followed as a function of time. The results are shown in Figure 2 where the evolution of $^{14}CO_2$ as nmoles/mg protein-N has been expressed as percentage conversion of substrate. In several independent experiments, cells grown on glutamate consistently released lower levels of $^{14}CO_2$ after 15 minutes incubation than cells grown on histidine. However, the curves became almost identical after 30 minutes incubation at 37°C. Experiments were performed using two concentrations of radioactive glutamate, 0.164 nmoles/ml and 0.246 nmoles/ml. The results are only presented for the lower concentration of radioactive substrate since no significant differences were observed in the kinetics at the two concentrations.

The pH of the buffer used to suspend the washed cells was found to influence the degradation of glutamic acid-5-^{14}C. Cells were grown

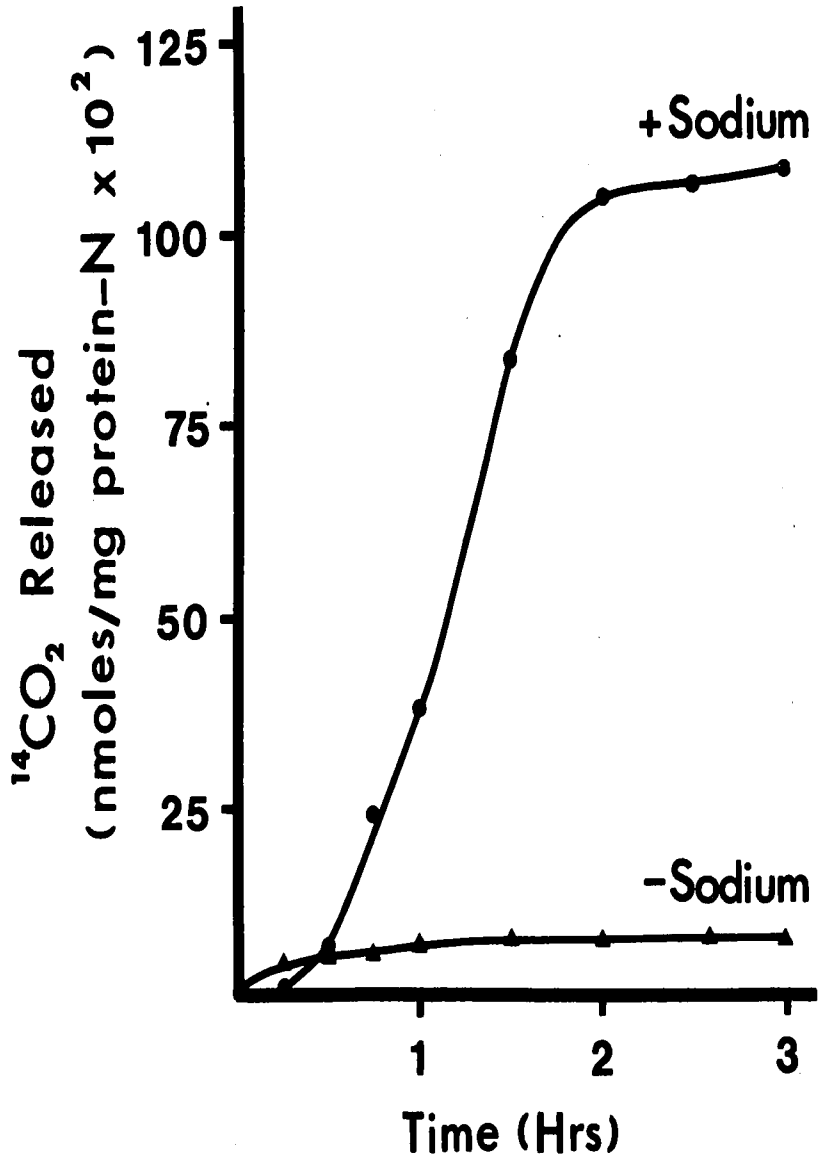

125
100
75
50
25
+Sodium
-Sodium
1
2
3
Time (Hrs)
$^{14}CO_2$ Released
(nmoles/mg protein-N x 10^2)

FIGURE 1

SODIUM STIMULATION OF GLUTAMIC ACID DEGRADATION

BY WASHED CELLS OF P. AEROGENES

Cells were grown for 10 hr on glutamate medium, washed three times in 0.1 M potassium phosphate buffer, pH 7.0, and added to reaction mixtures containing: 0.0625 M phosphate buffer, pH 7.0, 13.3 nmoles L-glutamic acid-5-^{14}C, and 3.1×10^{-3} M glutamic acid in a final volume of 8.0 ml. Sodium sulphate was added at 8.8×10^{-3} M when required.

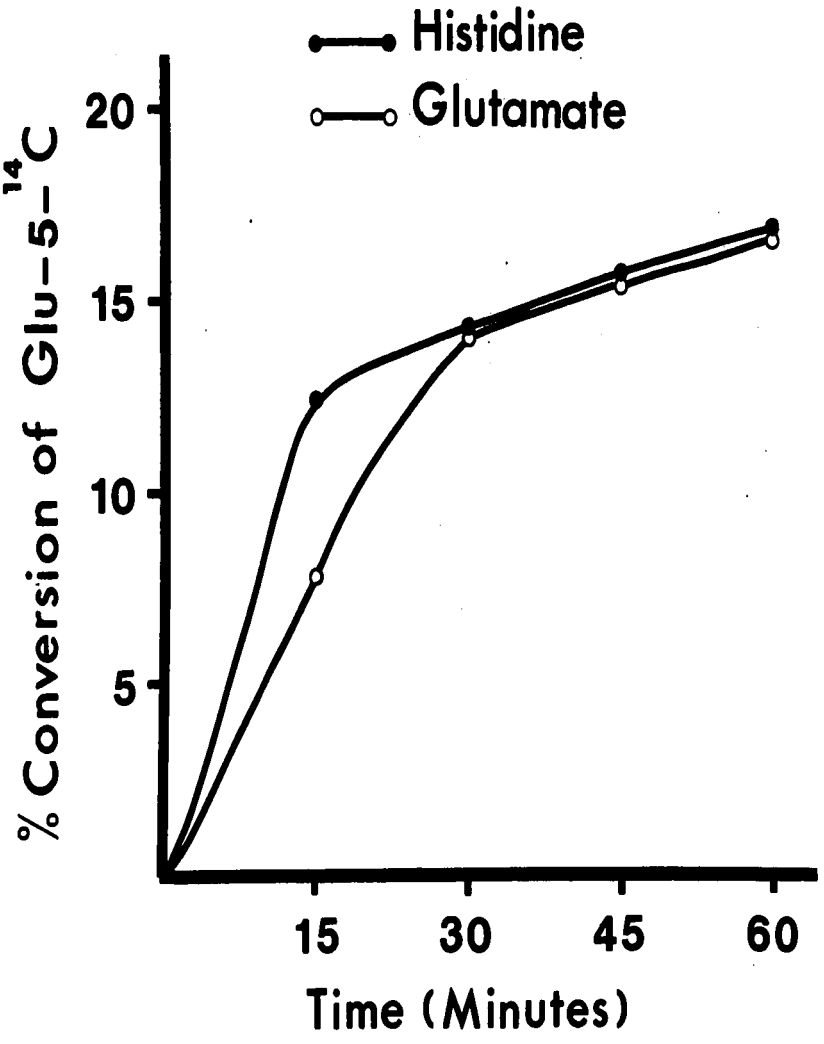

Histidine
Glutamate
% Conversion of Glu-5-^{14}C
20
15
10
5
15
30
45
60
Time (Minutes)

FIGURE 2

COMPARISON OF THE RATE OF GLUTAMATE DEGRADATION BY CELLS OF P. AEROGENES GROWN ON HISTIDINE OR GLUTAMIC ACID

Cells were grown for 10 hr on medium supplemented with glutamate or histidine, washed three times in 0.1 M phosphate buffer, pH 7.0, and added to reaction mixtures containing: 0.096 M potassium phosphate buffer, pH 7.0, 7×10^{-3} M Na_2SO_4, and 0.82 nmoles DL-glutamic acid-5-^{14}C in a final volume of 5.0 ml. The release of $^{14}CO_2$ calculated as nmoles/mg protein-N has been expressed as % conversion of substrate.

and washed as described above. Samples of washed cells were resuspended in 0.1 M potassium phosphate buffers of varying pH and the release of $^{14}CO_2$ after 30 minutes incubation at 37°C was plotted as a function of protein-N at several pH values. The results in Figure 3 indicate that the optimum evolution of $^{14}CO_2$ from glutamic acid-5-^{14}C by whole cells occurs at pH 6.5 in potassium phosphate buffer.

The effect of extracellular glutamate concentration of the degradation of specifically labelled glutamic acid was tested using cells from histidine- as well as glutamate-supplemented medium. Washed cells were suspended in phosphate buffer at pH 6.5 and added to reaction mixtures containing glutamate at concentrations varying from zero to 2 x 10^{-2} M. The release of $^{14}CO_2$ from DL-glutamic acid-5-^{14}C was followed for 90 minutes at 15 minute intervals. The results are presented in Figure 4 for histidine-grown cells and in Figure 5 for cells grown on glutamic acid. The conversion of DL-glutamic acid-5-^{14}C to $^{14}CO_2$ appears to be stimulated by extracellular glutamate only in the case of cells which were cultured on glutamate. The data showed that low concentrations (2.5 and 5.0 mM) of glutamic acid carrier enhanced glutamate degradation above the control level. The progressively lower amount of $^{14}CO_2$ produced as the extracellular concentration of glutamic acid increased was attributed to dilution of the radioactive glutamic acid prior to entry into the cell. The results using histidine-grown cells show no stimulation of glutamate conversion by non-radioactive carrier. The control levels in both systems appear to be fairly comparable although there is a definite levelling after 45 minutes in the cells grown on glutamate which is not observed with histidine-grown cells.

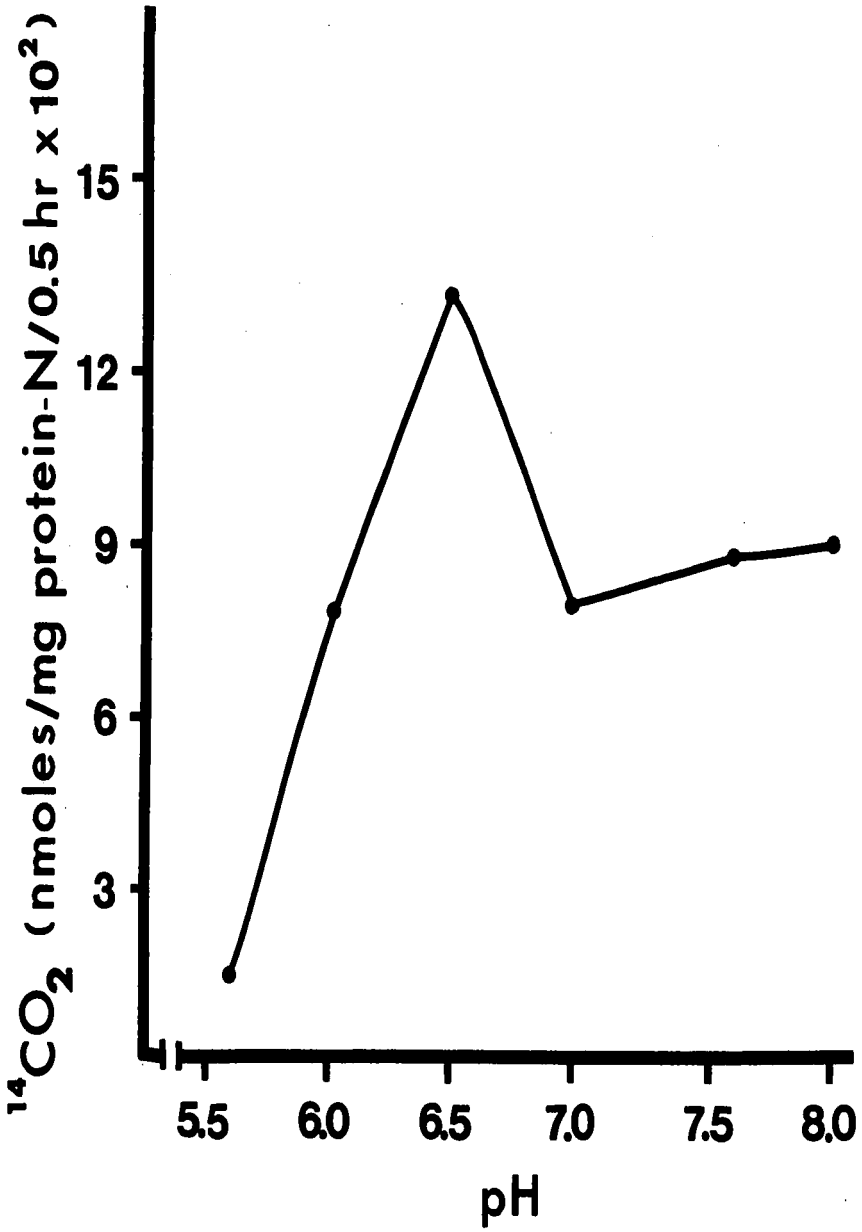

$^{14}CO_2$ (nmoles/mg protein-N/0.5 hr x 10^2)
15
12
9
6
3
5.5
6.0
6.5
7.0
7.5
8.0
pH

FIGURE 3

EFFECT OF pH ON THE DEGRADATION OF GLUTAMATE

BY WASHED CELLS OF P. AEROGENES

Cells were grown for 10 hr on glutamate supplemented medium, washed as before, and resuspended in 0.1 M potassium phosphate buffers of varying pH. Each reaction mixture contained the following: 0.82 nmoles DL-glutamic acid-5-^{14}C, 7 x 10^{-3} M Na_2SO_4, and 0.096 M potassium phosphate buffer of desired pH in a final volume of 5.0 ml.

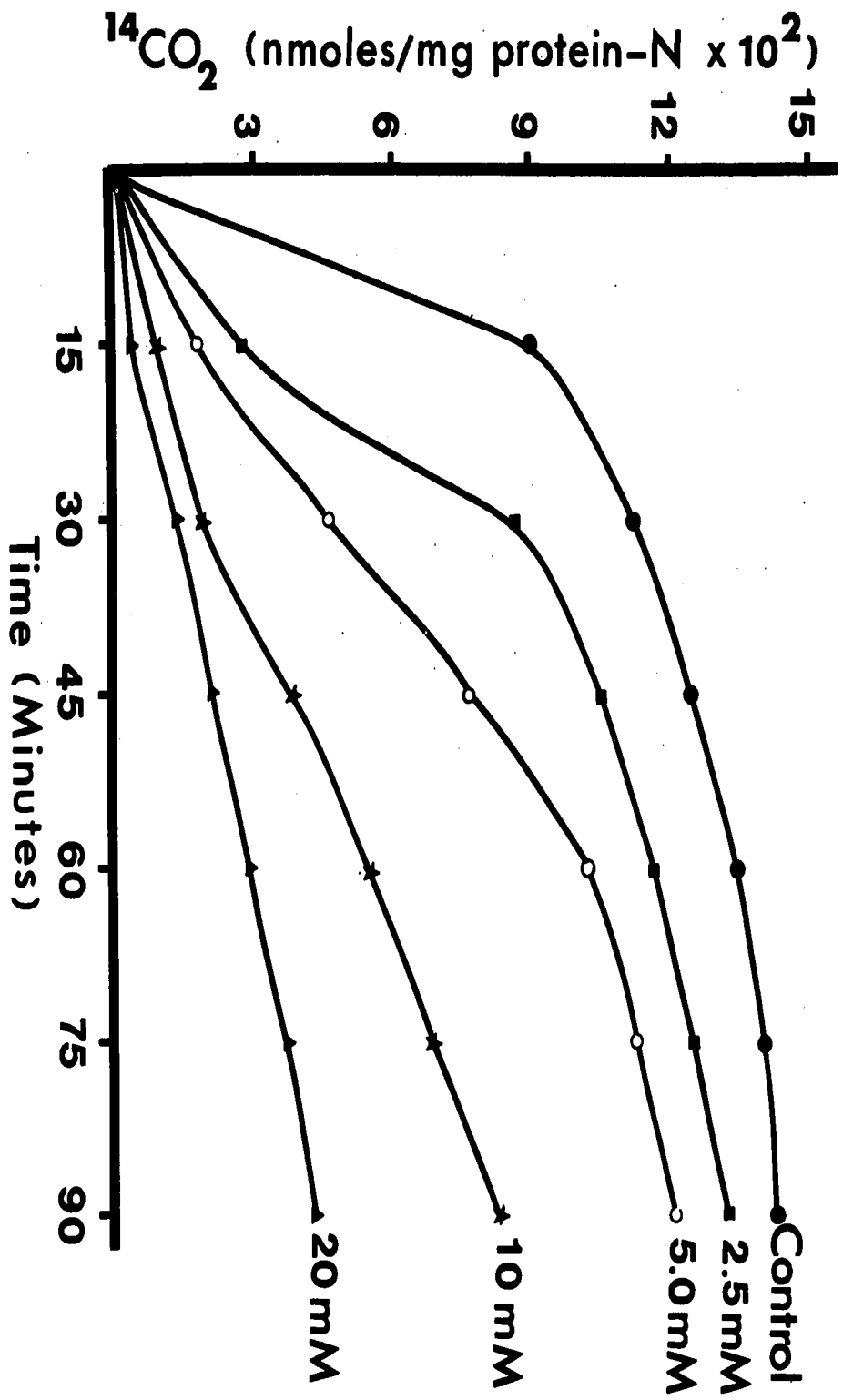

$^{14}CO_2$ (nmoles/mg protein-N x 10^2)
3
6
9
12
15
Time (Minutes)
15
30
45
60
75
90
20 mM
10 mM
5.0 mM
2.5 mM
Control

FIGURE 4

THE EFFECT OF EXTRACELLULAR GLUTAMATE CONCENTRATION ON THE RELEASE OF $^{14}CO_2$ FROM RADIOACTIVE GLUTAMATE BY CELLS CULTURED ON HISTIDINE

P. aerogenes cells were grown for 10 hr on histidine-supplemented medium, washed in 0.1 M potassium phosphate buffer, pH 6.5, and resuspended in the same buffer. Washed cells were added to reaction mixtures containing 0.82 nmoles DL-glutamic acid-5-^{14}C, 7×10^{-3} M Na_2SO_4, 0.096 M phosphate buffer, pH 6.5, and glutamic acid at concentrations varying from zero to 20×10^{-3} M in a final volume of 5.0 ml.

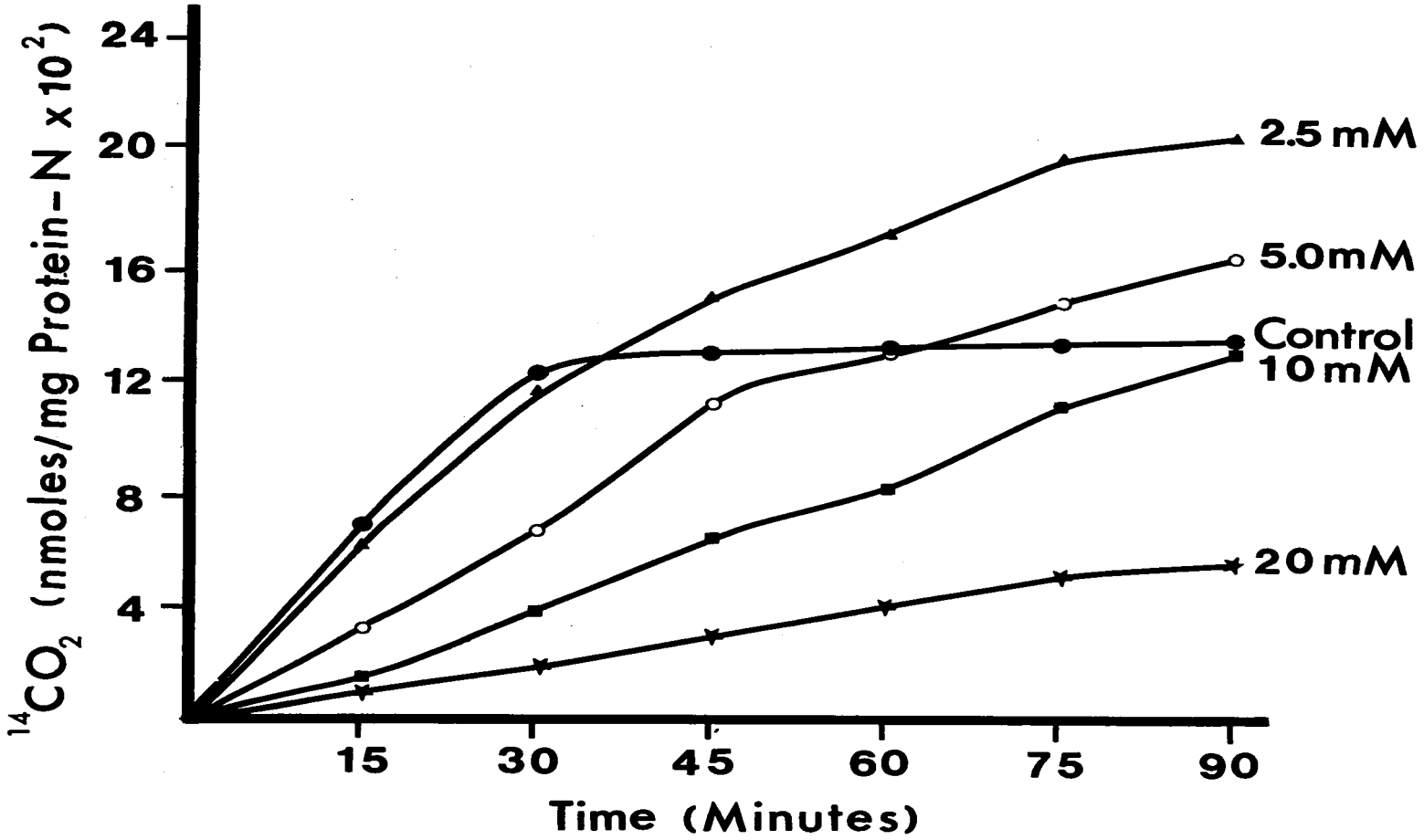

$^{14}CO_2$ (nmoles/mg Protein-N x 10^2)
24
20
16
12
8
4
15
30
45
60
75
90
Time (Minutes)
2.5 mM
5.0mM
Control
10 mM
20 mM

FIGURE 5

THE EFFECT OF EXTRACELLULAR GLUTAMATE CONCENTRATION ON THE RELEASE OF $^{14}CO_2$ FROM RADIOACTIVE GLUTAMATE BY CELLS CULTURED ON GLUTAMIC ACID

Cells of P. aerogenes were grown for 10 hr on glutamate-supplemented medium, washed in 0.1 M potassium phosphate buffer, pH 6.8, and resuspended in the same buffer. Washed cells were added to reaction mixtures containing 0.82 nmoles DL-glutamic acid-5-^{14}C, 7×10^{-3} M Na_2SO_4, 0.096 M phosphate buffer, pH 6.5, and glutamic acid at concentrations varying from zero to 20×10^{-3} M in a final volume of 5.0 ml.

III. The Enzymatic Degradation of Glutamic Acid by Cell-free Extracts of P. aerogenes

The relative efficiency of several methods of cell disruption was established in order to couple maximum cell breakage with minimum enzyme inactivation. Initially, the effect of lysozyme digestion of washed cells prior to sonic disruption was compared to sonic disintegration alone. Washed cells were suspended at 250 mg wet weight/ml of 0.05 M potassium phosphate buffer, pH 6.7, and divided into two equal portions. One portion was subjected to lysozyme digestion as described in Methods (Section V) prior to disruption in a Bronwill Biosonik set at maximum intensity for designated times at 4°C. Samples of 0.5 ml were withdrawn, the cells removed by centrifugation, and the supernatant fraction assayed for protein on triplicate 10 µl volumes by the method of Lowry (Lowry et al., 1951). Control samples were removed before sonic disruption to account for any cell leakage of protein and establish the base level of protein caused by lysozyme addition. The results presented in Figure 6 indicate that digestion with lysozyme renders the cells more susceptible to sonic disintegration. Further investigations of cell breakage techniques indicated that rupture in the Hughes' Press was the most effective method. The release of protein from a given weight of washed cells was 60% higher by Hughes' Press treatment than by sonic disruption after lysozyme pre-digestion. In cases where sonic disruption was employed, cells could be broken efficiently by low-temperature disruption in a Bronwill Biosonik III set a maximum intensity for 5 minutes, thereby avoiding the longer time intervals required in the

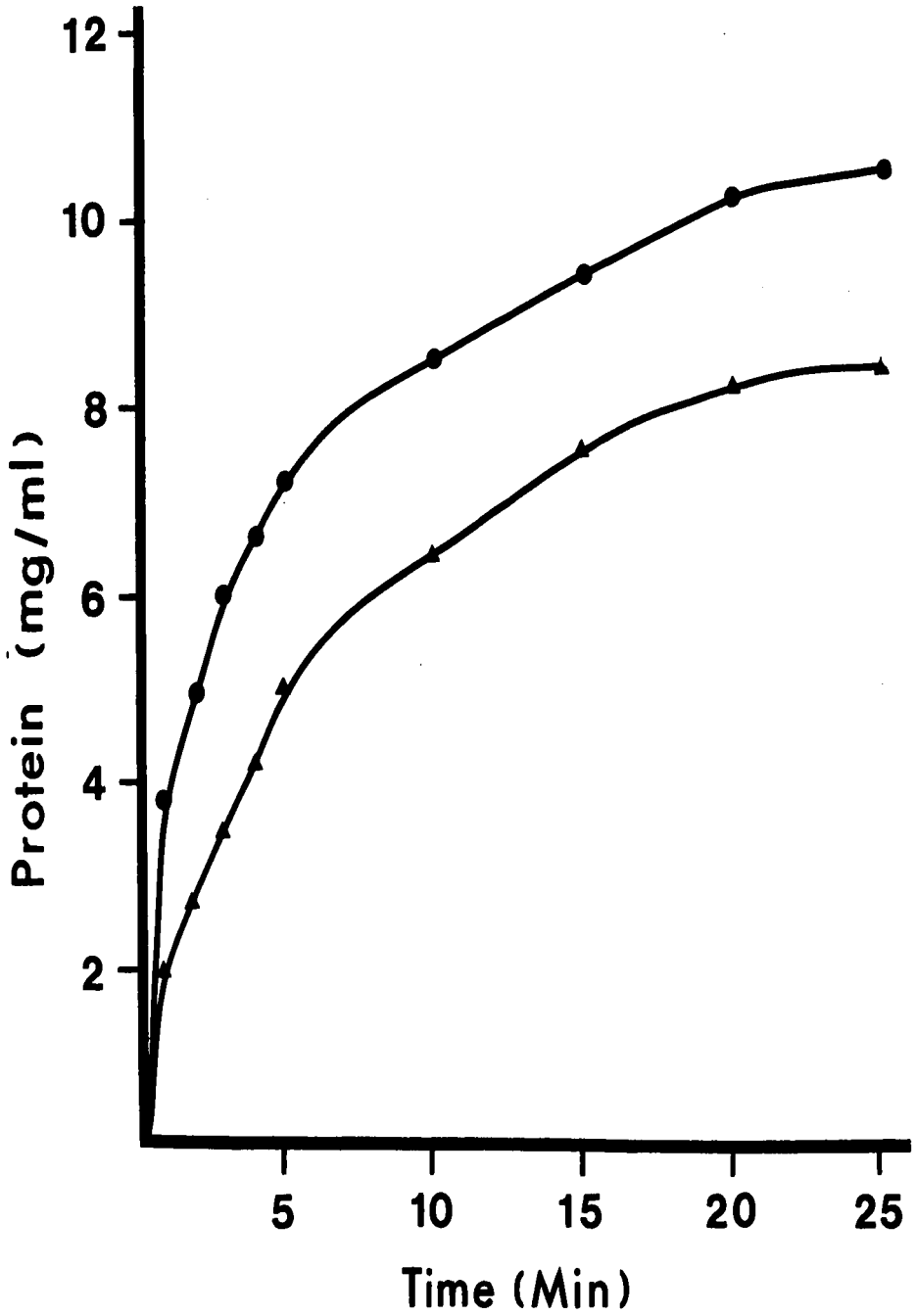

12
10
8
6
4
2
Protein (mg/ml)
5
10
15
20
25
Time (Min)

FIGURE 6

EFFECT OF PRE-DIGESTION WITH LYSOZYME ON THE SUSCEPTIBILITY OF P. AEROGENES TO SONIC DISRUPTION

Conditions for lysozyme digestion are presented in the text. Control samples were removed before sonic disruption to account for any cell leakage of protein and to establish the base level of protein caused by lysozyme addition.

Sonic disruption

Lysozyme pre-digestion + sonic disruption

smaller Bronwill Biosonik.

The preparation of crude cell-free extracts used for investigation of the enzymatic degradation of specifically labelled glutamic acid has been described in Material and Methods, Section V. The following compounds were added to crude cell extracts in an attempt to stimulate utilization of glutamic acid-5-^{14}C: Coenzyme A, ATP, FAD, NAD, NADP, pyridoxal phosphate, Mg^{++}, Na^{+}, mercaptoethanol, and glutathione. The reaction mixtures were flushed with nitrogen and any $^{14}CO_2$ released was collected as described in Methods. After incubation for desired time intervals, protein was removed by treatment with ethanol and samples of the concentrated reaction mixtures were analyzed by paper chromatography on strips as described in Methods. After varying the reaction mixtures with respect to pH, incubation temperature, and cofactor concentrations in addition to removing the additives, one at a time, the conversion of ^{14}C-glutamic acid was limited to a minimum number of radioactive products.

Under conditions of NAD-supplementation, a single radioactive product was found to accumulate from ^{14}C-glutamic acid. Cell extracts were prepared as described in Methods, Section V, and glutamic acid was degraded in a reaction mixture containing, per milligram protein: 1 µmole of L-(+)-glutamic acid, 10 µmoles potassium phosphate buffer, pH 6.5, and 0.1 µmole β-NAD. In order to produce radioactive reaction products, reaction mixtures were prepared using DL-glutamic acid-1-^{14}C, or DL-glutamic acid-5-^{14}C in place of unlabelled L-(+)-glutamic acid. All incubations were maintained at 37°C for 4 hr and chromatographic analysis was performed on paper strips as described in Methods. Results

for the conversion of specifically labelled glutamic acid in the presence and absence of NAD are presented in Figure 7. The results show that NAD was required for the metabolism of glutamic acid. All of the radioactivity could be recovered as either unused substrate or as product. The observation that the same NAD-dependent reaction product (R_f 0.63-0.65) was obtained from both carbon-1 or carbon-5 labelled glutamate (R_f 0.24) suggested that no decarboxylation had occurred.

When FAD was added at 0.1 μmole/mg protein to the reaction mixtures containing NAD and radioactive substrate, a second product appeared having a higher R_f (0.78) in n-butanol:acetic acid:water (12:3:5). This compound has been purified by ion exchange chromatography on Dowex but no positive identification was possible by comparison to standard carboxylic acids. Unfortunately, there was insufficient purified compound to permit chemical analysis.

IV. Purification and Identification of the NAD-dependent Reaction Product as α-Hydroxyglutaric Acid

Details of the procedures employed during the isolation and identification of the product which accumulated in NAD-supplemented reaction mixtures have been reported previously (Johnson and Westlake, 1969). Protein was removed from the reaction mixtures by precipitation with ethanol. The solvent was evaporated and the aqueous residue subjected to 18 hr continuous ether extraction under acid conditions as described previously. Such extraction separated the ether-soluble product from the excess substrate. The ether was removed by evaporation and the residue dissolved in water prior to purification by anion

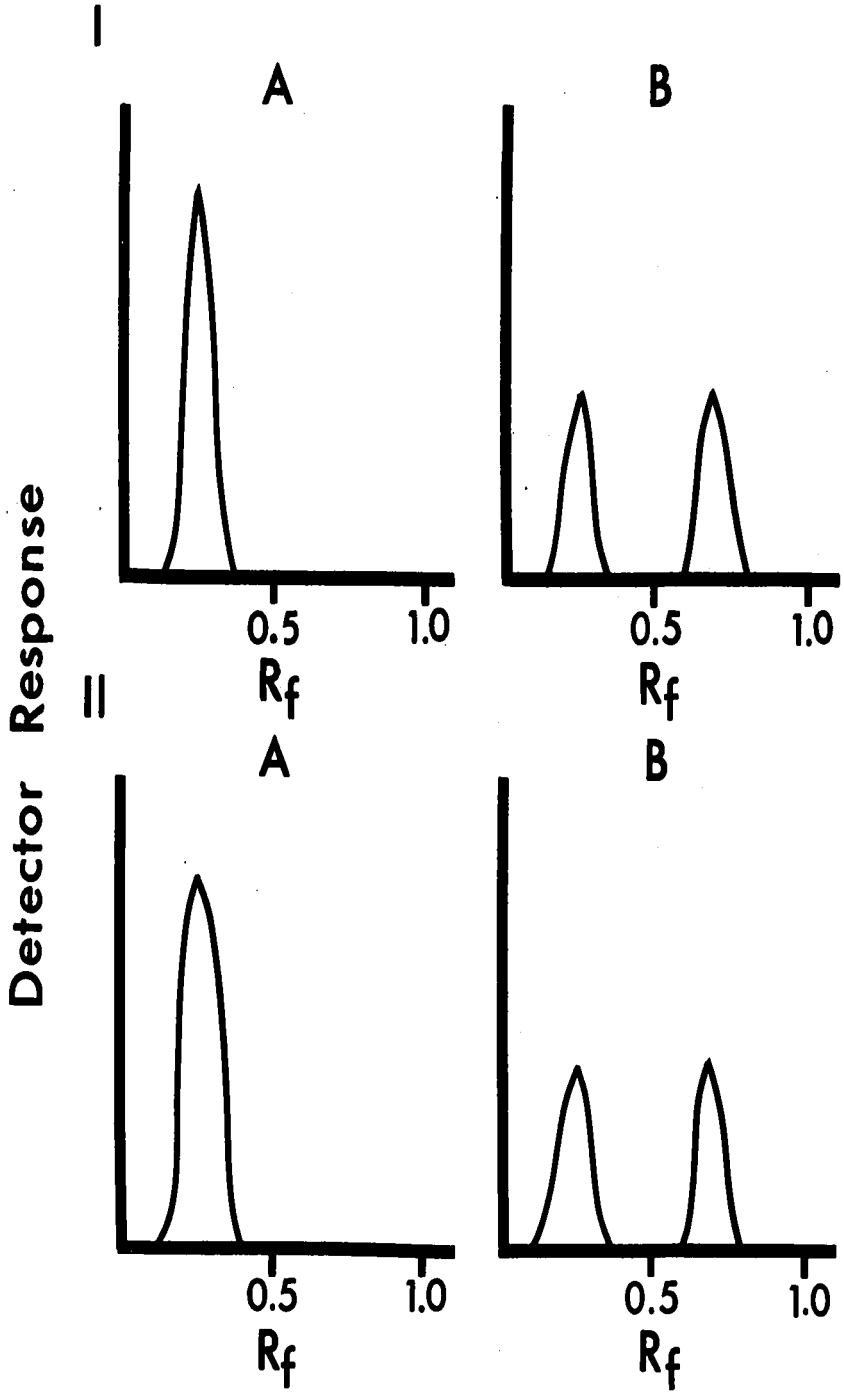

I
A
B
0.5
1.0
R_f
0.5
1.0
R_f
II
A
B
Detector Response
0.5
1.0
R_f
0.5
1.0
R_f

FIGURE 7

ACTIGRAPH TRACINGS SHOWING THE CONVERSION OF SPECIFICALLY LABELLED GLUTAMATE BY CRUDE CELL-FREE EXTRACTS OF P. AEROGENES

I. Conversion of DL-glutamic acid-1-^{14}C:

A. in the absence of NAD

B. in the presence of NAD

II. Conversion of DL-glutamic acid-5-^{14}C:

A. in the absence of NAD

B. in the presence of NAD

All reaction mixtures were incubated at 37°C for 4 hr and chromatographic analysis was performed on paper strips as described in Materials and Methods. The solvent used for development was n-butanol: acetic acid:water (12:3:5).

exchange chromatography using Dowex-1 acetate as described in Methods. Aliquots of 0.1 ml were removed from fractions and assayed for radioactivity by suspension in Bray's scintillation fluid. A typical fractionation diagram presented in Figure 8 shows that the fractions were well separated from one another with no obvious overlapping. The recoveries of radioactivity for each major fraction are presented in Table II. The radioactive fractions in each peak were pooled, evaporated to dryness, and resuspended in a small volume of distilled water.

After evaporation of the eluting acid from the pooled radioactive fractions (Figure 8), samples of the major fractions were chromatographed and the R_f values compared with the unknown products. Fraction I was identified chromatographically as a trace amount of ninhydrin-positive glutamic acid. Fractions II, III, and IV were not amino acids since they were ninhydrin-negative. The major radioactive peaks, fractions III and IV, exhibited an R_f of 0.70, the same as the R_f of the unknown after ether extraction and prior to purification.

The purified reaction product (Fraction III or IV) failed to form a 2,4-dinitrophenylhydrazone derivative indicating the lack of a free keto group. In addition, failure to bleach cold permanganate suggested the absence of unsaturated bonds.

Chromatographically, the purified reaction product behaved as a typical organic acid giving positive colour reactions with aniline-xylose, indicator sprays, and 2,6-dichlorophenolindophenol reagent. Furthermore, the product formed a yellow colour when sprayed with Altman's reagent, a typical reaction of several five-carbon dicarboxylic acids, but not the same colour reaction as with α-ketoglutarate or glutaconic acid,

TABLE II

RECOVERY OF RADIOACTIVITY IN ACIDIC FRACTIONS

ELUTED FROM DOWEX-1-ACETATE COLUMN

Fraction Number†	Total Activity (ncuries)	% of Total Radioactivity Recovered from Column*
I	10.71	3.52
II	19.91	6.54
III	86.06	28.29
IV	143.04	47.13

* Recovery: 315.9 ncuries -Total radioactivity added to column

304.0 ncuries -Total radioactivity eluted from column

† Refer to Figure 8

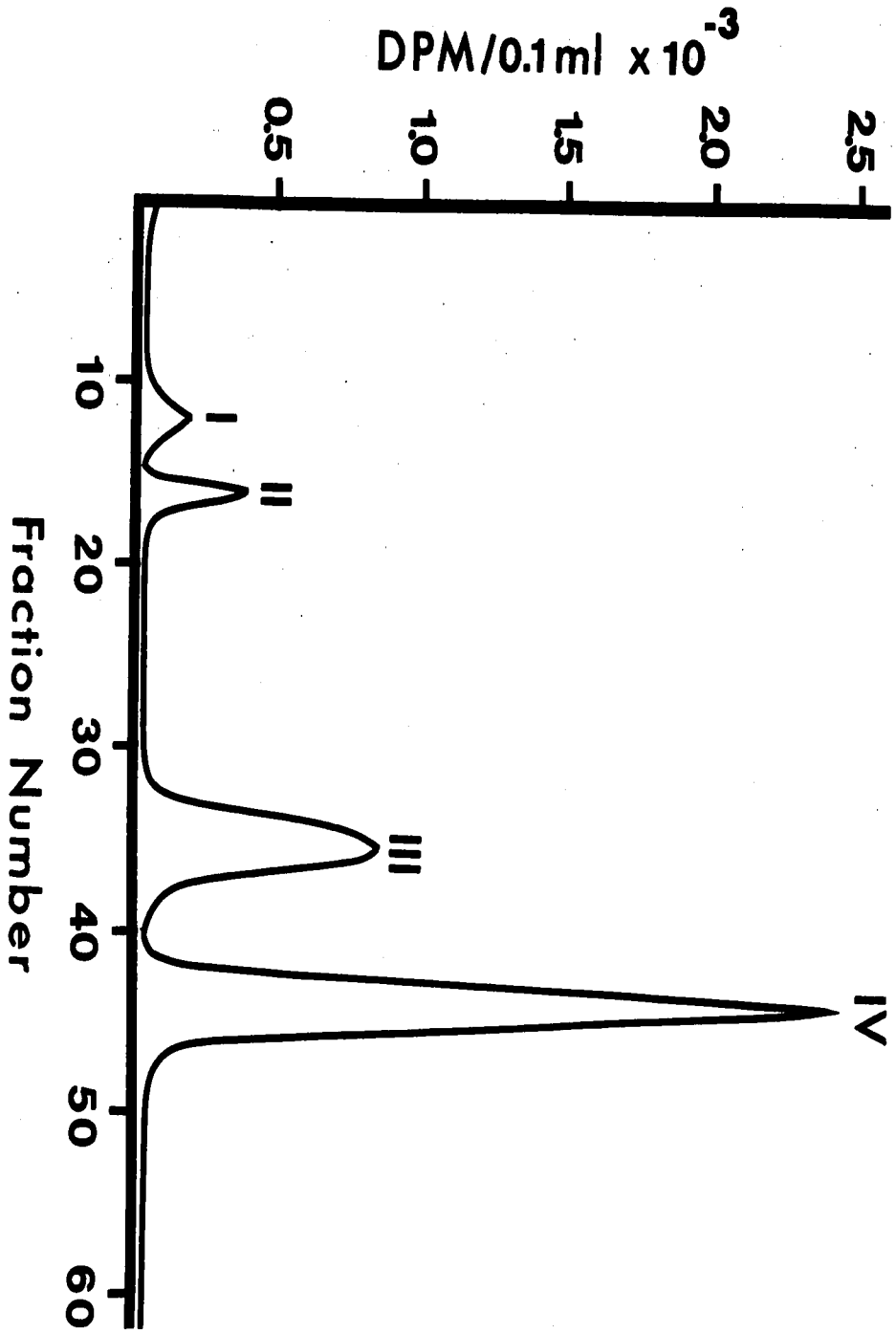

DPM/0.1ml x 10^-3
0.5
1.0
1.5
2.0
2.5
Fraction Number
10
20
30
40
50
60
I
II
III
IV

FIGURE 8

FRACTIONATION OF THE ETHER-SOLUBLE PORTION OF REACTION MIXTURES IN WHICH RADIOACTIVE GLUTAMATE WAS DEGRADED BY CELL-FREE EXTRACTS FROM P. AEROGENES

NAD-supplemented reaction mixtures were prepared as described in the text using DL-glutamic acid-5-^{14}C. Following incubation for 4 hr at 37°C and protein precipitation with ethanol, the ethanol was evaporated and the aqueous residue subjected to continuous ether extraction under acidic conditions for 18 hours. The ether was removed by evaporation, and the residue dissolved in water prior to application to a 3 x 15 cm column of Dowex-1-acetate. After elution using a gradient to 2.5 N acetic acid followed by a gradient to 6.0 N formic acid 0.1 ml samples of fractions were assayed for radioactivity as described in Materials and Methods.

two possible intermediates.

For positive identification of the major radioactive peak, fraction IV was subjected to more exhaustive structural analysis by means of mass spectrometry and infrared spectroscopy as described in Methods. The mass spectroscopic analysis showed the molecular weight to be 130 since the molecular ion peak appeared at m/e 130. By comparison to standard spectra (Budzikiewicz et al., 1967), the mass spectrum of the unknown shown in Figure 9 indicated that the compound was probably a γ-lactone. The most common feature of the γ-lactone mass spectrum arises from cleavage adjacent to oxygen to give a strong peak at m/e 85. The absence of a peak at m/e 115 indicates that the possibility of a branched-chain compound is remote, thereby eliminating mesaconic acid, citraconic acid, or citramalic acid. The infrared spectrum of the unknown compound (Figure 10) was found to be identical to that of DL-α-hydroxyglutaric acid in the γ-lactone form. Both infrared spectra are distinguished by a lack of stretching absorption in the 900-920 cm^{-1} region, and by a shift in the stretching vibration of the carbon-oxygen double bond from 1700-1725 cm^{-1} to 1760-1780 cm^{-1}, characteristics which are typical of a saturated γ-lactone (Cross, 1964). The infrared spectrum of fraction III was found to be identical to those of both fraction IV and DL-α-hydroxyglutaric acid in the γ-lactone form.

A commercial sample of DL-α-hydroxyglutaric acid (hemi-zinc salt) was combined with a sample of the ether extracted radioactive product to yield a mixture containing 0.432 meq total acidity and 1.46 μcuries total radioactivity. This sample was passed through Dowex-50 (H^+) to remove Zn^{++}, concentrated, and applied to a Dowex-1-formate

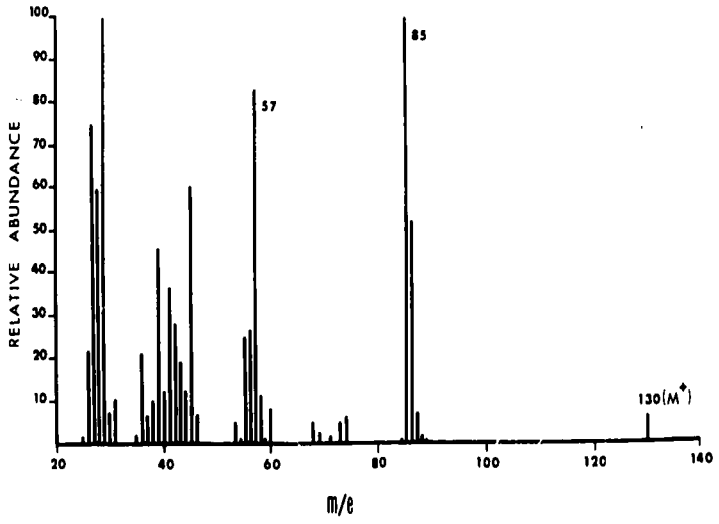

RELATIVE ABUNDANCE
100
90
80
70
60
50
40
30
20
10
57
85
130(M+)
20
40
60
80
100
120
140
m/e

FIGURE 9

MASS SPECTRUM OF THE RADIOACTIVE REACTION PRODUCT

ISOLATED BY CHROMATOGRAPHY ON DOWEX-1

The major radioactive compound (Fraction IV, Figure 8) isolated by chromatography of the ether-soluble fraction of NAD-supplemented reaction mixtures was subjected to mass spectroscopy. Analysis was performed by the Spectroscopy Laboratory, Department of Chemistry, University of Alberta, according to the specifications presented in Materials and Methods.

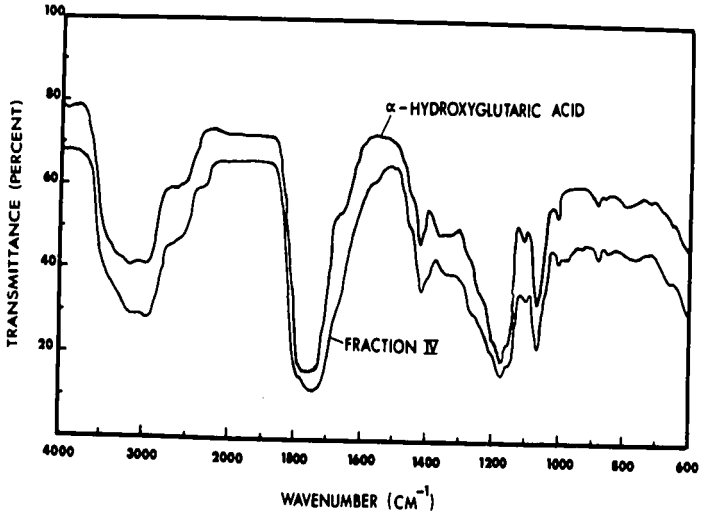

100
80
60
40
20
TRANSMITTANCE (PERCENT)
α-HYDROXYGLUTARIC ACID
FRACTION IV
4000
3000
2000
1800
1600
1400
1200
1000
800
600
WAVENUMBER (CM⁻¹)

FIGURE 10

INFRARED SPECTRA OF STANDARD α-HYDROXYGLUTARATE AND THE REACTION PRODUCT ISOLATED BY CHROMATOGRAPHY ON DOWEX-1

The infrared spectra were obtained for standard α-hydroxyglutarate and the product from NAD-supplemented reaction mixtures which was isolated by Dowex-1 chromatography (Fraction IV, Figure 8). Samples were prepared on KBr discs as described in Materials and Methods, and analysis was performed by the Spectroscopy Laboratory, Faculty of Pharmacy, University of Alberta.

column as described in Methods. The column was eluted with a formic acid gradient, and fractions tested for radioactivity, the radioactive fractions pooled, evaporated to dryness, and a sample of each radioactive peak assayed for acidity. The results showed a similar distribution of label and acidity into two peaks (Fractions 3 and 4, and fractions 6 and 7) as observed during the purification of the unknown product. The total acidity recovered from the column was 0.435 meq and the total radioactivity recovered was 1.44 μcuries. The ratio of acidities for the two peaks was 3.1 as compared to a ratio of 2.92 on the basis of radioactivity. Hence, the distribution of acidity could be directly correlated to the distribution of radioactivity. Samples from both peaks were found to behave identically to chromatographic analysis in the n-butanol:acetic acid:water (12:3:5) solvent system (Figure 11) and their infrared spectra were very similar both in the cyclic form (Figure 12) and the partially neutralized form (Figure 13). The separation of α-hydroxyglutarate into two peaks following cyclization and ion exchange chromatography would appear to be a phenomen of the purified compound as well as the experimentally derived material.

Since α-hydroxyglutaric acid can react as either an α-hydroxy acid or a γ-hydroxy acid upon conversion from the salt to the free acid, it is possible to form either lactides by bimolecular esterification of the α-hydroxy acid or lactones by spontaneous cyclization of the γ-hydroxy acid (Noller et al., 1965). Therefore, it is not surprising to find two peaks which react similarly on chemical analysis subsequent to acidic ether extraction and hence, lactone-lactide formation. The infrared spectra of partially neutralized samples in Figure 13 show a shift in

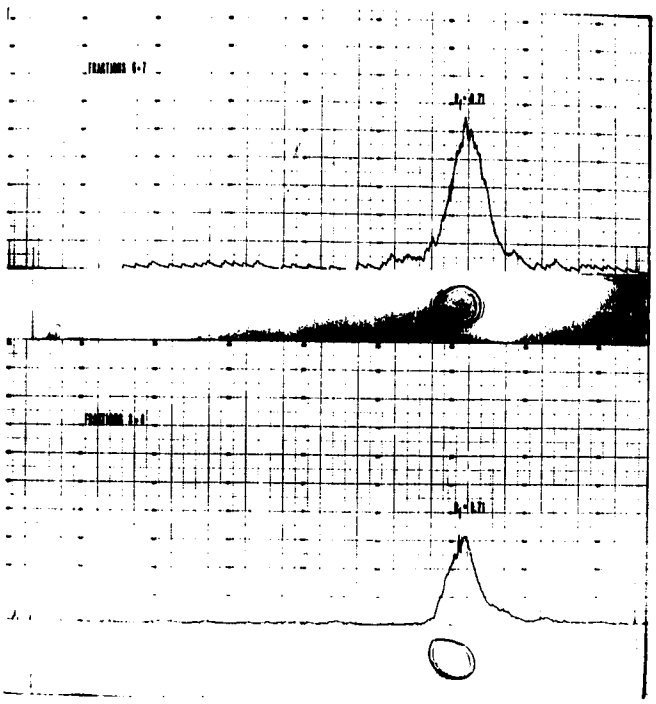
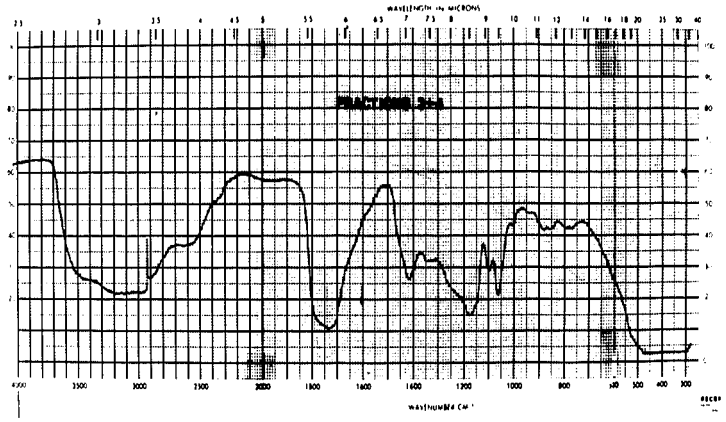


FIGURE 11

COMPARISON OF THE ACTIGRAPH TRACINGS OF THE TWO PEAKS RESULTING FROM FRACTIONATION OF α-HYDROXYGLUTARATE ON DOWEX-1-FORMATE

A commercial sample of DL-α-hydroxyglutaric acid was combined with a sample of the ether-extracted radioactive product to yield a mixture containing 0.432 meq total acidity and 1.46 μcuries total radioactivity. The mixture was applied to Dowex-1-formate and eluted with a formic acid gradient. Two peaks of acidity and radioactivity were observed after Dowex fractionation (Fractions 3 + 4, and Fractions 6 + 7). Samples from both peaks were chromatographed in n-butanol:acetic acid:water (12:3:5) and acidic areas were located using 0.04% bromophenol blue.



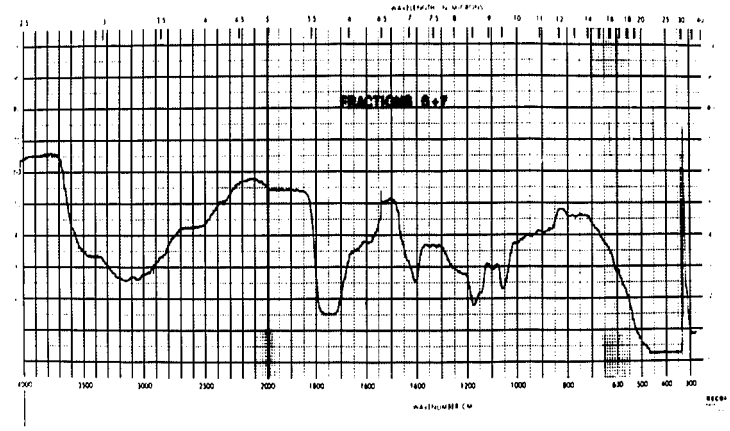
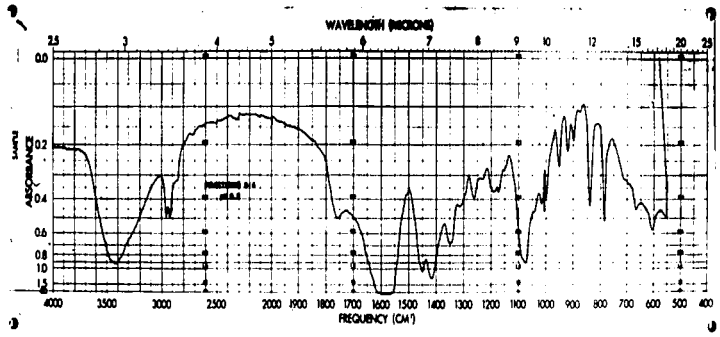


FIGURE 12

COMPARISON OF THE INFRARED SPECTRA OF THE TWO PEAKS RESULTING FROM FRACTIONATION OF α-HYDROXYGLUTARATE ON DOWEX-1-FORMATE

A commercial sample of DL-α-hydroxyglutarate was subjected to fractionation on Dowex-1-formate as described in the text and the legend to Figure 11. The eluting acid was evaporated from the two peaks (Fractions 3 + 4, and Fractions 6 + 7) prior to infrared analysis.


WAVELENGTH (MICRONS)
2.5 3 4 5 6 7 8 9 10 12 15 20 25
0.0
0.2
0.4
0.6
0.8
1.0
1.5
ABSORBANCE
4000 3500 3000 2500 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400
FREQUENCY (CM-1)

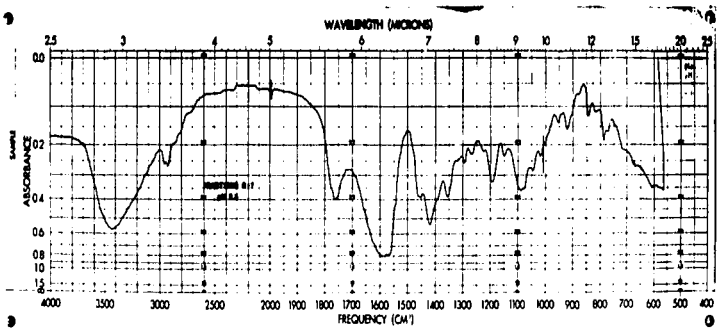

WAVELENGTH (MICRONS)
2.5 3 4 5 6 7 8 9 10 12 15 20 25
0.0
0.2
0.4
0.6
0.8
1.0
1.5
ABSORBANCE
4000 3500 3000 2500 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 600 500 400
FREQUENCY (CM-1)

FIGURE 13

COMPARISON OF THE INFRARED SPECTRA OF THE TWO PEAKS RESULTING FROM FRACTIONATION OF α-HYDROXYGLUTARATE ON DOWEX-1 AFTER PARTIAL NEUTRALIZATION

A commercial sample of DL-α-hydroxyglutarate was subjected to fractionation on Dowex-1-formate as described in the text and legend to Figure 11. The eluting acid was evaporated from the two peaks (Fractions 3 + 4, and Fractions 6 + 7), and samples were neutralized to pH 8.5 with NaOH prior to analysis by infrared spectroscopy.

carbonyl absorption to that of a carboxylate ion in the region of 1600 cm^{-1}. However, the neutralized sample continued to show part of a peak at 1750 cm^{-1} indicating incomplete lactone (lactide) hydrolysis.

V. Evidence Supporting the Assignment of α-Ketoglutarate and NADH As Pathway Intermediates

The most desirable hypothesis for the formation of α-hydroxyglutaric acid from glutamic acid involves oxidative deamination followed by reduction of the α-keto acid to the corresponding α-hydroxy acid as discussed in a recent communication (Johnson and Westlake, 1969). Since no apparent NADH formation could be demonstrated at pH 6.5, under conditions where α-hydroxyglutarate was produced, the reduction of α-ketoglutarate to α-hydroxyglutarate could be achieved by reoxidation of the reduced NAD produced during the initial reaction, assuming that α-ketoglutarate was the intermediate. Substantiation of this theory was achieved by investigating the ability of cell-free extracts to utilize radioactive α-ketoglutarate in the presence and absence of NADH.

Cell-free extracts were prepared as described in Section V of Methods. Reaction mixtures contained: 800 ncuries α-ketoglutarate-5-^{14}C (S.A. = 17.1 mc/mM), 400 μmoles potassium phosphate buffer, pH 7.0, and 10 mg of protein in a total volume of 5.0 ml. One μmole of NADH was added when required. Radioactive carbon dioxide was trapped in phenethylamine counting fluid during incubation for 60 minutes at 37°C. Protein was precipitated with ethanol, the supernatant evaporated to dryness, and the residue suspended in 1.0 ml of distilled water. Aliquots were removed for chromatographic analysis by comparison to standard

^{14}C-α-ketoglutarate and 0.5 ml was acidified with HCl and applied to a 1 x 10 cm Dowex-50 (H^+) column at pH 1.0. The column was then washed with three column volumes of 2 N HCl. The material from both the acid wash and the water wash was evaporated to dryness and suspended in a small volume of distilled water. The acid wash contained ^{14}C-glutamic acid which was identified by co-chromatography. An aliquot of the concentrated water wash was chromatographed with standards of α-ketoglutarate and α-hydroxyglutarate in the butanol:acetic acid:water (12:3:5) solvent system. The radioactive areas corresponding to these compounds were cut out, eluted, and estimated quantitatively as described in Methods. The distribution of radioactivity is presented in Table III.

The results indicate that NADH is required for the utilization of α-ketoglutarate since 4% of the ^{14}C-α-ketoglutarate was converted in the absence of NADH as compared to 84% conversion in the presence of NADH. Moreover, the data show that the radioactive substrate is converted to both glutamic acid and α-hydroxyglutarate, although not in equal proportions. The low level of conversion in the absence of added NADH suggests that crude extracts contain, as expected, base levels of NADH and ammonia. The concentration of ammonia is likely the limiting factor for the formation of glutamic acid-5-^{14}C from α-ketoglutaric acid-5-^{14}C in the presence of NADH.

The demonstration of the release of significant amounts of $^{14}CO_2$ from α-ketoglutarate-5-^{14}C and α-hydroxyglutarate-5-^{14}C by whole washed cells provided further positive evidence for the assignment of these compounds as pathway intermediates. Cells were grown for 10 hr on

TABLE III

DEGRADATION OF α-KETOGLUTARATE-5-^{14}C BY CELL-FREE EXTRACTS OF *P. AEROGENES* IN THE PRESENCE AND ABSENCE OF NADH

	+ NADH		- NADH	
	Radioactivity (ncuries)	% of Original	Radioactivity (ncuries)	% of Original
^{14}C-α-ketoglutarate added	800	100	800	100
^{14}C-glutamate produced	11	1	9	1
^{14}C-α-hydroxyglutarate produced	662	83	26	3
^{14}C-α-ketoglutarate remaining	125	16	768	96
Total Recovery	798	100	803	100
$^{14}CO_2$ *	Nil (98*)	-	Nil (77*)	-

* Expressed as cpm recovered/60 minutes of incubation at 37°C.

glutamate medium, washed three times in 0.1 M potassium phosphate buffer, pH 7.0, and added to reaction mixtures containing: 250 μmoles potassium phosphate buffer, pH 7.0, 7×10^{-3} M Na_2SO_4, and radioactive substrate as indicated in Table IV in a total volume of 3.0 ml. The release of $^{14}CO_2$ was followed as a function of time at 37°C and after 90 minutes incubation, the cells were removed by centrifugation and the radioactivity remaining in the supernatant was determined quantitatively. The kinetics of $^{14}CO_2$ release from carbon-5 labelled glutamate, α-ketoglutarate, and α-hydroxyglutarate are presented in Figure 14.

The results of Table IV and Figure 14 show significant release of $^{14}CO_2$ from α-ketoglutarate-5-^{14}C and DL-α-hydroxyglutarate-5-^{14}C, thereby providing evidence for their inclusion as pathway intermediates. The conversion of DL-glutamic acid-5-^{14}C was included as a control and the kinetics compare favorably to independent results shown in Figure 1. The low levels of $^{14}CO_2$ recovered from α-ketoglutarate-5-^{14}C and α-hydroxyglutarate-5-^{14}C suggest that the permeability of these compounds is limited. The data in Table IV confirm this possibility in that relatively high levels of radioactivity remain in the supernatant following removal of cells. Since label released as carbon dioxide was accounted for, the radioactivity remaining in the supernatant following removal of cells was assumed to be substrate which was not metabolized.

TABLE IV

THE METABOLISM OF RADIOACTIVE GLUTAMATE, α-KETOGLUTARATE, AND α-HYDROXYGLUTARATE BY WASHED CELLS OF P. AEROGENES

Substrate	nmoles added	nmoles $^{14}CO_2$ released/ 90 min.	% conversion to $^{14}CO_2$	% remaining*
DL-glutamic acid-5-^{14}C	119	42.2	35.5	55.9
α-ketoglutarate-5-^{14}C	8.95	2.04	22.8	66.3
DL-α-hydroxyglutarate-5-^{14}C	4.38	0.57	13.1	77.4

* Calculated on the assumption that the radioactivity remaining in the supernatant was attributed to ^{14}C-substrate. The % remaining in the supernatant was determined on the basis of nmoles from the specific activity of the radioactive substrate.

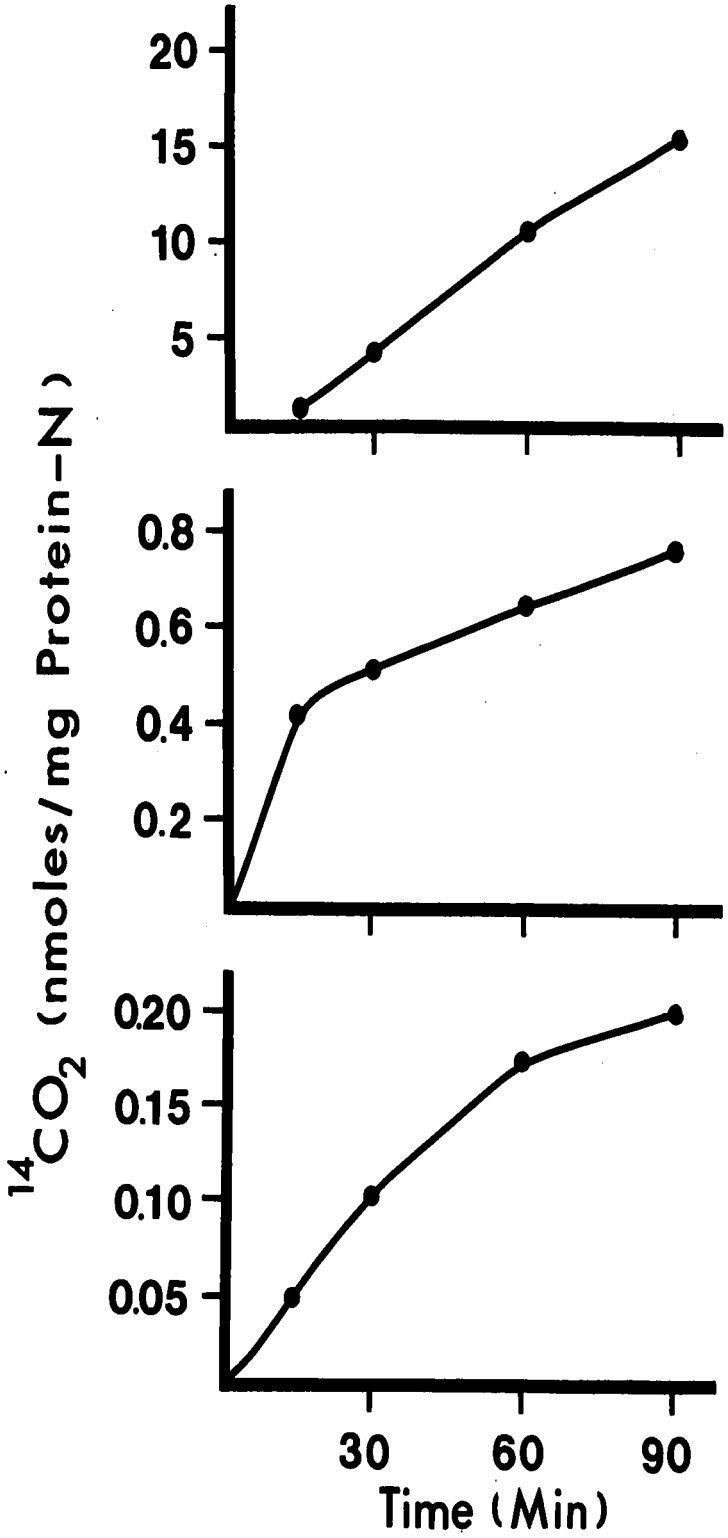

20
15
10
5
0.8
0.6
0.4
0.2
0.20
0.15
0.10
0.05
$^{14}CO_2$ (nmoles/mg Protein-N)
30
60
90
Time (Min)

FIGURE 14

METABOLISM OF GLUTAMATE, α-KETOGLUTARATE, AND α-HYDROXYGLUTARATE BY WASHED CELLS OF P. AEROGENES

Procedures for the washing of cells and preparation of reaction mixtures are presented in the text. The kinetics of $^{14}CO_2$ release from carbon-5 labelled glutamate, α-ketoglutarate, and α-hydroxyglutarate were determined at 37°C.

Graph	Substrate
Upper	DL-glutamic acid-5-^{14}C
Middle	α-ketoglutarate-5-^{14}C
Lower	DL-α-hydroxyglutarate-5-^{14}C

VI. Identification and Initial Fractionation of Two NAD-dependent Enzymes Involved in Glutamate Degradation

Two NAD-dependent enzymes which function in the metabolism of glutamate have been observed in crude cell-free extracts prepared according to the procedure in Section VII of Materials and Methods. One enzyme, glutamic dehydrogenase (E.C. 1.4.1.2), catalyzes the deamination of glutamate to α-ketoglutarate in the presence of diphosphopyridine nucleotide. The other enzyme, α-ketoglutarate reductase (α-hydroxyglutarate dehydrogenase)(E.C. 1.6.99.3), is responsible for the reversible oxidation-reduction reaction between α-hydroxyglutarate and α-ketoglutarate. For clarity, the term reductase will be employed to indicate the reduction of α-ketoglutarate in the presence of NADH as opposed to dehydrogenase to designate the oxidation of α-hydroxyglutarate to α-ketoglutarate. Both enzymes were assayed according to the procedure given in Methods, Section VIII.

Preliminary fractionation of the two enzymes from crude extracts was a two-step procedure involving ammonium sulphate precipitation followed by gel filtration on Sephadex. Crude extracts were subjected to ammonium sulphate precipitation at 4°C to 80% saturation using 10% increments as described in Section VII of Methods. Both enzymes were found to precipitate between 50 and 70% saturation with ammonium sulphate. Since no discrete separation of the two activities occurred, crude extracts adjusted to 10 mg protein/ml were treated with ammonium sulphate to 50% saturation. The precipitate was discarded, thereby removing considerable non-specific protein and facilitating more effective gel

filtration. The supernatant of the 0-50% fractionation was adjusted to 80% saturation and the resultant precipitate recovered by centrifugation. The precipitate was dialyzed against two 1-liter changes of 0.05 M tris-HCl buffer, pH 8.8, at 4°C.

The primary separation of the two enzyme activities was achieved by gel filtration with Sephadex G-200. The dialyzed preparation from the 50-80% ammonium sulphate fractionation was applied to a 2.5 x 100 cm Sephadex G-200 column maintained at 4°C and eluted with 0.05 M tris-HCl buffer, pH 8.8. A Buchler refrigerated fraction collector was employed for the collection of 120-drop fractions at 4°C. Each fraction was analyzed for protein by following E_{280} values in addition to enzyme assays for glutamic dehydrogenase or α-hydroxyglutarate dehydrogenase activities. A typical fractionation on Sephadex G-200 presented in Figure 15 illustrates that the two activities were well separated from one another with no significant overlapping. Data for recoveries, purification, and specific activities for each enzyme are presented in Tables V and XIII. The active fractions for each enzyme were pooled and concentrated in an Amicon Ultrafiltration Apparatus, Model 52 or 402, prior to further purification. Preparations of glutamic dehydrogenase were concentrated using a Diaflo XM-50 membrane whereas α-hydroxyglutarate dehydrogenase preparations required a PM-10 or UM-10 membrane. The maximum operating pressure of nitrogen used during ultrafiltration was 45 psi.

VII. Purification of Glutamic Dehydrogenase

Concentrated glutamic dehydrogenase from G-200 was applied to a 1.5 x 25 cm column of DEAE-cellulose equilibrated in 0.05 M tris-HCl buffer,

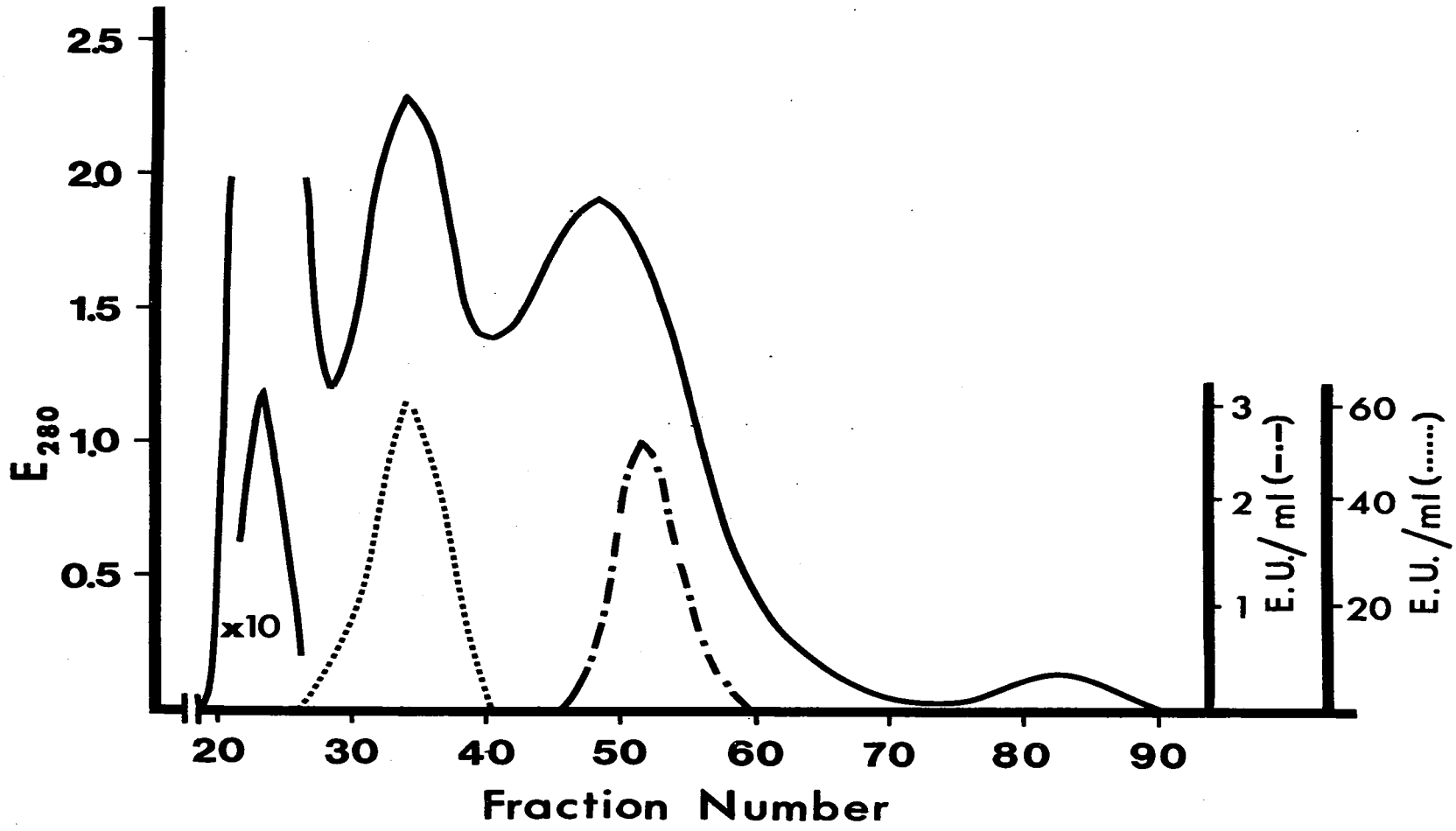

2.5
2.0
1.5
1.0
0.5
E_{280}
x10
3
2
1
E.U./ml (---)
60
40
20
E.U./ml (.....)
20
30
40
50
60
70
80
90
Fraction Number

FIGURE 15

SEPARATION OF GLUTAMIC DEHYDROGENASE AND α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES ON SEPHADEX G-200

Following ammonium sulphate fractionation of crude cell-free extracts of P. aerogenes, the enzyme preparation was applied to a 2.5 x 100 cm column of Sephadex G-200 maintained at 4°C. Procedures for sample preparation, application, and elution are described in the text. Fractions of 120-drops were collected at 4°C and assayed for protein and enzyme content.

Glutamic dehydrogenase

α-Hydroxyglutarate dehydrogenase

E_{280}

pH 8.8. A NaCl gradient composed of 200 ml of 0.05 M tris-HCl buffer, pH 8.8, and 200 ml of 0.5 M NaCl in the same buffer was used for elution. A Buchler refrigerated fraction collector was employed for the collection of 115-drop fractions at 4°C. Fractions were analyzed by following E_{280}, enzyme activity, and conductivity. A typical elution profile from DEAE-cellulose is presented in Figure 16. Glutamic dehydrogenase activity was eluted in a single peak at a NaCl concentration of 0.21 M. The active fractions were concentrated by ultrafiltration and dialyzed prior to chromatography on hydroxylapatite. Recovery and specific activity data are presented in Table V.

A 2.5 x 23 cm column of hydroxylapatite was prepared and equilibrated in 0.01 M potassium phosphate buffer, pH 7.6, prior to use. Concentrated enzyme from the DEAE-cellulose column was dialyzed against 2 liters of 0.01 M potassium phosphate buffer, pH 7.6, for 8 hr at 4°C. The enzyme was applied to the column and eluted using a linear gradient of increasing concentration of potassium phosphate buffer at constant pH (250 ml of 0.01 M plus 250 ml of 0.05 M buffer). Fractions were collected as above at 115 drops per fraction and analyzed for E_{280}, enzyme activity, and conductivity. A typical elution profile from an hydroxylapatite column is presented in Figure 17. Data presented in Table V indicate that the hydroxylapatite treatment results in an increase in specific activity as compared to post-DEAE-cellulose enzyme.

The enzyme recovered from hydroxylapatite was concentrated by ultrafiltration and dialyzed against 0.05 M tris-HCl buffer, pH 8.8 at 4°C. The enzyme was then applied to a 2.5 x 25 cm column of carboxymethyl cellulose (CM-cellulose) equilibrated with the same buffer. The

TABLE V

PURIFICATION TABLE FOR GLUTAMIC DEHYDROGENASE

FROM P. AEROGENES

Procedure	Total units ($\times 10^3$)	% Recovery	Specific Activity*	Purification
Crude Extract	4.52	100	4.85	1.00
$(NH_4)_2SO_4$	5.10	113	10.02	2.06
Sephadex G-200	7.12	157	22.20	4.57
DEAE-Cellulose	3.16	70	26.80	5.50
Hydroxylapatite	1.38	41	54.20	11.20
CM-Cellulose	1.13	25	64.50	13.30

* Specific activity was calculated as E. U./mg protein.

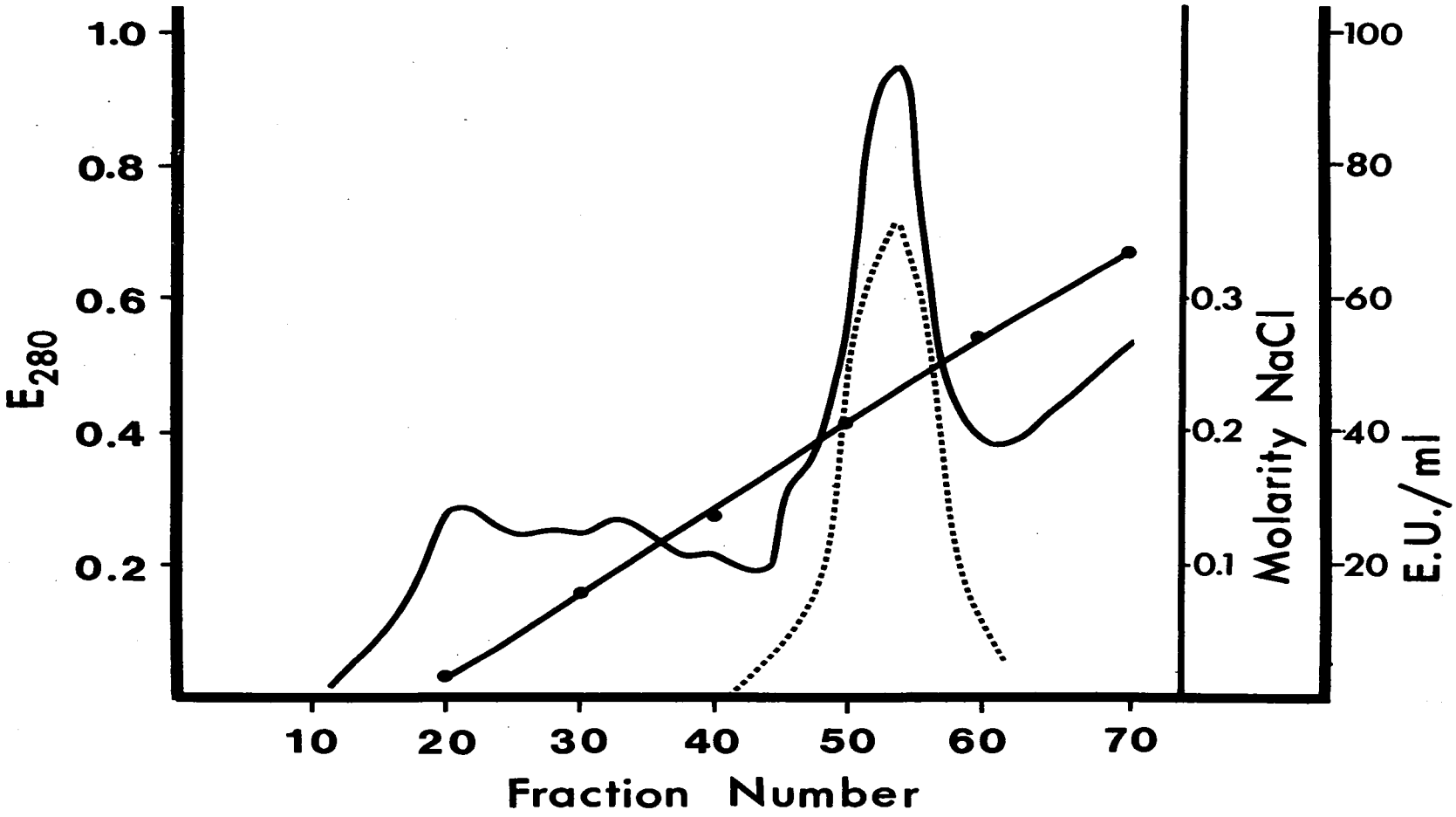
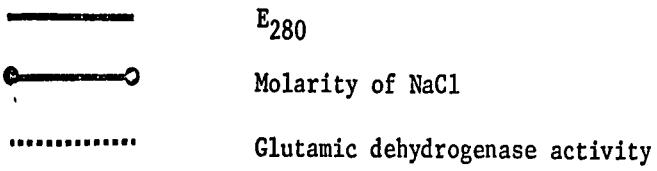

1.0
0.8
0.6
0.4
0.2
E_{280}
0.3
0.2
0.1
Molarity NaCl
100
80
60
40
20
E.U./ml
10
20
30
40
50
60
70
Fraction Number

FIGURE 16

ELUTION PROFILE FROM DEAE-CELLULOSE FOR GLUTAMIC DEHYDROGENASE OF P. AEROGENES

Concentrated glutamic dehydrogenase from Sephadex G-200 was applied to a 1.5 x 25 cm column of DEAE-cellulose equilibrated in 0.05 M tris-HCl buffer, pH 8.8. A NaCl gradient as described in the text was employed for elution and 115-drop fractions were collected at 4°C. A 10 µl volume of each fraction was analyzed for protein, enzyme activity, and conductivity.



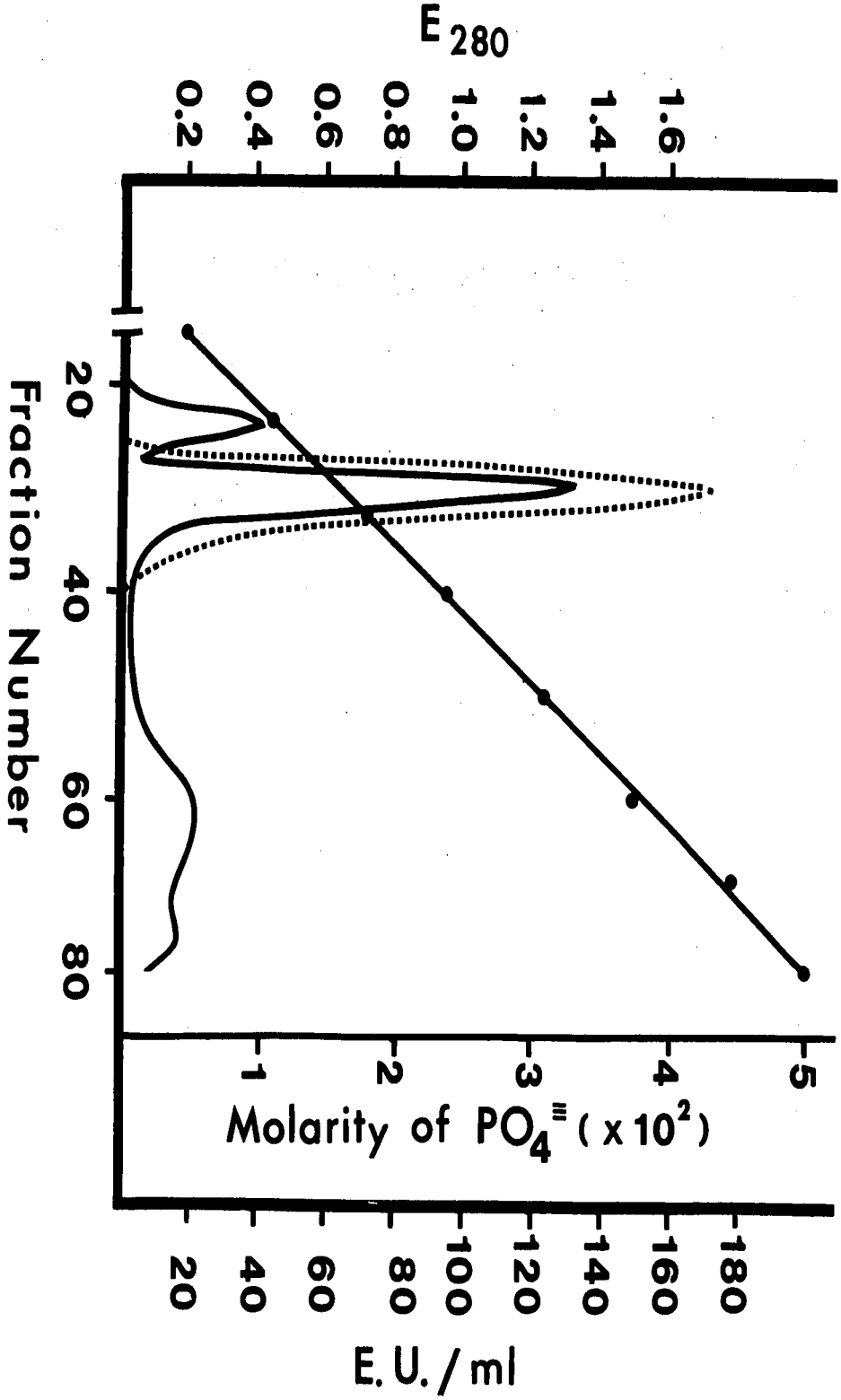
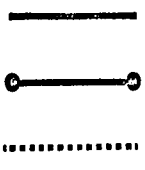

E 280
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Fraction Number
20
40
60
80
1
2
3
4
5
Molarity of $PO_4^{\equiv}$ (x 10^2)
20
40
60
80
100
120
140
160
180
E. U. / ml

FIGURE 17

ELUTION PROFILE FROM HYDROXYLAPATITE FOR GLUTAMIC DEHYDROGENASE OF P. AEROGENES

Concentrated glutamic dehydrogenase from DEAE-cellulose was applied to a 2.5 x 23 cm column of hydroxylapatite equilibrated in 0.01 M potassium phosphate buffer, pH 7.6. A phosphate buffer gradient as described in the text was employed for elution and 115-drop fractions were collected at 4°C. A 10 µl volume of each fraction was analyzed for protein, enzyme activity, and conductivity.



E_{280}

Molarity of phosphate buffer

Glutamic dehydrogenase activity

curve for enzyme activity followed a single protein peak eluted in the void volume of the column. The treatment on CM-cellulose produced a slight increase in specific activity as indicated in Table V. Subsequent concentration, dialysis, and gel filtration on Sephadex G-100 produced no significant increase in specific activity, an indication of the purity of the enzyme preparation.

The purification results presented in Table V show a 25% recovery and a 13-fold purification of the enzyme. The purification data will be discussed more thoroughly and compared to results cited in a recent publication (Kew and Woolfolk, 1970). The enzyme preparation of specific activity 65 enzyme units/mg protein was that used for sedimentation analysis and enzyme characterization unless otherwise specified.

VIII. Characterization of Glutamic Dehydrogenase

A. Identification of the Reaction Product

Since α-ketoglutarate was the anticipated product of glutamic acid dehydrogenation, an incubation mixture was prepared which contained 40 μmoles tris-HCl buffer, pH 8.8, 10 μmoles L-(+)-glutamic acid, 10 μmoles NAD, and 1 mg purified enzyme (S.A. = 65 units/mg) in a final volume of 1.5 ml. The mixture was incubated at 37°C for 60 minutes with equal additions of 10 μmole quantities of glutamic acid and NAD in buffer at 15, 30, and 45 minutes. Control experiments were performed in the absence of NAD. The incubations were terminated by the addition of 2,4-dinitrophenylhydrazine reagent (1 mg/ml in 2 N HCl) at a ratio of 2 volumes to 1 volume of reaction mixture. An additional 60 minutes of incubation at 25°C permitted the formation of α-keto acid 2,4-dinitro-

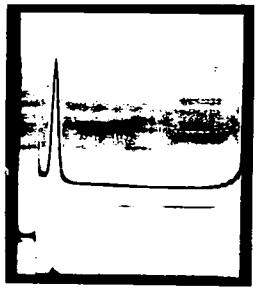
phenylhydrazones. The hydrazones were extracted three times, each time with a quantity of ether equal to the volume of the aqueous phase being extracted. The ether was evaporated from the extracted material and the hydrazones dissolved in ethyl acetate prior to examination by thin layer chromatography on silica gel H plates. The isoamyl alcohol:ammonia and n-butanol:ethanol:ammonia solvent systems were employed as described in Methods, Section VI-A. No detection reagent was necessary for localization of the bright yellow derivatives. In both solvents, the reaction product migrated to the same position as a standard sample of α-ketoglutarate-2,4-dinitrophenylhydrazone, therefore providing additional evidence that α-ketoglutarate is the product of the reaction catalyzed by glutamic dehydrogenase.

B. Sedimentation Analysis and Estimation of Molecular Weight

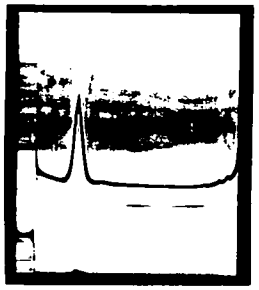
The Schlieren pattern obtained upon sedimentation velocity analysis of purified glutamic dehydrogenase in tris-HCl buffer is shown in Figure 18. The sedimentation velocity results give every indication of sample homogeneity since a single symmetrical peak appeared which persisted for most of its passage across the cell. Figure 19 shows the relationship of the logarithm of the distance sedimented to time for a 72 minute duration (Chervenka, 1969). Calculation of the slope of the regression line in Figure 19 (Steel and Torrie, 1960) and substitution into the equation

$$s = \frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{2.303}{60\omega^2}\frac{d \log x}{dt'}$$

results in a value for the sedimentation coefficient. The observed sedimentation rate (S_{obs}) for glutamate dehydrogenase was calculated



8



24

48

72

FIGURE 18

SEDIMENTATION PATTERN OF PURIFIED

GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

The enzyme preparation (4 mg/ml) in 0.05 M tris-HCl buffer, pH 8.8, was centrifuged at 60,000 rpm at 5°C. Sedimentation was from left to right. Photographs were taken at 8 minute intervals for 80 minutes after the rotor attained full speed.

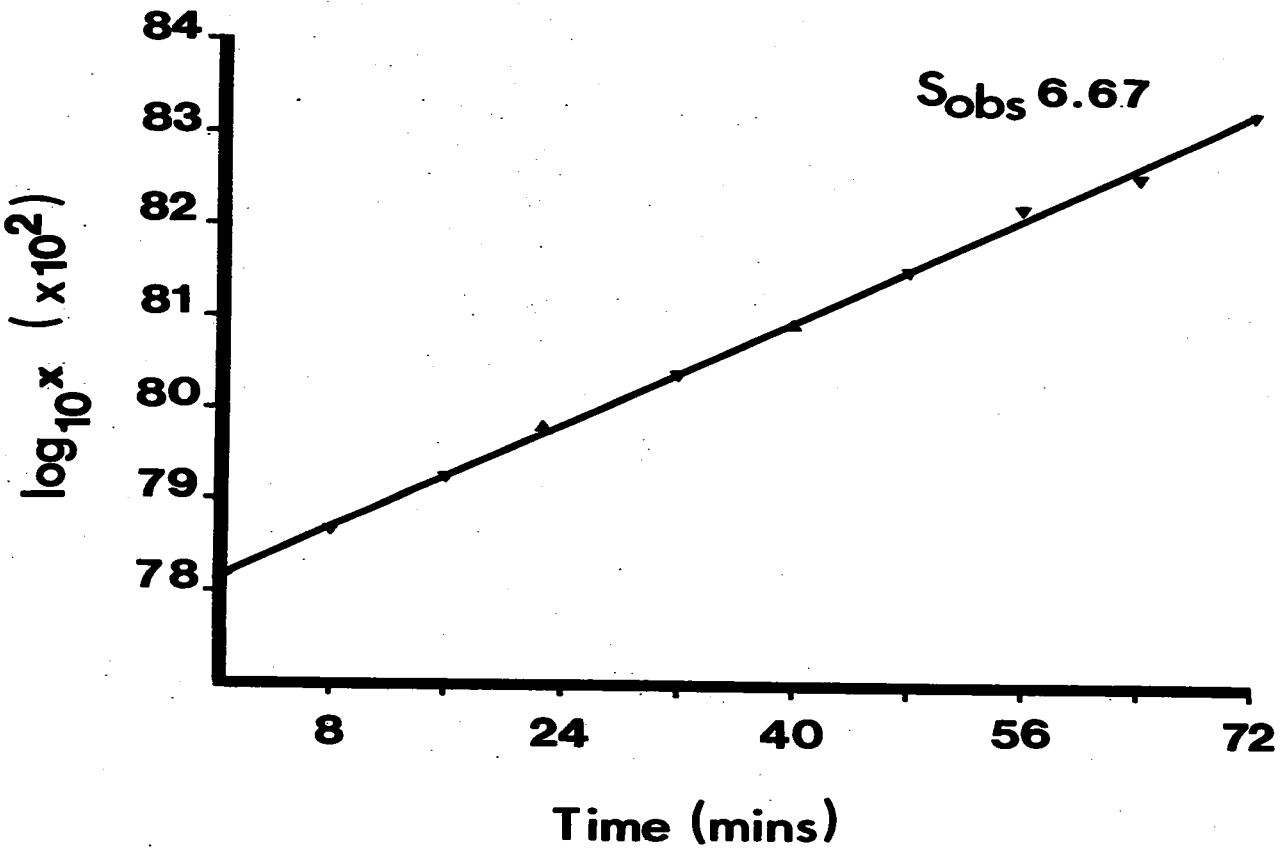

84
83
82
81
80
79
78
$\log_{10}x$ ($\times 10^2$)
S_{obs} 6.67
8
24
40
56
72
Time (mins)

FIGURE 19

DATA FOR THE DETERMINATION OF SEDIMENTATION COEFFICIENT FOR PURIFIED GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

The logarithm of distance sedimented was plotted against time for calculation of the sedimentation coefficient as described in the text. The conditions for sedimentation velocity centrifugation are stated in the legend to Figure 18.

x = distance measured on photographic plates before correction for camera lens magnification (cm)

t = time in minutes

as 6.67. The linear relationship of log x and time indicates that the sedimentation constant did not change during the course of the experiment. None of the observed sedimentation coefficients were converted to $S_{20,w}$ values since complete temperature dependent viscosity values for tris-HCl buffer were not available.

Data for the calculation of molecular weights were obtained by conventional sedimentation equilibrium experiments in preference to the classical sedimentation and diffusion method. The conventional method is based on the measurement of solute concentration throughout the centrifuge cell after distribution under equilibrium conditions. The equilibrium must be such that the solute concentration at the meniscus is not zero. Data were plotted as $\log_{10}$ c versus r^2 (Figure 20) for equilibrium determinations at varying initial solute concentrations. Non-linearity of log c vs. r^2 plots under equilibrium conditions at varying initial solute concentration indicated that the molecular weight is dependent on concentration, increasing with increasing concentration. Such results generally suggest heterogeneity of the solute preparation. Under conditions of polydispersity, the slope of the plot at any point is proportional to the apparent weight average molecular weight of the mixture at that position in the cell. Slope terms ($d\log c/dr^2$) were substituted into the following equation for computation of molecular weights:

$$M = \frac{2\ RT}{(1 - \bar{v}\rho)_\omega^2} \cdot \frac{2.303(d\log c)}{d(r^2)}$$

If one considers the molecular weight values calculated for the more concentrated position in the cell for independent equilibrium experiments,

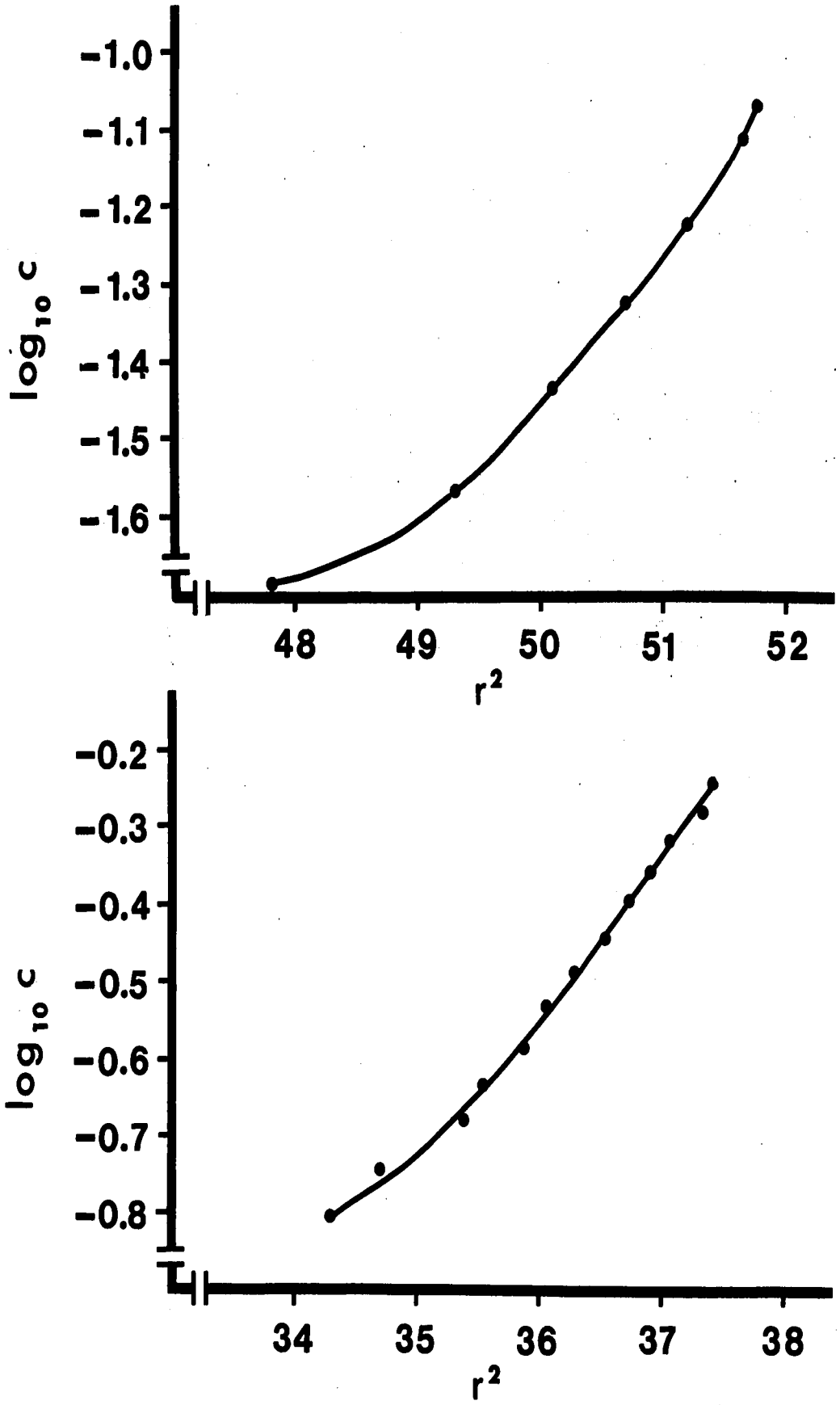

-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
log10 c
48
49
50
51
52
r2
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
log10 c
34
35
36
37
38
r2

FIGURE 20

PLOTS OF LOG_{10} C VERSUS r^2 FOR SEDIMENTATION EQUILIBRIUM EXPERIMENTS USING GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

Equilibrium sedimentation was performed as described in Materials and Methods at 278°K and a rotor speed of 4,840 rpm. The data were plotted as $\log_{10}$ c vs. r^2 for independent experiments with varying initial solute concentration.

r = radial distance in centimeters, corrected for camera lens magnification

c = concentration

Upper Plot: Protein concentration was 2 mg/ml

Lower Plot: Protein concentration was 6 mg/ml

an average molecular weight could be obtained for the mixture which was reasonably independent of protein concentration giving values of 1.68, 1.63, and 1.62 x 10^5 (Av. M. W. = 1.64 x 10^5) at protein concentrations of 3.1, 5.0, and 6.99 mg/ml. However, these results are of little value for molecular weight determinations of the purified protein since the calculations are an average of a heterogeneous mixture.

It was observed that as the initial solute concentration increased in equilibrium experiments, the $\log_{10}c$ vs. r^2 plot more closely approximated a linear relationship (Figure 20). The apparent linearity is probably caused by effects of non-ideality (downward curvature) becoming more obvious at higher concentrations and cancelling upward curvature produced by polydispersity.

The molecular weight of glutamic dehydrogenase from P. aerogenes was also estimated by calibrated gel filtration on Sephadex G-200 (Andrews, 1965). Standard proteins were employed for construction of a selectivity curve as described in Methods, Section IX. Calibration data including elution volumes and K_{av} calculations appear in Table VI. The plot of $\log_{10}$molecular weight versus K_{av} is presented in Figure 21. Unless otherwise specified, molecular weight values as described by Andrews were employed. Purified samples of glutamic dehydrogenase were applied to the calibrated column and their elution volumes converted to K_{av} values for molecular weight extrapolation from the selectivity curve. This procedure suggested a molecular weight of 266,000 for glutamic dehydrogenase. The purified dehydrogenase was eluted in a single symmetrical peak producing superimposable profiles of E_{280} and enzyme activity. Thus, there is no suggestion of heterogeneity in gel

TABLE VI

CALIBRATION DATA FOR SEPHADEX G-200

SELECTIVITY CURVE

Compound	Amount (mg)	MW (x 10^3)	$\log_{10}$ MW	V_e* (ml)	K_{av}
Blue Dextran 2000	1.0	-	-	59 (V_o)	-
Sucrose	3.0	-	-	176 (V_t)	-
Cytochrome c	2.0	12.4	4.0934	150	0.778
Ovalbumin	3.0	45	4.6532	121.5	0.534
Bovine Serum Albumin	5.0	67	4.8261	113	0.461
Serum Albumin Dimer	∝ 0.5	135	5.1303	97	0.325
Yeast Alcohol Dehydrogenase	2.0	151**	5.179	94	0.299
Catalase	0.1	195**	5.290	88	0.248
α-Hydroxyglutarate dehydrogenase	1.0	58.21	4.765†	116	0.487
Glutamic dehydrogenase	1.0	266	5.425†	81	0.188

* Average of two independent filtration experiments.

** As reported by von Tigerstrom, 1968.

† From K_{av} vs. log MW curve (Figure 21).

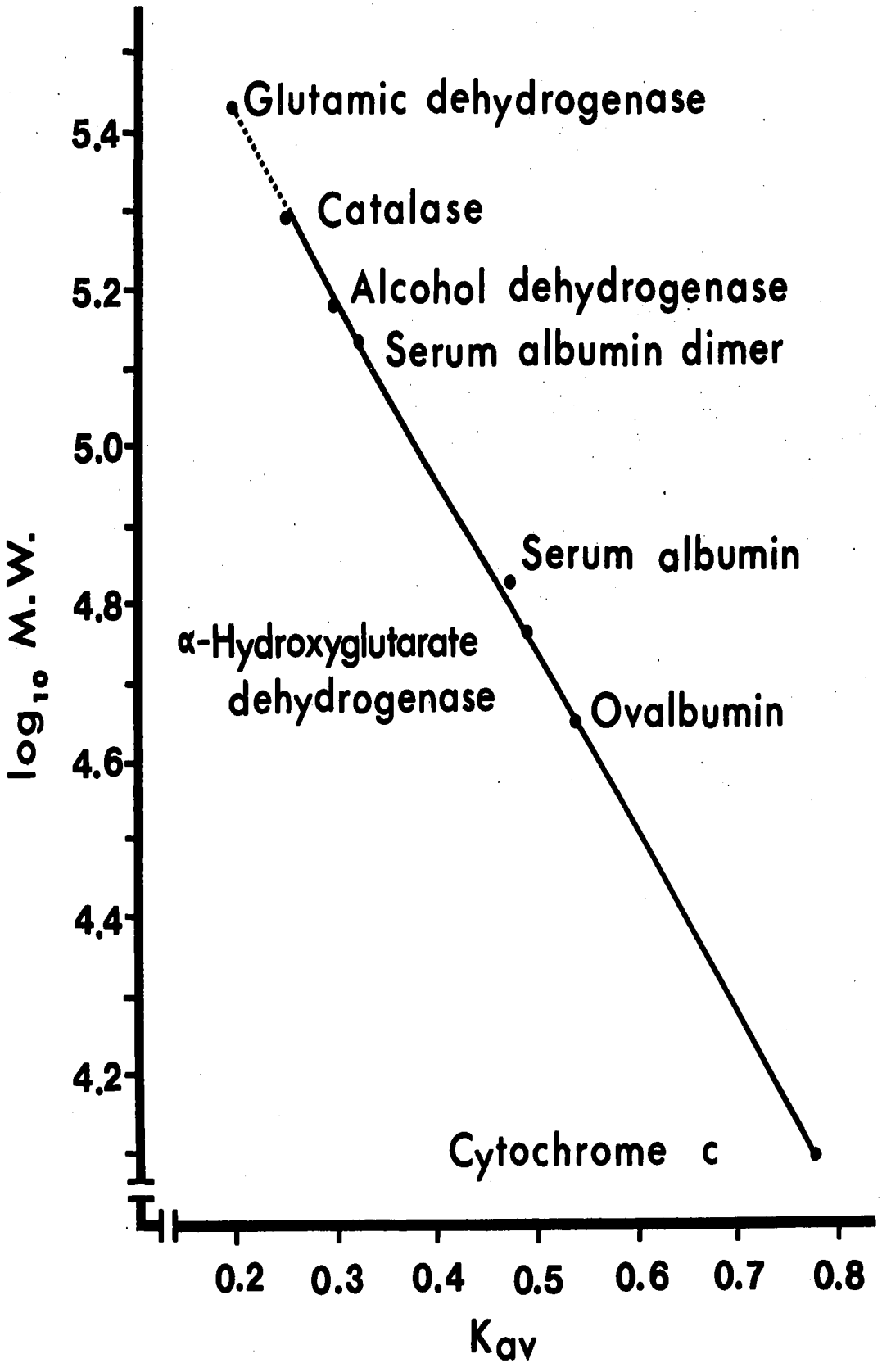

Glutamic dehydrogenase
Catalase
Alcohol dehydrogenase
Serum albumin dimer
Serum albumin
α-Hydroxyglutarate dehydrogenase
Ovalbumin
Cytochrome c
5.4
5.2
5.0
4.8
4.6
4.4
4.2
log10 M.W.
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Kav

FIGURE 21

SELECTIVITY CURVE FOR STANDARD PROTEINS USING SEPHADEX G-200

Procedures for column preparation, elution, and calibration are described in Materials and Methods. A list of standard proteins and assays appears in Table I, and elution volumes and K_{av} data are presented in Table VI. Purified preparations of glutamic and α-hydroxyglutarate dehydrogenase from P. aerogenes were applied to the calibrated column of Sephadex G-200 for molecular weight estimation.

filtration experiments as opposed to the results from sedimentation equilibrium analysis. The reliability of molecular weight estimations by calibrated gel filtration will be considered in the Discussion.

C. Kinetic Analysis

The kinetic analysis of glutamic dehydrogenase was limited to the determination of substrate Km values in order to ensure saturating conditions in the enzyme assay. Lineweaver-Burke plots are presented in Figure 22 for glutamic acid and NAD Km values respectively. The Km was found to be 1.3×10^{-3} M for glutamic acid and 2.5×10^{-4} M for NAD. The maximum velocity under saturating substrate conditions was 27.8 nmoles NADH formed/minute at 25°C.

D. General Enzyme Properties

The initial velocity was determined at various temperatures to obtain data for the temperature optimum under conditions of saturating substrate concentrations. The results presented in Figure 23 and Table VII show that the optimum temperature falls between 50 and 55°C.

Enzyme activity was determined in several buffers at a range of pH values on either side of the pKa for the buffer. Each assay mixture contained 40 μmoles of the designated buffer and pH, 5×10^{-3} M glutamic acid, 1×10^{-3} M NAD, and 0.29 μg of purified protein in a total volume of 1.0 ml. Initial velocities were determined at 25°C immediately after the addition of NAD. The results presented in Table VIIa were corrected for control levels of NAD reduction and expressed in terms of specific activity. No activity was observed at any pH value in either boric acid-borax buffer (pH 7.55 to 8.95) or borax-NaOH buffer (pH 8.95 to 11.2)

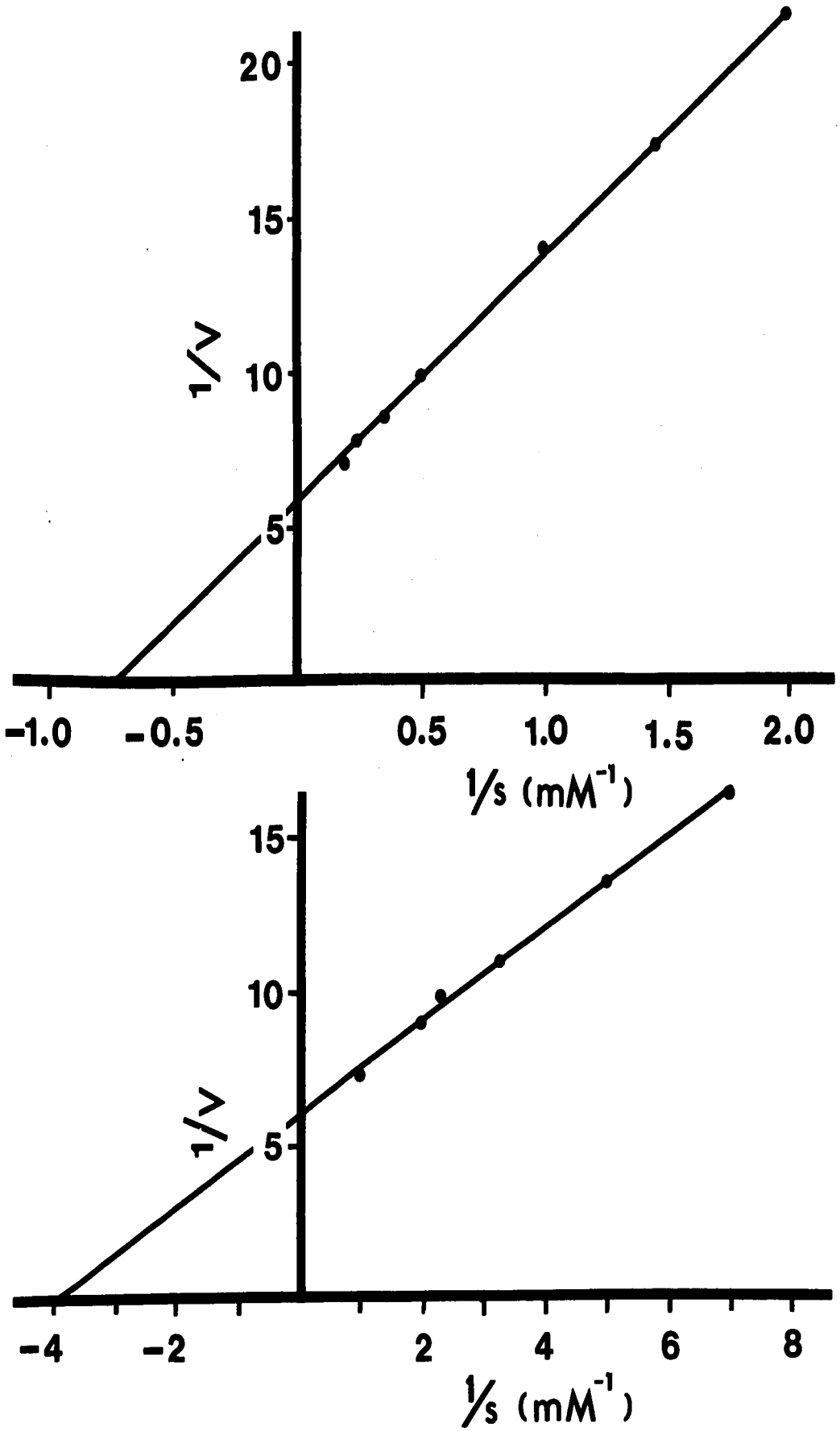

20
15
10
5
1/v
-1.0
-0.5
0.5
1.0
1.5
2.0
1/s (mM^{-1})
15
10
5
1/v
-4
-2
2
4
6
8
1/s (mM^{-1})

FIGURE 22

LINEWEAVER-BURKE PLOTS OF KINETIC DATA FOR GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

Upper Plot: Lineweaver-Burke plot for glutamic acid

Assay mixtures contained designated quantities of glutamic acid in addition to 40 µmoles tris-HCl buffer, pH 8.8, 1×10^{-3} M NAD, and 0.34 µg of purified enzyme (S. A. = 65 E. U./mg) in a total volume of 1.0 ml. Initial velocities were measured by the increase in absorbance at 340 nm caused by the formation of reduced NAD at 25°C. The Km value for glutamic acid was calculated at 1.3×10^{-3} M and the Vmax was 28.07 nmoles NADH formed/minute at 25°C.

Lower Plot: Lineweaver-Burke Plot for NAD

Assay mixtures contained designated quantities of NAD and 5×10^{-3} M glutamic acid in addition to buffer and enzyme as described above. Initial velocities were measured as above and the Km value for NAD was calculated at 2.5×10^{-4} M. The maximum velocity observed in this experiment was 27.58 nmoles NADH formed/minute at 25°C.

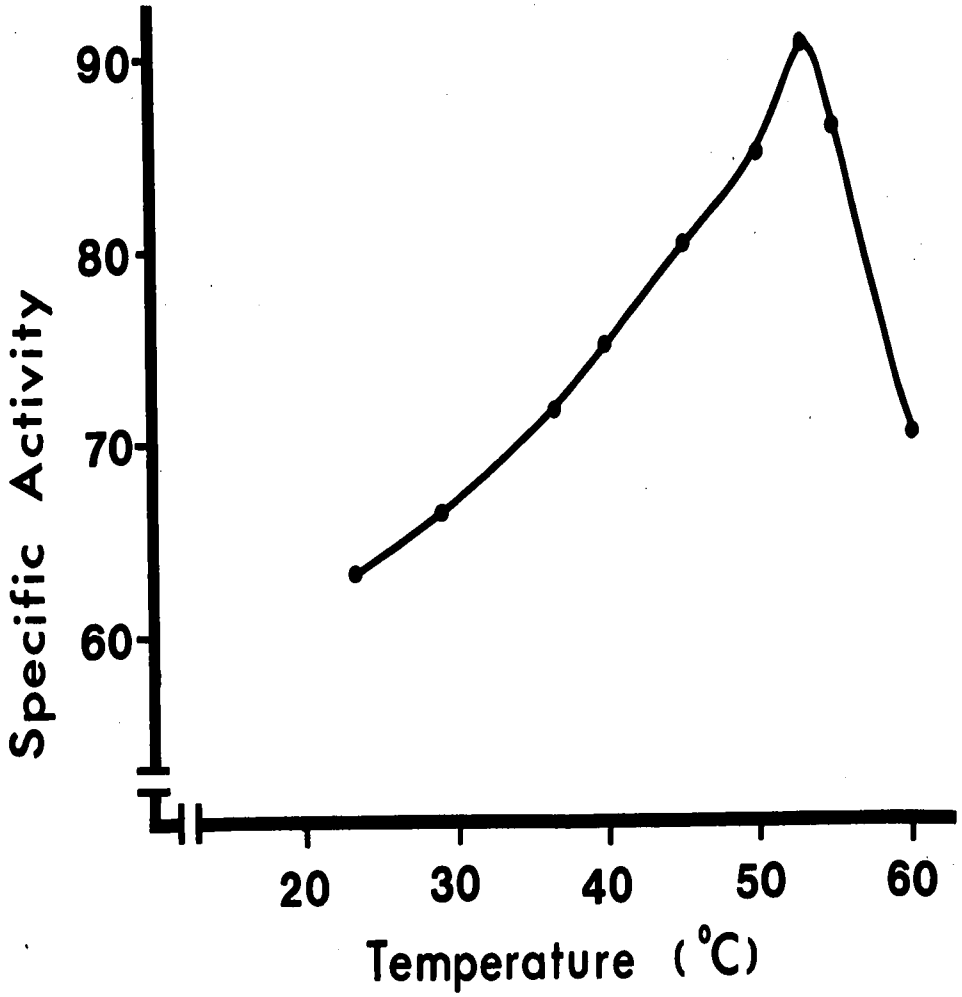

Specific Activity
90
80
70
60
20
30
40
50
60
Temperature (°C)

FIGURE 23

TEMPERATURE OPTIMUM CURVE FOR GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

Each assay mixture contained 40 µmoles tris-HCl buffer, pH 8.8, 5×10^{-3} M glutamic acid, 1×10^{-3} M NAD, and 0.22 µg protein in a total volume of 1.0 ml. Enzyme was added last after pre-incubation of substrate and buffer at the required temperature for 5 minutes. Results are expressed in terms of specific activity (µmoles NADH formed/minute/mg protein) at varying temperatures. All initial velocities were corrected for NAD reduction in the absence of glutamic acid.

TABLE VII

TEMPERATURE OPTIMUM DATA FOR GLUTAMIC DEHYDROGENASE FROM *P. AEROGENES*

Temperature	Specific Activity (μmoles/min/mg protein)
23.5	63.0
29	66.7
36.5	71.8
40	74.8
45	79.2
49.5	85.0
53	89.4
55	86.5
60	70.4

TABLE VIIa

OPTIMAL CONDITIONS OF BUFFER AND pH FOR GLUTAMIC DEHYDROGENASE ACTIVITY FROM P. AEROGENES

Buffer	pK_a*	Maximum S. A.**	pH of Maximum S. A.
Potassium phosphate	7.2	47.83	8.1
N-ethylmorpholine	7.67	60.07	8.4
Veronal	7.98	68.97	8.75
Tris-HCl	8.08	65.63	8.8
Glycine-NaOH	9.78	55.62	8.95

* pK_a values at 25°C as reported by McKenzie, 1969.

** Specific activity was calculated as E. U./mg protein.

suggesting the formation of inhibitory borate complexes. The results in Table VII indicate a high pH optimum in the region of pH 8.8 to 8.9 in either tris-HCl, glycine, or veronal buffer. An examination of the data in Table VII shows maximum activity in veronal at pH 8.75 followed by tris-HCl at pH 8.8 and N-ethylmorpholine at pH 8.4.

Several new hydrogen ion buffers having pK values between 6 and 8 (Good et al., 1966) were compared to tris-HCl at pH 8.0. A code of trivial and technical names for the buffers appears in Table VIII. All assay mixtures contained 40 μmoles of the designated buffer at pH 8.0, 5×10^{-3} M glutamic acid, 1×10^{-3} M NAD, and 0.28 μg protein in a final volume of 1.0 ml. Initial velocities were determined at 25°C immediately after the addition of NAD. The results presented in Table IX indicate that glutamic dehydrogenase was most active in tris-HCl buffer, contrary to the suggestion of Good that several of the new buffers may be superior to standard buffers now widely employed.

Purified preparations of glutamic dehydrogenase at 5 mg/ml were maintained in 0.05 M tris-HCl buffer, pH 8.8, at -20°C for several months with no significant loss in specific activity. In addition, successive freezing and thawing of the enzyme as many as ten times produced no loss in activity.

The presence of sodium chloride in the assay mixture was found to produce a 13.5% stimulation at concentrations of 2.5 to 5.0×10^{-2} M, although lower concentrations had no detectable effect. Therefore, all enzyme preparations recovered from NaCl gradients were dialyzed prior to assay for enzyme activity.

The thermal stability of glutamic dehydrogenase was determined

TABLE VIII

CODE TO BUFFER NOMENCLATURE

Trivial Name	Technical Name
Veronal	Sodium diethylbarbiturate
TRIS	Tris(hydroxymethyl)amino methane
TES	N-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid
Tricine	N-tris(hydroxymethyl)-methyl-glycine
Bicine	N,N-bis-(2-hydroxyethyl)-glycine
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

from 50 to 80°C. The experimental conditions are described in the legend to Figure 24 where the results are expressed in terms of percentage initial activity at various temperatures. The results indicate that glutamic dehydrogenase is particularly sensitive to heating for 10 minutes above 65°C. Heat treatment for 10 minutes at 80°C was sufficient to completely destroy enzyme activity.

The kinetics of heat inactivation are presented for temperatures of 65, 75, and 77°C in Figure 25. Linear inactivation kinetics were observed at all temperatures when the data were plotted as $\log_{10}$ percent velocity as a function of time at the designated temperatures. The results of experiments of thermal stability indicate that a carefully controlled heat treatment step could be employed in the purification sequence with no appreciable loss of activity.

Substrate specificity studies using glutamic dehydrogenase showed that the enzyme is specific for L-(+)-glutamic acid and NAD. No reaction was observed in the presence of 1.0×10^{-2} M D-glutamate, D- or L-aspartate, or glutamine. No activity was observed when 1, 2, or 5 mM NADP was substituted as the cofactor.

When the reaction was studied in the reverse direction, in the presence of 0.02 M α-ketoglutarate, 0.2 M NH_4Cl, and 4×10^{-4} M reduced cofactor (NADH or NADPH), the activity with NADPH was only 0.85% of that observed with NADH. These results compare favorably with the 1.1% activity with NADPH documented in a recent publication (Kew and Woolfolk, 1970).

Results for the activity of glutamic dehydrogenase in the presence of various ionic additives are presented in Table X. At a concentration

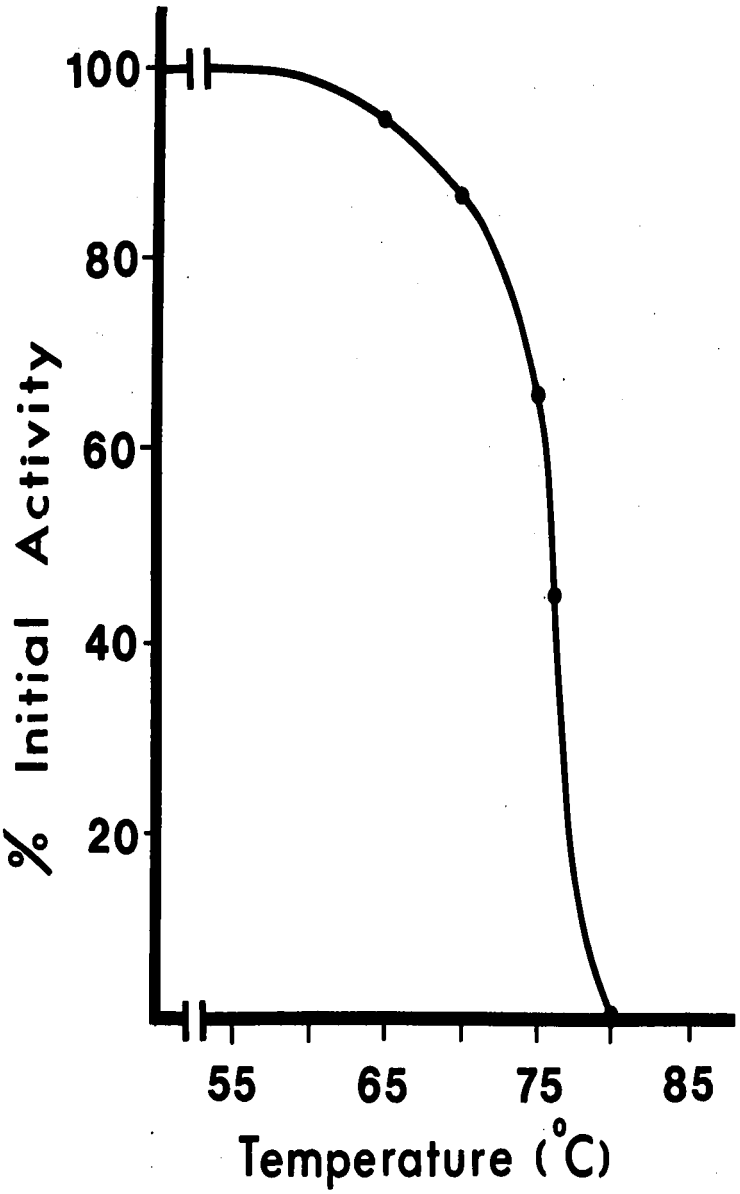

100
80
60
40
20
% Initial Activity
55
65
75
85
Temperature (°C)

FIGURE 24

THERMAL STABILITY CURVE FOR GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

Purified enzyme containing 100 µg protein/ml in 0.05 M tris-HCl buffer, pH 8.8, was subjected to 10 minutes of heat treatment at the designated temperatures. The activity remaining after heat treatment is expressed as percentage of the initial activity.

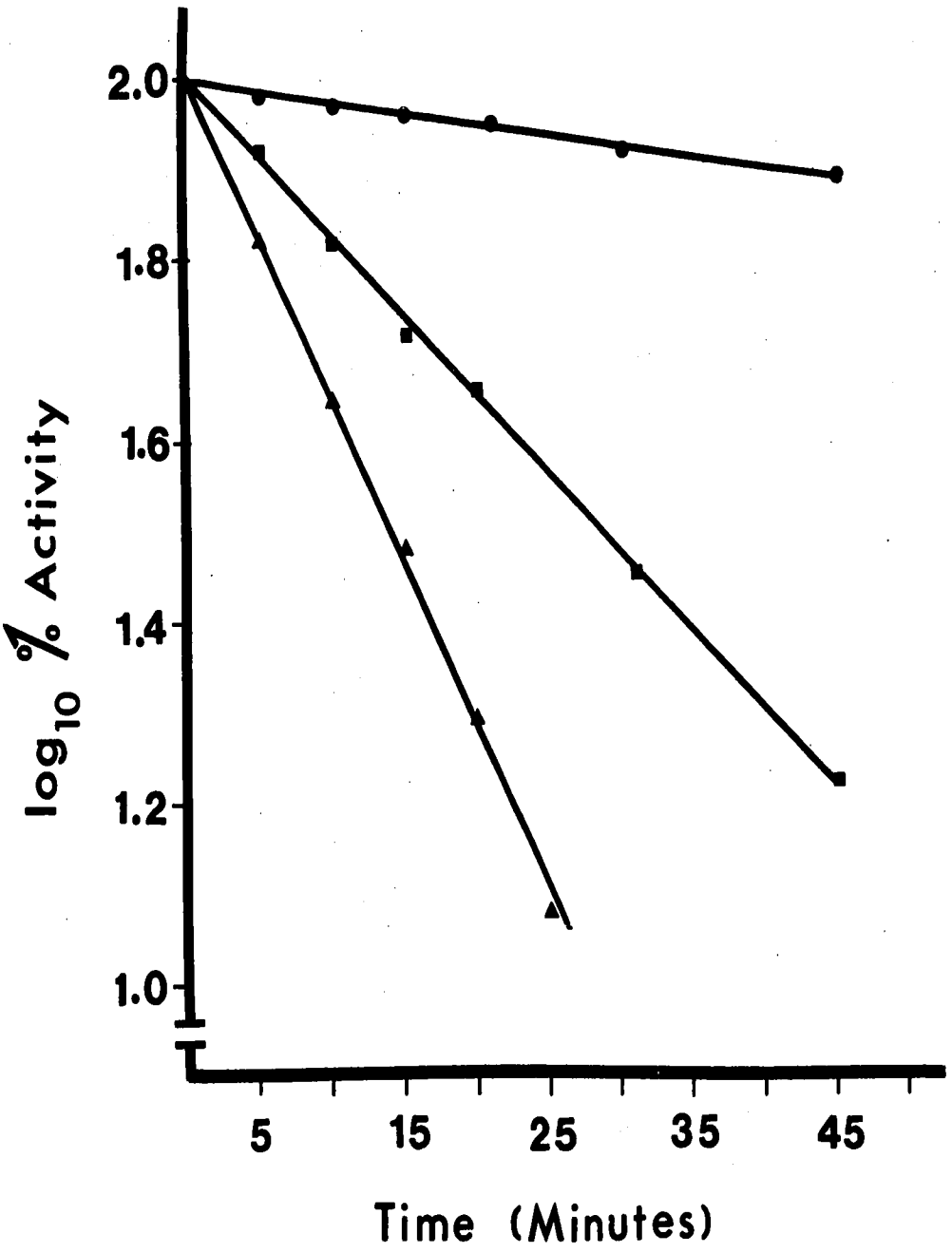
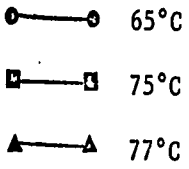

2.0
1.8
1.6
1.4
1.2
1.0
log10 % Activity
5
15
25
35
45
Time (Minutes)

FIGURE 25

HEAT INACTIVATION CURVE FOR GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

A purified enzyme preparation containing 100 µg protein/ml in 0.05 M tris-HCl buffer, pH 8.8, was subjected to 65, 75, and 77°C temperatures for various time intervals. The data are expressed as $\log_{10}$ percentage activity vs. time in minutes.



of 1×10^{-3} M, only Mg^{++}, Mn^{++}, and Ca^{++} were slightly stimulatory (7 to 8%). Varying degrees of inhibition were exhibited in the presence of Al^{+++}, Fe^{++}, Zn^{++}, Co^{++}, Cd^{++}, and Fe^{+++}. Other ions such as Na^{+}, K^{+}, Cs^{+}, and Rb^{+}, showed no effect on enzyme activity at 1mM concentration. The general trend of the results indicates that monovalent cations show little, if any, effect whereas divalent cations are either stimulatory or inhibitory. There was no inhibition observed by the anion in the presence of sodium arsenate.

The presence of chelating or reducing agents has little effect on glutamic dehydrogenase activity as illustrated in Table XI. High concentrations of EDTA (20 mM), cysteine (10mM), and a combination of mercaptoethanol and EDTA (10 mM + 10 mM) produced a slight increase in specific activity although never more than 10% of the control value. The only inhibition observed was in the presence of 10 mM dithioerythritol. In general, the enzyme appears to be relatively insensitive to the protective effects of reducing agents.

The results of inhibition studies are presented in Table XII. The most extensive inhibition was produced by various concentrations of organic mercurials including p-chloro- and p-hydroxymercuribenzoate. The metal chelators, 8-hydroxyquinoline and 1,10-phenanthroline, were not effective inhibitors with the exception of 8% inhibition in the presence of 1×10^{-3} M 1,10-phenanthroline. Iodoacetate produced some inhibition even though it is not a highly specific alkylating reagent. Examination of a more specific alkylating reagent, N-ethylmaleimide, illustrated some inhibition (9%) at a concentration of 1×10^{-3} M. No inhibition was observed with 1×10^{-3} M concentrations of malonate,

TABLE X

THE EFFECT OF IONS ON THE ACTIVITY OF GLUTAMATE DEHYDROGENASE FROM P. AEROGENES *

Additive	Relative Rate (%)
A. No Effect	
NH_4Cl (SO_4)	100
CsCl	100
LiCl	100
KCl	100
K_2HPO_4	100
RbCl	100
Na-arsenate	100
NaCl	100
$Na_2HPO_4 \cdot 7H_2O$	100
B. Activators	
$CaCl_2 \cdot 2H_2O$	107
$MnCl_2 \cdot 4H_2O$	107
$MgCl_2 \cdot 6H_2O$	108
C. Inhibitors	
$AlCl_3$	88
$FeSO_4$	84
$ZnCl_2$	83
$SnCl_2 \cdot 7H_2O$	80
$CoCl_2 \cdot 6H_2O$	78
$CdCl_2 \cdot 2.5H_2O$	75
$FeCl_3 \cdot 6H_2O$	72
$FeCl_2 \cdot 4H_2O$	46

* Assays were performed as described in Methods with the addition of the designated salts to a final concentration of 1×10^{-3} M.

TABLE XI

THE EFFECT OF CHELATING AND REDUCING AGENTS ON THE ACTIVITY OF GLUTAMIC DEHYDROGENASE FROM P. AEROGENES*

Additive	Final Concentration	S. A. (E.U./mg)	% Control Activity
None (Control)	-	64.93	100
EDTA	1×10^{-4} M	62.66	100
	1×10^{-3} M	65.65	100
	1×10^{-2} M	68.64	106
2-Mercaptoethanol	1×10^{-4} M	64.15	100
	1×10^{-3} M	65.65	100
	1×10^{-2} M	61.94	100
	2×10^{-2} M	64.15	100
2-Mercaptoethanol + EDTA **		70.91	109
Cysteine	1×10^{-4} M	63.44	100
	1×10^{-3} M	66.43	100
	1×10^{-2} M	69.35	107
Dithioerythritol	1×10^{-4} M	64.93	100
	1×10^{-3} M	62.66	100
	1×10^{-2} M	44.00	68
Reduced glutathione	1×10^{-4} M	63.44	100
	1×10^{-3} M	64.93	100
	1×10^{-2} M	64.15	100
Dithiothreitol	1×10^{-5} M	66.43	100
	1×10^{-4} M	63.44	100
	1×10^{-3} M	64.15	100

* Purified glutamic dehydrogenase was assayed as described in Methods in the presence of the designated additives. All of the chelating and reducing agents were adjusted to pH 8.8-9.0 prior to use.

** The final concentration of each component was 1×10^{-2} M.

TABLE XII

THE EFFECT OF INHIBITORS ON GLUTAMIC DEHYDROGENASE FROM P. AEROGENES*

Additive	Final Concentration	S. A. (E.U./mg)	% Inhibition
None (Control)	-	65.66	-
p-Chloromercuri-benzoate	1×10^{-3} M	45.61	30.5
	5×10^{-4} M	54.68	16.7
	1×10^{-4} M	65.01	-
p-Hydroxymercuri-benzoate	1×10^{-2} M	3.02	95.4
	5×10^{-3} M	13.78	79.0
	1×10^{-3} M	48.23	26.5
8-Hydroxyquinoline	1×10^{-3} M	62.02	-
	5×10^{-4} M	65.01	-
	1×10^{-4} M	65.01	-
1,10-phenanthro-line	1×10^{-3} M	60.29	8.2
	5×10^{-4} M	68.01	-
	1×10^{-4} M	67.49	-
Iodoacetate	1×10^{-3} M	51.23	22.0
	5×10^{-4} M	59.39	9.6
	1×10^{-4} M	65.9	-
N-ethylmaleimide	1×10^{-3} M	59.63	9.2
Malonate	1×10^{-3} M	67.18	-
Oxalacetate	1×10^{-3} M	68.01	-
Arsenite	1×10^{-3} M	67.49	-

* Purified enzyme was incubated with buffer in the presence of inhibitor for 60 minutes at 30°C prior to assay by the addition of substrates. The inhibitors were adjusted to pH 9.0 when possible.

oxalacetate, and arsenite. The results observed in the presence of malonate are not necessarily conclusive since higher concentrations of the inhibitor should have been tested in view of Cooper's demonstration of inhibition by 10^{-2} M malonate in the case of enzymes such as succinic dehydrogenase and malate dehydrogenase (Cooper, 1969).

Absorption data for purified glutamic dehydrogenase was obtained from dry weight measurements and determination of E_{280}. It was calculated that a purified solution of the enzyme at a concentration of 1.0 mg/ml in 0.05 M tris-HCl buffer, pH 8.8, had an absorbance of 0.94 at 280 nm. The ratio of $E_{280}:E_{260}$ was 1.72 .

IX. Purification of α-Hydroxyglutarate Dehydrogenase

Throughout the purification sequence, this enzyme was assayed in the direction of formation of reduced NAD and α-ketoglutarate from α-hydroxyglutarate as described in Methods, Section VIII. Concentrated α-hydroxyglutarate dehydrogenase from G-200 (Section VI) was applied to a 2.4 x 40 cm column of DEAE-cellulose equilibrated in 0.05 M tris-HCl buffer, pH 8.8, at 4°C. A NaCl gradient composed of 250 ml of 0.05 M tris-HCl buffer, pH 8.8, and 250 ml of 0.4 M NaCl in the same buffer was used for elution. A Buchler refrigerated fraction collector was employed to collect 100-drop fractions at 4°C. Fractions were analyzed for extinction at 280 nm, enzyme activity, and conductivity. A typical elution profile from DEAE-cellulose is presented in Figure 26. The α-hydroxyglutarate dehydrogenase activity was eluted at a NaCl concentration of 0.17 M as compared to 0.21 M for glutamic dehydrogenase. The active fractions were concentrated by ultrafiltration and dialyzed prior

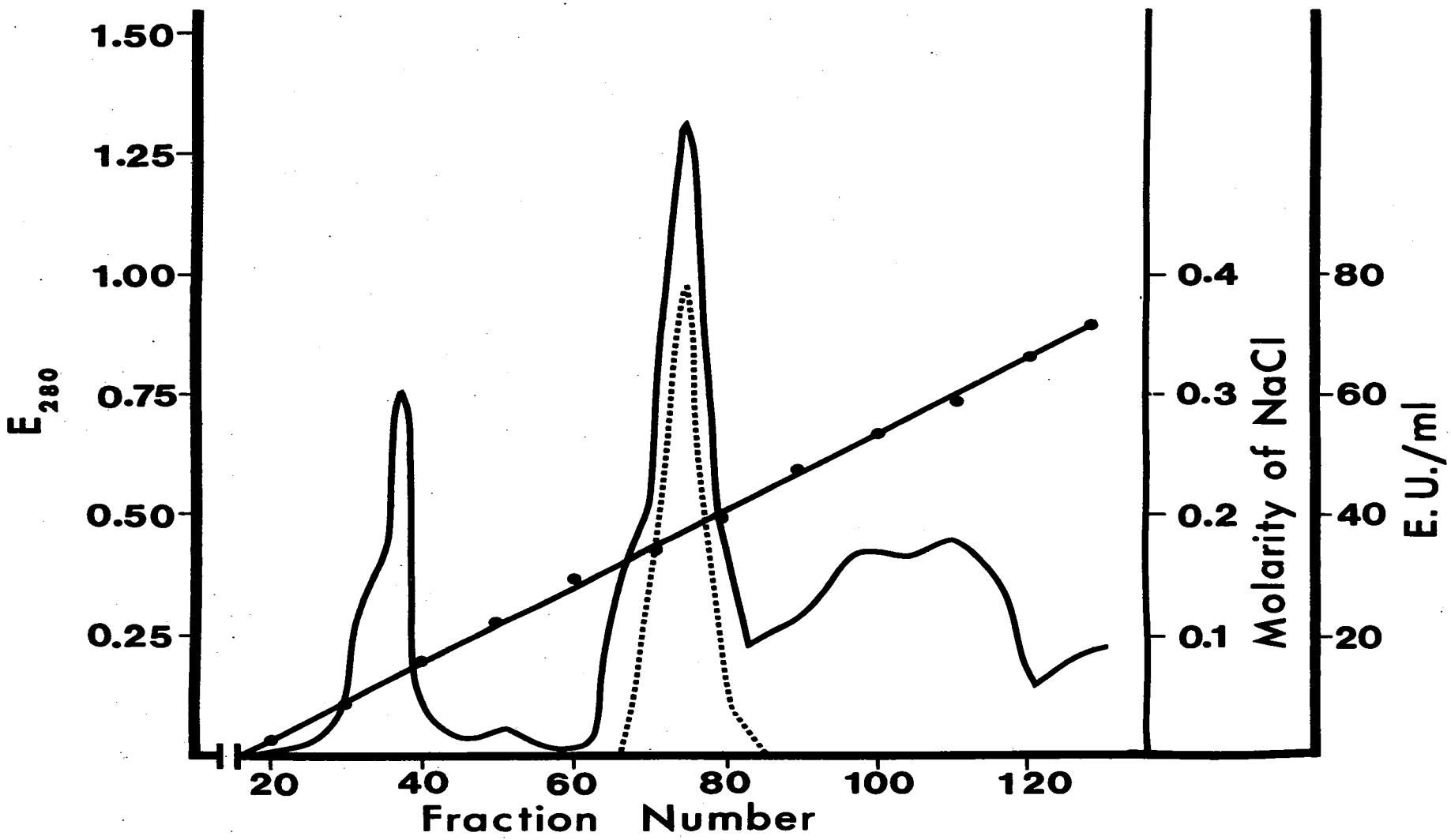
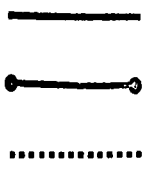

1.50
1.25
1.00
0.75
0.50
0.25
E_{280}
0.4
0.3
0.2
0.1
Molarity of NaCl
80
60
40
20
E.U./ml
20
40
60
80
100
120
Fraction Number

FIGURE 26

ELUTION PROFILE FROM DEAE-CELLULOSE FOR α-HYDROXYGLUTARATE DEHYDROGENASE OF P. AEROGENES

Concentrated α-hydroxyglutarate dehydrogenase from Sephadex G-200 was applied to a 2.5 x 40 cm column of DEAE-cellulose equilibrated in 0.05 M tris-HCl buffer, pH 8.8. A NaCl gradient as described in the text was employed for elution and 100-drop fractions were collected at 4°C. Samples of each fraction were analyzed for protein, enzyme activity, and conductivity.



E_{280}

Molarity of NaCl

α-Hydroxyglutarate dehydrogenase activity

to chromatography on DEAE-Sephadex A-50. Recovery and specific activity data are presented in Table XIII.

A 2.5 x 36 cm column of DEAE-Sephadex was equilibrated at 4°C in 0.05 M tris-HCl buffer, pH 8.8, prior to sample application. Since no enzyme was eluted after washing with 500 ml of the same buffer, a 500 ml gradient to 0.5 M tris-HCl buffer at the same pH was applied to the column. The enzyme activity was eluted in a single protein peak at the end of the tris-HCl gradient. Data for recovery and increase in specific activity presented in Table XIII show a 33% recovery and a 736-fold purification. Recovery figures were based upon activity in ammonium sulphate fractionations because of the increase in total units from the crude extract to the ammonium sulphate treatment. The enzyme preparation of specific activity 43 enzyme units/mg protein was used for sedimentation analysis and characterization unless otherwise specified.

X. Characterization of α-Hydroxyglutarate Dehydrogenase

A. Identification of the Reaction Product

Since α-ketoglutarate was also the expected product of α-hydroxyglutarate oxidation, incubation mixtures were prepared as described for glutamic dehydrogenase (Section VIII) with the substitution of 10 μmoles of D-α-hydroxyglutarate for glutamic acid and 1 mg of purified α-hydroxyglutarate dehydrogenase for glutamic dehydrogenase. Further substrate additions and conditions for derivative formation were identical to those described for glutamic dehydrogenase. Thin layer chromatographic analysis of the extracted 2,4-dinitrophenylhydrazones showed that the reaction product migrated to the same position as a standard sample of

TABLE XIII

PURIFICATION TABLE FOR α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Procedure	Total units ($\times 10^3$)	% Recovery†	Specific Activity*	Purification
Crude Extract	0.046	-	0.06	1
$(NH_4)_2SO_4$	3.55	100	8.12	139
Sephadex G-200	1.82	51	27.12	464
DEAE-Cellulose	1.43	40	36.43	618
DEAE-Sephadex	1.18	33	43.00	736

* Specific activity was calculated as E. U./ mg protein.

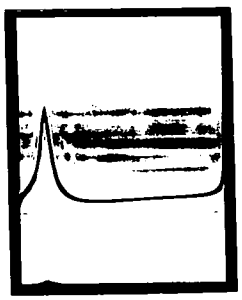
† Recovery based on total units in $(NH_4)_2SO_4$ fractionation because of the increase in total units from the crude extract to this stage.

α-ketoglutarate-2,4-dinitrophenylhydrazone in both solvents. This evidence established that the product of α-hydroxyglutarate oxidation was, as expected, α-ketoglutarate.

B. Sedimentation Analysis And Estimation of Molecular Weight

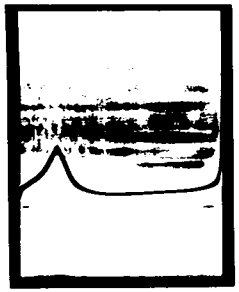
The Schlieren pattern obtained upon sedimentation velocity analysis of purified α-hydroxyglutarate dehydrogenase in tris-HCl buffer is illustrated in Figure 27. The relationship of the logarithm of the distance sedimented to time for a 36 minute duration is presented in Figure 28. Calculation of the slope of the regression line in Figure 28 and substitution into the equation previously given for determination of the sedimentation coefficient results in an observed sedimentation rate (S_{obs}) of 2.26. As before, the linear relationship of log x and time indicates that the sedimentation constant did not change during the course of the experiment. The sample did not appear to be homogeneous since a minor lighter component was observed during sedimentation velocity experiments. The observed sedimentation rate refers to the major peak.

Conventional sedimentation equilibrium analysis was performed as described in Methods to confirm the suspected heterogeneity. Data were plotted as $\log_{10} c$ vs. r^2 (Figure 29) and once again non-linearity was observed under conditions of thermodynamic equilibrium. The molecular weight was directly dependent on concentration. However, since the slope of the plot at any point is proportional to the apparent weight average molecular weight of the mixture at that position in the cell, the data were not discarded. The apparent weight average molecular weight in the more concentrated area of the cell was calculated from the slope at that



4

12



24

36

FIGURE 27

SEDIMENTATION PATTERN OF PURIFIED α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

The enzyme preparation (4 mg/ml) in 0.05 M tris-HCl buffer, pH 8.8, was centrifuged at 60,000 rpm at 5°C. Sedimentation was from left to right. Photographs were taken at 4 minute intervals for 36 minutes after the rotor attained full speed.

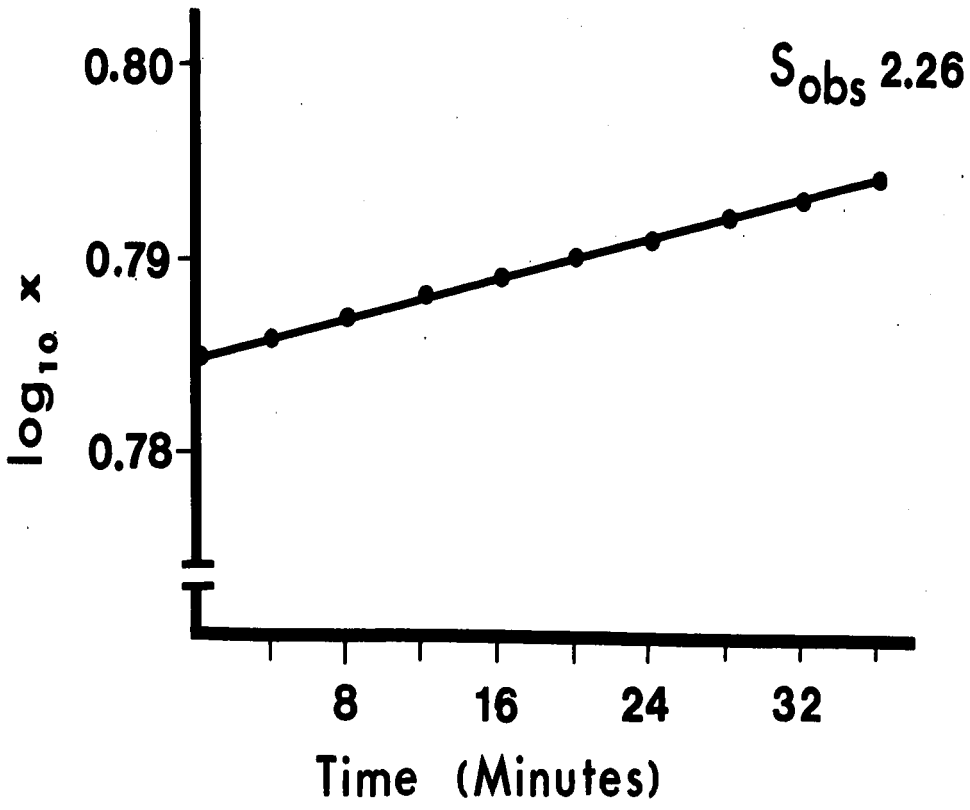

0.80
0.79
0.78
S_{obs} 2.26
$\log_{10} x$
8
16
24
32
Time (Minutes)

FIGURE 28

DATA FOR THE DETERMINATION OF SEDIMENTATION COEFFICIENT FOR PURIFIED α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

The logarithm of distance sedimented was plotted against time for calculation of the sedimentation coefficient as described in the text. The conditions for sedimentation velocity centrifugation are presented in the legend to Figure 27.

x = distance measured on photographic plates before correction for camera lens magnification (cm)

t = time in minutes

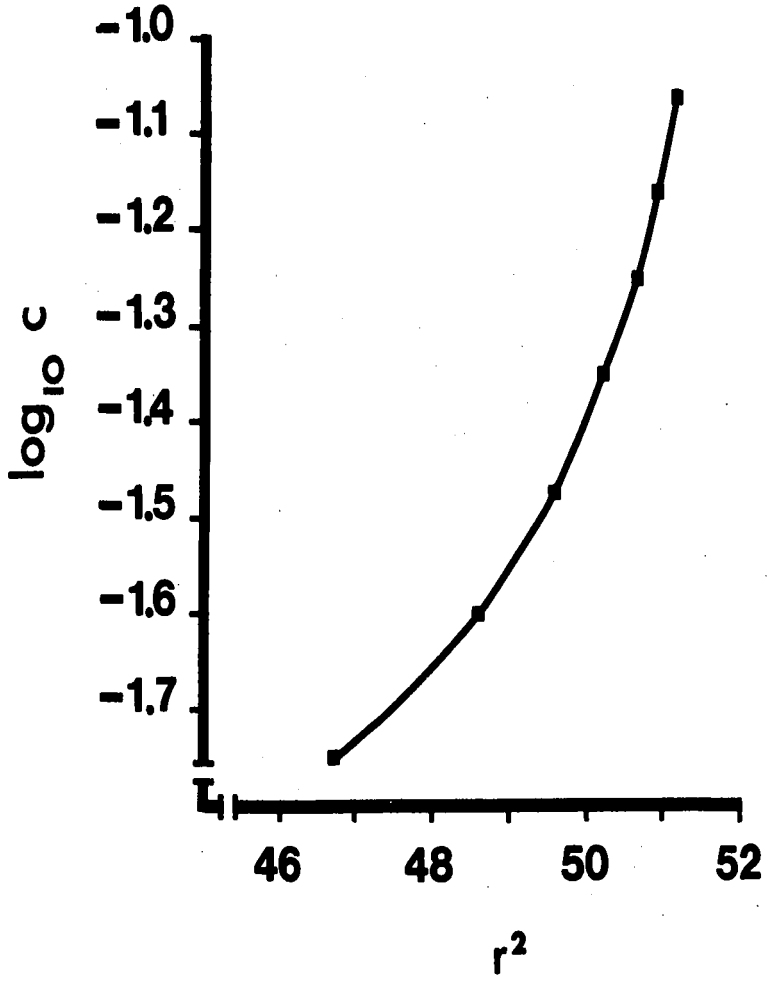

-1.0
-1.1
-1.2
-1.3
-1.4
-1.5
-1.6
-1.7
log10 c
46
48
50
52
r2

FIGURE 29

PLOT OF LOG_{10} C VERSUS r^2 FOR SEDIMENTATION EQUILIBRIUM EXPERIMENTS USING α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Equilibrium sedimentation was performed as described in Materials and Methods at 278°K, 2 mg protein/ml, and a rotor speed of 11,000 rpm. The data were plotted as $\log_{10}$ c vs. r^2.

r = radial distance in centimeters, corrected for camera lens magnification

c = concentration

and found to be 61,000. The protein concentration at that point in the cell corresponded to 3.1 mg/ml. Details of the theory of conventional sedimentation equilibrium analysis have been presented in Section VIII under the characterization of glutamic dehydrogenase.

The molecular weight of α-hydroxyglutarate dehydrogenase from P. aerogenes was also estimated by calibrated gel filtration on Sephadex G-200 as previously described (Section VII, characterization of glutamic dehydrogenase). Calibration data including elution volumes and K_{av} calculations for standard proteins appear in Table VI and the selectivity curve is presented in Figure 21. Purified samples of α-hydroxyglutarate dehydrogenase were applied to the calibrated column and their elution volumes converted to K_{av} values for molecular weight interpolation from the selectivity curve. This procedure suggested a molecular weight of 58,000 for α-hydroxyglutarate dehydrogenase. The purified dehydrogenase was eluted in a single symmetrical peak producing superimposable profiles of E_{280} and enzyme activity. Thus, there is no suggestion of heterogeneity in gel filtration experiments as opposed to the results from sedimentation analyses.

C. Kinetic Analysis

The kinetic analysis of α-hydroxyglutarate dehydrogenase was more complete than that for glutamic dehydrogenase. Lineweaver-Burke plots for Km determinations for D-α-hydroxyglutarate and NAD are presented in Figures 30 and 31 respectively. The Km value for D-α-hydroxyglutarate is 1.1×10^{-3} M and 1.9×10^{-4} M for NAD. The average maximum velocity under saturating conditions of substrate concentration

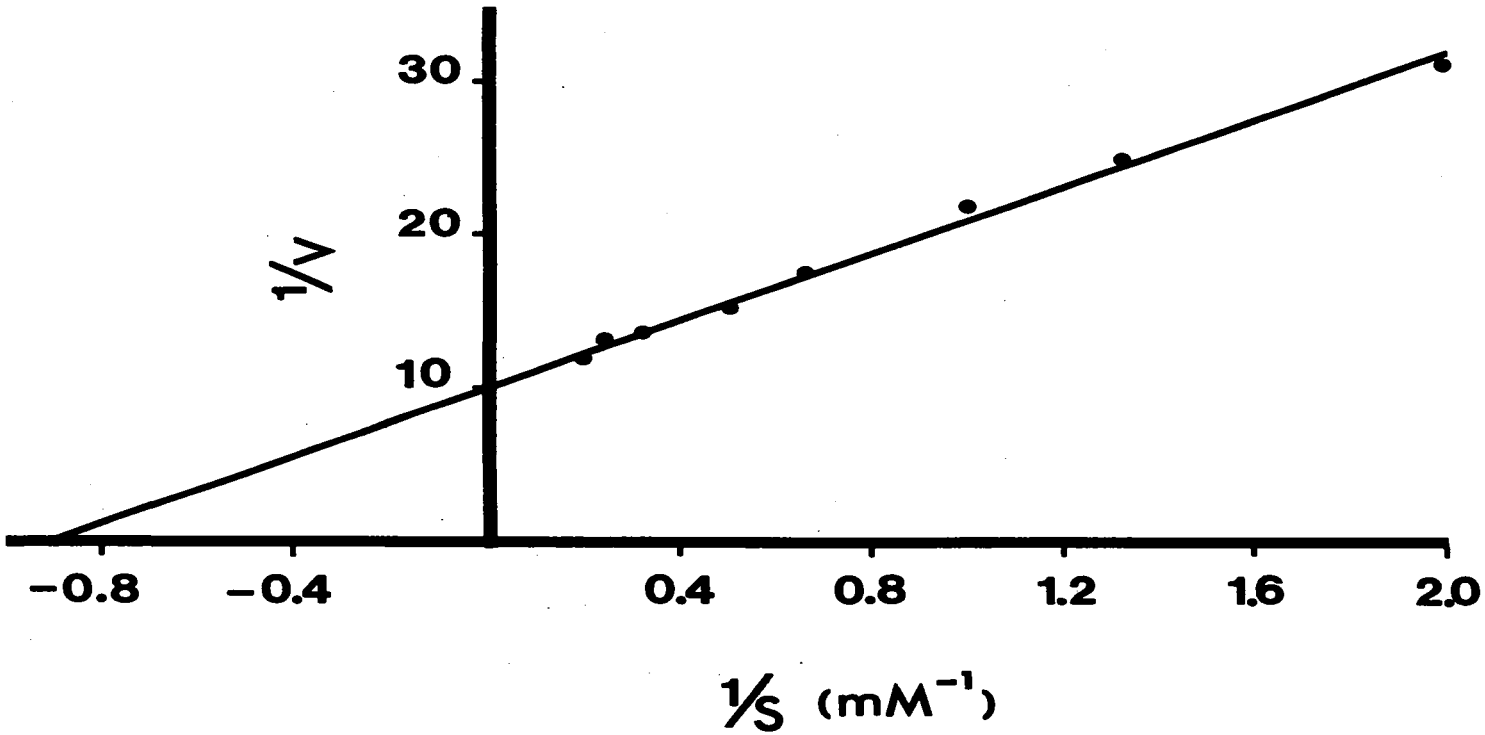

30
20
10
1/v
−0.8
−0.4
0.4
0.8
1.2
1.6
2.0
1/S (mM⁻¹)

FIGURE 30

DETERMINATION OF Km VALUE OF D-α-HYDROXYGLUTARATE USING α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Assay mixtures contained designated quantities of D-α-hydroxyglutarate in addition to 40 μmoles tris-HCl buffer, pH 8.8, 1×10^{-3} M NAD, and 0.32 μg of purified enzyme (43 E. U./mg) in a total volume of 1.0 ml. Initial velocities were measured by the increase in absorbance at 340 nm caused by the formation of reduced NAD at 25°C. The Km value for D-α-hydroxyglutarate was calculated at 1.1×10^{-3} M and the Vmax was 16.13 nmoles NADH formed/minute at 25°C.

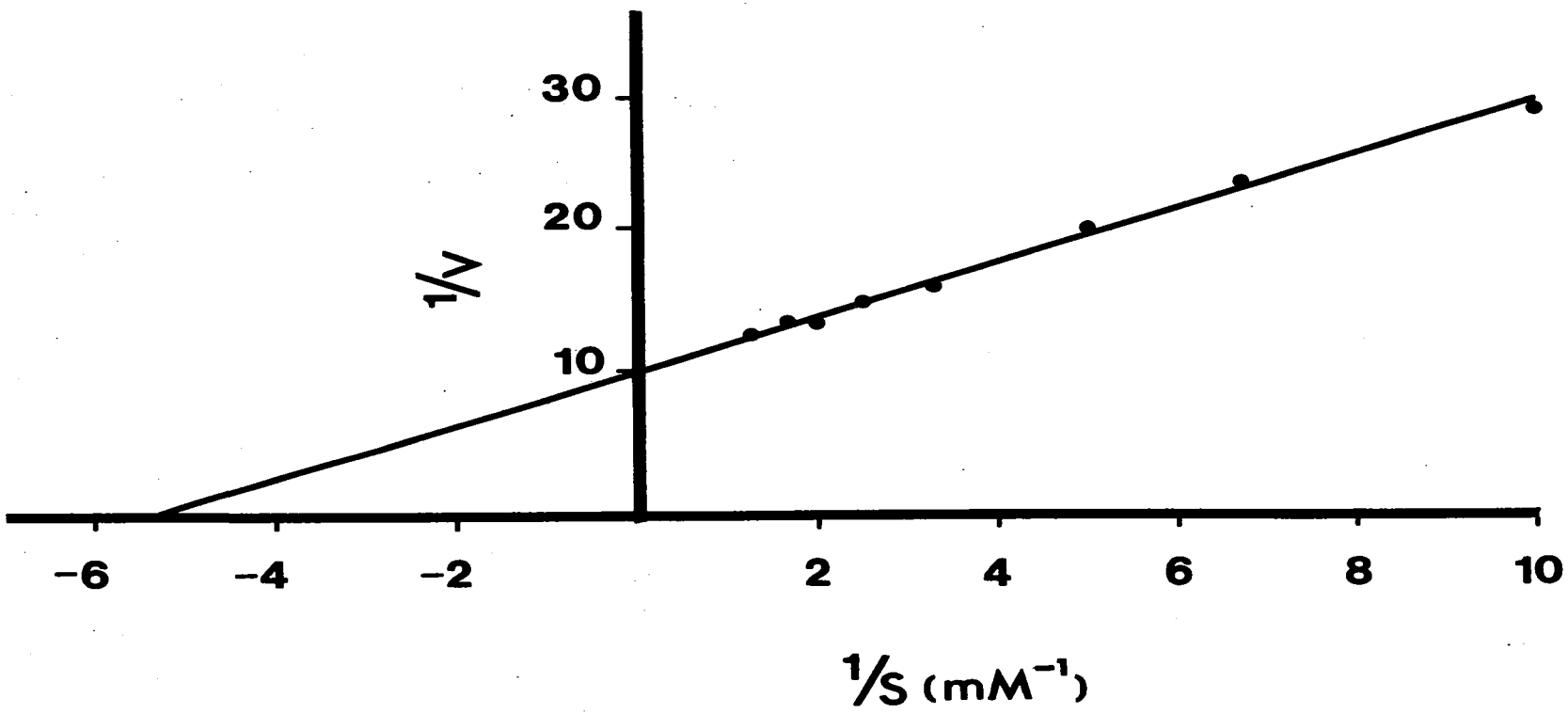

30
20
10
1/v
−6
−4
−2
2
4
6
8
10
1/S (mM⁻¹)

FIGURE 31

DETERMINATION OF Km VALUE FOR NAD USING α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Assay mixtures contained designated quantities of NAD and 5×10^{-3} M D-α-hydroxyglutarate in addition to 40 μmoles of tris-HCl buffer, pH 8.8, and 0.32 μg of purified enzyme in a total volume of 1.0 ml. Initial velocities were determined at several NAD concentrations. The Km was calculated at 1.9×10^{-4} M for NAD and the Vmax was 15.65 nmoles NADH formed/minute at 25°C.

was 15.89 nmoles NADH formed/minute at 25°C.

L-α-hydroxyglutarate was tested as a substrate for the enzyme at concentrations varying from 0.5 to 10 mM. At 10 mM substrate concentration, the initial velocity in the presence of the L-isomer was only 3.4% that observed with 10 mM D-α-hydroxyglutarate. The low level of activity in the presence of the L-isomer suggests that the enzyme is specific for the D-form and that the L-substrate was probably contaminated with a small quantity of the D-isomer.

Experiments were also performed to determine whether L-α-hydroxyglutarate was actually inhibitory to the enzyme. L-α-hydroxyglutarate was added at final concentrations varying from 1.25 to 25 mM to reaction mixtures containing buffer, 2.5×10^{-3} M D-α-hydroxyglutarate, and 1×10^{-3} M NAD. Initial velocities were determined after the addition of purified enzyme. The addition of L-α-hydroxyglutarate produced no change in the specific activity of the enzyme, suggesting that the L-isomer neither stimulates nor inhibits the reaction at the concentrations tested. These results showed that the D-form of α-hydroxyglutarate was the most desirable substrate. However, since no inhibition was produced by the L-isomer, a DL-mixture could be employed for routine assays with appropriate consideration of the fact that only the D-isomer is enzymatically active.

Whereas kinetic data for α-hydroxyglutarate and NAD followed normal Michaelis-Menton kinetics at all substrate concentrations, results for both α-ketoglutarate and NADH suggest inhibition by high concentrations of substrate. Preliminary experiments were performed in all cases to ensure non-inhibitory concentrations for the substrate held constant

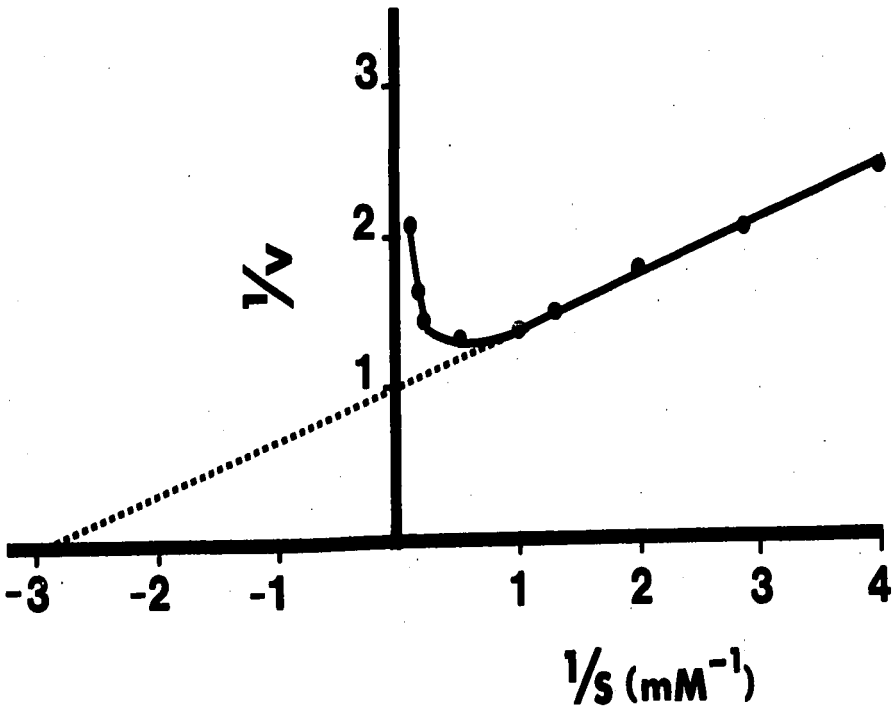
during determinations of apparent Km values. Lineweaver-Burke plots for α-ketoglutarate and NADH are presented in Figure 32. Inhibition was observed when the α-ketoglutarate concentration exceeded 1 to 2 mM or when the NADH concentration was above 0.5 mM. The apparent Km value was 3.6×10^{-4} M for α-ketoglutarate and 9.4×10^{-5} M for NADH. Both Km values were obtained by extension of the linear section of the Lineweaver-Burke plots. In the absence of substrate inhibition, the theoretical average Vmax would be 159.7 nmoles NADH utilized/minute at 25°C. However, the maximum observed velocity was 121.6 nmoles NADH utilized/minute at 25°C.

Since the reaction catalyzed by α-hydroxyglutarate dehydrogenase is a reversible reaction, the equilibrium constant may be calculated from the kinetic data employing the Haldane relationship (Haldane, 1930). The kinetic constants were substituted into the following equation:

$$K = \frac{V_f \, K_m^{\,C} \, K_m^{\,D}}{V_r \, K_m^{\,A} \, K_m^{\,B}}$$

where V_f and V_r are the maximum velocities in the forward and reverse directions, respectively, $K_m^{\,A}$ and $K_m^{\,B}$ are the Michaelis constants of the substrates in the forward reaction and $K_m^{\,C}$ and $K_m^{\,D}$ are the Michaelis constants of the substrates in the reverse reaction.

The reduction of α-ketoglutarate to α-hydroxyglutarate coupled with oxidation of the reduced cofactor was assumed to be the forward reaction. For purposes of calculation of the equilibrium constant, the Vmax in the forward direction was assumed to be 121.6 nmoles NADH utilized per minute at 25°C since this was the maximum observed velocity in the


3
2
1
1/v
-3
-2
-1
1
2
3
4
1/S (mM⁻¹)

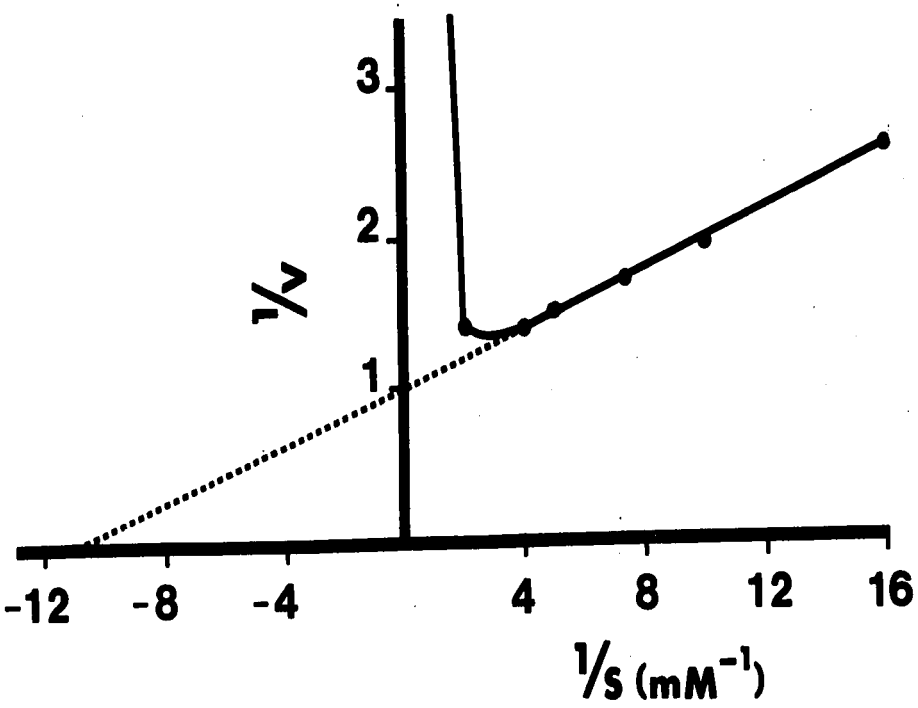

3
2
1
1/v
-12
-8
-4
4
8
12
16
1/S (mM⁻¹)

FIGURE 32

DETERMINATION OF Km VALUES FOR α-KETOGLUTARATE AND NADH USING α-KETOGLUTARATE REDUCTASE FROM P. AEROGENES

Upper Plot: Lineweaver-Burke plot for α-ketoglutarate

Assay mixtures contained designated quantities of α-ketoglutarate in addition to 40 μmoles tris-HCl buffer, pH 8.8, 5×10^{-4} M NADH, and 0.32 μg of purified enzyme (43 E. U./mg) in a total volume of 1.0 ml. Initial velocities were determined at 25°C and the apparent Km calculated by extension of the linear section of the double reciprocal plot. The Km calculated by this procedure for α-ketoglutarate was 3.6×10^{-4} M and the Vmax was 161.3 nmoles NADH utilized/minute at 25°C in the absence of substrate inhibition.

Lower Plot: Lineweaver-Burke plot for NADH

Assay mixtures contained designated quantities of NADH and 1×10^{-3} M α-ketoglutarate in addition to buffer and enzyme as described above. Initial velocities were determined at 25°C, and the apparent Km calculated by extension of the linear section of the double reciprocal plot. The Km calculated by this procedure for NADH was 9.4×10^{-5} M and the Vmax was 158.07 nmoles NADH utilized/minute at 25°C.

absence of substrate inhibition. Hence,

$$K = \frac{121.62 \times 1.1 \times 10^{-3} \times 1.9 \times 10^{-4}}{15.89 \times 3.6 \times 10^{-4} \times 9.4 \times 10^{-5}}$$

and $K = 47.3$.

Since the apparent equilibrium constant is greater than 1, the equilibrium of the reaction lies to the right hand side and the forward reaction is favored. The standard free energy change (ΔG°) was calculated from the equilibrium constant using the relationship of $\Delta G^\circ = -RT\ln K$. At 25°C, the free energy change was -2.28 kcal/mole. These results confirm the validity of the original assumption that the forward reaction is in the direction of α-ketoglutarate reduction since this reaction is favored kinetically. From the kinetic data, it would appear that it is more correct to refer to the enzyme as a reductase than a dehydrogenase since the direction of α-ketoglutarate reduction is favored.

In order to demonstrate inhibition by reaction products, initial velocities were determined in the presence of varying concentrations of the reaction product and increasing concentrations of the related substrate. Details of the assay mixtures are presented in the legends to the appropriate figures where data are expressed as plots of reciprocal initial velocity versus inhibitor concentration.

Product inhibition by α-hydroxyglutarate was found to be competitive with respect to α-ketoglutarate. The dissociation constant for the enzyme-product complex ($\bar{K}_i'$) was calculated from the 1/V vs. i plot as 5.95×10^{-3} M. Similarly, inhibition by NAD was also competitive with respect to NADH and the $\bar{K}_i'$ was 2.25×10^{-3} M. These results are

presented in Figures 33 and 34. The inhibition was termed "competitive" since double reciprocal plots of the data at increasing inhibitor concentrations resulted in an increase in the apparent Km value coupled with no change in the Vmax.

Product inhibition by α-ketoglutarate and NADH was competitive with respect to the related substrate. The results are presented in Figures 35 and 36. The $\bar{K}_i'$ was 3.5×10^{-5} M for α-ketoglutarate and $3.5\text{-}4.0 \times 10^{-6}$ M for NADH.

The relative affinities of the enzyme for substrates and products as reflected by the Km and $\bar{K}_i'$ values for the respective compounds are very important parameters when considered from the aspect of pathway control. The enzyme has a greater affinity for α-ketoglutarate and NADH as substrates, as suggested by their lower Km values in comparison to α-hydroxyglutarate and NAD. In addition, the relatively low $\bar{K}_i'$ values for α-ketoglutarate and NADH indicate that they are very potent inhibitors of the reverse reaction from α-hydroxyglutarate. All of the kinetic data indicate that the enzyme has a greater affinity for α-ketoglutarate and NADH, favoring the results of the equilibrium constant and thus, enzyme catalysis in the direction of α-keto acid reduction. The significance of substrate inhibition and the role of physiological concentrations of reduced cofactor will be considered in the Discussion.

D. General Enzyme Properties

The initial velocity was determined at various temperatures to obtain data for the temperature optimum of the enzyme under conditions of saturating concentrations. Assay conditions are presented in the legend

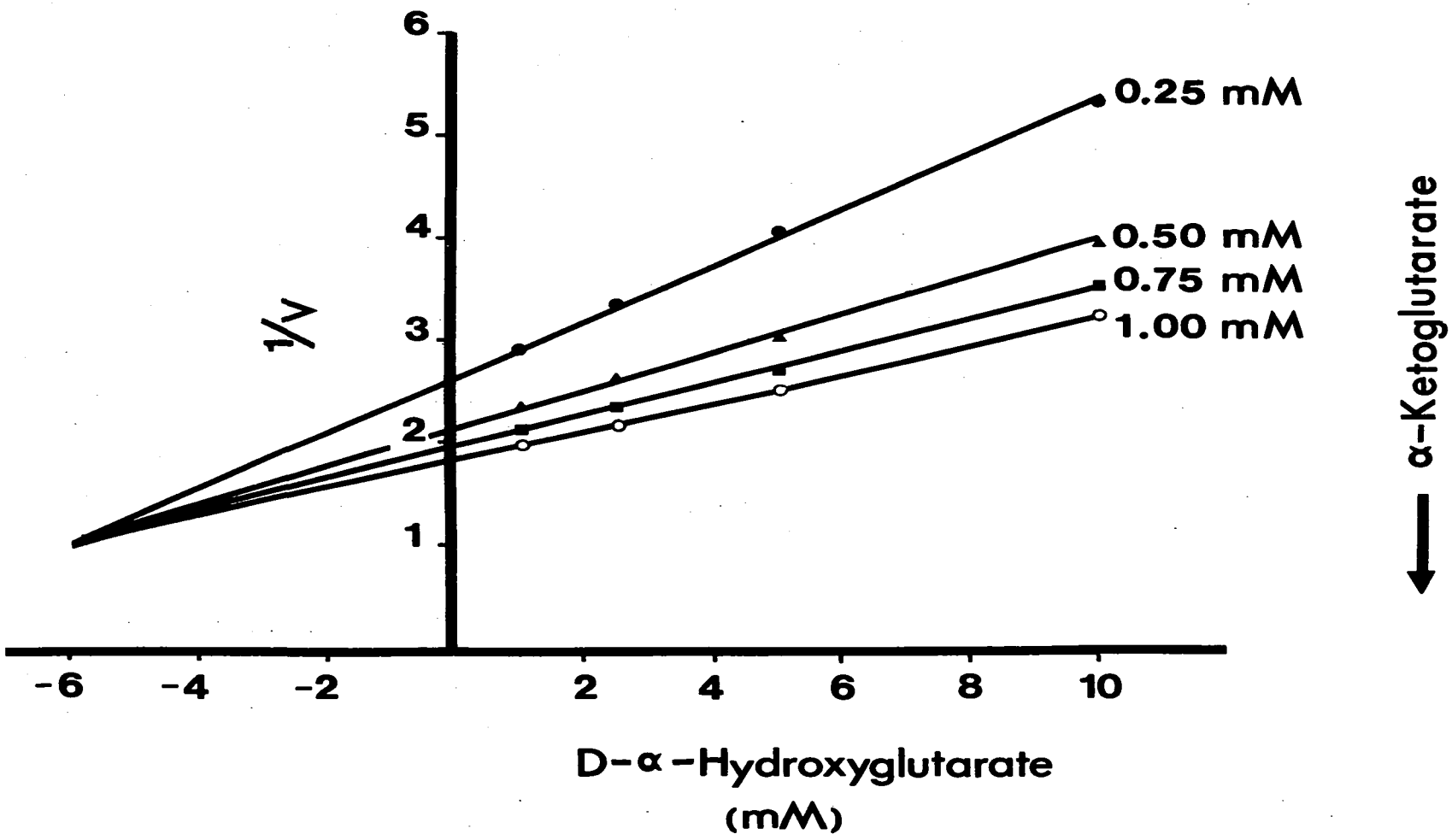

6
5
4
3
2
1
1/v
0.25 mM
0.50 mM
0.75 mM
1.00 mM
α-Ketoglutarate
-6
-4
-2
2
4
6
8
10
D-α-Hydroxyglutarate
(mM)

FIGURE 33

PRODUCT INHIBITION OF α-KETOGLUTARATE REDUCTASE FROM P. AEROGENES BY α-HYDROXYGLUTARATE

Assay mixtures contained 40 μmoles tris-HCl buffer, pH 8.8, 5×10^{-4} M NADH, designated quantities of α-ketoglutarate varying from 2.5×10^{-4} M to 1×10^{-3} M, 0.32 μg purified enzyme, and inhibitor (D-α-hydroxyglutarate) at final concentrations of 1, 2.5, 5, and 10 mM. Initial velocities were determined at 25°C. Data are plotted as $1/v_o$ vs. [i] at increasing concentrations of substrate (α-ketoglutarate). The $\bar{K}i'$ for D-α-hydroxyglutarate was calculated to be 5.95×10^{-3} M.

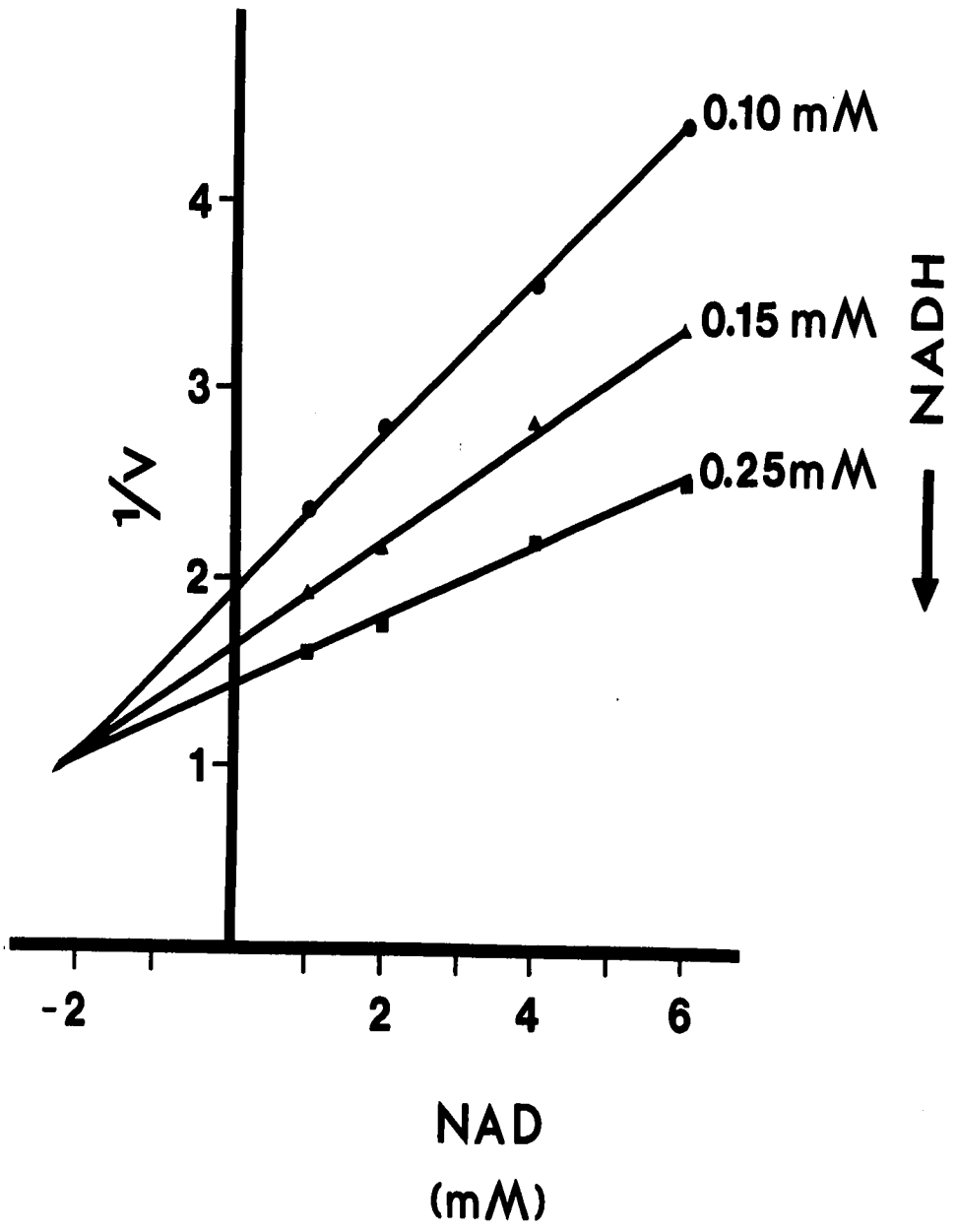

0.10 mM
0.15 mM
0.25mM
NADH
1/v
4
3
2
1
-2
2
4
6
NAD
(mM)

FIGURE 34

PRODUCT INHIBITION OF α-KETOGLUTARATE REDUCTASE FROM P. AEROGENES BY NAD

Assay mixtures contained 40 μmoles of tris-HCl buffer, pH 8.8, 1×10^{-3} M α-ketoglutarate, designated quantities of NADH varying from 1×10^{-4} M to 5×10^{-4} M, 0.32 μg purified enzyme, and inhibitor (NAD) at final concentrations of 1,2, 4, and 6 mM. Initial velocities were determined at 25°C. Data are plotted as $1/v_o$ vs. [i] at increasing concentrations of substrate (NADH). The $\bar{K}_i'$ was calculated to be 2.25×10^{-3} M for NAD.

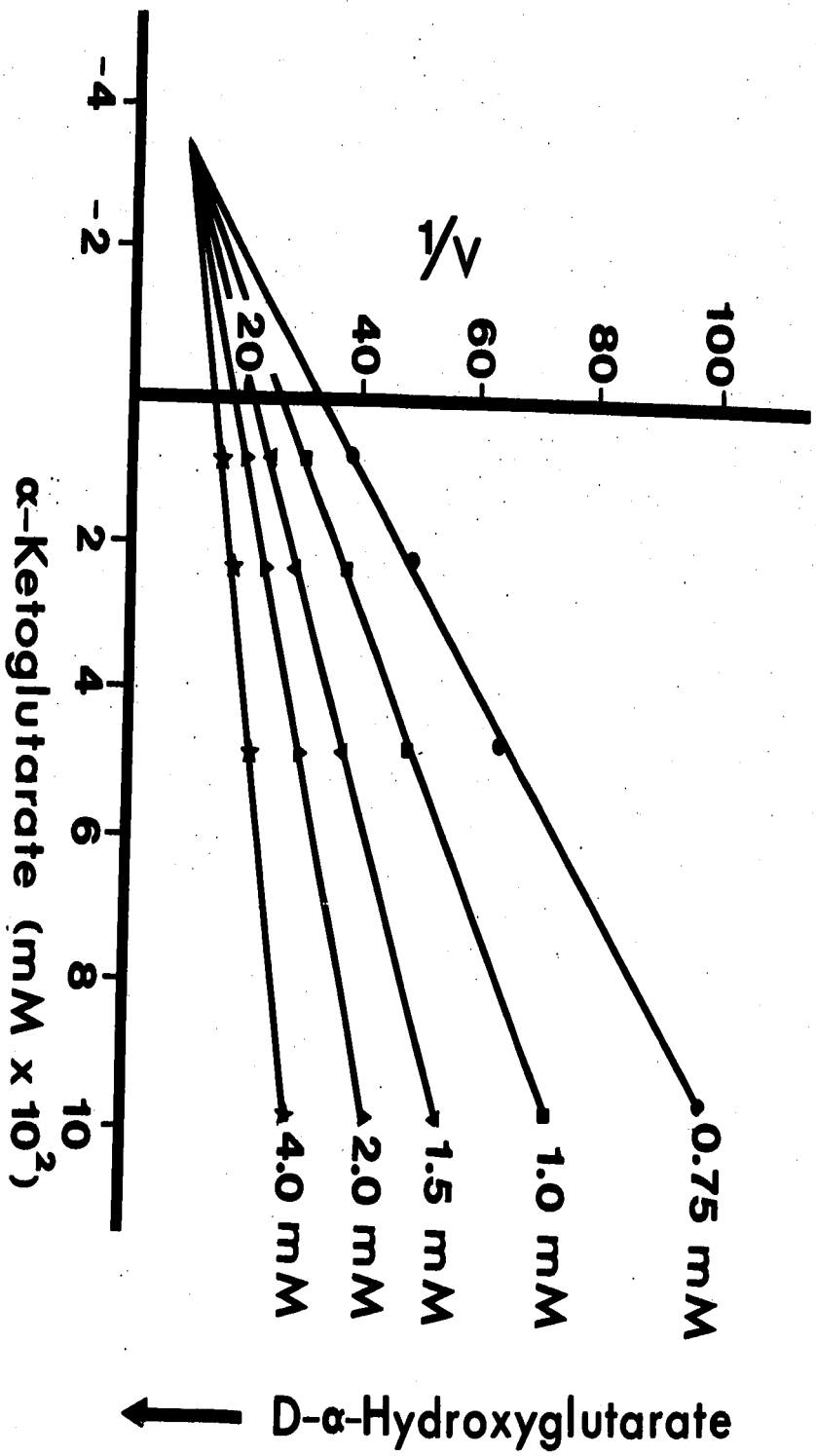

1/v
-4
-2
20
40
60
80
100
2
4
6
8
10
α-Ketoglutarate (mM x 10²)
4.0 mM
2.0 mM
1.5 mM
1.0 mM
0.75 mM
D-α-Hydroxyglutarate

FIGURE 35

PRODUCT INHIBITION OF α-HYDROXYGLUTARATE DEHYDROGENASE OF P. AEROGENES BY α-KETOGLUTARATE

Assay mixtures contained 40 μmoles tris-HCl buffer, pH 8.8, 1×10^{-3} M NAD, designated quantities of D-α-hydroxyglutarate varying from 7.5×10^{-4} M to 4.0×10^{-3} M, 0.32 μg purified enzyme, and inhibitor (α-ketoglutarate) at final concentrations of 0.01, 0.025, 0.05, and 0.1 mM. Initial velocities were determined at 25°C. Data are plotted as $1/v_o$ vs. [i] at increasing concentrations of substrate (α-hydroxyglutarate). The $\bar{K}_i'$ for α-ketoglutarate was calculated to be 3.5×10^{-5} M.

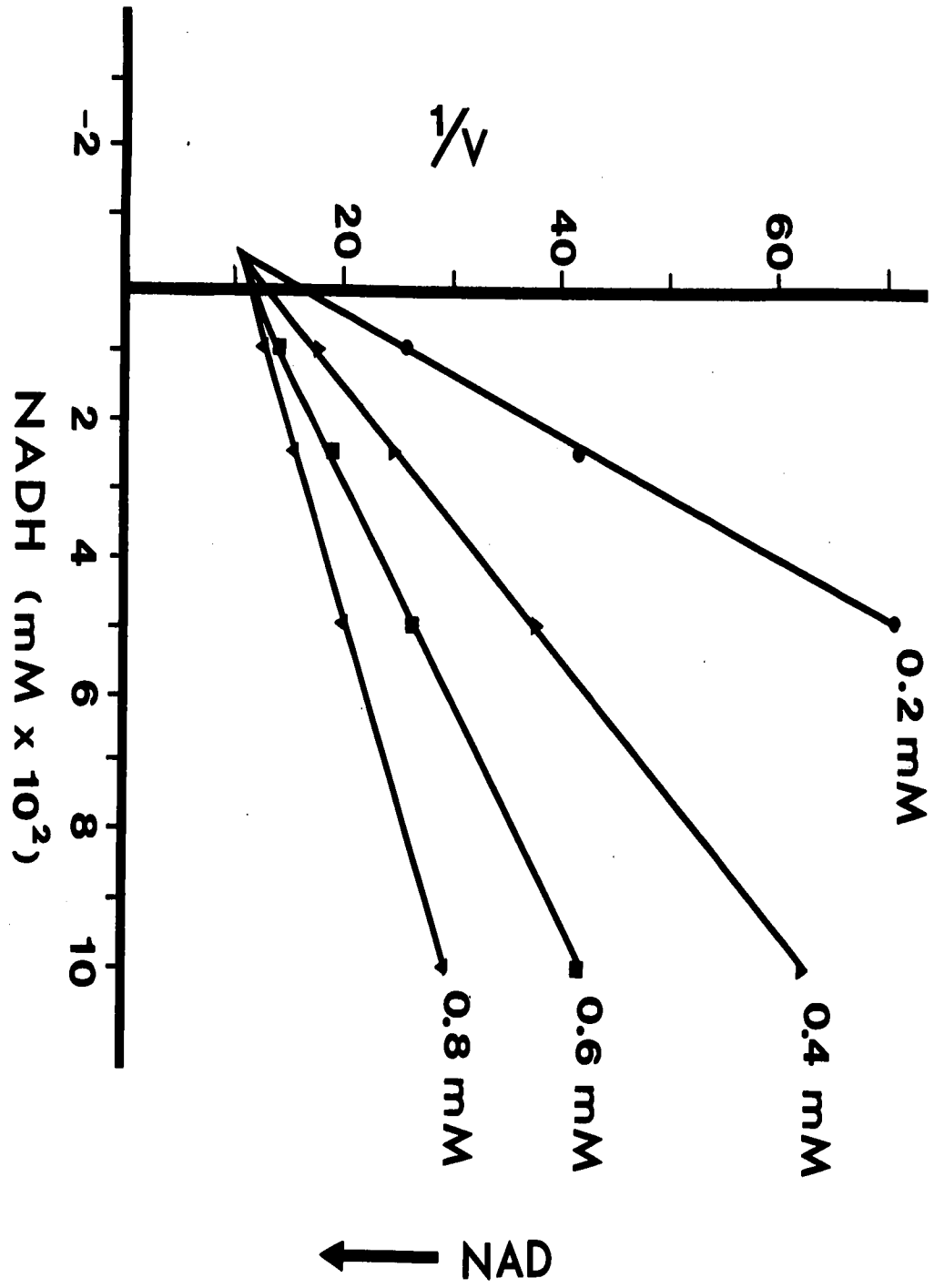

1/V
-2
20
40
60
2
4
6
8
10
NADH (mM x 10²)
0.2 mM
0.4 mM
0.6 mM
0.8 mM
NAD

FIGURE 36

PRODUCT INHIBITION OF α-HYDROXYGLUTARATE DEHYDROGENASE OF P. AEROGENES BY NADH

Assay mixtures contained 40 μmoles tris-HCl buffer, pH 8.8, 5×10^{-3} M D-α-hydroxyglutarate, designated quantities of NAD varying from 0.2 mM to 0.8 mM, purified enzyme (0.32 μg), and inhibitor (NADH) at final concentrations of 0.01, 0.025, 0.05, and 0.10 mM. Initial velocities were determined at 25°C. Data are plotted as $1/v_o$ vs. [i] at increasing concentrations of substrate (NAD). The $\bar{K}_i'$ for NADH was calculated to be $3.5\text{-}4.0 \times 10^{-6}$ M.

to Figure 37. The optimum temperature falls between 46 and 50°C.

Enzyme activity was determined in several buffers at a range of pH values on either side of the pKa for the buffer. Each assay mixture contained 40 μmoles of the designated buffer and pH, 5×10^{-3} M D-α-hydroxyglutarate, 1×10^{-3} M NAD, and 0.44 μg of protein in a total volume of 1.0 ml. Initial velocities were determined at 25°C immediately after the addition of NAD. The results presented in Table XIV were corrected for control levels of NAD reduction and expressed in terms of specific activity. No activity was observed at any pH value in either boric acid-borax buffer (pH 7.55 to 8.95) or borax-NaOH buffer (pH 8.95 to 11.2) suggesting again, the formation of inhibitory borate complexes. The results in Table XIV indicate a high pH optimum in the region of pH 9.0 to 9.6 in either veronal buffer or glycine buffer. A summary of the data shows maximum activity in veronal buffer at pH 9.1 to 9.5 followed by glycine-NaOH and tris-HCl at pH 8.6 to 9.0

The previously described hydrogen ion buffers having pK values between 6 and 8 (Good et al., 1966) were compared to activity in tris-HCl at pH 8.0. All assay mixtures contained 40 μmoles of the designated buffer at pH 8.0, 5×10^{-3} M D-α-hydroxyglutarate, 1×10^{-3} M NAD, and 0.44 μg protein in a total volume of 1.0 ml. Initial velocities were determined at 25°C immediately after the addition of NAD. The results presented in Table XV indicate that α-hydroxyglutarate dehydrogenase was most active in tris-HCl buffer, similar to the data obtained for glutamic dehydrogenase in Table IX. The same trend for relative % activity in the various buffers is expressed by both enzymes.

Purified preparations of α-ketoglutarate reductase at 3-5 mg/ml

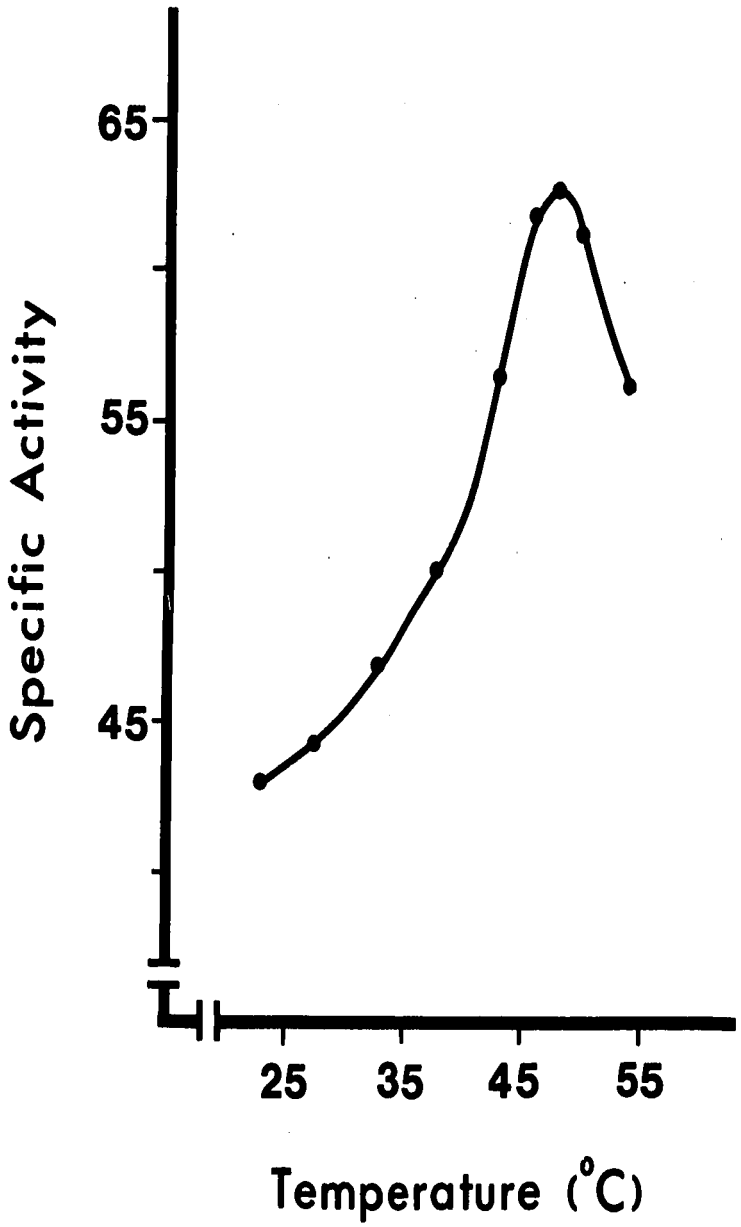

Specific Activity
65
55
45
25
35
45
55
Temperature (°C)

FIGURE 37

TEMPERATURE OPTIMUM CURVE FOR α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Each assay mixture contained 40 μmoles tris-HCl buffer, pH 8.8, 5×10^{-3} M NAD, and 0.70 μg protein in a total volume of 1.0 ml. Enzyme was added last after pre-incubation of substrate and buffer at the required temperature for 5 minutes. Results are expressed in terms of specific activity at varying temperatures. All initial velocities were corrected for NAD reduction in the absence of α-hydroxyglutarate.

TABLE XIV

OPTIMAL CONDITIONS OF BUFFER AND pH FOR α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Buffer	pK_a*	Maximum S. A.**	pH of Maximum S. A.
Potassium phosphate	7.2	29.33	8.1
N-ethylmorpholine	7.67	40.32	8.35
Veronal	7.98	49.85	9.1-9.5
Tris-HCl	8.08	43.26	8.6-9.0
Glycine-NaOH	9.78	49.12	9.6

* pKa values at 25°C as reported by McKenzie, 1969.

** Specific activity was calculated as E. U./mg protein.

TABLE XV

ACTIVITY OF α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES IN SEVERAL HYDROGEN ION BUFFERS AT pH 8.0

Buffer†	pKa*	Specific Activity	Relative % Activity
Tris	8.3	33.73	100
TES	7.5	24.19	72
Tricine	8.15	23.46	69
Bicine	8.35	21.99	65
HEPES	7.55	21.26	63

† Each assay contained 40 μmoles of buffer at pH 8.0.

* pKa values at 20°C as described by Calbiochem.

in 0.05 M tris-HCl buffer, pH 8.8, were stable at 4°C or -20°C for at least two months. More dilute protein preparations slowly lost activity over a period of several weeks at 4°C. This loss of activity could not be prevented by storage in 10 or 20% glycerol or 10 or 20% ethylene glycol. In addition, the enzyme was not sensitive to freezing and thawing since no significant loss of activity was observed after ten successive treatments.

The presence of sodium chloride in the assay mixture was found to produce a slight stimulation (11%) at concentrations of 2.5 to 5.0 x 10^{-2} M although lower concentrations had no detectable effect. Enzyme preparations recovered from NaCl gradients were routinely dialyzed prior to assay.

The thermal stability of α-ketoglutarate reductase was determined from 35 to 65°C. The experimental conditions are described in the legend to Figure 38 where results are expressed in terms of percentage initial activity remaining at various temperatures. The results indicate that α-hydroxyglutarate dehydrogenase is not as resistant to heat as glutamic dehydrogenase since heat treatment for 10 minutes at 60 to 65°C was sufficient to destroy the enzyme whereas temperatures near 80°C were required to inactivate glutamic dehydrogenase.

The kinetics of heat inactivation are presented for temperatures of 50, 55, and 57°C in Figure 39. Linear inactivation kinetics were observed at all temperatures when the data were plotted as $\log_{10}$percent velocity as a function of time.

The results of experiments of thermal stability indicate that the enzyme is not as heat-resistant as glutamic dehydrogenase.

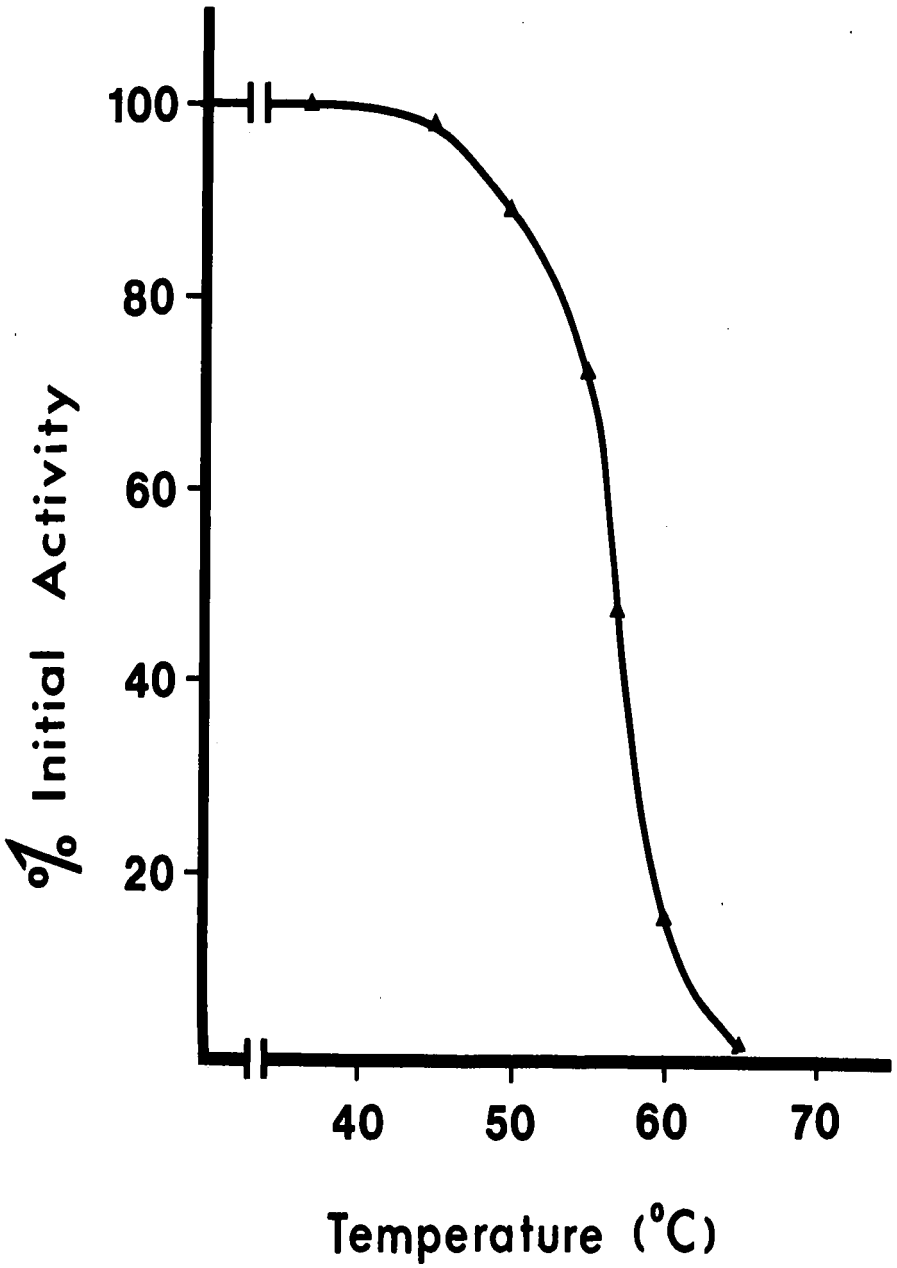

100
80
60
40
20
% Initial Activity
40
50
60
70
Temperature (°C)

FIGURE 38

THERMAL STABILITY CURVE FOR α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Purified enzyme containing 100 μg protein/ml in 0.05 M tris-HCl buffer, pH 8.8, was subjected to 10 minutes of heat treatment at the designated temperature. The activity remaining after heat treatment is expressed as percentage of the initial activity.

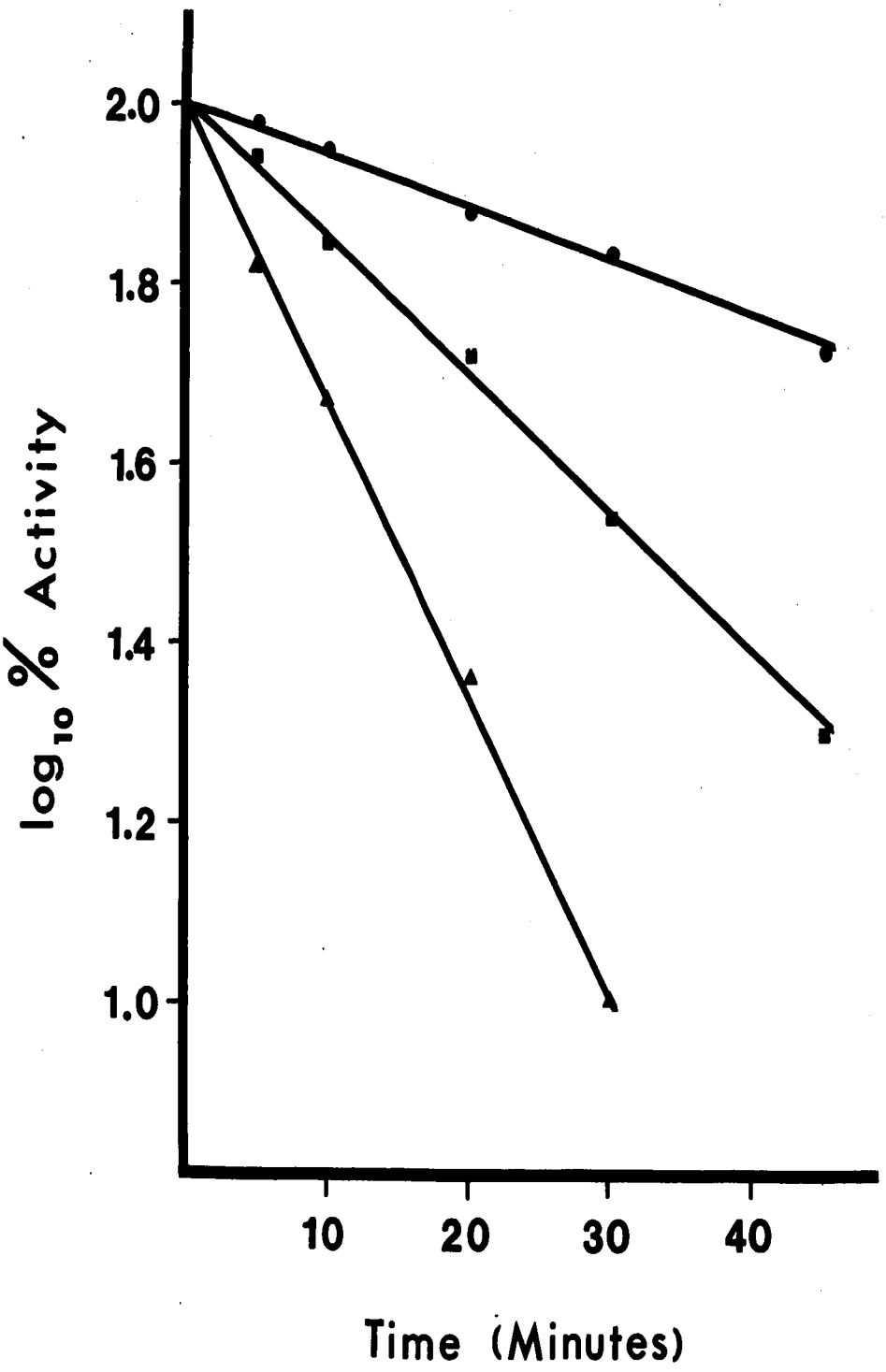

2.0
1.8
1.6
1.4
1.2
1.0
log10 % Activity
10
20
30
40
Time (Minutes)

FIGURE 39

HEAT INACTIVATION CURVE FOR α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

A purified enzyme preparation containing 100 µg/ml protein in 0.05 M tris-HCl buffer, pH 8.8, was subjected to 50,55, and 57°C temperatures for various time intervals. The data are expressed as $\log_{10}$ percentage activity vs. time in minutes.

●——○ 50°C

■——□ 55°C

▲——△ 57°C

This feature could be advantageous in a purification sequence for glutamic dehydrogenase since a carefully controlled heat treatment at 65 to 70°C would destroy contaminating reductase activity while retaining dehydrogenase activity.

Experiments were performed with α-hydroxyglutarate dehydrogenase to determine whether the presence of substrates protected the enzyme from heat inactivation. Enzyme at 100 µg protein/ml was incubated at 55°C for 10, 20, and 30 minute time intervals. The loss of activity in the control samples was identical to that observed in the presence of either 10 mM D-α-hydroxyglutarate or 2 mM NAD. The results indicated that no protection was provided by either substrate at the concentrations tested.

The substrate specificity of the enzyme for analogues of α-hydroxyglutarate was determined using purified α-hydroxyglutarate dehydrogenase. All substrates were tested at a final concentration of 10 mM. Assay mixtures contained 40 µmoles tris-HCl, pH 8.8, 1×10^{-3} M NAD, and 10 µg purified protein in a final volume of 1.0 ml. All substrates were adjusted to pH 9.0 prior to use. The following compounds were tested for activity relative to D-α-hydroxyglutarate: DL-α-hydroxyglutarate, DL-α-hydroxy-n-caproate, DL-α-hydroxybutyrate, DL-β-hydroxybutyrate, γ-hydroxybutyrate, L-malate, DL-malate, DL-glycerate, DL-lactate, glycolate, DL-α-hydroxy-n-hexacosanoate, and fumarate. There was no activity observed with any of the substrates tested other than D- and DL-α-hydroxyglutarate. The activity in the presence of the DL-compound was 51.8% of that observed with the D-isomer. These results suggest that the DL-preparation is a racemic mixture containing approximately

equal proportions of D and L forms since earlier studies showed that the L-isomer produced no inhibition at relatively high concentrations.

The substrate specificity of α-ketoglutarate analogues was determined using purified preparations of α-ketoglutarate reductase. All α-keto acid substrates were tested at a final concentration of 1 mM in the presence of 2.5×10^{-4} M NADH in order to prevent possible substrate inhibition as observed with higher concentrations of α-ketoglutarate and NADH. Assay mixtures contained substrates as described above, 40 μmoles of tris-HCl buffer, pH 8.8, and 10 μg of purified protein in a final volume of 1.0 ml. All substrates were neutralized to pH 9.0 prior to use. The following compounds were tested for activity relative to α-ketoglutarate: α-ketoisocaproate, α-ketoisovalerate, pyruvate, phenylpyruvate, 5-keto-D-gluconate, p-hydroxyphenylpyruvate, and oxaloacetate. None of the compounds tested other than α-ketoglutarate reacted with the enzyme, suggesting a high degree of substrate specificity.

Reduced NADP was tested for activity as a substitute for NADH in the direction of α-ketoglutarate reduction. Assay mixtures contained 1.0×10^{-3} M α-ketoglutarate and 2.5×10^{-4} M reduced cofactor in addition to 40 μmoles tris-HCl, pH 8.8, and 10 μg enzyme. The activity with NADPH was only 2.9% that observed with NADH at the same concentration. These results suggest that the enzyme has a higher degree of specificity for the substrate than for the cofactor.

Results for the activity of α-ketoglutarate reductase in the presence of various ionic additives are presented in Table XVI. At concentrations of 1×10^{-3} M, none of the additives were stimulatory.

TABLE XVI

THE EFFECT OF IONS ON THE ACTIVITY OF α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES*

Additive	Relative Rate (%)
A. No Effect	
NH_4Cl	100
$CaCl_2 \cdot 2H_2O$	100
CsCl	100
LiCl	100
$MgCl_2 \cdot 6H_2O$	100
$MnCl_2 \cdot 4H_2O$	100
K_2HPO_4	100
RbCl	100
Na-arsenate	100
NaCl	100
$Na_2HPO_4 \cdot 7H_2O$	100
B. Inhibitors	
$CdCl_2 \cdot 2.5H_2O$	93
KCl	92
$AlCl_3$	89
$FeCl_3 \cdot 6H_2O$	83
$CoCl_2 \cdot 6H_2O$	80
$SnCl_2 \cdot 7H_2O$	73
$ZnCl_2$	71
$FeSO_4$	55
$FeCl_2 \cdot 4H_2O$	45

* Assays were performed as described in Methods with the addition of the designated salts to a final concentration of 1×10^{-3} M.

Varying degrees of inhibition were observed in the presence of Cd^{++}, K^{+}, Al^{+++}, Fe^{+++}, Co^{++}, Sn^{++}, Zn^{++}, and Fe^{++}. Other ions such as Ca^{++}, Li^{+}, Mg^{++}, and Rb^{+} showed no effect on enzyme activity at 1 mM concentrations. Most of the monovalent cations tested showed no effect whereas divalent cations were inhibitory. No inhibition was observed by the anion in the presence of sodium arsenate.

The presence of chelating or reducing agents had little effect on α-ketoglutarate reductase activity as illustrated in Table XVII. Only 10 mM cysteine produced a slight (3%) increase in specific activity over the control. Inhibition of enzyme activity was observed in the presence of 10 mM EDTA, and 1 and 10 mM dithioerythritol. Otherwise, the enzyme appears relatively insensitive to the protective effects of reducing agents.

The results of inhibition studies are presented in Table XVIII. The most extensive inhibition was produced by various concentrations of organic mercurials including p-chloro- and p-hydroxymercuribenzoate. The metal chelator, 8-hydroxyquinoline, produced no inhibition at the concentrations tested. Only 11% inhibition was observed at 1×10^{-3} M 1,10-phenanthroline indicating that the compound was not a particularly effective inhibitor. The enzyme was only sensitive to a specific alkylating reagent such as N-ethylmaleimide since no inhibition was observed in the presence of iodoacetate at concentrations varying from 1×10^{-4} M to 1×10^{-3} M. No inhibition was observed with 1×10^{-3} M concentrations of malonate, oxalacetate, and arsenite. Comparison of the inhibition pattern for α-ketoglutarate reductase to that for glutamic dehydrogenase in Table XII shows a striking similarity in the

TABLE XVII

THE EFFECT OF CHELATING AND REDUCING AGENTS ON THE ACTIVITY OF α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES*

Additive	Final Concentration	S. A. (E.U./mg)	% Control Activity
None (Control)	-	42.45	100
EDTA	1×10^{-4} M	42.45	100
	1×10^{-3} M	41.68	100
	1×10^{-2} M	34.21	81
2-Mercaptoethanol	1×10^{-4} M	41.89	100
	1×10^{-3} M	40.13	100
	1×10^{-2} M	41.16	100
	2×10^{-2} M	40.89	100
2-Mercaptoethanol + EDTA **		34.97	83
Cysteine	1×10^{-4} M	41.16	100
	1×10^{-3} M	42.18	100
	1×10^{-2} M	43.47	103
Dithioerythritol	1×10^{-4} M	41.16	100
	1×10^{-3} M	39.36	94
	1×10^{-2} M	37.82	90
Reduced glutathione	1×10^{-4} M	41.42	100
	1×10^{-3} M	43.48	100
	1×10^{-2} M	42.96	100
Dithiothreitol	1×10^{-5} M	43.73	100
	1×10^{-4} M	41.68	100
	1×10^{-3} M	41.42	100

* Purified glutamic dehydrogenase was assayed as described in Methods in the presence of the designated additives. All of the chelating and reducing agents were adjusted to pH 8.8-9.0 prior to use.

** The final concentration of each component was 1×10^{-2} M.

TABLE XVIII

THE EFFECT OF INHIBITORS ON α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES*

Additive	Final Concentration	S. A. (E.U./mg)	% Inhibition
None (Control)	-	39.94	-
p-Chloromercuri-benzoate	1×10^{-3} M	14.85	62.8
	5×10^{-4} M	23.62	40.9
	1×10^{-4} M	30.82	22.9
p-Hydroxymercuri-benzoate	1×10^{-2} M	4.54	88.6
	5×10^{-3} M	8.13	79.7
	1×10^{-3} M	17.82	55.4
8-Hydroxyquinoline	1×10^{-3} M	40.51	-
	5×10^{-4} M	40.67	-
	1×10^{-4} M	39.94	-
1,10-phenanthro-line	1×10^{-3} M	35.49	11.2
	5×10^{-4} M	37.23	6.8
	1×10^{-4} M	38.46	3.7
Iodoacetate	1×10^{-3} M	38.18	-
	5×10^{-4} M	39.94	-
	1×10^{-4} M	40.51	-
N-ethylmaleimide	1×10^{-3} M	35.20	11.9
Malonate	1×10^{-3} M	39.10	-
Oxalacetate	1×10^{-3} M	40.67	-
Arsenite	1×10^{-3} M	39.58	-

* Purified enzyme was incubated with buffer in the presence of inhibitor for 60 minutes at 30°C prior to assay by the addition of substrates. The inhibitors were adjusted to pH 9.0 when possible.

behavior of the two enzymes with the exception of inhibition by iodoacetate in the case of glutamic dehydrogenase.

Absorption data for purified α-hydroxyglutarate dehydrogenase was obtained from dry weight measurements and determination of E_{280}. It was calculated that a purified solution of the enzyme at a concentration of 1.0 mg/ml in 0.05 M tris-HCl buffer, pH 8.8, had an absorbance of 1.02 at 280 nm. The ratio of E_{280}:E_{260} was 1.81.

Indications of the presence of flavin prosthetic groups in α-hydroxyglutarate dehydrogenase were obtained by the difference spectrum of the enzyme recorded after reduction with D-α-hydroxyglutarate. The difference spectrum as calculated from the absorption spectra is presented in Figure 40 and details of the reduction procedure are given in the legend. Reduction of flavins with borohydride, dithionite, or enzymatically in the presence of substrate causes bleaching of the light absorption peaks in the region of 370 and 350 nm (Mahler, 1966). The difference spectrum in Figure 40 is characteristic of flavoproteins having maxima at 450 and 380 nm after bleaching by D-α-hydroxyglutarate. These results demonstrate the flavoprotein nature of the enzyme although positive identification of the flavin constituent as FAD or FMN is not possible within the scope of this experimentation.

XI. Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis of crude extracts and purified protein was performed as described in Material and Methods, Section IX. Details of specific staining procedures also appear in Methods. The R_e values were calculated for protein and enzyme bands by considering the distance

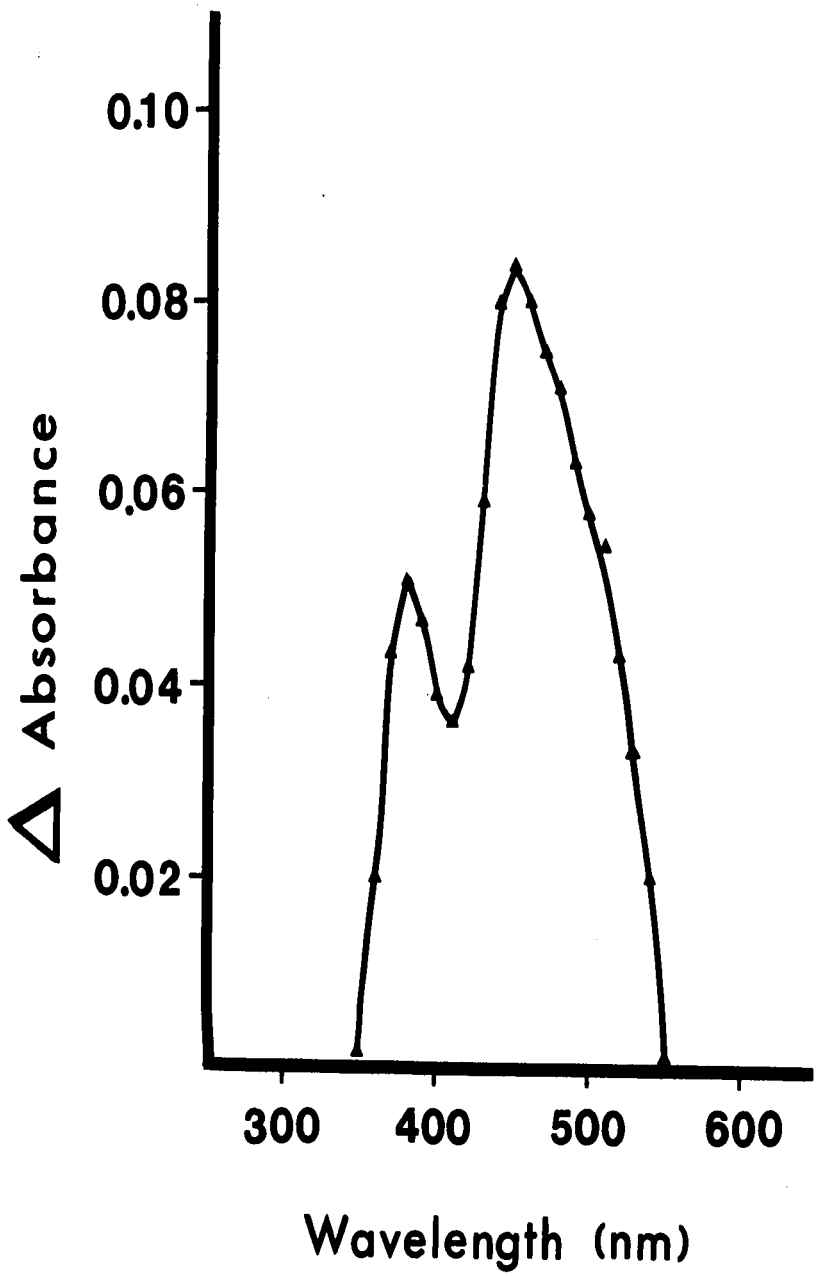

0.10
0.08
0.06
0.04
0.02
Δ Absorbance
300
400
500
600
Wavelength (nm)

FIGURE 40

DIFFERENCE SPECTRUM BETWEEN OXIDIZED AND REDUCED FORMS OF α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Purified enzyme was concentrated by ultrafiltration to 9.3 mg protein/ml in 0.05 M tris-HCl buffer, pH 8.8. Absorption spectra were obtained using a 1 cm light path in a Beckman Model DB-G Spectrophotometer equipped with a scanner and recorder. The enzyme was reduced by the addition of D-α-hydroxyglutarate to a final concentration of 5×10^{-3} M. The spectrum for the reduced form was obtained immediately after the addition of α-hydroxyglutarate. The difference in absorbance between the oxidized and reduced forms was calculated at 10 nm intervals and plotted as a function of wavelength.

migrated into the small pore gel relative to the entire length of the separating gel.

Figure 41 illustrates the disc gel electrophoretic behavior of crude cell extracts and demonstrates the localization techniques for specific enzymes as well as non-specific protein bands. The results indicate that the major band for glutamic dehydrogenase activity (R_e 0.32) corresponds to the most predominant protein in the crude extract. However, the assay for glutamic dehydrogenase activity in situ suggests that there is more than one band of activity in the crude extract. Although the major band of enzyme activity exhibited an R_e of 0.32, a minor band is also seen at an R_e value of 0.28. The α-hydroxyglutarate dehydrogenase activity was located as a single band and corresponded to a protein band in the same relative position.

Figure 42 illustrates the electrophoretic mobility of purified glutamic dehydrogenase. There is one major (0.31) and two minor bands (0.28, 0.123) which stain as protein whereas only one major (0.31) and one minor band (0.28) appear in the enzyme assay. Therefore, the results suggest that the protein of R_e 0.123 is a contaminant and that both of the other bands are active protein. These data correlate well to suggested heterogeneity from sedimentation analysis but a further problem is posed in that more than one band of enzyme is present. The discussion of these results should consider either multiple enzyme forms or non-specific enzyme alteration although the presence of both bands of enzyme in the crude extract tends to eliminate the latter possibility. Purification of the enzyme in the presence of a protease inhibitor would perhaps establish the validity of these hypotheses.

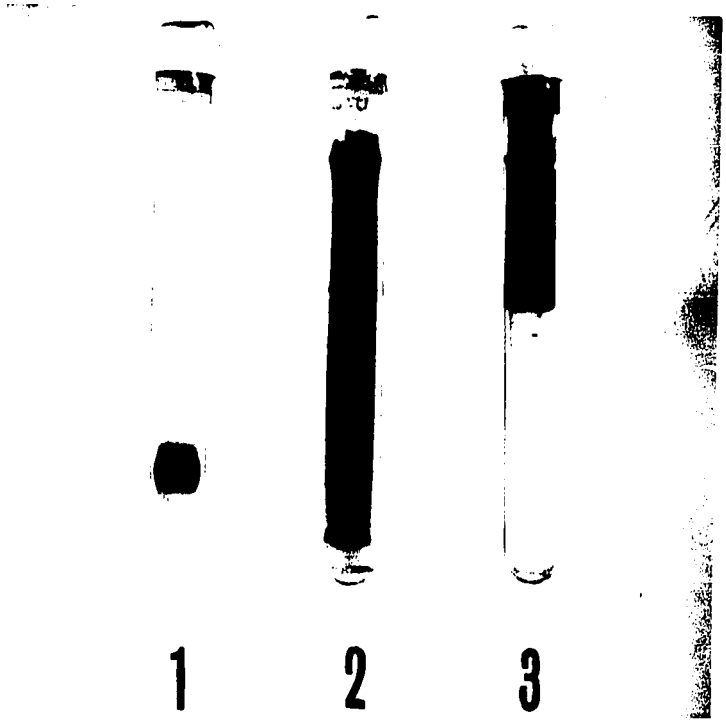

1
2
3

FIGURE 41

POLYACRYLAMIDE GEL ELECTROPHORESIS OF CRUDE CELL EXTRACTS FROM P. AEROGENES

Electrophoresis and staining procedures were performed as described in Materials and Methods. Each tube contained 15 µg of protein. Photographs were taken of gels suspended in 7% acetic acid with the small pore gel to the bottom of the picture.

Tube 1 - Specific assay for α-hydroxyglutarate dehydrogenase

Tube 2 - Non-specific protein stain

Tube 3 - Specific assay for glutamic dehydrogenase

1 2

FIGURE 42

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED GLUTAMIC DEHYDROGENASE FROM P. AEROGENES

Electrophoretic conditions were described in Materials and Methods. Gels for in situ enzyme assays contained 3 µg purified enzyme in contrast to 15 µg for gels stained non-specifically for protein.

Tube 1 - Specific assay for glutamic dehydrogenase activity

Tube 2 - Protein stain

The results for electrophoresis of purified α-hydroxyglutarate dehydrogenase appear in Figure 43. In this case, a single band of enzyme activity corresponds to a single band of protein (R_e = 0.75). The only indication of heterogeneity of this protein preparation arises from sedimentation equilibrium experiments and may perhaps be caused by denaturation during the relatively long period of time required for establishment of equilibrium.

XII. Additional Enzymes Assayed in Crude Extracts of P. aerogenes

Cell extracts were assayed for histidase, L-malic dehydrogenase (NAD-dependent), and α-ketoglutarate dehydrogenase activities in addition to glutamic dehydrogenase and α-ketoglutarate reductase.

Crude extracts of P. aerogenes grown in histidine- or glutamate-supplemented medium were assayed for histidase activity as described in Methods, Section VIII. The specific activity in histidine-induced cells was 3.14×10^3 units/mg protein as compared to 28.6 units/mg for glutamate-induced cells. These results indicate that an active histidase induced by the presence of L-histidine in the medium is present. The base level of histidase activity in glutamate induced cells is most probably caused by the histidine content of the complex medium constituents. Since histidase shows a requirement for glutathione in P. fluorescens (Mehler, 1953), crude extracts were prepared from 15 hr cells as described in Methods except for the presence of 1×10^{-3} M glutathione during all stages of extract preparation. The high pH of the assay (9.2) is sufficient to prevent interference by any urocanase present in the extract.

1 2

FIGURE 43

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED α-HYDROXYGLUTARATE DEHYDROGENASE FROM P. AEROGENES

Electrophoretic conditions were described in Materials and Methods. Gels for in situ enzyme assays contained 20 µg purified enzyme in contrast to 15 to 20 µg for gels stained non-specifically for protein.

Tube 1 - Specific assay for α-hydroxyglutarate dehydrogenase activity

Tube 2 - Protein stain

L-Malate dehydrogenase activity was determined in freshly prepared extracts of P. aerogenes grown on glutamate medium. When assayed as described in Methods, the enzyme had a specific activity of 3.8 units/mg protein in crude extracts. It was questionable whether this activity was, in fact, caused by malate dehydrogenase or was actually a non-specific oxidation of L-malate in the presence of NAD by α-hydroxyglutarate dehydrogenase. However, since no activity towards L-malate could be demonstrated by purified α-hydroxyglutarate dehydrogenase and the dehydrogenase was shown to be specific for the D-isomer of α-hydroxyglutarate, NAD-dependent degradation of malate by crude preparations was attributed to catalysis by malate dehydrogenase.

Activity of the α-ketoglutarate dehydrogenase enzyme complex in crude extracts of P. aerogenes was compared to the same enzyme activity in aerobically grown Escherichia coli. Since α-ketoglutarate reductase also functions as an NADH oxidase, crude extracts could not be assayed for the enzyme complex by the NAD reduction assay described by Reed (Reed, 1969). Therefore, assays were based on the colorimetric determination of ferrocyanide produced by oxidative decarboxylation of α-ketoglutarate with ferricyanide as electron acceptor (Reed, 1966). E. coli B was grown in a salts medium containing glutamate and pyruvate as described by Korkes (Korkes, 1955). Sodium pyruvate was filter-sterilized prior to addition to the medium. E. coli was grown 5 hr at 37°C with vigorous aeration whereas P. aerogenes was grown for 12 hr at 37°C in the glutamate medium as outlined in Methods. Cells were harvested, washed twice with 0.02 M potassium phosphate buffer, pH 7.0, and subjected to sonic disruption for 5 minutes at 4°C in the Biosonik III.

Cell debris was removed by centrifugation at 40,000 x g for 30 minutes. The specific activity of α-ketoglutarate dehydrogenase in crude extracts of E. coli B grown aerobically was 3.2 units/mg protein. Identical assays on crude extracts from P. aerogenes showed a specific activity of only 0.04 units/mg protein. These results suggest that P. aerogenes does not possess a very active α-ketoglutarate dehydrogenase complex, providing further evidence that the majority of the α-ketoglutarate produced by oxidative deamination of glutamate probably is utilized as substrate for α-ketoglutarate reductase.

DISCUSSION

The fermentation of glutamic acid and histidine by Peptococcus (Micrococcus) aerogenes ATCC 14963 (Whiteley, 1957a; Barker, 1961; Horler et al., 1966a; McConnell et al., 1967) was confirmed by radiotracer experiments following the disappearance of substrates specifically labelled with carbon-14. The metabolism of histidine-α-^{14}C by P. aerogenes has been shown to proceed through urocanic acid to glutamate, at which point the degradative sequences for histidine and glutamic acid presumably become equivalent (McConnell et al., 1967). Since the carbon dioxide released during glutamic acid fermentation by P. aerogenes is derived exclusively from carbon-5 of glutamic acid, the fermentation process could be easily followed by the appearance of $^{14}CO_2$ from glutamic acid-5-^{14}C.

The evolution of $^{14}CO_2$ from glutamic acid-5-^{14}C by washed cell suspensions has been shown to be quite slow, reaching a maximum after two hours of incubation. An explanation of such results required consideration of amino acid permease systems which are found to be constitutive in bacteria for most amino acids(Kepes and Cohen, 1962). In particular, the glutamic acid concentrating systems of Staphylococcus aureus (Gale, 1954) and lactobacilli (Holden and Holman, 1959) are characterized by a slow uptake of glutamic acid which reaches a maximum after a minimum time of one hour. In addition, the uptake of glutamate by these organisms has been shown to be essentially irreversible, with no significant exit of glutamate after washing the cells. Therefore, it is suggested that the relatively slow uptake of glutamic acid observed in

other systems may be the factor limiting the rate of $^{14}CO_2$ release from glutamic acid-5-^{14}C by P. aerogenes. Furthermore, the amino acid supplemented during growth was found to influence the kinetics of $^{14}CO_2$ release from glutamic acid-5-^{14}C by washed cells. Consistently, a lag was observed in the production of $^{14}CO_2$ by cells grown on glutamic acid when compared to cells cultured on histidine (Figure 2). It has been suggested that the difficult case of active glutamate transport is mediated by a process which is distinct from the neutral amino acid systems (Heinz et al., 1965).

The observation that sodium is required for the complete and rapid utilization of glutamic acid by P. aerogenes (Westlake et al., 1967) was confirmed in early experiments using cell suspensions (Figure 1). The most probable explanation for the sodium effect involves cationic stimulation of the transport-permease system for glutamic acid. The participation of sodium ion in amino acid and sugar transport has been well documented in higher organisms (Crane, 1965; Stein, 1967; Christensen et al., 1967; Albers, 1967). However, it is becoming increasingly evident that sodium stimulated transport is also very important in bacteria. Recent findings of a sodium transport effect have been reported in A. aerogenes (O'Brien, 1969), a marine Pseudomonad (Wong, 1969), and E. coli (Frank, 1969). One cannot exclude the possibility that the cation provides metabolic stimulation during the degradation of glutamate after transport across the cell membrane. However, the observation that sodium influenced glutamate utilization by intact cells of E. coli B could not be demonstrated in broken cell preparations of the same organism (Kahana, 1969) suggests that the stimulation of glutamate oxidation occurs at the

transport level. The enzymes isolated from P. aerogenes which were involved in glutamate catabolism were not appreciably affected by sodium. These results further substantiate the probability of sodium stimulation during glutamate permeation in preference to sodium effects on the metabolic processes.

Active cell-free extracts of P. aerogenes were prepared which metabolized glutamic acid to α-hydroxyglutaric acid. Factors affecting the formation of this intermediate were studied by following the conversion of glutamic acid labelled with carbon-14 in the carbon-1 or carbon-5 positions. The results of these experiments revealed that the production of α-hydroxyglutaric acid from glutamic acid by cell-free extracts was NAD-dependent.

The formation of α-hydroxyglutaric acid from glutamic acid could involve a lyase reaction followed by hydration across the double bond, hydrolytic deamination, or oxidative deamination followed by reduction of the α-keto acid to the corresponding α-hydroxy acid. The first possible mechanism, desaturative deamination prior to hydration (Stephenson, 1949), would account for the formation of glutaconic acid from glutamate by whole cells of this organism (Horler et al., 1966b). However, the probability of this reaction occurring is unlikely as lyase deaminations do not generally require NAD as a cofactor. A sequence involving "hydrolytic deamination" (Virtanen and Erkama, 1938) would imply direct conversion of the α-amino acid to the α-hydroxy acid. This possibility is also remote since microbial deamination by hydrolysis generally occurs only in the cleavage of amides and imides. Data from the experiments using cell-free systems of P. aerogenes suggested that a sequence of

oxidative deamination followed by enzymatic reduction provided the most plausible mechanism. This hypothesis was substantiated by the observation that specifically labelled α-ketoglutarate was utilized by crude extracts in the presence of reduced NAD. The requirement for NADH in the metabolism of α-ketoglutarate suggested that the reduction of α-ketoglutarate to α-hydroxyglutarate was most likely achieved by reoxidation of the reduced NAD produced during the deamination of glutamate . Moreover, washed cell suspension of P. aerogenes released significant amounts of $^{14}CO_2$ from α-ketoglutarate-5-^{14}C and α-hydroxyglutarate-5-^{14}C, providing further evidence for their assignment as pathway intermediates.

The results suggesting that α-ketoglutarate was an intermediate in glutamate fermentation do not agree with data previously documented for P. aerogenes (Whiteley, 1957b). Whiteley reported that α-ketoglutarate was fermented by cells or extracts of Micrococcus lactilyticus but not by P. aerogenes. Furthermore, succinyl-CoA was found as in intermediate product in the fermentation of α-ketoglutarate by M. lactilyticus, presumably formed by oxidative decarboxylation of α-ketoglutarate. The present data for P. aerogenes indicate that α-ketoglutarate is readily metabolized by extracts and less efficiently by washed cells. In addition, the product of metabolism by crude extracts of P. aerogenes in the presence of NADH has been shown to be α-hydroxyglutarate, suggesting reduction of the α-keto acid rather than decarboxylation as in the case of M. lactilyticus.

The discovery of NAD-dependent glutamic dehydrogenase activity in crude extracts of P. aerogenes during the present investigation provided confirmation of a pathway involving oxidative deamination of

glutamate. This enzyme was subjected to a purification sequence involving ammonium sulphate fractionation, gel filtration, and ion exchange chromatography. However, once the enzyme remained at constant specific activity, only a thirteen fold purification had been effected. These results could indicate that the preparation, although purified to constant specific activity, was not pure, that the enzyme was present in high concentrations, or that the enzyme was extremely active. The results of disc-gel electrophoresis of crude extracts indicate that the band corresponding to glutamic dehydrogenase activity is one of the most abundant proteins in the extract. This alone could account for the low purification. Sedimentation velocity analysis of the purified enzyme gave every indication that the protein was homogeneous since a single symmetrical peak appeared which persisted for most of its passage across the cell. However, both disc gel electrophoresis and equilibrium ultracentrifugation suggested polydispersity of the purified protein preparation. Upward curvature observed in log c versus r^2 plots at varying initial solute concentrations indicated solute heterogeneity rather than non-ideality (Chervenka, 1969). Furthermore, polyacrylamide gel electrophoresis showed by in situ enzyme assay that the purified protein preparation contained more than one band of enzyme activity in addition to a minor protein contaminant. These results tend to eliminate the possibility that polydispersity in equilibrium experiments was caused by denaturation during the relatively long period of time required to attain equilibrium. The presence of more than one band of enzyme in crude as well as purified preparations may suggest multiple enzyme forms or non-specific enzyme alteration by proteolytic modification which, in addition

to producing multiple forms of an enzyme, may decrease enzyme stability. Recent reports have described proteolytic modification in other systems including yeast hexokinase (Schulze and Colowick, 1969), yeast pyruvate decarboxylase (Juni and Heyme, 1968), and Pseudomonas aeruginosa exonuclease (Bryan, personal communication, 1970). Purification of glutamate dehydrogenase from P. aerogenes in the presence of a protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) (Fahrney, 1963) would perhaps establish whether the enzyme was subject to proteolytic alteration.

Whereas there is little evidence for regulation of glutamate biosynthesis in bacteria (Umbarger, 1969), the catabolism of glutamate is usually subject to some form of regulatory control. The effects of purine nucleotides on glutamate dehydrogenase in plants, fungi, and animals have been well reviewed (Frieden, 1965). Most microorganisms such as E. coli possess a single NADP-dependent glutamate dehydrogenase (Halpern, 1960; Liess, 1966). In these microbes, the presence of glutamate in the medium induces aspartase activity which decomposes aspartate, the product of glutamate-aspartate transaminase. However, some organisms, in addition to the NADP-linked glutamate dehydrogenase, possess an NAD-dependent dehydrogenase which is induced by glutamate and generally under allosteric control by AMP and ADP. The presence of two specific glutamate dehydrogenases has been reported in Thiobacillus novellus (LeJohn, 1968), Neurospora crassa (Stachow, 1964), and Saccharomyces cerevisiae (Holzer, 1963). Kaplan supports the concept that reduced NADP generated by the NADP-specific dehydrogenase functions as a source of reducing power for biosynthetic processes (Kaplan et al., 1956; Kaplan, 1963), whereas the reduced NAD is oxidized by electron-transport to generate ATP.

It would appear that P. aerogenes is a member of the group in which glutamate induces an NAD-dependent glutamate dehydrogenase. However, in the purified enzyme preparations of glutamic dehydrogenase from P. aerogenes which contained more than one NAD-dependent dehydrogenase, no NADP-linked activity could be demonstrated. These results suggest that multiple forms of glutamate dehydrogenase observed in purified preparations from P. aerogenes are artifactitious, arising from protein modification or as a consequence of purification as reported with ribonuclease (Shapira, 1960) and glyceraldehyde phosphate dehydrogenase (Boross, 1960).

The results of the present investigation with glutamate dehydrogenase from P. aerogenes have been partially confirmed in a recent publication (Kew and Woolfolk, 1970). Kew and Woolfolk have also observed an unusually high specific activity for NAD-linked glutamate dehydrogenase from this organism coupled with only a twenty-fold purification required to obtain enzyme homogeneity. These data are in agreement with the present results which suggest very high levels of glutamate dehydrogenase in P. aerogenes. The purification procedure employed by Kew and Woolfolk was considerably different from that described in this text and involved streptomycin precipitation and heat treatment followed by sequential ammonium sulphate fractionations. Their purified protein was homogeneous to analytical gel electrophoresis. Although Kew and Woolfolk recorded no precautions to avoid proteolytic modification, they observed a single NAD-linked glutamate dehydrogenase activity in crude and purified preparations, Therefore, it would appear that the degree of purification required to achieve homogeneity was not

misleading and does, in fact, provide an accurate indication of the proportion of glutamate dehydrogenase in P. aerogenes. Personal communication with C. A. Woolfolk, Department of Molecular and Cell Biology, University of California, Irvine, California, indicated that he has investigated the glutamate dehydrogenase from P. aerogenes with respect to nucleotide specificity and overall stoichiometry. To this end it has been found that the equilibrium favors glutamate oxidation and the enzyme will react only with NAD in this direction. However, the nucleotide specificity is very unusual in the reverse direction since either reduced NADP or NAD will function as coenzyme during the formation of glutamate from α-ketoglutarate.

The most interesting enzyme activity observed in crude extracts of P. aerogenes during the present investigation was an NAD-dependent α-hydroxyglutarate dehydrogenase. The product of the reaction catalyzed by this enzyme was identified chromatographically as α-ketoglutarate. Furthermore, the reaction was found to be reversible since the enzyme also possessed the ability to reduce α-ketoglutarate to α-hydroxyglutarate in the presence of NADH. The α-hydroxyglutarate dehydrogenase has been shown to be distinct from malate dehydrogenase activity also present in crude extracts of P. aerogenes. The enzyme, like glutamate dehydrogenase, is induced in cells grown on either glutamate or histidine, thus providing more evidence for the equivalence of catabolic sequences for glutamate and histidine (McConnell et al., 1967) in this organism.

In general, enzymes which reduce α-keto acids or oxidize α-hydroxy acids have not been well documented. A recent publication describes α-hydroxyglutarate oxidoreductase activity in Pseudomonas putida as an

inducible, membrane-bound enzyme of the electron transport particle (Reitz and Rodwell, 1969). The authors theorize that the enzyme functions during L-pipecolate metabolism as a dehydrogenase, oxidizing α-hydroxyglutarate to α-ketoglutarate which in turn would be transaminated to glutamate. However, attempts to demonstrate the formation of α-hydroxyglutarate from any of its immediate precursors such as α-ketoadipate or L-α-aminoadipate have not seen success in the P. putida system. Pyridine nucleotide linked reduction of α-ketoglutarate to α-hydroxyglutarate by rat tissue has been attributed to the activity of an isozyme, lactic dehydrogenase X (Schatz and Segal, 1969). In addition, a relatively non-specific D-α-hydroxy acid dehydrogenase from anaerobically cultured yeast has also been purified and described (Cremona, 1964; Cremona and Singer, 1966). The dehydrogenase had a wide range of specificity since the purified enzyme would oxidize D-lactate, D-α-hydroxybutyrate, and D-malate in addition to DL-glycerate.

Since little information is available concerning α-ketoglutarate reductase activity functioning in a catabolic sequence, it was desirable to determine the equilibrium of the reaction catalyzed by the enzyme from P. aerogenes. When the kinetic studies were interpreted by the Haldane relationship, the results indicated that the forward reaction in the direction of α-ketoglutarate reduction was favored. Therefore, it would appear that the catabolic sequence originating from glutamate does proceed via α-ketoglutarate to α-hydroxyglutarate as previously postulated (Johnson and Westlake, 1969). Since the first two enzymes of the sequence have been shown to be NAD-dependent, the reduced cofactor generated by oxidative deamination of glutamate is the most logical source of coenzyme

for the reductase.

During the preliminary kinetic analysis of the α-ketoglutarate reductase from P. aerogenes, it became evident that the enzyme was subject to substrate inhibition for both α-ketoglutarate and reduced NAD. Whereas normal Michaelis-Menton kinetics were observed at low concentrations of both substrates, the velocity decreased at higher concentrations suggesting that excess substrate results in the formation of abortive substrate-enzyme complexes which resist the binding of further substrate molecules. It would appear that inhibition of the reductase by excess substrate is not the only form of control at this level in P. aerogenes. Not only do the kinetic data suggest that the enzyme has a greater affinity for α-ketoglutarate and NADH than for α-hydroxyglutarate and NAD, but α-ketoglutarate and NADH are potent competitive inhibitors of α-hydroxy acid oxidation. In addition, sufficiently high concentrations of α-hydroxyglutarate or NAD function to inhibit the reduction of α-ketoglutarate, thereby maintaining simple feedback control. These results are all consistent with calculations for the equilibrium of the reaction and favor enzymic catalysis in the direction of α-keto acid reduction.

The purified preparations of glutamate dehydrogenase and α-ketoglutarate reductase from P. aerogenes shared several common properties. Both enzymes showed similar, although not identical pH optima, buffer preference, relative insensitivity to chelating and reducing agents, and response to inhibitors. The dehydrogenase was more resistant to high temperatures than the reductase, suggesting that a heat treatment step could have been included in the purification sequence for glutamate dehydrogenase. The substrate specificity of α-hydroxyglutarate

dehydrogenase was particularly interesting in that the purified enzyme would only oxidize the D-isomer of α-hydroxyglutarate and showed no activity towards malate or lactate, closely related analogues. In addition, no α-keto acid other than α-ketoglutarate was reduced by the enzyme and reduced NAD was the preferred cofactor since only 3% of the total activity was observed in the presence of reduced NADP.

Neither the dehydrogenase or the reductase from P. aerogenes required the presence of a reducing agent for enzymatic activity or a chelator to protect from heavy metal ion inhibition. High concentrations of EDTA or cysteine provided slight stimulation of glutamate dehydrogenase whereas cysteine was the only protective agent which affected reductase activity. Moreover, both enzymes were inhibited by organic mercurials such as p-chloromercuribenzoate and p-hydroxymercuribenzoate in addition to N-ethylmaleimide, an alkylating reagent. The results of these experiments suggest that although sulphydryl groups are not likely involved at the active center of these enzymes, they may play an important role in the tertiary protein structure.

Both the glutamate dehydrogenase and the α-ketoglutarate reductase from P. aerogenes were sufficiently stable to permit purification and ultracentrifugal analysis. As discussed previously, the dehydrogenase was heterogeneous to gel electrophoresis as well as sedimentation equilibrium analysis. Hence, the molecular weight value for glutamate dehydrogenase from P. aerogenes was estimated at 266,000 by calibrated gel filtration on Sephadex G-200. This value compares favorably to the 250,000 molecular weight for glutamate dehydrogenase previously documented (Frieden, 1963). Consideration of the tertiary structure of glutamate

dehydrogenase indicated that the enzyme was generally composed of four chains, contained no disulphide bonds, and possessed four binding sites per molecule (Schachman, 1963) The α-ketoglutarate reductase from P. aerogenes which appeared homogeneous to polyacrylamide electrophoresis, was shown to be polydisperse after sedimentation equilibrium analysis. The heterogeneity may have been caused by denaturation during the fifty-four hours required to achieve equilibrium. Hence, the equilibrium data were not suitable for molecular weight calculations and the molecular size was estimated at 58,000 by calibrated gel filtration. This value is approximately one-half that reported for other α-keto acid reductases. For example, the molecular weight estimated by sucrose gradient methods and by Sephadex filtration for D-α-hydroxy acid dehydrogenase of yeast was 105,000 (Iwatsubo and Curdel, 1963). Similarly, sedimentation equilibrium analysis of mammalian lactic dehydrogenase which catalyzes α-ketoglutarate reduction suggested a value of 125,000 for the molecular weight (Schatz, 1969). There was no evidence reported in either the D-α-hydroxy acid or lactic dehydrogenase systems to suggest that the enzyme was composed of two subunits. However, one must consider that the molecular weights reported here may not be accurate since gel diffusion methods of molecular weight estimation are known to have numerous sources of variation (Ackers, 1964). Factors such as electrostatic interaction of the protein and the gel, hydration of the protein, molecular asymmetry, and gel heterogeneity in addition to experimental inaccuracies may influence the molecular weight and therefore provide inconclusive results.

The flavoprotein nature of the α-ketoglutarate reductase from

P. aerogenes has been demonstrated in the present investigation. This feature is not unusual since results using D-α-hydroxy acid dehydrogenase purified from anaerobically grown yeast also suggest the presence of flavin prosthetic groups in the enzyme (Cremona, 1964). The difference spectrum indicating flavoprotein properties in the yeast system was obtained after reduction of the enzyme with D-lactate. However, the flavin component was not identified as FAD or FMN.

The results of limited cofactor additions to crude extracts of P. aerogenes indicated that in the presence of NAD and FAD, radioactive glutamate was converted to α-hydroxyglutarate and an additional product. The second unknown compound was purified and is probably a dicarboxylic acid since it was formed with equal facility from carbon-1 or carbon-5 labelled precursor. Unfortunately, there was not a sufficiently large amount of the purified compound to permit infrared analysis. However, co-chromatography of the available material indicated that it was not α-keto- or α-hydroxyglutarate, crotonic acid, glutaconic acid, succinic acid, citraconic acid, or mesaconic acid. The unknown compound was hydrolyzed in 0.5 N KOH at 37°C for one hour to hydrolyze thioesters, if present (Numa et al., 1964). The hydrolyzed and original samples gave identical R_f values when chromatographed in several solvents indicating that the compound was not a thioester. Furthermore, the compound was eluted from Dowex-1 prior to α-hydroxyglutarate, providing further evidence that it is not a dicarboxylic acid such as fumarate or α-keto-glutarate (Van Korff, 1969). Previous experiments using washed cell suspensions of P. aerogenes have implicated glutaconic acid as a pathway intermediate of glutamate fermentation (Horler et al., 1966b). However,

during the present investigation using crude extracts, the formation of glutaconic acid has not been demonstrated either by radiotracer techniques from specifically labelled glutamate, α-ketoglutarate, or α-hydroxyglutarate or by chemical estimation. It has been suggested that α-hydroxyglutarate, by its very nature, is sensitive to pyrolytic dehydration (Sonntag, 1961). Hence, the appearance of glutaconate subsequent to steam distillation of volatile acids may be an artifact. The conversion of α-hydroxyglutarate to glutaconate is a very logical sequence but would require the formation of a Coenzyme-A derivative in order to differentiate the carbon-1 and carbon-5 ends of the molecule. The involvement of a Coenzyme-A derivative of glutaconic acid may well explain the failure to demonstrate the formation of the free acid.

It would appear that in biological systems where glutaconate functions as a metabolic intermediate, it is always as the activated derivative. This is demonstrated by glutaric acid metabolism in P. fluorescens (Numa et al., 1964), α-aminoadipate degradation in liver, (Borsook, 1948), and tryptophan catabolism in mammaliam systems (Besrat et al., 1969). A common feature of these pathways is specific decarboxylation of glutaconyl-CoA to form carbon dioxide and crotonyl-CoA. As previously discussed (Horler et al., 1966b), the inclusion of glutaconyl-CoA and crotonyl-CoA on the pathway of glutamate degradation could easily produce the unique labelling pattern observed in the original studies of glutamate fermentation (Horler et al., 1966a). It may be necessary to synthesize the CoA derivative of glutaconic acid in order to demonstrate conclusively whether or not it is a pathway intermediate.

As reported at the 1970 meeting of the American Society for Microbiology, it is unusual to find large quantities of glutamic dehydrogenase in anaerobic systems (Lerud and Whiteley, 1970). Lerud surveyed several anaerobes for glutamate dehydrogenase activity as well as α-ketoglutarate reductase activity. Glutamate dehydrogenase activity was high in P. aerogenes and Acidaminococcus VR-4 with non-detectable amounts in C. tetanomorphum, C. welchii, and low levels in anaerobically grown E. coli. Moreover, α-ketoglutarate reductase activity was also present in anaerobes demonstrating glutamate dehydrogenase activity. Specifically, reductase activity was found in approximately equal amounts in P. aerogenes and Acidaminococcus with none detectable in C. welchii or E. coli. In addition, the specific activity of α-ketoglutarate reductase in crude extracts of P. aerogenes was found to vary with the concentration of glutamate in the medium, reaching a maximum at 0.5% Lerud reported some kinetic data for the α-ketoglutarate reductase from P. aerogenes including a K_m of 10 mM and a K_i of 30 mM for α-hydroxyglutarate. These values are significantly higher than those determined in the present investigation for D-α-hydroxyglutarate (K_m = 1.1 mM; K_i = 5.95 mM). The discrepancies in the kinetics results probably occur because Lerud used a DL-mixture of α-hydroxyglutarate without considering that the enzyme was stereospecific for the D-isomer.

Since α-hydroxyglutarate has been shown to arise from α-ketoglutarate, other possible fates of the keto acid must be considered. Therefore, the activity of α-ketoglutarate dehydrogenase complex was determined in extracts of P. aerogenes and compared to control experiments using E. coli. The results demonstrated negligible activity of

the complex in P. aerogenes, eliminating the possibility that the α-keto-glutarate formed by oxidative deamination of glutamate is decarboxylated to carbon dioxide and succinyl-CoA. Therefore, crude extracts of P. aerogenes appear to deaminate glutamate to α-ketoglutarate and then reduce the keto acid to α-hydroxyglutarate.

The metabolic sequence proposed above is plausible enough but is very difficult to explain in terms of energy metabolism. It is known that glutamate stimulates growth but the mechanism by which this is achieved is terms of energy production may only be hypothesized. In anaerobes, no electron transport phosphorylations have been demonstrated and therefore, anaerobic microorganisms must accomplish ATP formation by substrate level phosphorylations which may be limited to a single type of reaction (Decker et al., 1970). Since there are only a limited number of energy-rich intermediates associated with substrate level phosphorylations, such as acetyl phosphate, carbamyl phosphate, or phosphoenol pyruvate, the growth substrate is generally converted to one of these compounds. During the production of such energy-rich intermediates in anaerobes, the hydrogen balance is maintained by product coupling of intramolecular redox processes, a phenomenon which is not observed in aerobic systems. Trace amounts of hydrogen often evolved during glutamate fermentation may arise from reduced pyridine nucleotides as demonstrated in Clostridium kluyveri (Decker et al., 1970).

During the fermentation of glutamic acid to acetate and butyrate by C. tetanomorphum, a five-carbon unit known as citramalate is cleaved to acetate and pyruvate. This sequence provides a substrate for an ATP-synthesizing reaction since pyruvate is decarboxylated to acetyl-CoA

and converted to acetylphosphate by phosphotransacylation. The hydrolysis of acetylphosphate, an energy-rich intermediate, would supply ATP by substrate level phosphorylation.

In P. aerogenes , however, the catabolism of glutamate to α-hydroxyglutarate would not lead to subsequent ATP production or net gain or loss of reducing equivalents. In addition, radiotracer studies in this organism (Horler et al., 1966a) have shown that butyrate could be formed from the first four carbons of glutamate, presumably without rupture of the chain. Energetically, therefore, it becomes necessary to postulate either a Coenzyme A or enzyme-bound intermediate in the pathway of glutamate fermentation in P. aerogenes. Either of these mechanisms would differentiate the carbon-1 and carbon-5 ends of glutaconic acid and still permit butyrate formation from the first four intact carbons of glutamate. The decarboxylation of glutaconyl-CoA to produce crotonyl-CoA followed by reduction of crotonyl-CoA to butyryl-CoA ($\Delta G_o'$ = -18.6 kcal/reaction)(Decker et al., 1970), would yield sufficient energy for ATP synthesis from both the reduction and subsequent phosphotransacylation to butyryl-phosphate, a high-energy intermediate. However, if glutaconate were merely an enzyme-bound intermediate so as to allow decarboxylation at carbon-5 and the release of crotonic acid, energy for ATP synthesis would still be available since the $\Delta G_o'$ of crotonate reduction is -18.2 kcal/reaction (Decker et al., 1970). An additional ATP would be formed by β-oxidation of crotonate to form two molecules of acetate with a $\Delta G_o'$ of -8.1 kcal/reaction and this reaction would also account for some incorporation of two-carbon units into butyrate as previously observed (Horler et al., 1966a). The

involvement of free saturated carboxylic acids is doubtful since the dehydrogenation of these compounds is so endergonic (e.g., $\Delta G_o'$ of butyrate oxidation is +10.1 kcal/reaction) that ATP synthesis would not be possible. Therefore, a rational explanation of the metabolic sequence in P. aerogenes to α-hydroxyglutarate from glutamate in terms of energy production is not possible until more of the pathway intermediates have been identified.

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