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Title: SYNTHESIS OF GLUTAMIC-γ-SEMIALDEHYDE IN ESCHERICHIA COLI: ISOLATION OF A LOW MOLECULAR WEIGHT COMPOUND REQUIRED FOR IN VITRO SYNTHESIS

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Preliminary work on a crude in vitro system for glutamic-γ-semialdehyde synthesis in Escherichia coli has been described. The cell extract synthesis of GSA is inhibited by proline; other allosteric characteristics observed include sigmoidal substrate variation and inhibition plots of activity. The membrane fraction and a low molecular weight compound in the soluble fraction were components of the cell extract required for in vitro synthesis. Emphasis of this work was on the isolation and characterization of the low molecular weight compound. An isolation procedure is described by which relatively large amounts of this cofactor can be partially purified. Some characteristics of this compound have been determined, and a possible mode of action is described.

Synthesis of Glutamic-γ-Semialdehyde in *Escherichia Coli*: Isolation of a Low Molecular Weight Compound Required for *in vitro* Synthesis

by

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Date thesis is presented November 10, 1967

Typed by Opal Grossnicklaus for Judith Ann Hartz

Dedicated

to my parents

Acknowledgement

I would like to express my appreciation to Dr. Baich for her patience and guidance through the course of this work.

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INTRODUCTION

Proline is synthesized in E. coli by way of the following series of reactions (1, 27, p. 251-252, 38, 39, 43, 46):

(1) L-Glutamic acid → L-Glutamic-γ-semialdehyde (GSA)

(2) GSA → Δ'-Pyrroline-5-carboxylic acid (PCA)

(3) PCA → L-Proline

Reaction 3 is catalyzed by an NADH or NADPH dependent enzyme, PCA reductase (23, 46). Step 2 is a nonenzymatic reaction with the equilibrium favoring the cyclic form (38, 43). Reaction 1 is of primary interest because control of proline synthesis occurs at this point.

Control of proline synthesis is inferred because proline is not overproduced by E. coli. Proline inhibits GSA production in resting cells (3, 33, 36). The rate of GSA production varied in cells that had been grown in limited or excess amounts of proline (36). PCA reductase was not inhibited by proline nor was its synthesis repressed (3). From these observations it was concluded that end-product inhibition is exerted in the first step of proline synthesis and that the early enzyme or enzymes of proline synthesis are repressed by proline. Feedback control by endproduct inhibition

is a relatively rapid means of controlling the action of an enzyme. The affected enzyme is generally the first enzyme of the specific sequence; the inhibitor is the endproduct of that sequence and not necessarily structurally similar to the substrate. Repression is a slower means of control exerted when a product of a sequence of reactions acts in some way to inhibit formation of an enzyme or enzymes in that reaction sequence (8, 31, 37).

GSA production has been characterized in whole cells but previous attempts to observe the synthesis of GSA in extracts of E. coli have been unsuccessful (24, 25, 33). Strecker found that in whole cells GSA production is an aerobic process which requires glutamate or glutamine and is stimulated by pyruvate and possibly AMP and ADP. Cell extracts prepared by grinding with various abrasives, sonication, or treating with organic solvents were inactive. Incubation with glutamate, pyruvate, and adenine nucleotides supplemented with cofactors and boiled extracts of yeast, liver and bacteria in various combinations were also unsuccessful (33, 35).

The cell free synthesis of PCA has been reported for pleuropneumonialike organisms (30). A soluble extract prepared by sonication formed PCA when Mg^{++}, glutamate, and ADP or ATP were supplied. The reaction product was reduced to proline by cell extracts in the presence of NADPH. Inhibitors which interfered with phosphorylation prevented PCA formation. These organisms are

different from E. coli in that they do not deaminate, decarboxylate, or transaminate with glutamate. Glutamic acid or glutamine is required for growth of the organism.

Cell free synthesis of other semialdehydes involved in amino acid metabolism have been described. The semialdehydes are formed by activation of the carboxyl group which is followed by reduction of the activated substrate. The carboxyl group can be activated by phosphate as in aspartic semialdehyde and N-acetylglutamate semialdehyde formation, or by adenylphosphate as in δ-adenylaminoadipate semialdehyde formation. Aspartic acid is converted to β-aspartyl phosphate in the presence of ATP, Mg^{++} and enzyme. The product is reduced by a TPNH dependent enzyme to aspartic semialdehyde (5, 6). Such a method would not be suitable for free glutamic acid reduction since it has been shown that γ-carboxyl derivatives e.g. phosphate anhydrides, thioesters, lactones and peptides, are highly reactive compounds which cyclize readily. Pyrrolidone carboxylate is formed by γ-carboxyl activated glutamate and this compound is not involved in the glutamate-proline pathway (9, 13, 17, 28, 32, 43).

An enzyme bound glutamic acid could be activated by phosphate. A model for the mechanism occurs in glutamine synthesis. Studies with mammalian glutamine synthetase show that Mg^{++} or Mn^{++} and ATP are required for binding glutamate to the enzyme. The ATP

and inorganic phosphate formed remain bound to the enzyme. No intermediates have been found, and partial reactions have not been demonstrated. The enzyme-glutamate complex can react with NH_3 forming glutamine or, if the enzyme is denatured, pyrrolidone carboxylate is formed (18, 19).

To prevent cyclization in a free amino acid, the amino group can be blocked prior to activation. In ornithine synthesis the α-amino N of glutamate is acetylated, N-acetylglutamate is phosphorylated and reduced to give N-acetyl GSA. Again ATP, Mg^{++} and TPNH are required for reactions parallel to those for aspartic semialdehyde formation (4, 20, 40). Reed presents evidence that cell extracts of 55-1 can produce GSA in this manner but in vivo C^{14} incorporation data show that this is not a major pathway, a result which agrees with the earlier work of Vogel (25, 41).

Another possibility for the mechanism of the activation reaction is the formation of an organic phosphate ester as occurs in lysine biosynthesis in yeast. In this system δ-adenylaminoadipic acid is formed in an aminoadipic acid dependent ATP pyrophosphate exchange system. The adenylated intermediate is reduced, then hydrolyzed to form the semialdehyde and AMP.(21, 29). (In E. coli lysine is synthesized via diaminopimelic acid and semialdehyde formation is not involved (12).)

Work in this laboratory has shown in vitro synthesis of GSA

using as an enzyme source strain 55-1, a proline auxotroph of E. coli. Strain 55-1 lacks PCA reductase and as a result PCA accumulates. PCA is detected by formation of a yellow compound with ortho-aminobenzaldehyde in neutral solution.

The purpose of these experiments was to describe the in vitro system and to isolate a low molecular weight compound required for the reaction.

MATERIALS AND METHODS

Materials

Potassium pyruvate, adenosine-5'-triphosphate, adenosine-5'-diphosphate, adenosine monophosphate, thiamine pyrophosphate chloride, L-glutamic acid, L-glutamine, and glyoxylic acid were obtained from Sigma Chemical Co. L-Proline was obtained from General Biochemicals. Mannex DEAE cellulose Type 20 and other amino acids were obtained from Mann Research Laboratories Inc. Whatman Column Chromedia cellulose powder was obtained from W. & R. Balson Ltd. Bio Gel P-2 and Dowex 50-X-8 resin were obtained from Bio-Rad Laboratories.

E. coli strains 55-1 and W-2, proline auxotrophs of strain W, were used. Strain W-2 does not produce GSA and strain 55-1 produces GSA but lacks PCA reductase. These strains are maintained in stock cultures in the lab. 55-1 and W-2 were obtained from Dr. D. F. Bacon of the Institute of Microbiology, Rutgers.

Cells of strain 55-1 were used as the source of enzyme for the cell free synthesis of GSA. Cells were grown in 100 liter batches in a stainless and steel Type 400 Fermenter using a glucose-inorganic salts medium supplemented with 0.1 mg per ml proline (42). Harvested cells were washed three times with 0.1 M potassium phosphate

buffer at pH 7 and stored at -10° C.

The glucose-inorganic salts medium, minimal medium E, 50 times concentrated, is prepared by dissolving 10 g $MgSO_4 \cdot 7H_2O$, 100 g citric acid$\cdot H_2O$, 500 g K_2HPO_4, 175 g $NaNH_4HPO_4 \cdot 4H_2O$, and 2 ml $CHCl_3$ in 670 ml H_2O. After dilution for use the pH is 7.

Methods

The Assay

Preparation of Cell Extract. About 70-75 g wet weight cells were thawed and aliquots of the cells suspended in a small amount of buffer were poured into an Eaton Press which had been kept at -20° C for 30 minutes. The half-filled press was kept in dry ice for five to ten minutes to ensure complete freezing of cells. The apparatus was placed in a Carver Laboratory Press and 10,000 psi were applied until the cells lysed.

The lysed cells were suspended in 2.5 ml buffer per gram original wet weight. The suspension was chilled in ice and sonicated at 6.5 amps for two 60 second intervals using a Branson sonifier. This solution was centrifuged ten minutes at 4000 × g in a Sorvall RC2B refrigerated centrifuge. The precipitate was discarded and the supernatant was centrifuged at 20,000 × g for ten minutes. This precipitate was washed three times with buffer and then was

suspended in 0.75 ml buffer per gram original wet weight cells. This fraction is designated the E or protein fraction. The fraction was shell frozen in 3 ml aliquots in screw capped test tubes and stored at -10° C.

The supernatant was centrifuged at 20,000 × g for two hours and the precipitate discarded. The supernatant is designated wS or whole soluble fraction. 15 ml aliquots of the wS fraction were heated in a boiling water bath for 1.5 minutes and the denatured protein was removed by filtration. The filtrate was called $^{\Delta}$S. Alternatively the wS fraction was dialyzed with agitation against distilled water in a ratio of 1:8 for an average of five hours at 4° C. This fraction was called dialysate. Dialysate concentrated by evaporation was called dS.

The Reaction Mixture. Each sample contained 125 mmoles phosphate, 40 mmoles pyruvate, 2.85 mmoles glutamic acid and 0.16 ml E (110 mg/ml protein) in a total volume of 1.5 ml. The S fraction was added to this mixture. The mixture was incubated at 37° C for 12 hours and the reaction was stopped by addition of 0.1 ml 10% trichloroacetic acid.

Detection of Reaction Product. GSA formation was measured after the method of Albrecht and Vogel (2). 1.0 ml of 3.6 M sodium acetate and 0.5 ml of ortho-aminobenzaldehyde (OAB), 4 mg per ml, were added to each tube. The protein was precipitated by

centrifugation at 20,000 × g for ten minutes. Optical density at 440 mμ was read in the Beckman DB Spectrophotometer using a cuvette with a 1 cm light path.

To correlate absorbency with μmoles GSA formed, a microbiological assay and OAB reaction were run with a standard GSA solution synthesized by the method of Strecker (34). The standard GSA solution was neutralized and diluted 1:10. Aliquots were taken for the OAB reaction. Tubes were brought to constant volume with buffer and sodium acetate and OAB were added. Optical density was determined after 30 minutes.

E. coli strain W-2 was used for the microbiological assay. Growth curves were obtained which compared aliquots of an 8.7×10^{-7} M proline solution with the neutralized GSA solution. Minimal medium E samples (5.0 ml) supplemented with proline or GSA and brought to constant volume with distilled water, were autoclaved in capped 25 ml glass test tubes. When the tubes were cool, 0.1 ml 25% sterile glucose and 0.1 ml of a W-2 suspension were added. The W-2 suspension was made by transferring aseptically a single colony from an overnight culture streaked on a nutrient agar plate and suspending this colony in sterile diluted medium E. The tubes were incubated at 37° for 20 hours. Growth was determined in a Klett-Summerson Photoelectric Colorimeter using the 660 mμ filter.

The concentration of GSA was estimated by comparing growth

in GSA with growth in proline. The μmoles GSA per optical density unit were determined from a plot of absorbency versus ml GSA. Under these conditions, 1 μmole of GSA is equivalent to an absorbency of 1.5 (Figure 1 to 3 and Table 1).

Isolation of the Soluble Factor

Column Chromatography. The heated soluble fraction, ΔS was further purified by passing through carboxy-methyl cellulose, DEAE cellulose, cellulose, Dowex-50-X-8 or Bio-Gel P-2 columns. Conditions are described in the results section. Aliquots of fractions were tested for activity in the GSA assay. Those fractions giving activity were combined and lyophilized. When propanol/water was used as the solvent system for the cellulose column, 10 to 12 fractions were combined, the solvent was removed and the residue was brought up into a small volume of water, aliquots of which were taken for analysis.

The dialysate or dS fraction was passed through Dowex-50 H^+ form which retained the active factor. The activity was eluted with I N HCl and this eluent was evaporated to dryness. The residue was dissolved in a small amount of water and the number of components was determined by thin layer chromatography.

Paper Chromatography. Whatman #3 MM paper (18 × 22 inches) was washed with distilled water and dried in air prior to

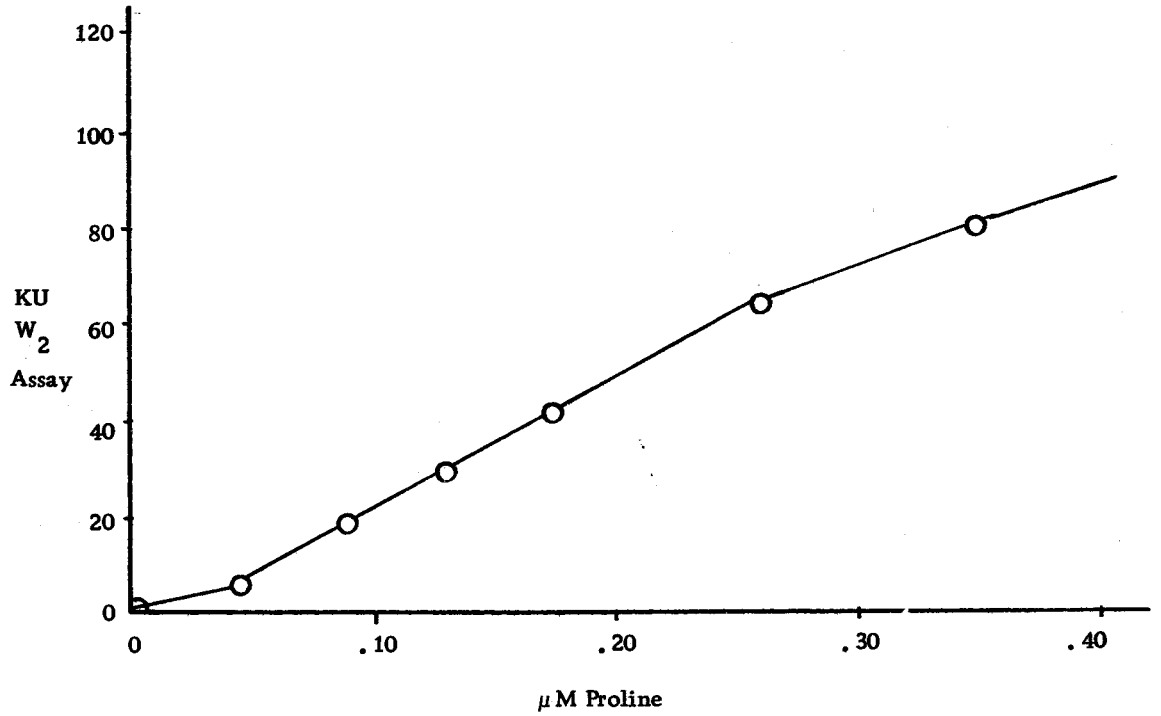

Figure 1. Growth in Klett units versus μ moles proline.

Table 1. Growth in Klett units versus ml GSA solution.

ml GSA solution	W_2 assay Klett units	From Figure 1 Equivalent μM proline
0	0	-
.05	18	.086
.10	29	.126
.15	39	.164
.20	48	.198
.30	63	.252
.40	82	.36
.50	92	.426

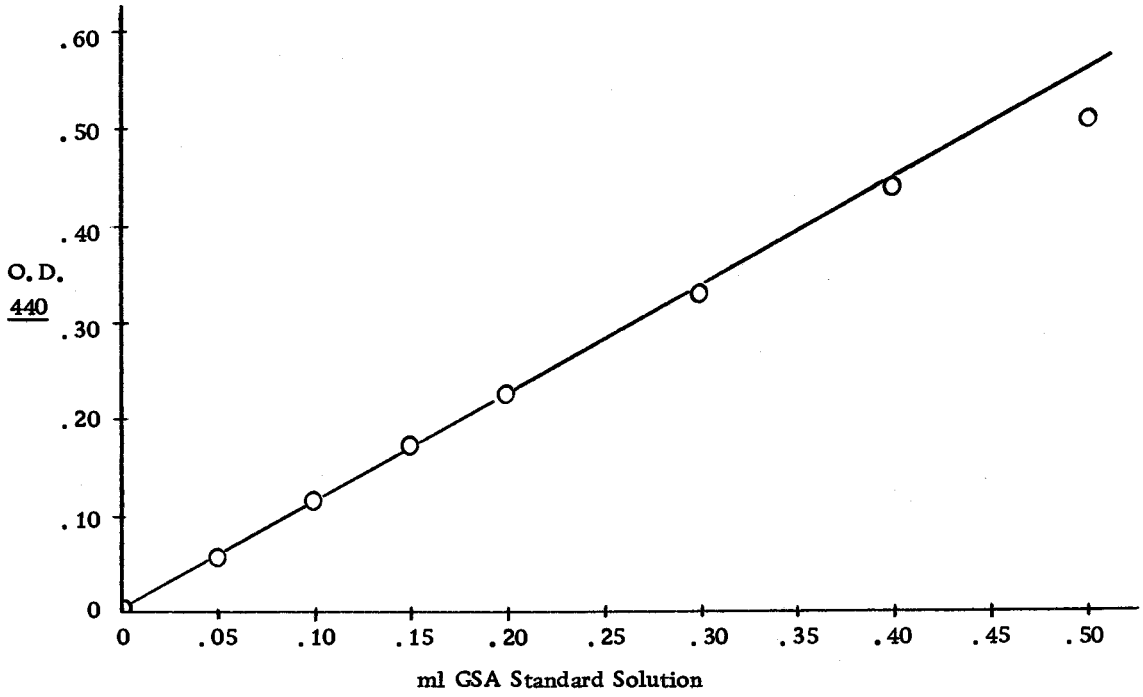

Figure 2. Absorbency at 440 mμ versus ml GSA solution.

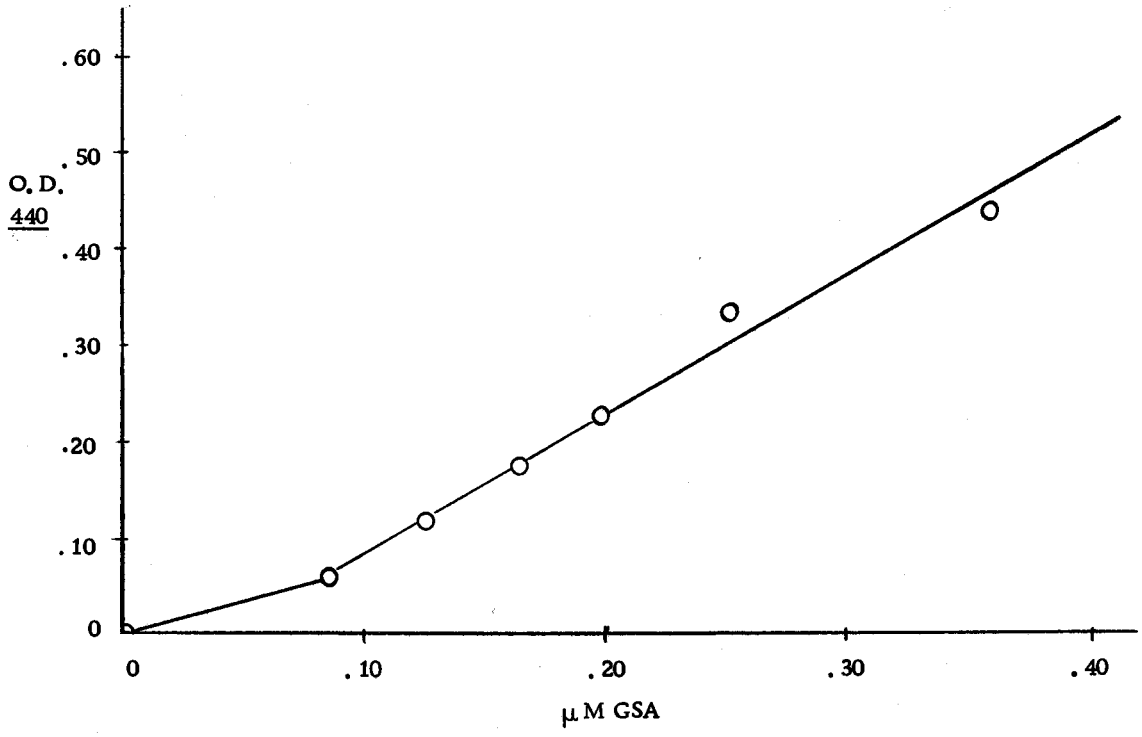

Figure 3. Absorbency at 440 mμ versus μM GSA.

use. Sheets were cut to four widths, 17 × 19.5 or 17.5 or 15.5 or 11.5 inches. The solution was applied in a line 3/4 inch above the bottom edge and extending to 1/2 inch of each side. A cylinder was made by sewing the side edges together with thread. Four cylinders could be placed in one chromatography jar. Elution by ascending chromatography proceeded for 24 hours in sealed jars. The chromatograms were dried in air and bands were detected with a short wave UV lamp. The bands were cut from the paper and eluted with distilled water by descending chromatography. These solutions were lyophilized and brought to the volume of solution originally applied to the paper.

Separation by Solubility Properties. One hundred ml dialysate was evaporated to about 5 ml on a hot plate and 100 ml of 100% ethanol was added. The mixture was centrifuged and the supernatant was evaporated to dryness and then washed with chloroform. The chloroform insoluble residue was brought up in a small volume of water. This fraction is referred to as the partially purified dialysate (PPD).

Preparative Thin Layer Chromatography. Kontes Chromaflex plates ground to depths of 500 or 1000 microns were used. Silica Gel H obtained from Brinkman Instruments, Inc. was washed with concentrated HCl and then rinsed with water to neutrality before spreading the plates. Alternatively, the plates were spread with

unwashed silica gel, developed in methanol - HCl (9:1) and dried prior to applying the sample (23, p. 30, p. 228). The PPD was applied in a line 2 cm above the bottom edge of the plate. The plates were developed for 12 to 15 cm. Bands were detected with UV light and scraped into centrifuge tubes where the gel was washed with 1 N HCl. A preliminary centrifugation with a desk top centrifuge removed most of the silica gel. The supernatant was centrifuged at 23,000 × g for 20 minutes. This supernatant was evaporated to dryness on a hot plate and the residue brought up in a small volume of water for assay.

Test for Homogeneity. A saturated solution of the active band from the preparative thin layer chromatograms was analyzed using the Millipore electrophoresis apparatus. The sample, about 0.6 μl, was developed in formic-acetic acid buffer, pH 2, for six to 16 minutes. After the slide dried it was sprayed with ninhydrin to detect the amino acids.

Characterization of the Active Material

Solubility of an active fraction was determined by drying then washing the residue with various solvents. The solutions were filtered and evaporated. The residue was dissolved with water or a portion of the solid material was put in an assay tube.

Heat stability was tested by placing the dialysate or wS in a

boiling water bath for varied time periods and then checking these fractions for activity in the assay. The dialysate was autoclaved before storing to prevent bacterial contamination with no loss of activity.

Reducing activity of aldehyde and sulfhydryl groups was tested with the triphenyl tetrazolium chloride in basic solution (7, p. 202-203).

Nitrogen was determined by the method of Lang (15). Phosphate was determined by the method of Fiske and SubbaRow.(16). Quantitative ninhydrin was determined by the method of Yemm and Cocking (45).

UV spectra were obtained using a Cary Model 11 Recording Spectrophotometer.

Melting temperature was determined with a Büchi Schmelzpunktbestimmungsapparat.

Amino acid analysis was performed by R. Howard of Dr. R. R. Becker's laboratory on a Beckman Model 120 B Amino Acid Analyzer.

An emission spectrum was run by T. Tiffany of this department on a Bausch and Lomb 1.5 Meter grating type Spectrograph.

RESULTS AND DISCUSSION

The purpose of these experiments was the isolation and characterization of a low molecular weight compound required for synthesis of GSA in the cell extract system. During the course of isolation some difficulties were encountered in obtaining consistent activity in the assay. It was thought necessary to look at some aspects of the assay at various purification steps and see how the system was affected. It was also hoped that more details of the nature of the cofactor could be obtained by such analysis.

The components of the reaction mixture were varied in relation to each other, various inhibitors and activators of whole cell assays were added to the cell extract system, and some conditions of the assay were determined. These results are described in the following section. Results of isolation procedures and some characteristics of the compound are given in the subsequent sections.

The standard reaction mixture contained 125 mmoles phosphate, 40 mmoles pyruvate, 2.8 mmoles glutamate, enzyme and cofactor in 2.0 ml total volume.

Variation of Enzyme

A hyperbolic curve is obtained when E is varied in the system containing partially purified dialysate (Figure 4). A Lineweaver-Burk

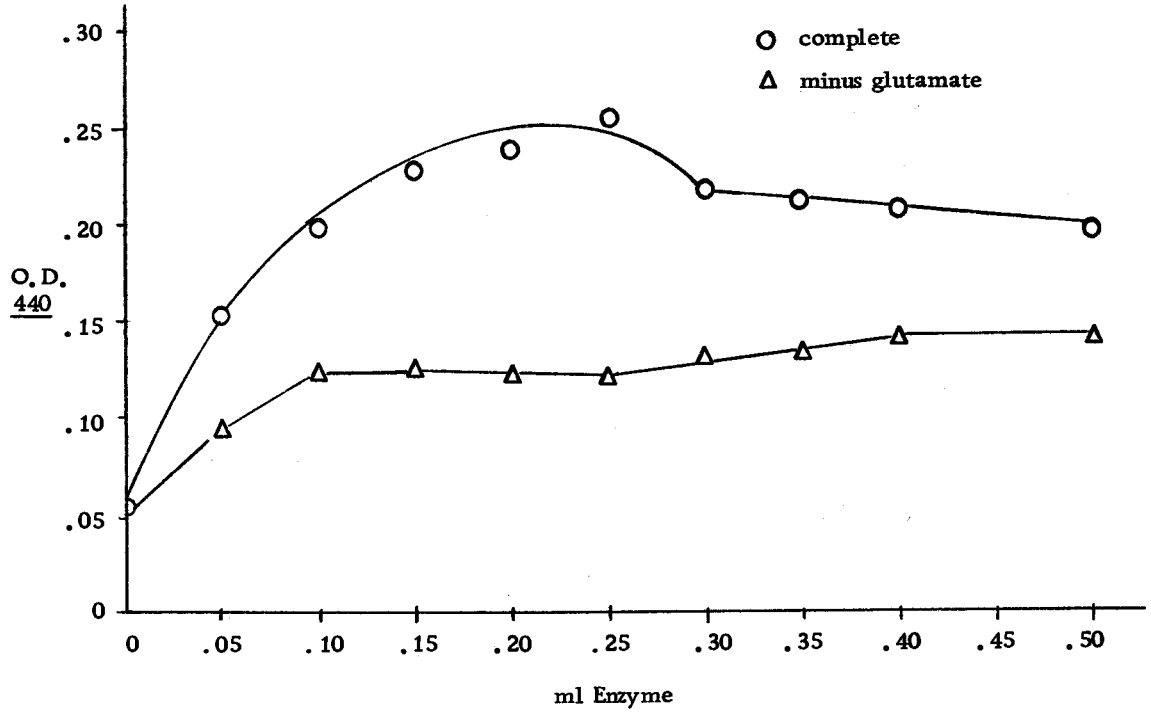

Figure 4. E titration of PPD system.

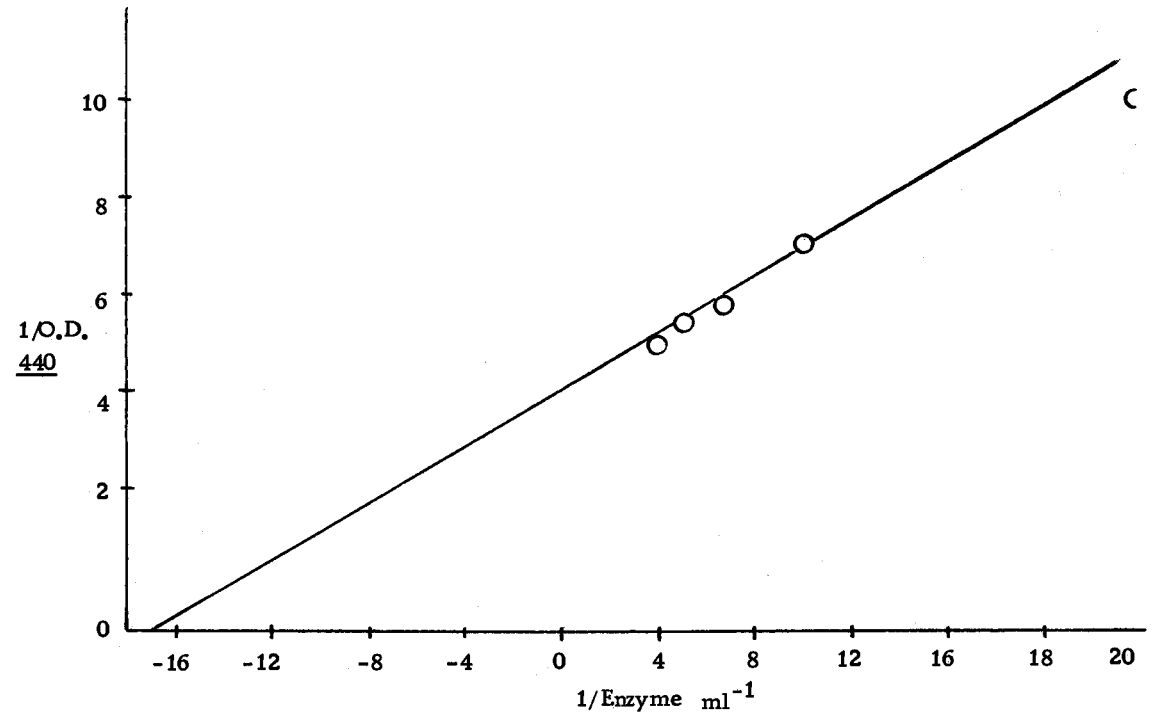

Figure 5. Lineweaver-Burk plot of data of Figure 4.

plot of the data is linear (Figure 5). These plots support the idea of enzyme-substrate complex formation.

Variation of Substrate

Sigmoidal curves, a characteristic of allosteric proteins, were obtained when glutamic acid was varied. Lineweaver-Burk plots of data were hyperbolic and substrate-activity plots such as Figures 8 and 9 were used to obtain the K_m and V_{max} values (Figures 6-9). Glutamine was found to be as good a substrate as glutamic acid and the same pattern of activity was obtained (Table 2). In a crude system where the assay is not a kinetic study the significance of the K_m is not clear, but the K_m can be used as a qualitative means of characterization of the assay.

Effect of Cofactor Variations

1) Under these assay conditions the whole soluble fraction (wS) promotes GSA formation over a narrow range of concentration, i.e. from 0.04 to 0.10 ml or about 8 to 20 mg protein. Maximum activity was obtained with 0.07 ml or about 14 mg protein (Figure 10).

When wS was heated and the coagulated protein removed, the supernatant gave 20% of the activity of wS (Figure 11). When ΔS, the filtrate of heated wS, was varied there was less inhibition at higher concentrations than was observed at high wS concentrations

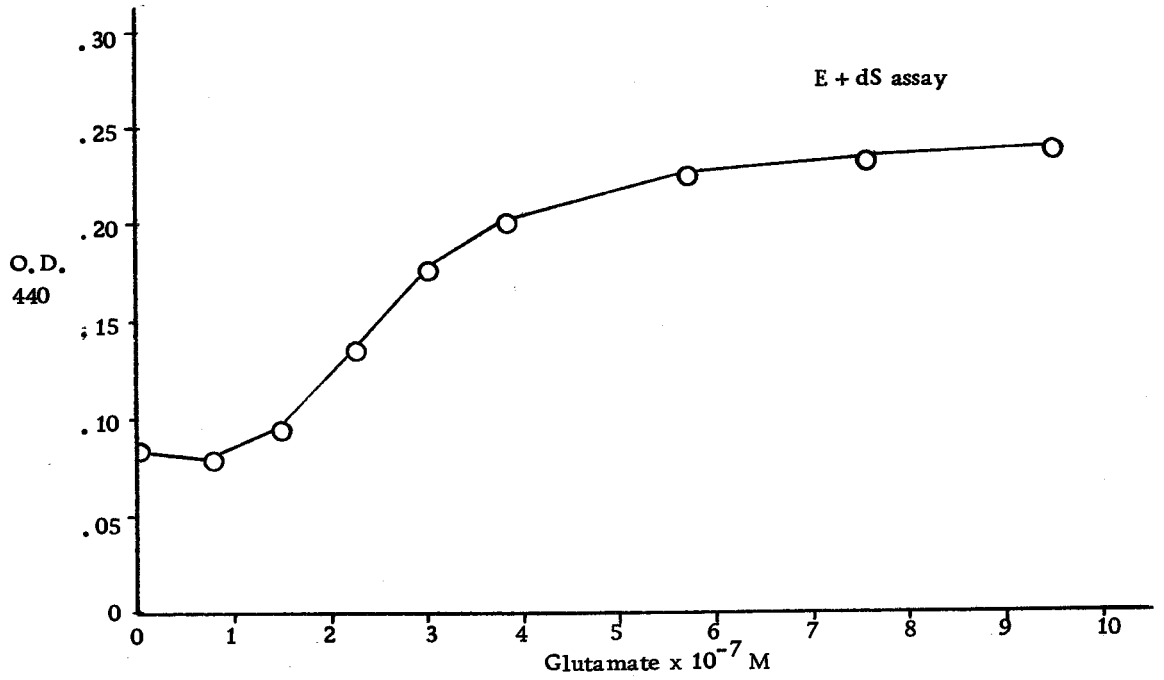

Figure 6. The effect of glutamate variation.

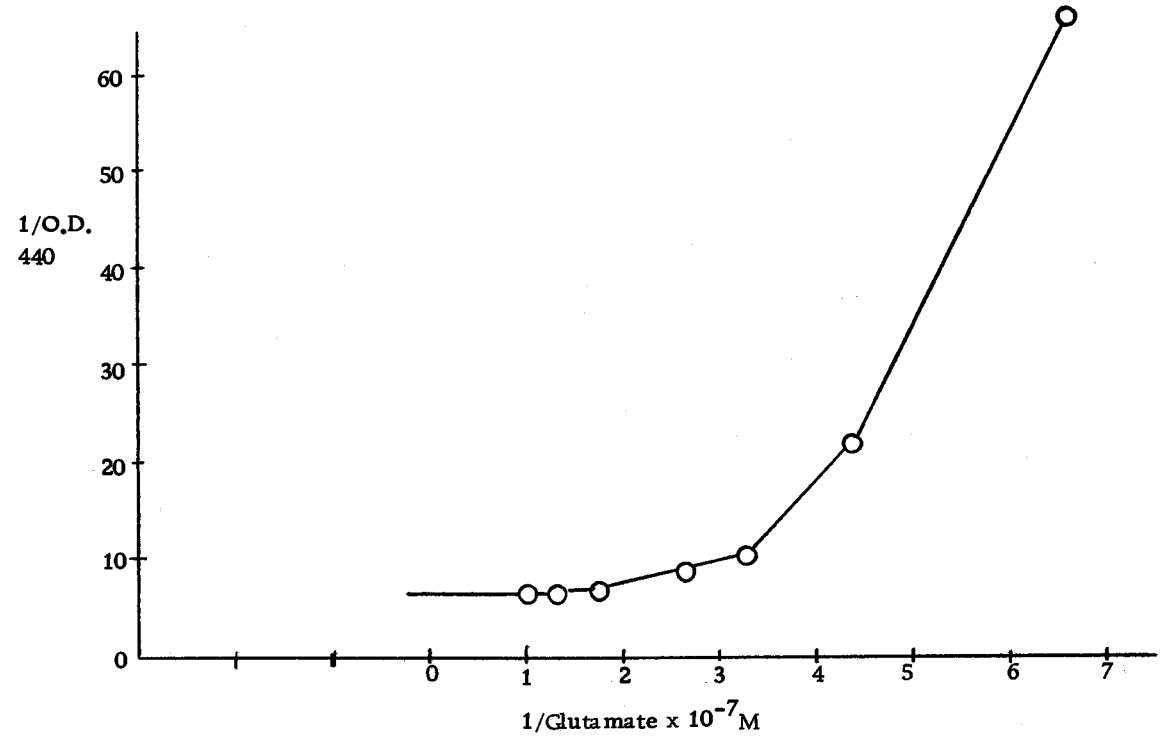

Figure 7. Lineweaver-Burk plot of data of Figure 6.

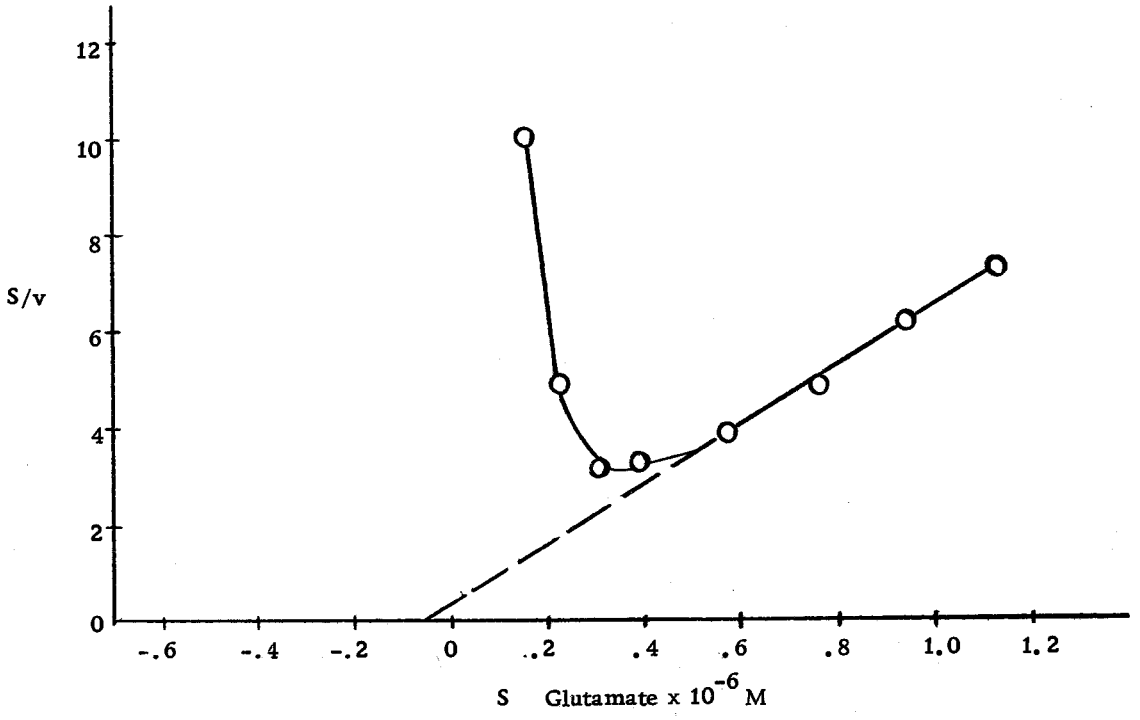

Figure 8. Data from Figure 6.

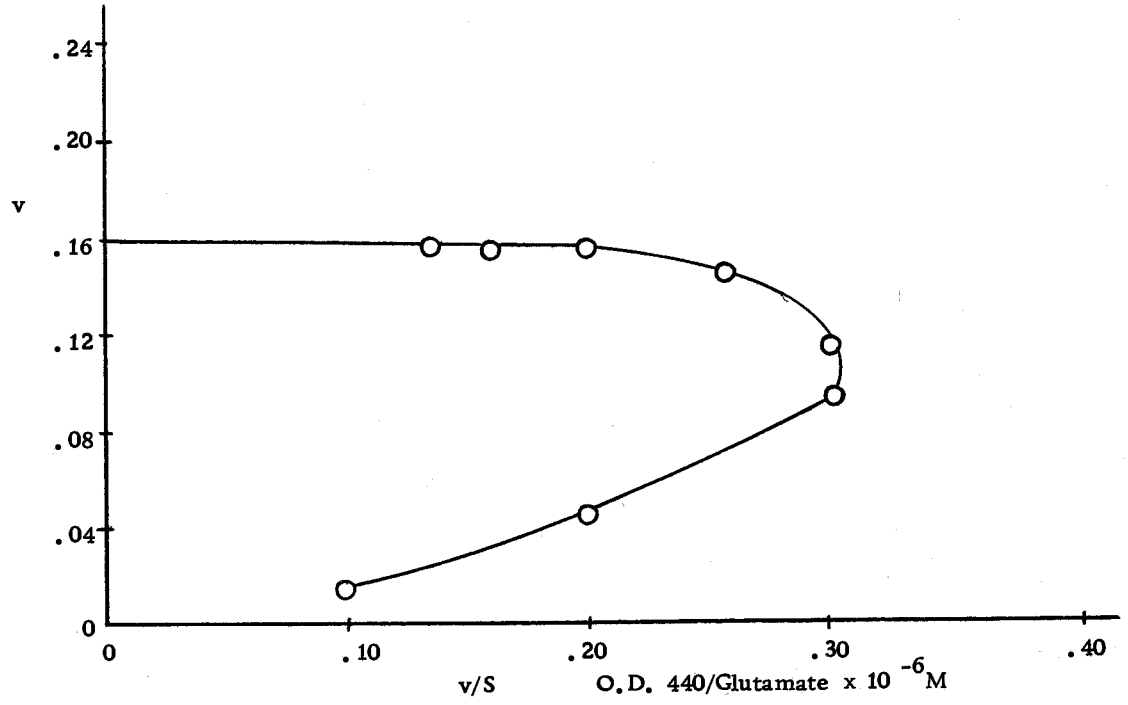

Figure 9. Data from Figure 6.

Table 2. K_m values obtained from glutamate and glutamine variation.

Assay	K_m value	Glutamate
E + wS	2.3×10^{-7} M to 1.2×10^{-6}M	
E + dS	0.8×10^{-7} M	
E + PPdS	0.4×10^{-7} M	

	K_m value	Glutamine
E + wS	5.5×10^{-7} M	

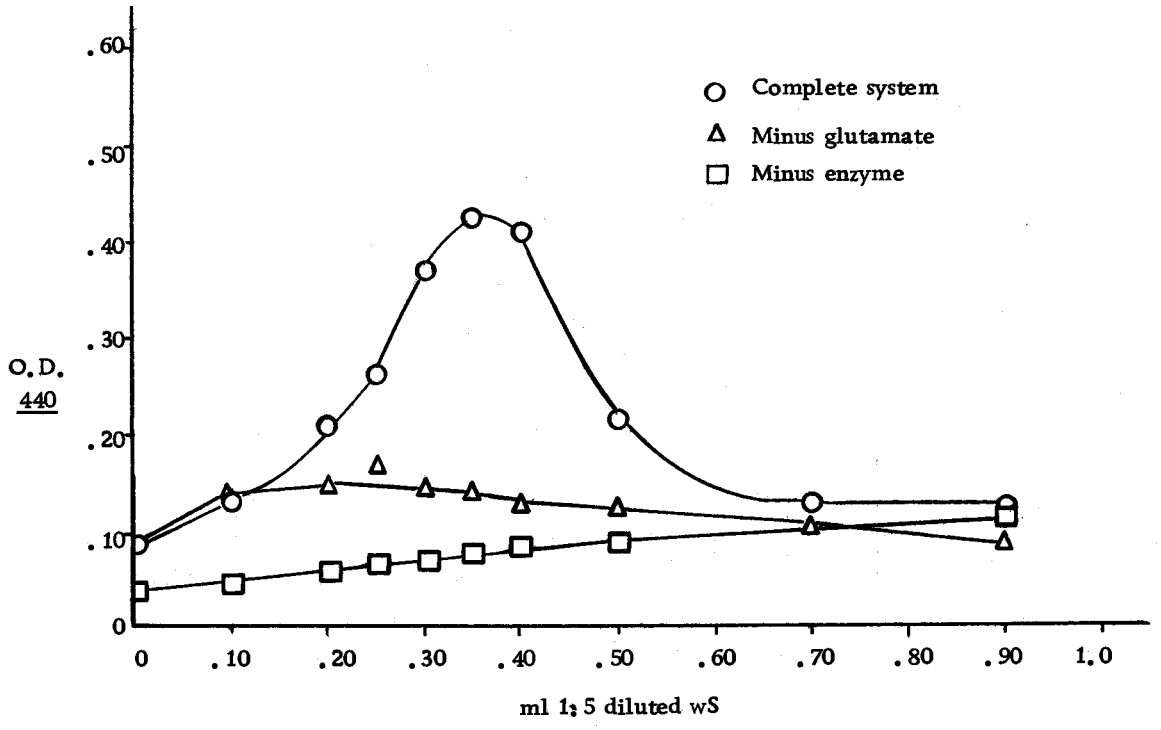

Figure 10. wS titration of E.

(Figure 12).

When wS was dialysed against distilled water at 4° C, the GSA promoting activity was found in the dialysate. The high molecular weight material (HMW) from dialysis stimulated, then inhibited dialysate activity as the HMW concentration was increased. The inhibition decreased upon prolonged dialysis of the HMW or disappeared upon heating the HMW before addition to the assay. The HMW in absence of dialysate gave only slight activity in the assay (Table 3).

The data indicate that there are at least two components of the whole soluble fraction which are directly involved in GSA production. A low molecular weight compound is capable of promoting GSA formation in a membrane fraction of E. coli; and some high molecular weight material, probably protein, stimulates or inhibits GSA production depending on its concentration.

2) Variation of concentrated dialysate (dS) gives an activity plot that has two distinct linear portions before inhibition occurs. (Figure 13). Heating the dialysate had no effect on its activity (Figure 14). When the dialysate was evaporated to dryness and then charred, the residue had no activity in the assay. When heated with concentrated nitric acid and neutralized the activity was again lost. It was concluded that the compound is a low molecular weight organic compound or an inorganic compound inactive in an oxidized or

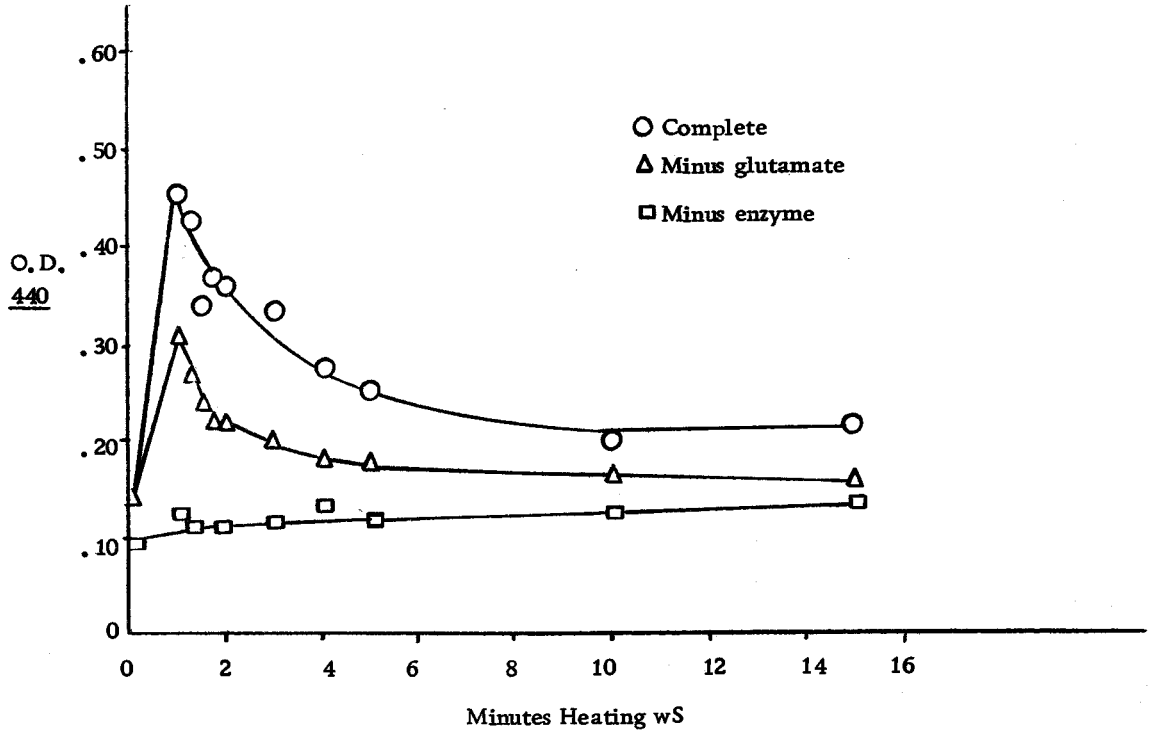

Figure 11. Activity of filtrate of heated wS vs. time heating.

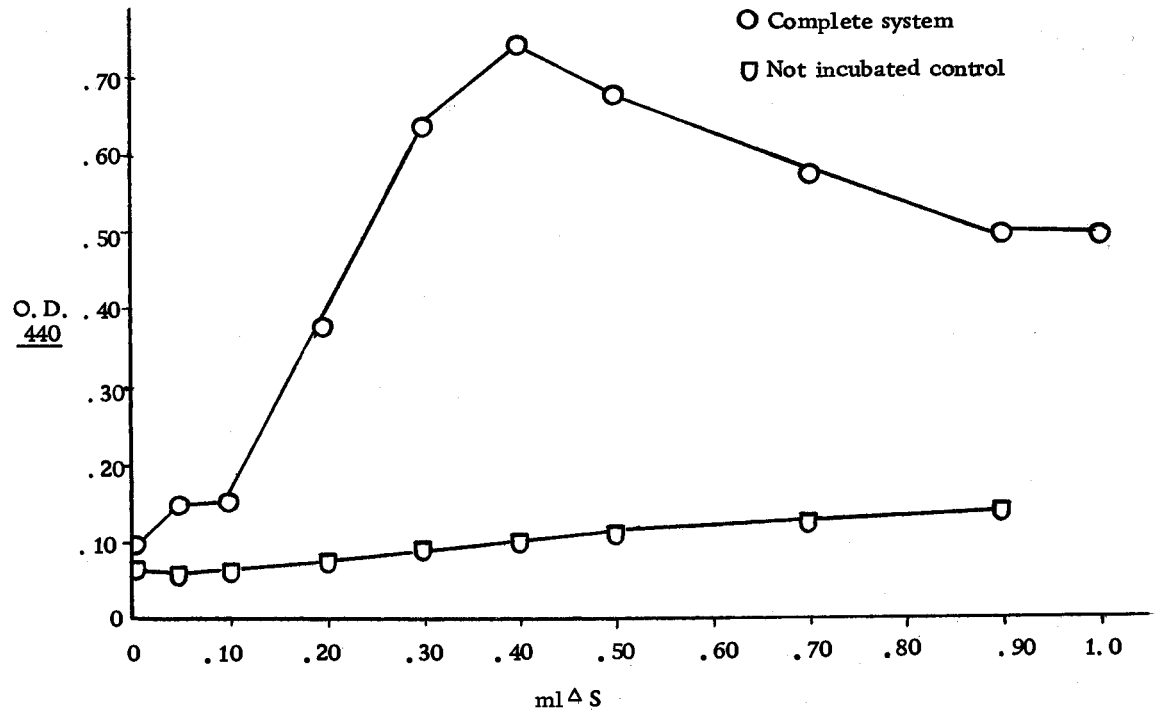

Figure 12. Δ S titration of E. Δ S is filtrate from 1.5 min heating of wS.

Table 3. Dialysis.

Fraction		O.D. at 440 mμ complete	- glutamate	- E
Dialysate 6 hrs	0.10 ml	0.85	0.175	0.148
	0.05 ml	0.43	0.16	0.06
HMW 6 hrs	0.05 ml	0.16	0.10	0.06
Dialysate 6 hrs +	0.10 ml			
HMW 6 hrs	0.05 ml	0.18	0.12	0.44
Dialysate 24 hrs	0.10 ml	0.20	0.13	0.04
HMW 24 hrs	0.05 ml	0.13	0.09	0.08
Dialysate 24 hrs +	0.10 ml			
HMW 24 hrs	0.05 ml	0.68	0.17	0.05
Dialysate 6 hrs +	0.10 ml			
HMW 24 hrs	0.05 ml	0.53	0.12	0.32
Dialysate 6 hrs +	0.10 ml			
HMW 30 hrs	0.05 ml	0.44	0.14	0.35
HMW 30 hrs	0.05 ml	0.13	0.09	0.05

Twenty-five ml wS was dialyzed against 200 ml H_2O for 6 hrs at 4° C. A portion of the high molecular weight material was heated in a boiling water bath for 1.5 minutes and the filtrate was used for assay.

Additions		complete	- glutamate	- E
Dialysate		0.32	0.09	0.08
Unheated HMW	0.10 ml	0.15	0.09	0.08
Heated HMW	0.15 ml	0.24	0.13	0.11
Dialysate +				
heated HMW	0.00 ml	0.32	0.09	0.08
	0.05 ml	0.39	0.12	0.11
	0.10 ml	0.43	0.12	0.15
	0.15 ml	0.49	0.14	0.19
	0.20 ml	0.52	0.17	0.20
Dialysate +				
unheated HMW	0.00 ml	0.32	0.09	0.08
	0.05 ml	0.42	0.11	0.13
	0.10 ml	0.21	0.12	0.10
	0.20 ml	0.11	0.10	0.10

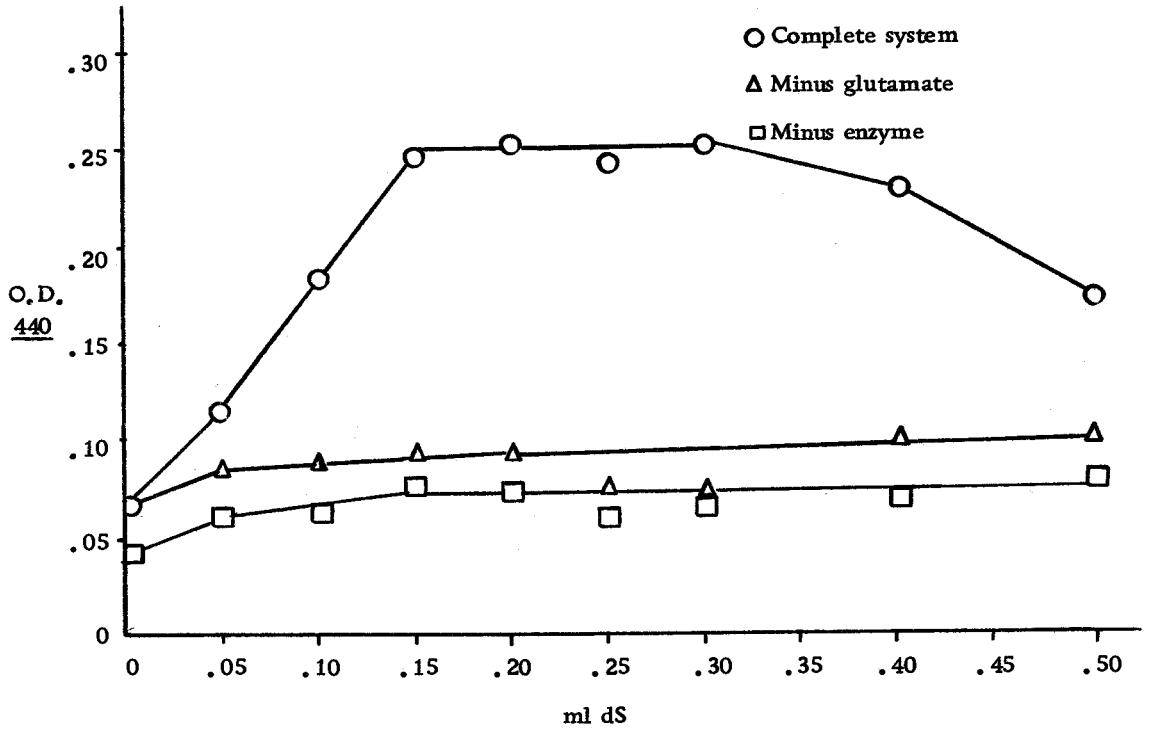

Figure 13. dS titration of E.

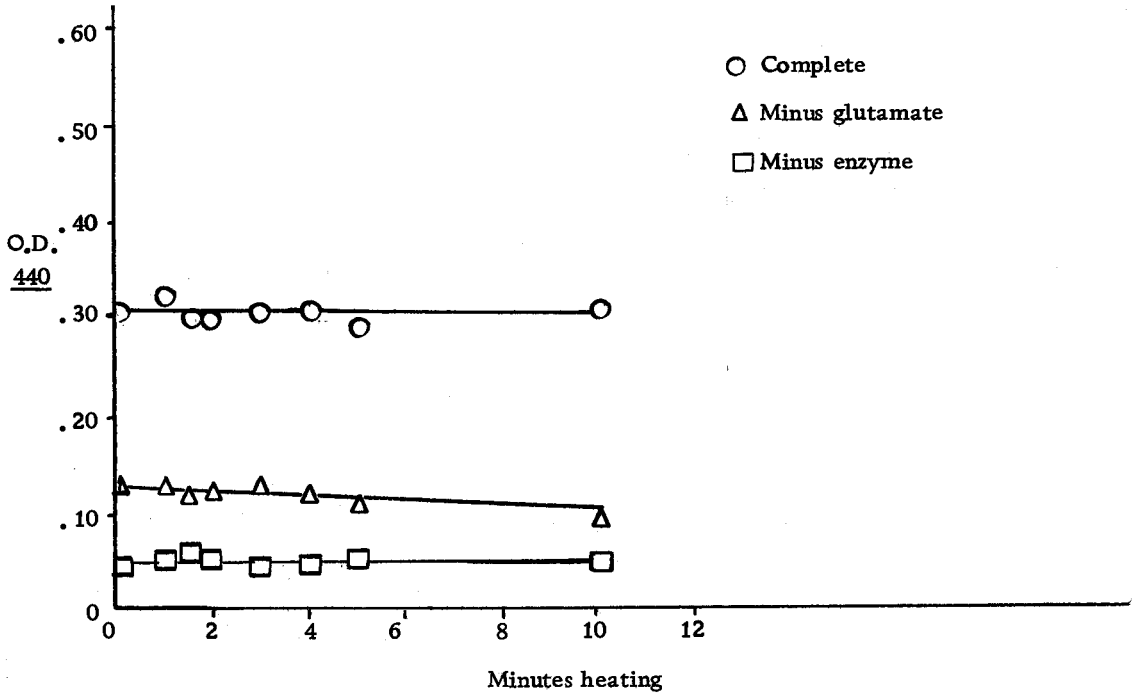

Figure 14. Activity of dialysate versus time heating.

complexed state.

Analysis of Cofactor Activation

Theory

An attempt was made to determine the nature of a reaction that would explain the form of the curve obtained with dialysate variation. For such a graph one formula defines one region of x values, a second formula another region, and the two have the same value at the inflection of the slope. Such a plot can be called a titration curve and the point of change of slope is the equivalence point. This type of curve can be distinguished from a hyperbolic curve by a Lineweaver-Burk plot. For the titration curve the horizontal portion of the two plots (titration and Lineweaver-Burk) will have reciprocal values, the slope of the straight line following the horizontal portion in the Lineweaver-Burk plot will have a value which is a reciprocal of the slope of the line of the first plot. The point of inflection in the two plots will have reciprocal values and the straight line of the Lineweaver-Burk plot should pass through the origin when extrapolated back. The hyperbolic curve would give a straight line in the Lineweaver-Burk plot which does not pass through the origin and whose slope is not a reciprocal value of the slope of the first plot (26, p. 116-119) (Appendix I).

In reactions involving activation, a cofactor can function by activating enzyme or substrate. There are two possible modes of action by which a cofactor activates an enzyme. The cofactor can react with the enzyme and this complex reacts with a substrate (I), or alternatively the enzyme reacts with substrate and the E-S complex is activated by the cofactor (II). In the following schemes E designates enzyme, S substrate, A activator, Ea activated enzyme, Sa activated substrate, Ca activated enzyme-substrate complex, C enzyme-substrate complex and P products.

I. $E + A \rightarrow Ea + S \rightarrow Ca \rightarrow Ea + P$

II. $E + S \rightarrow C + A \rightarrow Ca \rightarrow Ea + P$

III. $S + A \rightarrow Sa + E \rightarrow Ca \rightarrow E + A + P$

Model rate equations for these methods indicate that the first would give a titration curve where the equivalence point is independent of substrate. In the second case a hyperbolic curve would be obtained or if the cofactor were tightly bound, a titration curve would be obtained where the equivalence point is dependent upon substrate concentrations. If substrate were activated prior to enzyme-substrate complex formation then variations of substrate and activator would give similar plots, since both would enter the complex at the same time (26, p. 120-134).

Experimental

The partially purified dialysate was varied at three nonsaturating glutamate levels. Figure 15 shows that under these conditions the activity is dependent upon both glutamate and cofactor, and that glutamate is the limiting component. These data satisfy criteria for a titration curve, since titration plot and Lineweaver-Burk plot have reciprocal values for slope and horizontal portions, and the extrapolated line of the Lineweaver-Burk plot passes through the origin (Figure 16, Table 4).

GSA formation was linear with increasing concentrations of glutamate when glutamate was varied at different levels of partially purified dialysate (Figure 17). The saturation point at 3.5×10^{-7} M glutamate was independent of cofactor concentration, except for the lowest value of cofactor. The activity increased with increasing amounts of cofactor just as it did for increasing amounts of glutamate in the cofactor titration. A Lineweaver-Burk plot of the data indicates that this is also a titration curve (Figure 18, Table 4).

While initial rates are not measured, product formation is dependent upon substrate concentration, and such data can suggest a working hypothesis.

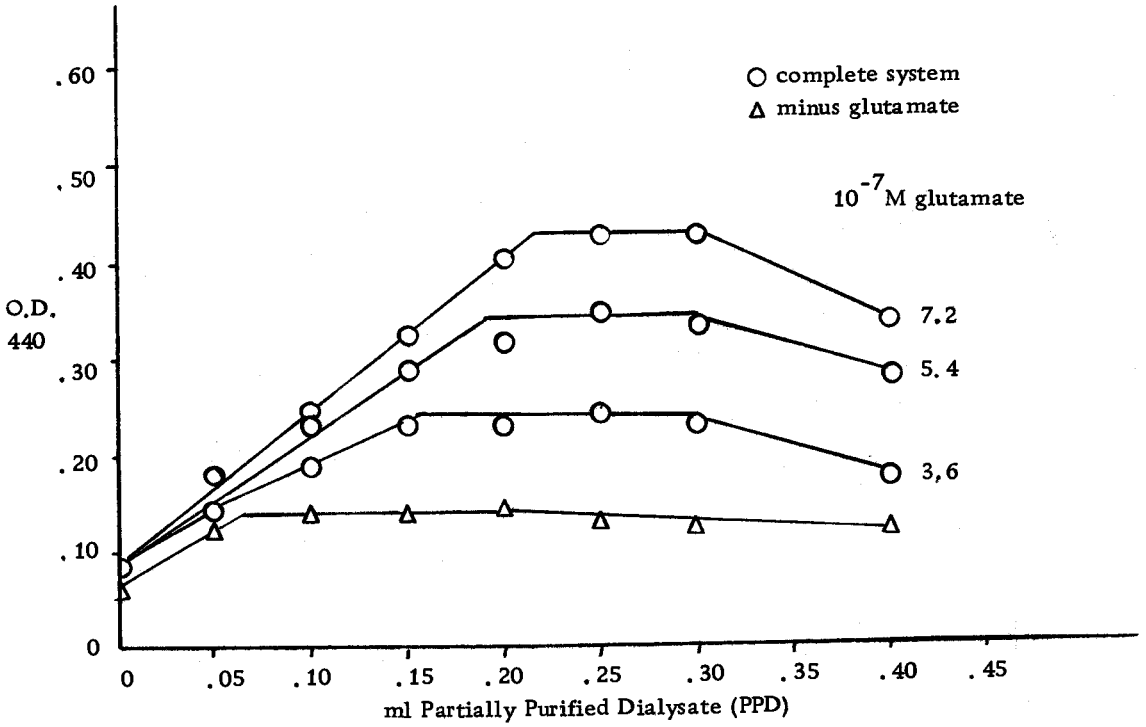

Figure 15. Effect of glutamate concentrations in PPD variations.

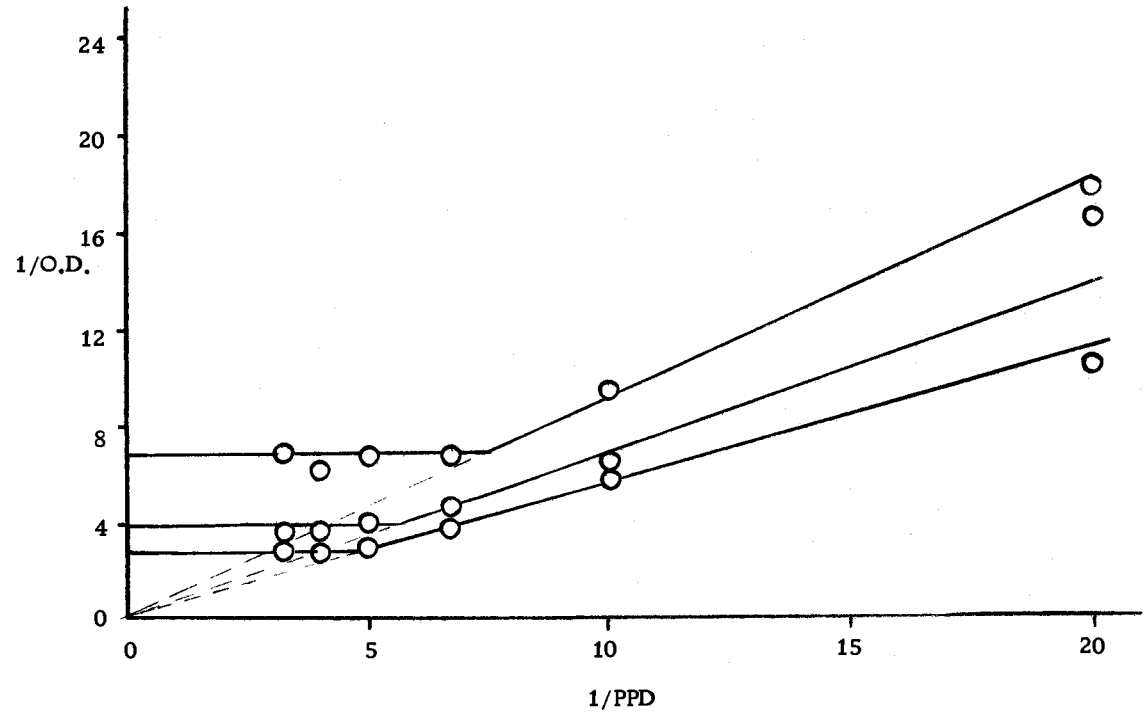

Figure 16. Lineweaver-Burk plot of data of Figure 15.

Table 4. Values for slopes, intercepts, inflection points obtained from cofactor and substrate titration and Lineweaver-Burk plots.

Glutamate	Cofactor filtration slope	Figure 15 activity	inflection point
3.6×10^{-7} M	1.0	0.15	0.14
5.4×10^{-7} M	1.4	0.26	0.18
7.2×10^{-7} M	1.6	0.35	0.21

Glutamate	Cofactor Lineweaver-Burk plot Figure 16 slope	1/slope	intercept	1/intercept	inflection point (I. P.)	1/I. P.
3.6×10^{-7} M.	0.91	1.1	7.0	.14	7.6	.13
5.4	0.67	1.5	4.0	.25	5.8	.17
7.2	0.55	1.8	2.8	.35	5.0	.20

PPD (ml)	Substrate titration Figure 17 slope	activity	inflection point	1/I. P.
0.06	0.045	0.16	3.45	.28
0.08	0.048	0.18	3.5	.29
0.10	0.052	0.21	3.6	.29

PPD (ml)	Substrate Lineweaver-Burk plot Figure 18 slope	1/slope	intercept	1/intercept	inflection point
0.06	23.3	0.043	6.2	0.16	.29
0.08	21.3	0.047	5.6	0.18	.28
0.10	18.3	0.055	5.0	0.20	.28

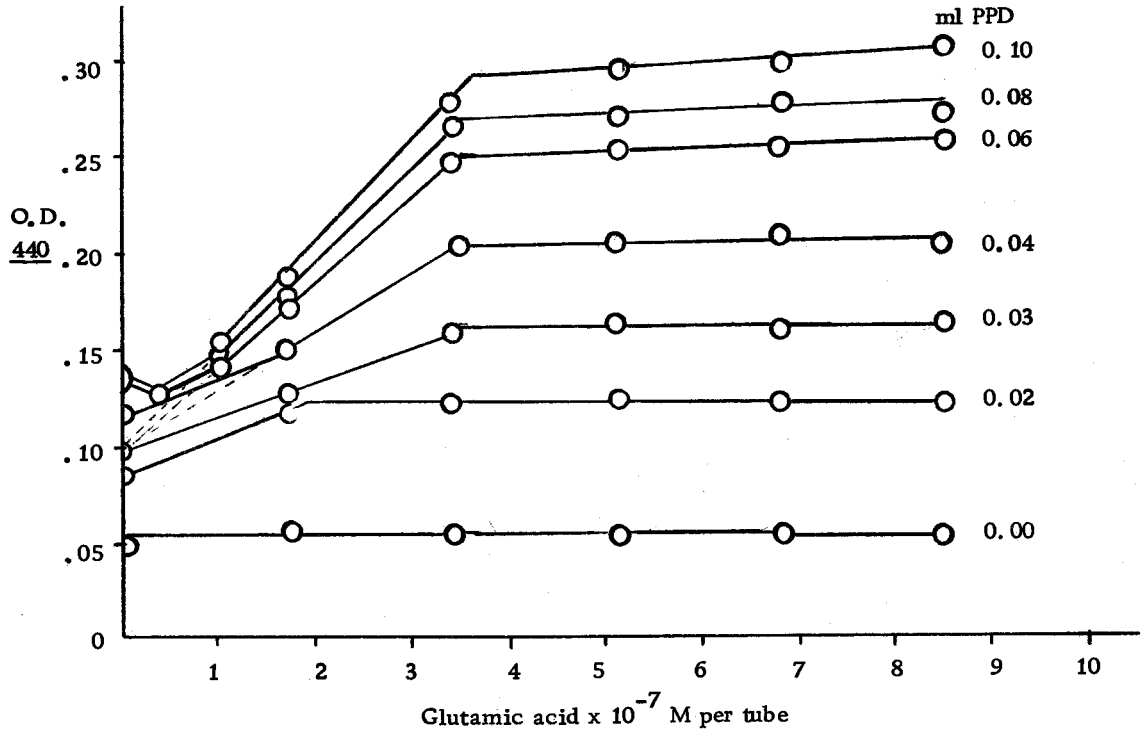

Figure 17. Glutamic acid variations at different levels of PPD.

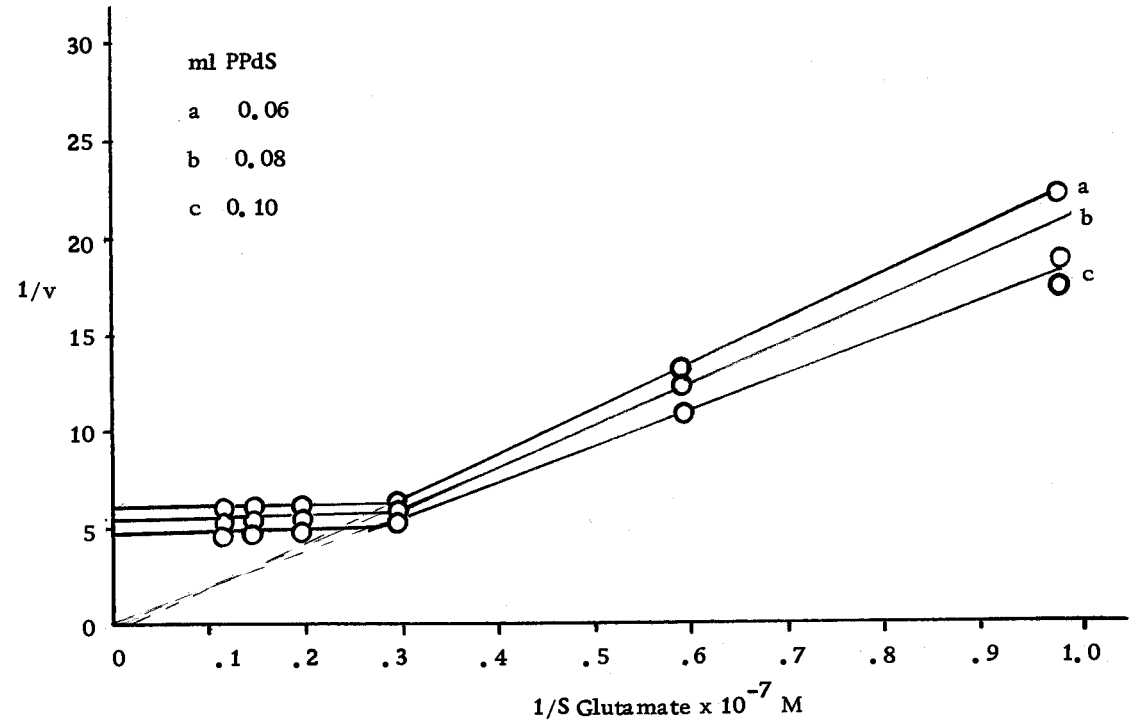

Figure 18. Lineweaver-Burk plot of data corrected for 1 zero point (0. 095) from Figure 17.

Conclusions

The cofactor titration data could satisfy scheme II, but since variation of substrate also gives a titration curve, scheme III is suggested. The distinguishing characteristic of scheme III is the symmetry of substrate and activator data. Substrate and cofactor enter the enzyme complex at the same time, so what is true of one should be true of the other. Titration curves should be superimposible.

In this system, glutamate is present in the partially purified fraction. It may be responsible for GSA production in the absence of glutamate in the substrate titration curve, and it would also affect the cofactor titration data. The substrate titration data suggests that at a low level of cofactor, the cofactor does affect the equivalence point. This may be a case where a threshold value of cofactor is required for activity, and after this value is reached then small changes in amount of cofactor would result in large changes of GSA production.

Scheme III is favored where the substrate is activated prior to enzyme-substrate complex formation. A titration curve is seen for a mechanism in which the activated substrate is tightly bound to the enzyme (Appendix I).

Effects of Inhibitors and Activators

In whole cells the conversion of glutamic acid to GSA is an aerobic process, stimulated by lactate, pyruvate, glucose and formate, pyruvate being most effective. Additional stimulation was obtained with AMP or ADP in presence of pyruvate. Similar results were found using glutamine as a substrate (33, 35).

The effects of these compounds on the cell-free system are shown in Table 5 and Figure 19. Either pyruvate or glucose was necessary for the reaction. Effects of ATP addition were ambiguous; at times its addition would stimulate the activity and at other times it was inhibitory. Of the other compounds tested, adenine, AMP, ADP and thiamine had no effect on the cell-free system, while formate and acetate were inhibitory.

L-Proline inhibited GSA production in experiments with whole cells (3, 33, 36). Streptomycin was also found to inhibit GSA production in whole cells (11). Proline and streptomycin had similar effects on the cell-free synthesis. Both stimulated at lower concentrations (1 to 7×10^{-9} M proline and 0.1 to 1.6×10^{-8} M streptomycin) and inhibited at higher concentrations. Using the whole soluble system, wS, complete inhibition was obtained with 4×10^{-8} M streptomycin or 1.2×10^{-8} M proline. With $^{\Delta}$S, 1.3×10^{-8} M streptomycin inhibited completely (Figures 20 and 21). In the PPD

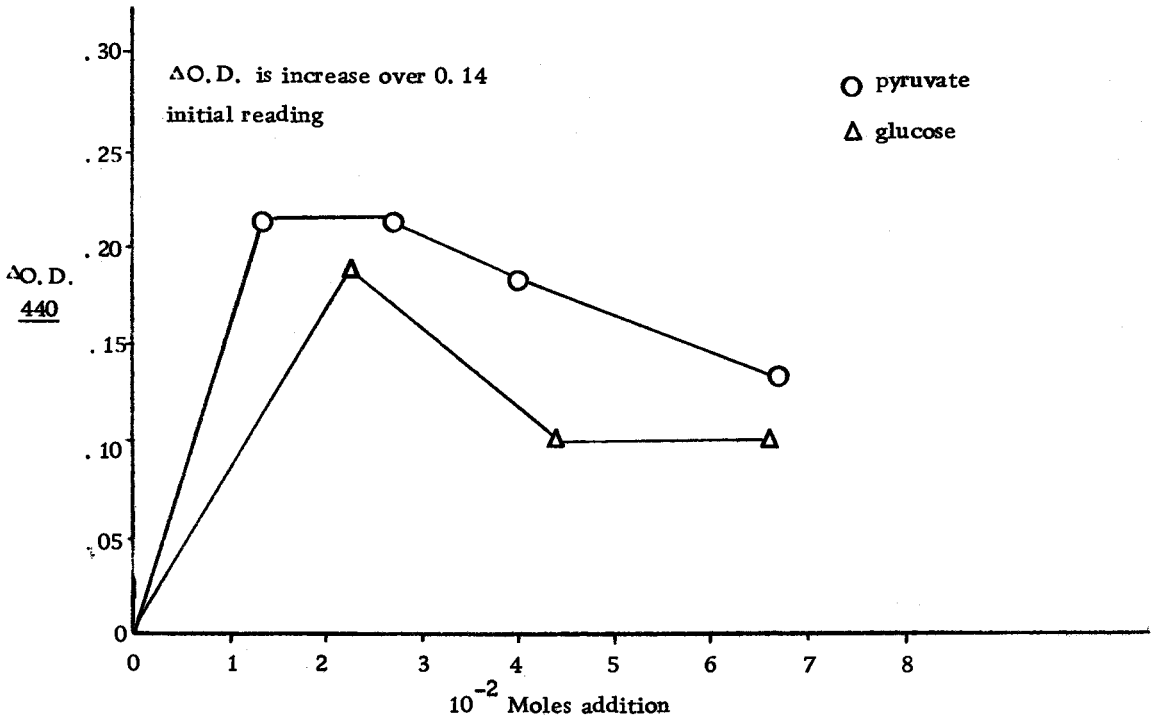

Figure 19. Addition to E + wS assay.

Table 5. Additions to E + ΔS assay.

Additions to glutamate + E in phosphate buffer	ml ΔS per assay tube. 0.1 ml ΔS	0.2 ml ΔS	0.3 ml ΔS	0.4 ml ΔS
None	100%	100%	100%	100%
3.3×10^{-2} M pyruvate	123	114	135	153
4.3×10^{-4} M AMP	93	90	86	80
1.7×10^{-3} M ATP	90	69	84	62
3.7×10^{-3} M formate	95	90	93	73
0.15 mg thiamine	103	75	87	80
Pyruvate	123%	114%	135%	153%
" + AMP	121	103	126	72
" + ATP	106	95	131	116
" + formate	126	102	123	120
" + thiamine	131	113	129	137

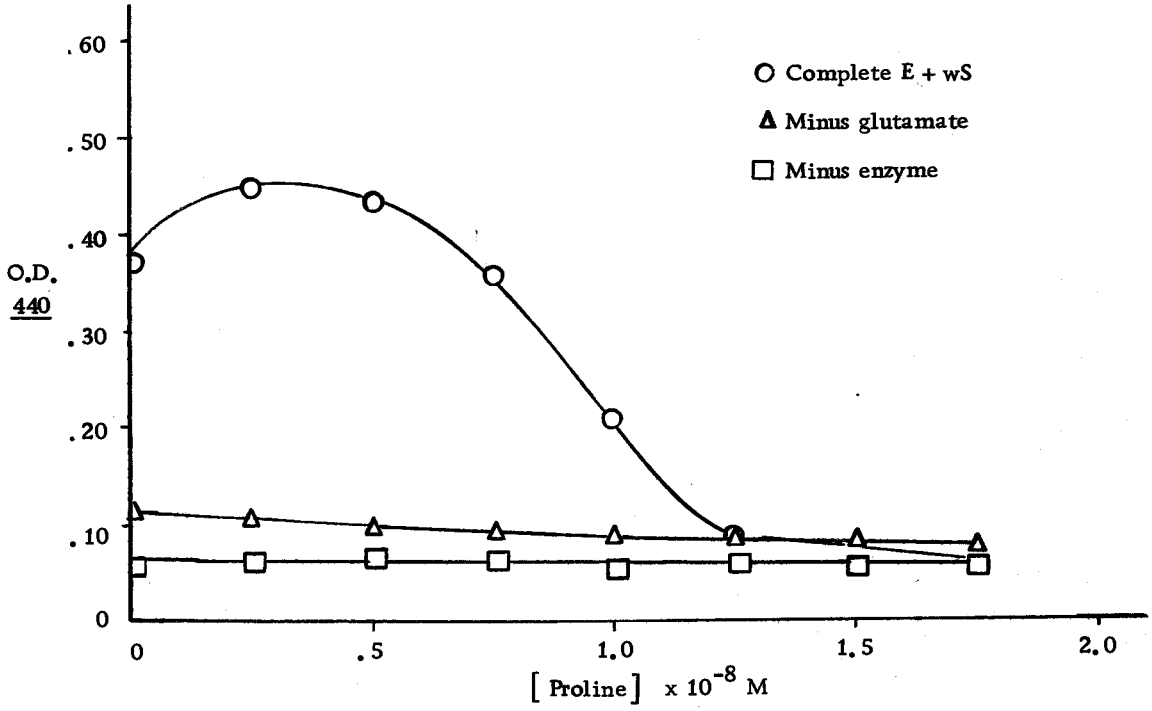

Figure 20. Effect of proline addition to E+wS assay system.

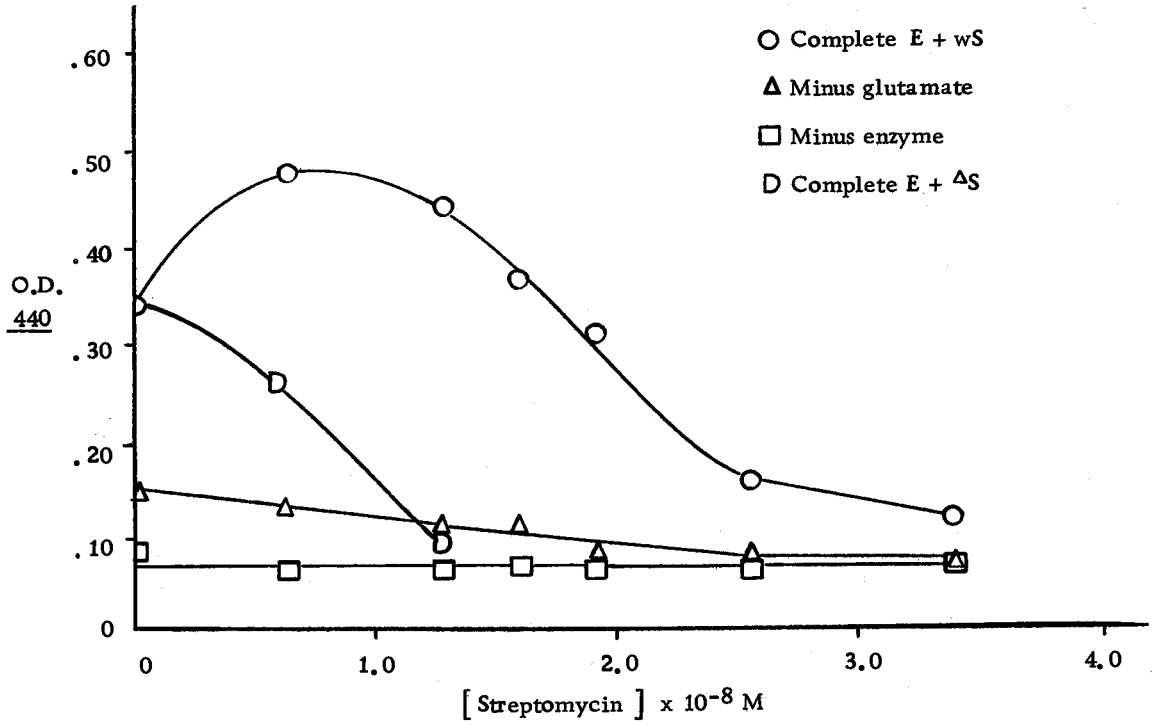

Figure 21. Effect of streptomycin in E+wS assay system.

system, proline stimulated at 0.5 to 1.3×10^{-8} M and was completely inhibitory at 5×10^{-8} M. Sigmoidal inhibition curves characteristic of an allosteric effect were found.

Further work is necessary to determine if these quantitative differences are significant, but the data do point out that proline inhibits the synthesis of GSA. Slight variations in amounts of endogenous proline present in the crude fractions used in the assay could greatly affect GSA formation.

Mn^{++}, Ni^{++} and Co^{++} stimulated GSA production in a system which contained Mg^{++}, ATP and thiamine, in addition to pyruvate and glutamate in phosphate buffer. Fe^{++}, Fe^{+++}, Cu^{++}, and Zn^{++} stimulated GSA production only slightly or had no effect.

Mg^{++}, Mn^{++} and Co^{++} were examined in the simplified incubation mixture containing phosphate, pyruvate, and glutamate (Table 6). Mg^{++} was inhibitory at high concentrations and had no effect at lower concentrations. Mn^{++} had no effect alone, but in the presence of Co^{++} it stimulated GSA production. Co^{++} addition gave complex results as shown in Figures 22 and 23. Figure 22 is similar to an activity-substrate plot for an enzyme with more than two active sites (26, p. 64-82). A 1/activity versus $1/(Co^{++})^2$ plot is linear for production of GSA in the ΔS system. This may indicate the reaction observed involves two cobalt ions (Figure 24)(44).

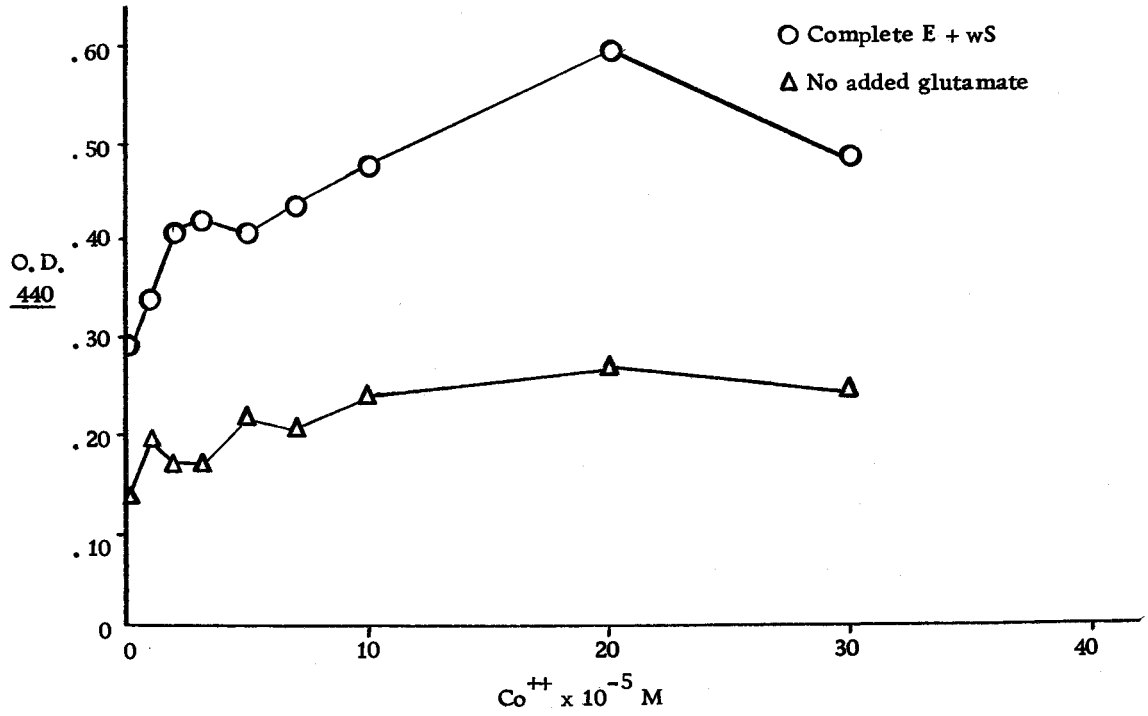

Figure 22. Co^{++} addition to E+wS assay system.

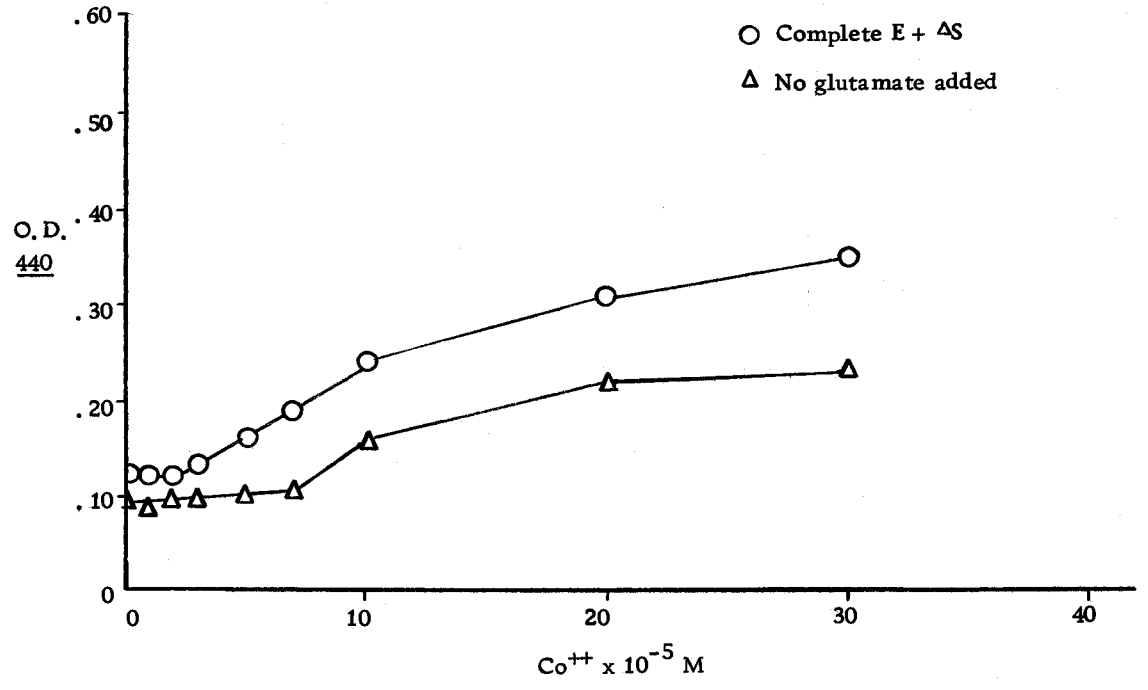

Figure 23. Co^{++} addition to E + ΔS assay system.

Table 6. Effect of cation addition to assay system.

Cation added	Concentration	Effect on assay
Mg^{++}	0.5 to 8 x 10^{-5} M	no effect on E + ΔS
	3.75 x 10^{-4} M	inhibitory to E + ΔS
Mn^{++}	1.9 x 10^{-4} M	no effect in E + ΔS
	1.9 x 10^{-3} M	no effect in E + ΔS
Mn^{++} in presence of Co^{++}	0.63 to 6.3 x 10^{-4} M	stimulated E + ΔS
Co^{++}	1.0 x 10^{-5} M to	
	3.0 x 10^{-4} M	stimulated E + wS and E + ΔS

If enzyme-substrate complex consists of more than one substrate molecule as in sequence: $nS + E \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ES_n \xrightarrow{k_2} E + P$ the rate is given by $v = V_{max} \frac{(S)^n}{(S)^n + K_m}$. In a case where n = 2

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{S^2} \qquad (44)$$

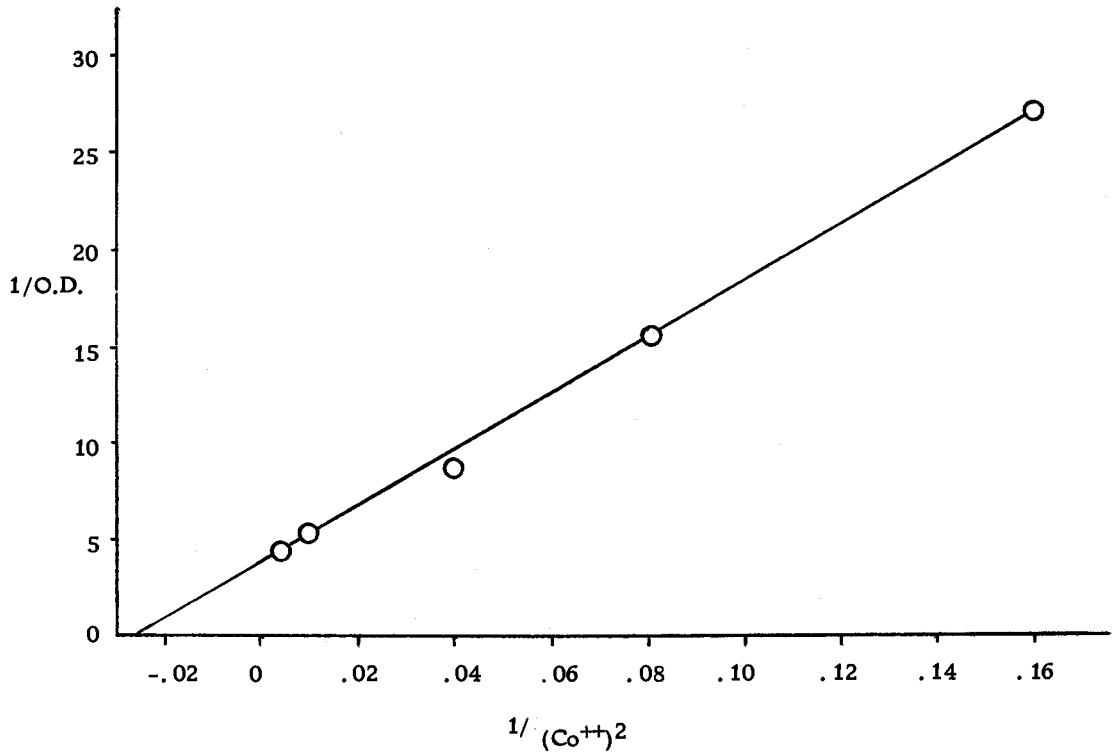

Figure 24. Double reciprocal plot of data, Figure 23. Co^{++} variation in E + ΔS assay.

Conditions of the Assay

The optimum pH for the reaction was 7.0 (Figure 25). The optimum temperature was 37° C, the optimum growth temperature of the organism (Figure 26).

To test for any buffer effect in the assay, cells were washed with saline and then phosphate or tris-HCl buffers were used for extract preparation. With cell extract prepared in phosphate buffer, tris buffer was as good as the phosphate buffer for the assay system. It appears that phosphate was required for activity of the tris-prepared enzyme assayed in tris buffer. This suggests that phosphate may be involved in the reaction (Table 7).

The time course of activity, Figure 27, shows that the reaction product is detectable after four hours and that the reaction is complete after 11 hours when wS is used as a cofactor. In experiments with whole cells, a lag in GSA production was attributed to endogenous proline which had to be metabolized before GSA was produced (33, 36). This could apply to the cell extracts since proline oxidase is also present in the membrane fraction of E. coli (14).

Isolation Procedures

First attempts to isolate the soluble factor utilized paper chromatography. ΔS, or alcohol extracts of ΔS, were applied directly to

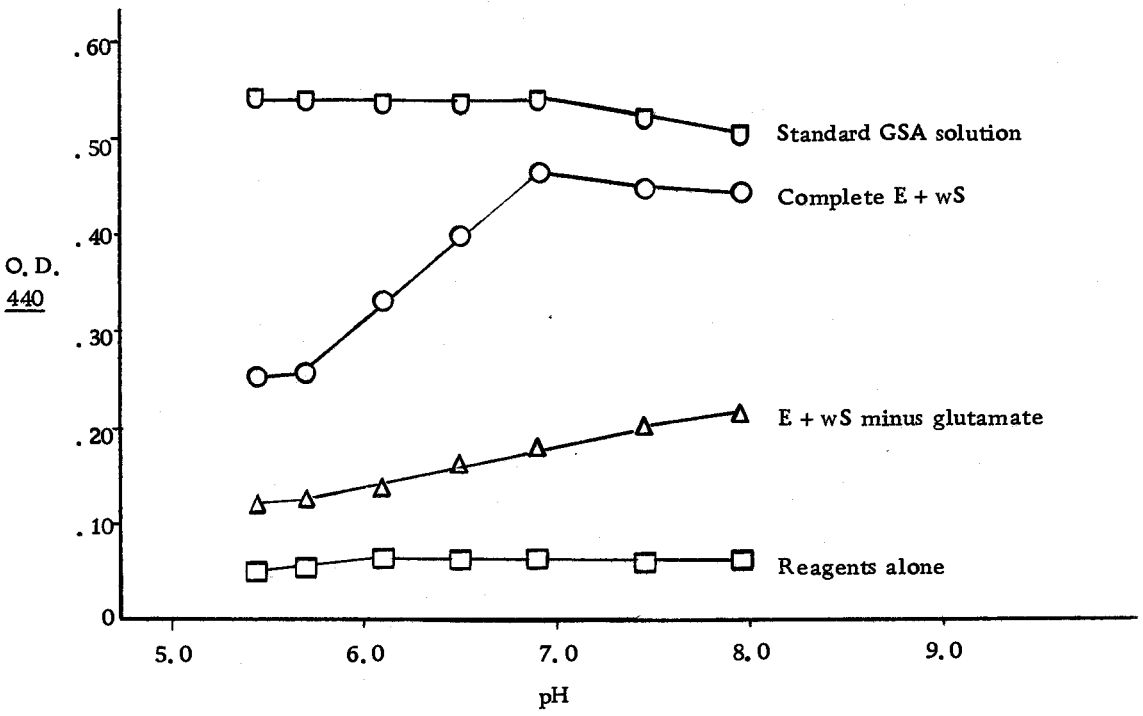

Figure 25. Effect of pH on GSA formation.

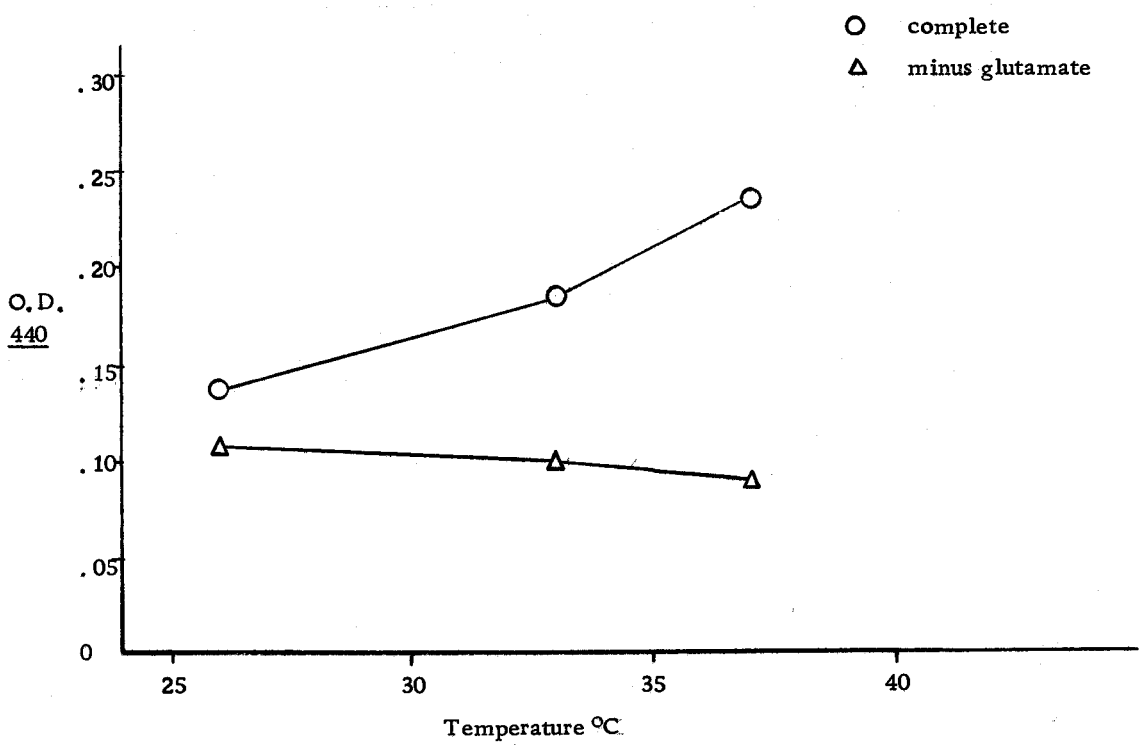

Figure 26. Effect of temperature on GSA formation.

Table 7. Cells washed with saline. Half of cells were prepared in Tris and half prepared in phosphate buffer. E plus Δ S Assay.

Phosphate prepared enzyme	
Phosphate buffer assay	Tris buffer assay
complete 0. 15	complete 0. 18
Tris prepared enzyme	
Phosphate buffer assay	Tris buffer assay
complete 0. 15	complete 0. 085
	complete plus 0. 2 ml
	phosphate buffer 0. 135

Table 8. Microbial assay of reaction product.

Additions	Klett units	
	55-1	W2
None	3	4
50 γproline	73	103
complete GSA assay	5	59
complete minus glutamate	5	17

E plus wS assay was incubated 12 hrs at 37° C. The reaction was stopped with trichloroacetic acid and the protein removed. 0. 5 ml supernatant was used in microbiological assay with 55-1 and W2. W2 can utilize GSA for growth, 55-1 cannot. Growth was determined by turbidity in a Klett-Summerson colorimeter. Microbiological assays of the reaction product verified that GSA is formed in the cell extract system.

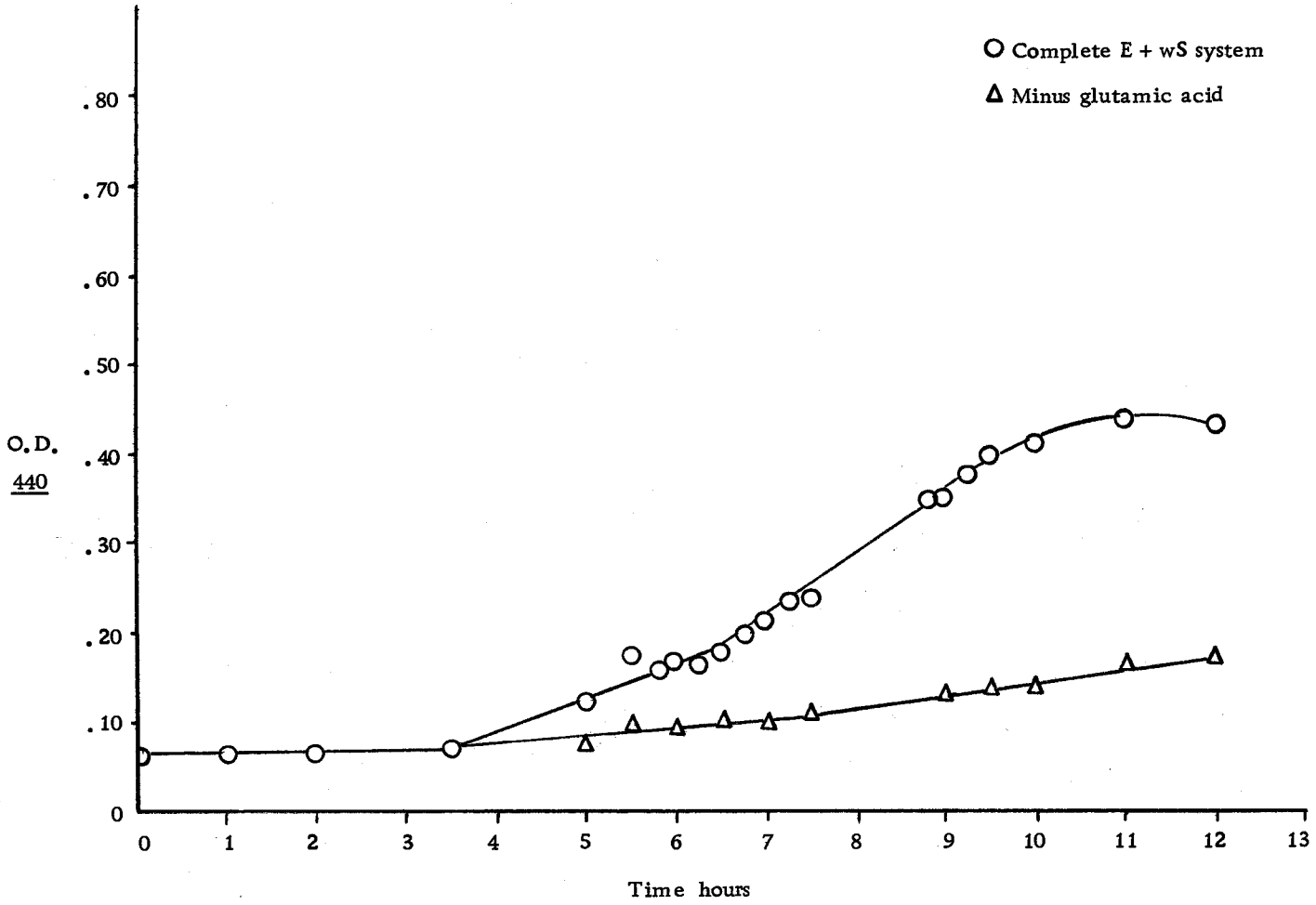

Figure 27. Time course of GSA formation in wS system.

the paper. The chromatogram was developed, all bands were eluted and tested for activity in the assay. Only small amounts of material could be used, three or four such chromatographings were required to get good separation, and yields were not large. All fractions chromatographed could be retained however, and tested for their combined influence in the assay. It was found that there were two bands, separated by a third, which were capable of activity alone in the assay. Together their effect was additive. This suggested that there may be two compounds involved, or that one compound in two ionic forms was being isolated.

ΔS was also separated on cellulose columns as listed in Table 9. The DEAE column appeared to be the most useful and convenient. The cofactor traveled with the ninhydrin reacting compounds; these fractions were combined, lyophilized and then applied to paper chromatograms or to a Bio Gel P-2 column. Several paper chromatograms were required again for good separation of ninhydrin reacting compounds.

The most effective isolation procedure involved dialysis of the whole soluble fraction followed by ethanol or acetone extraction of the evaporated dialysate (Table 9). The active factor was retained on Dowex-50-X-8 in H^+ form, but elution of cofactor varied over a wide range in the batch-like procedure, and active fractions still contained a number of amino acids.

Table 9. Chromatography of the soluble fractions. Columns were equilibrated with eluting solvent prior to sample application.

Medium	S fraction applied	Solvent system	Activity recovered	Purification
cellulose column (10.0 x 4.7 cm)	Δ S	propanol/water 80/36	70%	-
CM-cellulose column (9 x 4.7 cm)	Δ S	0.1 M phosphate buffer pH 7	34%	-
DEAE cellulose column (7 x 4.5)	Δ S	H_2O to 0.1 M phosphate pH 7	21%	-
	Δ S	0.1 M phosphate pH 7	105%	-
Bio Gel p-2 column (41 x 1.3 cm)	DEAE active eluent	H_2O	58-94%	6 ninhydrin 7 UV bands
Paper chromatogram	DEAE active eluent	Butanol/acetic acid/ water 4:1:1 (BAW)	140% in two bands	7 ninhydrin 10 UV bands
	BAW paper chromatogram	propanol/water 80:36	39%	5 ninhydrin 7 UV bands
	Dialysate	propanol/water 80:36	55%	14 ninhydrin 18 UV bands
Dowex-1-acetate column (16 x 1 cm)	propanol/water chromatogram	0.1 M sodium acetate pH 4.0	52%	-
Dowex-50-X-8 (7 x 4.5 cm)	dialysate	gradient H_2O to 2 N HCl	56-66%	6 ninhydrin 4 UV bands

Continued on next page

Table 9. Continued.

R_f values of the active fraction	

A. Column Chromatography.

Activity traveled with ninhydrin reacting compounds; these compounds were the first components eluted from the Dowex-1-acetate and cellulose columns.

B. Paper Chromatography.

Solvent System	R_f
butanol/acetic acid/water 4:1:1	.27 to .45
propanol/water 80:36	.36 to .60

Silica gel chromatography was found to be a better procedure for further separation. In early work with thin layer chromatography, it was difficult to recover activity applied to the silica gel. When preparative scale chromatography with washed silica gel was used, activity was recovered when the gel was eluted with HCl. The acid was removed by evaporation. When the ethanol extract of the dialysate was applied to the thick layer, separation was as effective as paper chromatography and much less tedious.

The homogeneity of the isolated fraction was difficult to estimate. Thin layer chromatography, generally used to determine the number of UV detectable and ninhydrin reacting components present, was not sensitive enough to detect components in the active fraction. Microscale electrophoresis of the fraction obtained by thin layer chromatography separated six ninhydrin positive compounds. An amino acid analysis was run on a concentrated sample of the solution by Dr. Becker of this department, with a Beckman Model 120 B Amino Acid Analyzer (Table 11). Components detected in this analysis were substituted for the soluble factor as described in the next section.

Substitutions for the Soluble Factor

None of the compounds tested substituted for the cofactor (Table 12). Glycine and glyoxylate were artifacts (Table 13).

Table 10. Solubility properties.

Fraction	Activity	TLC of fraction
Δ S	100%	-
ethanol soluble	77%	-
Dialysate	100%	5 ninhydrin, 4 UV bands
ethanol soluble	65%	4 ninhydrin, no UV bands
ethanol insoluble	26%	5 ninhydrin, 1 UV band
chloroform wash	0.25%	no ninhydrin, 4 UV bands
ether wash	0.0%	-
Dialysate	100%	-
acetone soluble	106%	-
acetone insoluble	6%	-
Ethanol soluble fraction of dialysate	100%	-
TLC active band	49-70%	nothing detected

Quantitative ninhydrin and phosphate analysis

Fraction	GSA activity O.D./ml	10^{-4} M NH_2/ml	10^{-4} M NH_2/O.D.	μ mole organic phosphate/ml
Dialysate	0.30/ml	9.30	31	-
Ethanol sol.	3.4/ml	70.0	21	1.0 μ mole/ml
TLC active band	2.5/ml	15.0	6.0	0.12 μ mole/ml

Table 11. Amino acid analysis of active band from thick layer chromatogram.

Amino acid	μ M/ml	% total NH_2	amino acid	μ M/ml	% total NH_2
cysteic	trace (T)	-	alanine	3.9	34.4%
aspartic	T	-	half cystine	T	-
threonine	0.22	1.9%	methionine	T	-
serine	T	-	isoleucine	T	-
glutamic	0.67	5.9%	lysine	0.055	0.4%
proline	0.47	4.1%	ammonia +	4.05	35.7%
glycine	1.95	17.2%	ethanolamine		

Table 12. Substitutions for the soluble factor.

No color formation:

pyridoxal phosphate

NADH

NAD

FAD

FAD plus NAD

ferrodoxin

CoA

phosphatidylethanolamine

*NH_4Cl

*ethanolamine

glycerol

*found in amino acid analysis of the active band

guanine
cytidine
uracil
thymine
adenine

*alanine
*isoleucine
*lysine
ornithine
aminobutyrate
aminobutyrate
phenylalanine
*threonine
glutamine
arginine
norleucine

*cysteine
*cystine
valine
*serine
tryptophan

histidine
*methionine
asparagine
homocystine
homoserine
allocystathionine
leucine
*aspartate
hydroxyproline
norvaline
homocystathiolactone

Color formation in the assay:

glycine

glyoxylic acid

proline

Co^{++}

Table 13. Glycine and glyoxylate substitutions for the cofactor O.D. 440.

Substitution		complete incubation mix	complete - glutamate	complete - enzyme
glyoxylate	0	.067	.045	.034
mg/2.0 ml solution	.5	.145	.101	.094
1.35×10^{-5} M	1.0	.252	.164	.211
	2.0	.355	.237	.384
	4.0	.528	.312	.625
glycine	0	.058	.054	.042
mg/2.0 ml solution	.5	.07	.065	.058
1.33×10^{-5} M	1.0	.082	.081	.073
	2.0	.115	.109	.112
	4.0	.155	.159	.116

Proline (10^{-8} M) stimulated GSA formation which was dependent upon glutamate and the enzyme fraction (Figure 28). The plot of the data does not take the same form as cofactor titration nor does extent of activity approach that of the cofactor. One explanation of proline stimulation could be that whole cells present in E utilize the amino acid for growth, and subsequently the glutamate is used for GSA production in the cells.

When Co^{++} was substituted for the cofactor, a similar titration curve was obtained as when the cofactor was varied. However, the extent of activity in this system was not quantitatively comparable to the cofactor system (Figures 29, 30). The level of activity obtained with Co^{++} substitution varied considerably. In the presence of Co^{++}, proline inhibited GSA formation at a concentration that was stimulatory in the absence of Co^{++}.

Characterization of the Cofactor

Preliminary work showed the cofactor to be a low molecular weight compound. The cofactor travels with amino acids and inorganic phosphate on a gel filtration column for low molecular weight compounds. It requires phosphate for elution from a DEAE column. The compound is either a simple organic compound or an inorganic compound inactive in an oxidized state.

The compound is stable to heat, hot HCl, and hot NaOH. It is

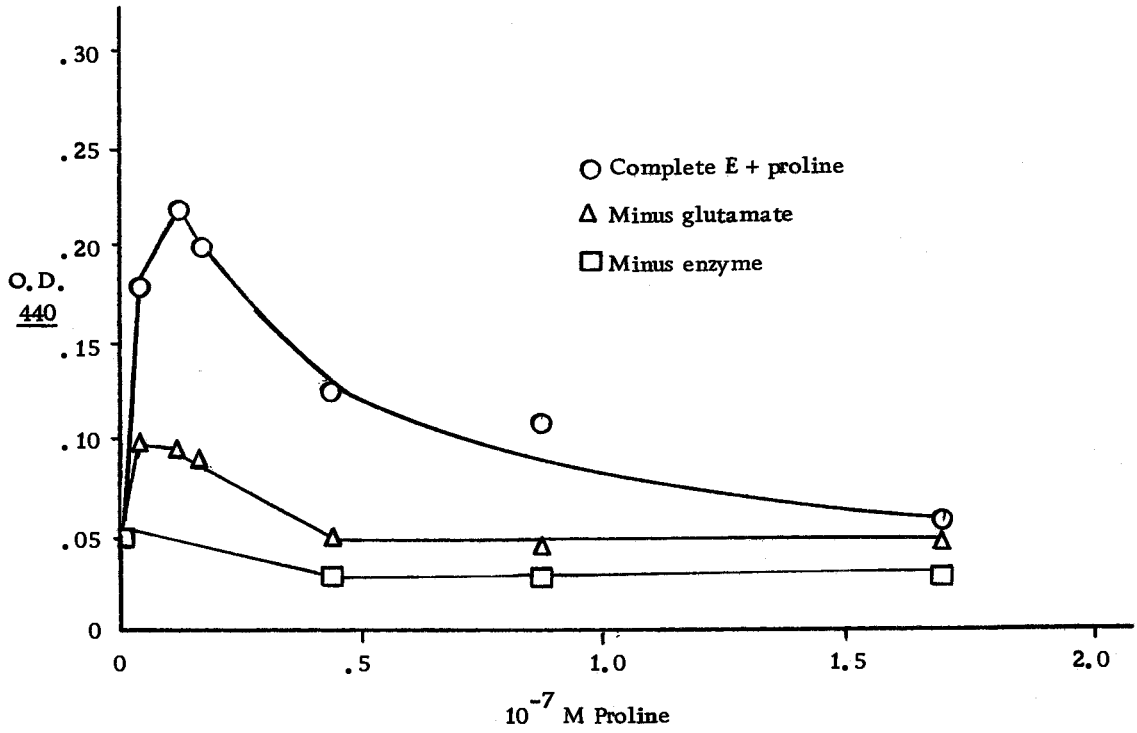

Figure 28. Proline substitution for cofactor.

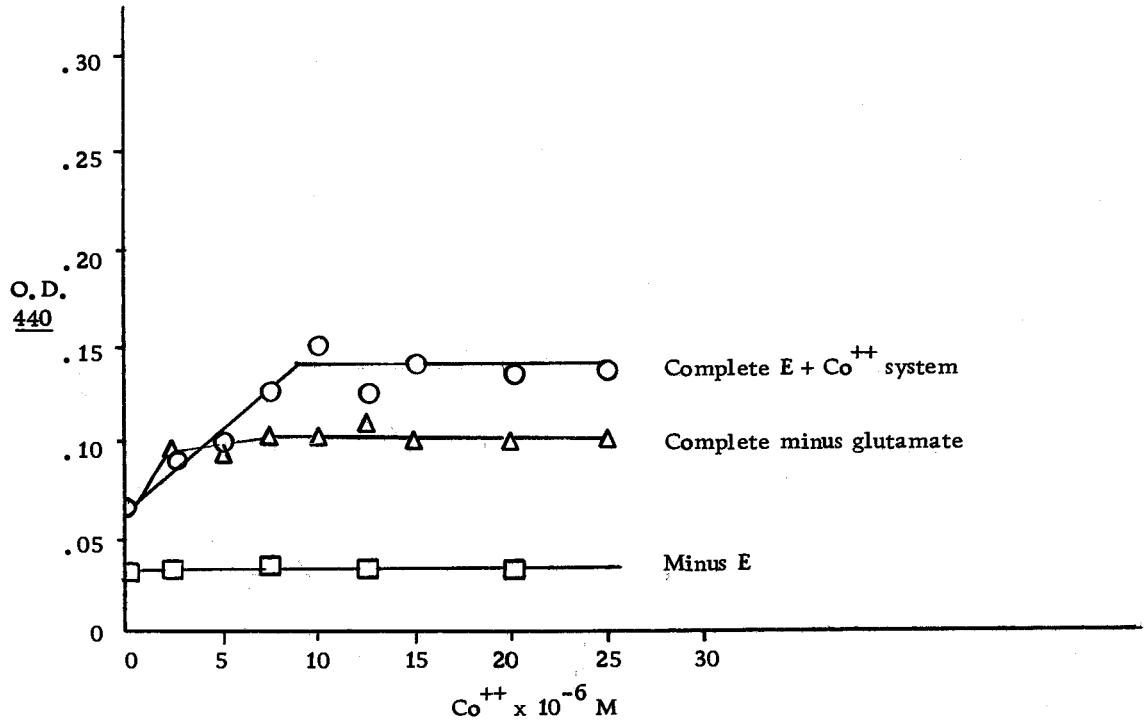

Figure 29. Co^{++} substitution for the cofactor.

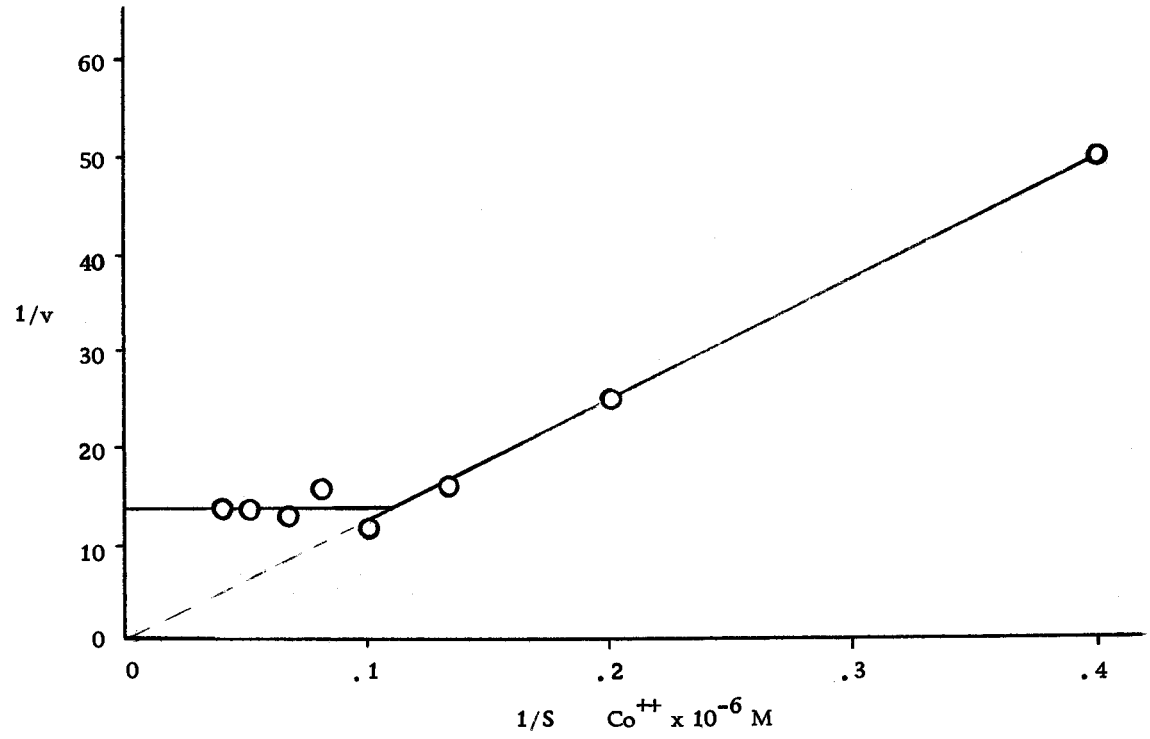

Figure 30. Lineweaver-Burk plot of data of Figure 29.

Co^{++} substitution for the cofactor

Figure 29.

slope	0.0083
intercept	0.075
inflection point	8.8

Figure 30. Lineweaver-Burk plot of Figure 29.

slope	125	1/slope	0.0080
intercept	13.8	1/intercept	0.072
inflection point	0.11	1/inflection point	9.1

soluble in water, HCl, NaOH, 95% ethanol and acetone, and is insoluble in ether and chloroform.

The compound does not appear to be an active reducing agent, since no activity was detected with the triphenyl tetrazolium chloride test, which can detect 7-10 μg/ml active reducing function. Aldehyde and sulfhydryl groups are absent.

There was no visible or ultra-violet absorbing material in the cofactor fraction detectable with the Cary recording spectrophotometer.

The cofactor does not appear to be an amino acid, since amino acids detected in the active fraction could not substitute for the cofactor in the assay system.

Since the cofactor was bound to a cation exchange column, either a positively charged organic compound, or the cofactor is a metal ion. An emission spectrum run with PPD and the TLC active band indicated that K, P, Ni, and Al were present. Co was detectable.

CONCLUSIONS AND SUMMARY

The cell extract synthesis of glutamic-γ-semialdehyde shows characteristics of an allosteric system. Sigmoidal plots of activity, indicating cooperative interactions, were obtained when the substrate was varied. The whole soluble fraction was effective over a narrow range of concentration in promoting GSA synthesis indicating that small changes in enzyme-activator concentration produced large changes in enzyme activity. Proline, an endproduct of the reaction sequence, not structurally similar to the substrate or reaction product, inhibited the reaction. Sigmoidal inhibition curves were obtained. This is consistent with observations that endproduct control of proline synthesis in whole cells occurs in the first reactions in formation of GSA in the proline sequence.

The membrane fraction and a low molecular weight compound in the soluble fraction were components of the cell extract required for *in vitro* synthesis. A high molecular weight component of the soluble fraction, probably protein, stimulated or inhibited GSA formation depending on its concentration. Glutamate, pyruvate, and possibly phosphate were also required for *in vitro* synthesis.

If one assumes that GSA formation is similar to other known semialdehyde syntheses where the γ-carboxyl group is activated prior to reduction, then roles of the components of this assay system

can be postulated. The activation step is enzyme dependent, the cofactor can react with the substrate, perhaps mediate its binding to the enzyme for activation. Pyruvate, metabolized by the enzymes of the membrane fraction, could supply ATP, if that is involved in activation. The activated glutamate could remain bound to the enzyme, or perhaps an adenylated phosphate intermediate is formed preventing the activated glutamate from cyclizing. The membrane fraction would probably supply any reduced TPN needed for the reduction step. The high molecular weight component of the soluble fraction might originally be a protein component of the membrane fraction which has been partially solubilized by the sonication procedure in the preparation.

A procedure was developed for isolation of relatively large amounts of cofactor. The whole soluble fraction was dialysed against distilled water and the dialysate concentrated by evaporation. The cofactor could be extracted from the residue with acetone. The supernatant of the acetone soluble fraction was evaporated to dryness and the residue was brought up in a small volume of water. Further purification can be obtained with thick layer silica gel or paper chromatography.

Characterization of the cofactor indicated that it was a stable, simple organic compound or an inorganic compound such as a metal ion. Amino acids did not substitute for this cofactor, nor did other

complex cofactors. Co^{++} has a marked effect on the assay. The effect of proline on Co^{++} activation and the effect of the high molecular weight component on Co^{++} activation bear further investigation.

Difficulties were encountered in isolating a low molecular weight compound where the means of identification was its activity in promoting GSA synthesis. The compound lost activity with time, purity was difficult to achieve and some uncertainty existed that characteristics determined were those of the active compound. Some problems with the assay were due to the presence of endogenous proline, which inhibits GSA formation.

Viable cells were shown to be present in the E fraction when E was incubated on nutrient agar plates. The total effect of whole cells in the cell extract system is not known, but it has been shown that the cofactor is required for GSA production in the cell extract system which contains glutamate and pyruvate. Whole cells require only glutamate and glucose or pyruvate for GSA production.

Preliminary work on a crude in vitro system for glutamic-γ-semialdehyde synthesis in Escherichia coli has been described. Emphasis of this work was on the isolation and characterization of a low molecular weight compound required for this synthesis. An isolation procedure is described by which relatively large amounts of cofactor can be partially purified. Some characteristics of this compound have been determined, and a possible mode of action is

described. The effect of viable cells in the enzyme fraction and presence of endogenous proline present important problems for future work on this assay system.

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APPENDICES

Appendix I

Scheme for activated substrate combining with enzyme in case where amount of activator which is tied up on enzyme in activated enzyme-substrate complex is significant relative to total activator (26, p. 116-134). An example of such a mechanism would be a magnesium salt of a phosphate ester which combines with and is acted upon by a Mg-activated enzyme.

The scheme:

$$S + A \underset{-1}{\overset{1}{\rightleftharpoons}} Sa;\quad E + Sa \underset{-2}{\overset{2}{\rightleftharpoons}} Ca;\quad Ca \xrightarrow{3} E + A + P$$

E = enzyme

S = substrate

A = activator

Sa = activated substrate

Ca = activated enzyme-substrate complex

P = products

Total amounts of substrate, activator and enzyme are conserved.

$Et = E + Ca$ $\qquad K_1 = \frac{k_{-1}}{k_1}$

$At = A + Sa + Ca$

$St = S + Sa + Ca$ $\qquad K_2 = \frac{k_{-2} + k_3}{k_2}$

Sa is determined by: $S + A \underset{-1}{\overset{1}{\rightleftharpoons}} Sa$; $At = A + Sa + Ca$; $St = S + Sa + Ca$

$$SA = K_1 Sa;\quad At = \frac{K_1 Sa}{S} + Sa + Ca;\quad Ca = \frac{E_t Sa}{K_2 Sa};\quad Sa = \frac{Ca K_2}{Et - Ca}$$

$$St = S + Sa + Ca = S + \frac{K_2 Ca}{Et - Ca} + Ca$$

$$S = St - Ca - \frac{K_2 Ca}{Et-Ca}$$

$$At = Ca + \frac{CaK_2}{Et-Ca} + \left[\frac{K_1 K_2 Ca}{Et-Ca}\right] / \left[St-Ca - \frac{K_2 Ca}{Et-Ca}\right]$$

$$St = Ca + \frac{CaK_2}{Et-Ca} + \left[\frac{K_1 K_2 Ca}{Et-Ca}\right] / \left[At - Ca - \frac{K_2 Ca}{Et-Ca}\right]$$

At and St enter Ca simultaneously as Sa, thus they are symmetrical. Anything true of At will be true of St, they form superimposible curves.

At is a function of Ca. rate $v = k_3 Ca$; the relative rate, $r = v/V$

$$r = \frac{k_3 Ca}{K_3 Et} = \frac{Ca}{Et} \qquad Ca = rEt$$

Rate r is rate relative to maximum possible rate.

Set Ca = rEt, substitute this into At and eliminate Et where possible.

$$At = rEt + \frac{rEtK_2}{Et-rEt} + \left[\frac{K_1 K_2 rEt}{Et - rEt}\right] / \left[St-rEt - \frac{K_2 rEt}{Et-rEt}\right]$$

$$At = rEt + \frac{K_2 r}{1-r} + \frac{K_1 K_2 r}{(St-rEt)(1-r)-k_2 r}$$

To find a value of r for partial saturation value when At is large the denominator of the third term is set equal to zero. The solution is a quadratic equation and the root applicable to a real solution is found.

$$r_a = \tfrac{1}{2}\left[(1 + (St+K_2)/Et) - \sqrt{\frac{(1 + St + K_2)^2}{Et} - \frac{4\,St}{Et}}\right]$$

$$r = v/k_3Et \qquad v = k_3Ca \qquad r_a = \frac{Va}{k_3Et}$$

Partial saturation rates are obtained by $k_3Et(rs)$ or $k_3Et(ra)$

$$Va = \tfrac{1}{2}k_3[\,Et + St + K_2) - \sqrt{(Et + St + K_2)^2 - r\,StEt}\,]$$

$$Vs = \tfrac{1}{2}k_3[\,(Et + At + K_2) - \sqrt{(Et + At + K_2)^2 - 4\,AtEt}\,]$$

If there is tight binding of the activated substrate to the enzyme then K_2 is nearly zero and both curves will be of the titration type. This distinguishes this model from models of enzyme activation.

Assume $K_2 = 0$:

$$Vs = \tfrac{1}{2}k_3\,[\,(Et + At) - \sqrt{(Et + At)^2 - 4\,AtEt}\,]$$

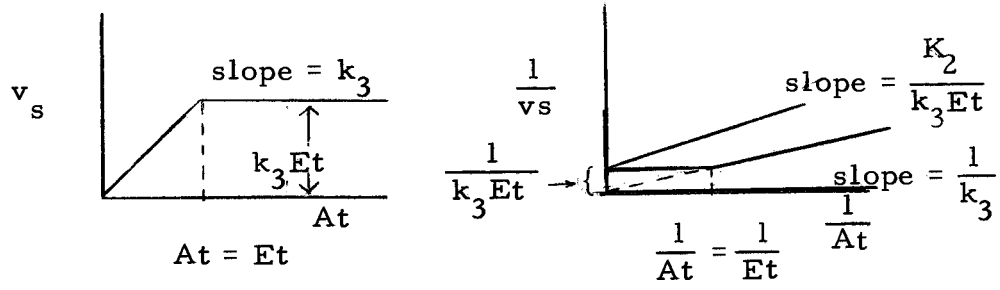
$$= \tfrac{1}{2}k_3[\,(Et + At) - \sqrt{(Et + At)^2}\,]$$

$$= k_3At \text{ when } At \text{ is less than } Et$$

$$= k_3Et \text{ when } At \text{ is greater than } Et$$

$$Va = k_3St \text{ when } St \text{ is less than } Et$$

$$= k_3Et \text{ when } St \text{ is greater than } Et$$



at substrate saturation