PURIFICATION OF FORMYLGLYCINAMIDE RIBOTIDE AMIDOTRANSFERASE FROM CHICKEN LIVER

by

Kiyoshi Mizobuchi

S.B., Kyoto University (1959)

SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September, 1964

Signature of Author..... Signature redacted
Department of Biology
Division of Biochemistry
August 24, 1964

Certified by..... Signature redacted
Thesis Supervisor

Accepted by..... Signature redacted
Chairman, Departmental
Committee on Graduate Students

ABSTRACT

Purification of Formylglycinamide Ribotide Amidotransferase from Chicken Liver

by

Kiyoshi Mizobuchi

Submitted to the Department of Biology, Division of Biochemistry, on September, 1964, in partial fulfillment of the requirements of the degree of Master of Science.

The present work was undertaken to develop a procedure for the isolation in relatively large amounts of highly purified FGAR amidotransferase (2-formamido-N-ribosylacetamide 5'-phosphate: L-glutamine amidoligase (ADP), EC. 6.3.5.3) from chicken liver for use in study of the reactive site of L-azaserine, o-diazoacetyl- L-serine, a stereospecific inhibitor of FGAR amidotransferase. The procedure for the purification of the enzyme involved extraction from acetone powder, ammonium sulfate fractionation, heat treatment, acidification, negative and positive calcium phosphate gel and DEAE-cellulose chromatography.

The analyses by starch gel electrophoresis and sucrose gradient centrifugation showed that the sample prepared by the method was about 70 to 90 per cent pure.

The enzyme was stable within a rather narrow range of pH (about 6.0 to 7.2). However, the addition of the substrate could protect the enzyme against inactivation at higher pH, whereas the incubation of enzyme alone in buffer of higher pH caused the loss of the activity.

The azaserine reactive site on FGAR amidotransferase in chicken liver was compared with that in Salmonella typhimurium. The products after the digestion of azaserine labeled enzyme in chicken liver with papain followed by pronase were identified as N-{2-(L-2-amino-2-carboxyethylthio) acetyl}-DL-serine and N-[2-{L-2-(L-valyl) amino-2-carboxyethylthio}acetyl]-DL-serine, respectively. These results were the same as those obtained with the Salmonella system.

Thesis Supervisor: John M. Buchanan

Title: Professor and Head of the Division of Biochemistry

ACKNOWLEDGEMENT

The guidance and encouragement of Dr. John M. Buchanan were of the most value to the author throughout his graduate training, and are gratefully acknowledged.

Particular thanks are also due to Drs. Thayer C. French and Igor B. Dawid, from whom the author learned much of the knowledge of experimental biochemistry.

The author (1961 to 1964) was supported financially by the National Institute of Health. This is also gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

I. INTRODUCTION

It has been well known that glutamine serves an important metabolic function in a number of enzymatic reactions. Among the biosynthetic reactions involving the transfer of the amide nitrogen of glutamine to give rise to "C-N" bond are the de novo syntheses of formylglycinamidine ribonucleotide from formylglycinamide ribonucleotide (1), of guanosine-5'-phosphate from xanthosine-5'-phosphate (2) (3), of nicotinamide adenine dinucleotide from nicotinic acid adenine dinucleotide (4), of cytidylic nucleotide from uridylic nucleotide (5), and of 5-phosphoribosylamine from 5-phosphoribosylpyrophosphate (6). The enzymes that catalyze the above reactions have been classified as "C-N bond ligase with glutamine", EC. 6.3.5 * (7). These enzymes with the exception of 5-phosphoribosylamine synthetase, require adenosine triphosphate (ATP) as the activator of the reactions. However, in the case of 5-phosphoribosylamine synthesis, the activation of ribose-5-phosphate by ATP occurs in a separate reaction to form the important metabolite 5-phosphoribosylpyrophosphate (8) (9).

Certain diazo compounds, in particular, o-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON), which bear a structural resemblance to the substrate, glutamine, have been found

* According to the Enzyme Commission of the International Union of Biochemistry (7), 5-phosphoribosylamine synthetase has been classified as a group of EC. 2.4.2, pentosyltransferase. However, the characteristics of this enzyme reaction are essentially the same as the others mentioned.

to be effective inhibitors of several of this class of enzyme (3) (4) (5) (10). The facts that azaserine inhibits FGAR amidotransferase (formylglycinamido ribotide amidotransferase or 2-formamido-N-ribosylacetamide-5'-phosphate; L-glutamine amidoligase (ADP), EC. 6.3.5.3) competitively at the site specific for glutamine, and that, once bound to the enzyme, it reacts covalently with a functional group at the site and makes possible a comparative study of the active site of glutamine on these enzymes from the view point of the analogous situation of chymotrypsin (or phosphoglucomutase)-diisopropylfluorophosphate system (11). Moreover, the inhibition of these amidotransferases by p-chloromercuribenzoate has been observed widely, indicating that they may be sulfhydryl enzymes (2) (12).

The ultimate goals of the investigation are to delineate the mechanism of the process of these similar enzyme reactions, and to elucidate the structural characteristics of the active site by the use of glutamine analogues. For such purpose, FGAR amidotransferase has been selected, because it has been extensively studied from the view points of enzymology and protein chemistry by Dr. Buchanan and his coworkers (1) (10) (12) (13) (14) (15).

The first enzymological studies on FGAR amidotransferase described some properties of a fraction that was purified 45 fold from pigeon liver (1). The stoichiometry of the reaction was demonstrated as the following equation:

$$\text{formylglycinamide ribotide} + \text{glutamine} + \text{ATP} + H_2O \xrightarrow{Mg^{++}} \text{formylglycinamidine ribotide} + \text{ADP} + \text{Pi} + \text{glutamate}$$

The Michaelis constants Km for glutamine and for FGAR were determined to be 6.2×10^{-4}M and 6.5×10^{-4}M, respectively. Incubation of P^{32}-labeled orthophosphate or C^{14}-labeled glutamic acid with the enzymatic system did not lead to the synthesis of radioactive ATP or glutamine. It is therefore believed that the reaction catalyzed by FGAR amidotransferase is essentially irreversible (12).

As has been mentioned already, FGAR amidotransferase is inhibited by azaserine or DON in the manner of competitive and partially non-competitive inhibition with the substrate, glutamine (10) (16). However, the incubation of such inhibitors with the enzyme in the absence of glutamine causes rapid formation of an inhibitor-enzyme complex which does not dissociate even upon addition of glutamine. These results suggest that such inhibitors react with the glutamine site on the enzyme. Therefore the determination of azaserine reactive site on the enzyme has been carried out in Dr. Buchanan's laboratory (12) (14) (15).

Preliminary experiments on the azaserine-reactive site in the chicken liver enzyme showed that the product after the acid hydrolysis of chymotryptic digestion of radioactive azaserine-labeled enzyme is S-carboxymethylcysteine (12).

French, Dawid and Buchanan (13) (14) (15) have extended this preliminary experiment to the enzyme of Salmonella typhimurium. In the use of about 50% pure enzyme, they showed that the product after digestion of azaserine-labeled enzyme with papain followed by pronase was N-{2-(L-2-amino-2-carboxylethylthio) acetyl}-L-serine, CS,

which was identical to chemically synthesized CS. The acid hydrolysis of one mole of CS yielded one mole of S-carboxymethylcysteine and one mole of serine. Therefore, it was believed that in the preliminary experiments with chicken liver by Buchanan et al., S-carboxymethylcysteine was formed from CS by acid hydrolysis.

Furthermore, the isolation and analysis of a pentapeptide at the site of binding of azaserine revealed the following sequence: ala-leu-gly-val-cysteine.

The evidence that azaserine reacts with a specific sulfhydryl group of the enzyme is also in accord with the indirect evidence of the inhibition of the enzyme by certain sulfhydryl reagents, such as p-chloromercuribenzoate.

To extend these experiments, the chicken liver system has been chosen. On the other hand, large quantities of highly purified enzyme are required for this type of experiment, so a purification procedure for the enzyme has been developed by the present author. The procedure which yields about 70 to 90% pure enzyme will be presented in Part-1 of this thesis.

Preliminary experiments on the azaserine reactive site of FGAR amidotransferase from chicken liver have been attempted for comparison with the results obtained from the Salmonella system. The results presented in Part-2 show that the reactive site is a specific sulfhydryl group in the chicken liver as well as in the bacterial enzyme.

II

PART-1. PURIFICATION OF FGAR AMIDOTRANSFERASE FROM CHICKEN LIVER

EXPERIMENTAL

Materials

Twice reprecipitated ammonium salt of formylglycinamide ribotide (FGAR) was enzymatically prepared by a modification of the method of Hartman, Levenberg and Buchanan (17). Disodium ATP was obtained from Pabst Laboratories and adjusted to pH 8.0 in an ice bath with KOH by use of a Beckman Zeromatic Electrode. L-Glutamine was purchased from Schwarz Bio Research, Inc. Disodium EDTA (Eastman Product), adjusted to pH 6.5 at room temperature, was used as a chelating agent if necessary.

FGAR amidotransferase free-2-formamido-N-ribosyl-acetamide-5'-phosphate cycloligase (ADP) (EC. 6.3.3.1, AIR synthetase) was prepared from pigeon liver by the method of Levenberg and Buchanan (18) and kept dry at -20° C until used.

Calcium phosphate gel was prepared by the method of Singer and Kearney (19) and was adjusted to pH 7.2 with acetic acid. Calcium phosphate gel-cellulose columns were prepared by the method of Deeb and Hagan (20). DEAE-cellulose (Brown Co.) was washed according to the method of Peterson and Sober (21) (22).

Assay of the activity of FGAR amidotransferase-

The standard assay of FGAR amidotransferase involved the measurement of the rate of formation of 5-aminoimidazole ribonucleotide (AIR) by the method of Bratton and Marshall for analysis of diazotizable amines. AIR-synthetase was included among the components

of the assay system to convert formylglycinamidine ribonucleotide to AIR. The reaction mixture contained in a total of 0.3 ml: 0.4 μmole of L-glutamine; 0.14 μmole of the ammonium salt of FGAR, previously adjusted to pH 5.6; 4.0 μmoles of ATP, pH 8.0; 4.0 μmoles of $MgCl_2$; 6.0 μmoles of KCl; 6.0 μmoles of Tris-HCl buffer, pH 8.0 at 38° C; suitable amounts of AIR synthetase dissolved in 0.03 M potassium phosphate buffer, pH 7.4 and FGAR amidotransferase. The mixture was incubated for 15 minutes at 38° C and then chilled in an ice bath. Then 0.1 ml of 20% trichloroacetic acid solution in 1.33 M potassium phosphate buffer, pH 1.40, was added to stop the reaction, and the precipitate formed was removed by centrifugation. The following chemicals were mixed at room temperature with the supernatant solution without disturbing the protein pellet: 0.05 ml of 0.1% $NaNO_2$; after 3 minutes, 0.05 ml of 0.5% ammonium sulfamate; and after 3 minutes 0.05 ml of 0.1% N-1-(naphthyl)ethylene-diamine dihydrochloride. After 10 minutes the solution was read at 500 mμ with a microcell attachment of the Beckman DU spectrophotometer. As the blank of the assay system, the reaction mixture was incubated without the addition of FGAR amidotransferase.

Under these conditions the activity of the enzyme versus amount of enzyme added (Fig. 1) or the activity of the enzyme versus incubation time (Fig. 2) was linear until the absorbancy at 500 mμ reached approximately 0.6.

For the development of this assay system, the effect of the concentration of each substrate and the effect of pH were examined. In the final conditions of the assay all substrates, except FGAR, were

included at saturating concentration. FGAR, however, was used at about one third of the saturating concentration. It should be noted here that the ratio of concentrations of ATP to $MgCl_2$ was important for the assay system. Fig. 3-a) and b) show the results of the effect of various ATP concentrations (or $MgCl_2$ concentrations) on the assay system at the constant concentrations of $MgCl_2$ (or ATP) in the reaction mixture. As the ratio of ATP to $MgCl_2$ increased, ATP inhibited the assay strongly. However, no such inhibition was observed even in relatively high concentration of ATP, when the ratio of ATP to $MgCl_2$ was 1.0 (Fig. 3-c). In contrast, high concentrations of L-glutamine and FGAR so far tested did not inhibit the assay system.

FGAR amidotransferase exhibited optimal activity in a narrow range around pH 8.0 (Fig. 4). The decrease of the apparent activity at higher pH, such as 8.6, probably results from the denaturation of the enzyme (See later section).

With some preparations of AIR synthetase, the addition of excess amounts of this enzyme to the reaction mixture caused the inhibition of the apparent activity of FGAR amidotransferase in an unknown manner. (Fig. 5, Preparation-2). This inhibition was also observed even after AIR synthetase was extensively dialyzed against 0.03 M potassium phosphate buffer, pH 7.4. Thus, it should also be emphasized that with each new preparation of AIR synthetase it is necessary to check the optimal concentration of enzyme added to the reaction mixture.

One unit of enzyme activity is defined as that amount required to catalyze the formation of product equivalent to absorbancy of 1.0 at 500 mμ per 0.55 ml (final volume of the reaction mixture). The

Fig. 1. Relationships between relative amounts of FGAR amidotransferase and activity.

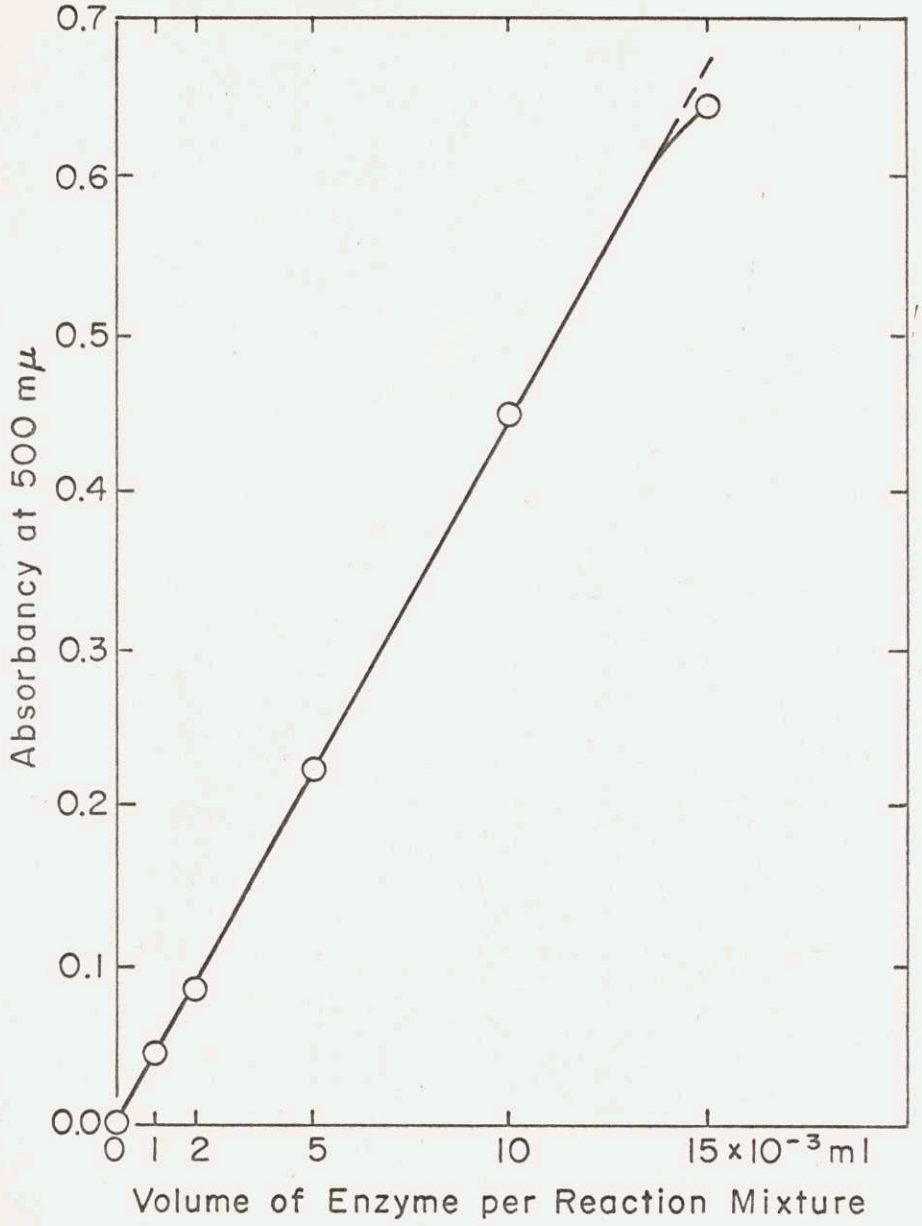

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbancy at 500 mμ
0
1
2
5
10
15 x 10⁻³ ml
Volume of Enzyme per Reaction Mixture

Fig. 2. Effect of incubation time on the assay system. The reaction mixture described in the text was incubated at 38° C for various times. In (A) and (B), 0.001 ml and 0.002 ml of the enzyme solution were used for the assay, respectively.

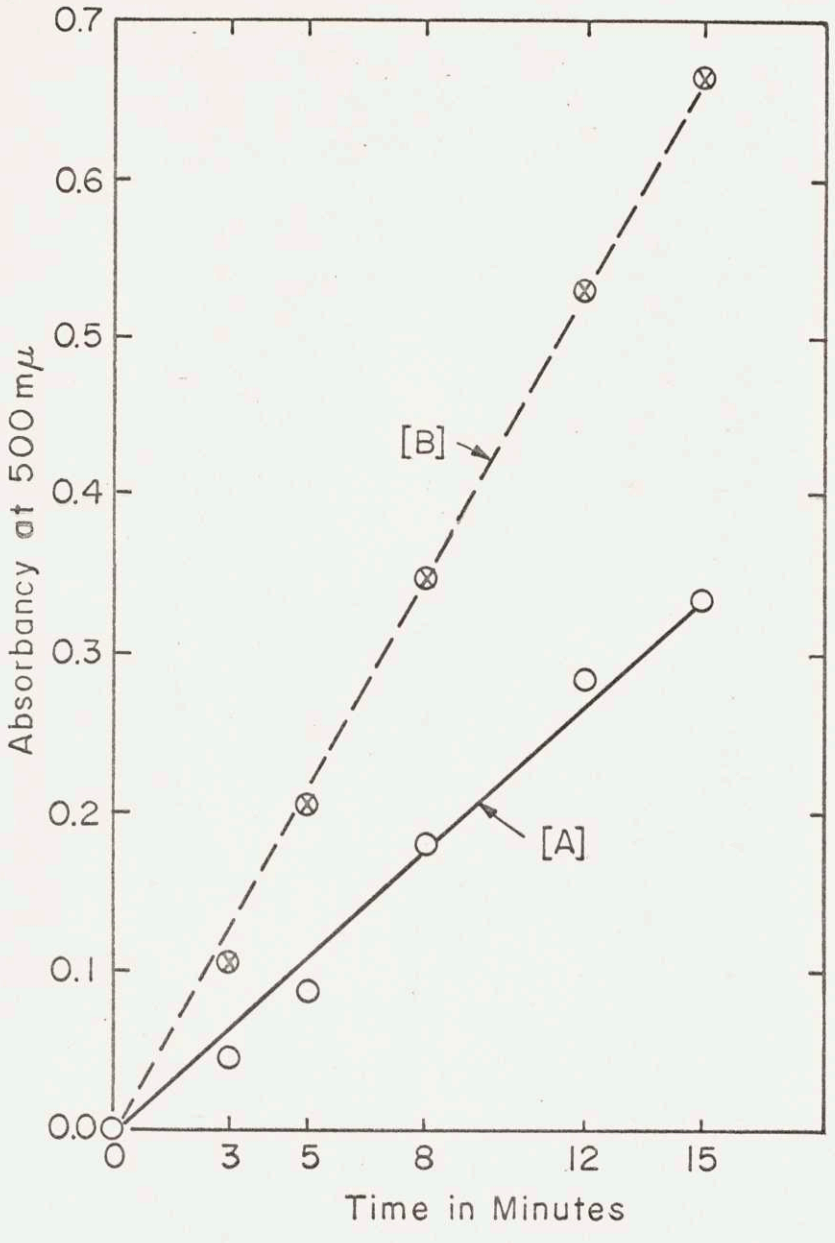

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbancy at 500 mμ
[B]
[A]
0
3
5
8
12
15
Time in Minutes

Fig. 3. Effects of ATP and Mg^{++} on the assay system.

a) Effect of various concentrations of ATP on the assay system. The concentrations of glutamine, FGAR, potassium chloride and Tris-HCl, pH 8.0 were the same as those in the standard assay system. However, 2.0 μmoles (curve A) and 4.0 μmoles (curve B) of $MgCl_2$ were used in this experiment. The arrows designate that the ratio of ATP to $MgCl_2$ is 1.0.

b) Effect of various concentrations of $MgCl_2$ on the assay system. The assay system employed was the same as the standard assay system except for the concentration of $MgCl_2$. The arrow designates that the ratio of ATP to $MgCl_2$ is 1.0.

c) Effect of the ratio of ATP to $MgCl_2$ on the assay system. The molar ratio of ATP to $MgCl_2$ is constant, 1.0.

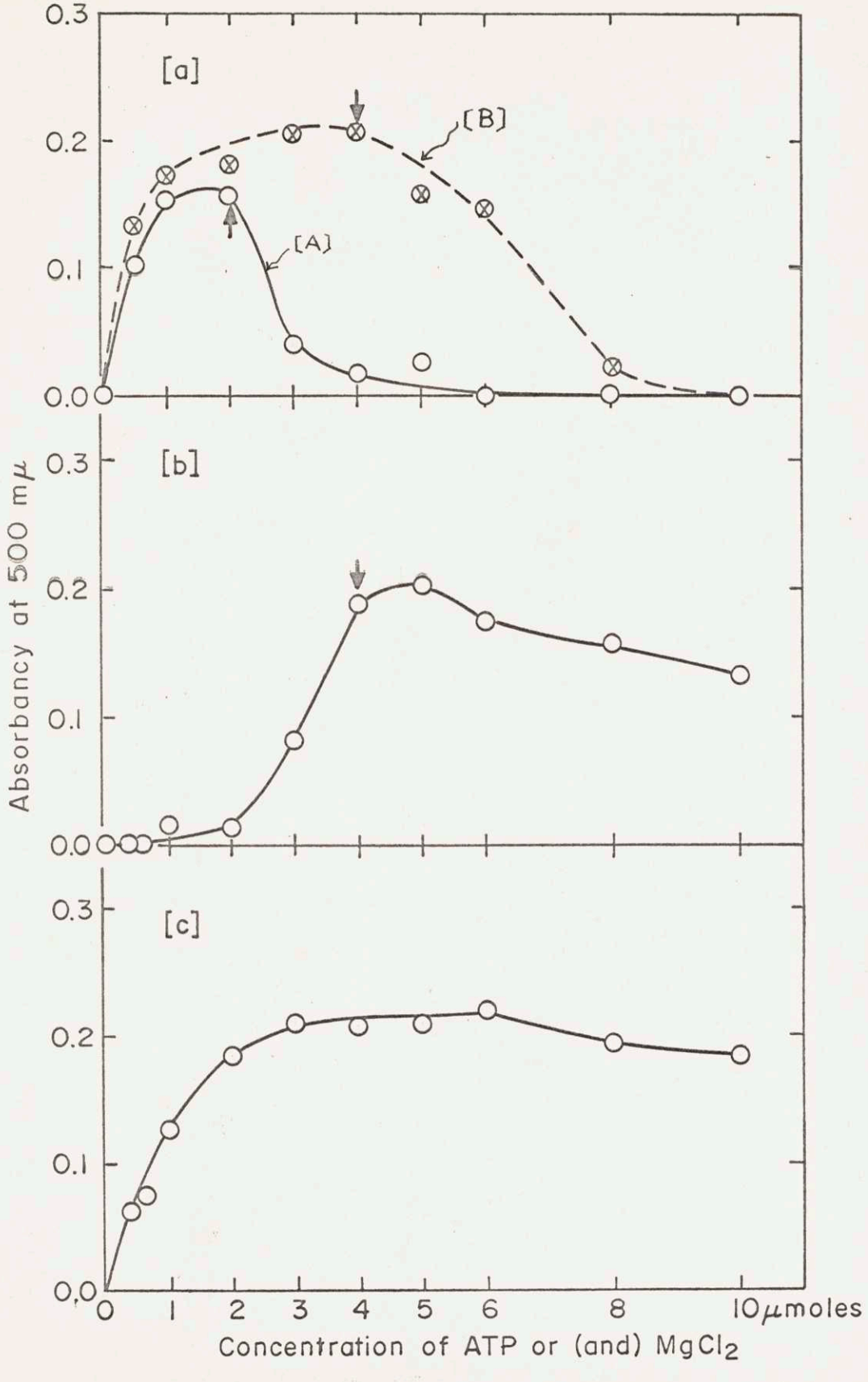

[a]
[B]
[A]
[b]
[c]
0.3
0.2
0.1
0.0
Absorbancy at 500 mμ
0 1 2 3 4 5 6 8 10μmoles
Concentration of ATP or (and) MgCl2

Fig. 4. Optimal pH of FGAR amidotransferase.

⊗, ○ and ▲ represent acetate, potassium phosphate and Tris-HCl buffer, respectively. The concentration of the buffers employed in the experiments was 6.0 μmoles per the reaction mixture.

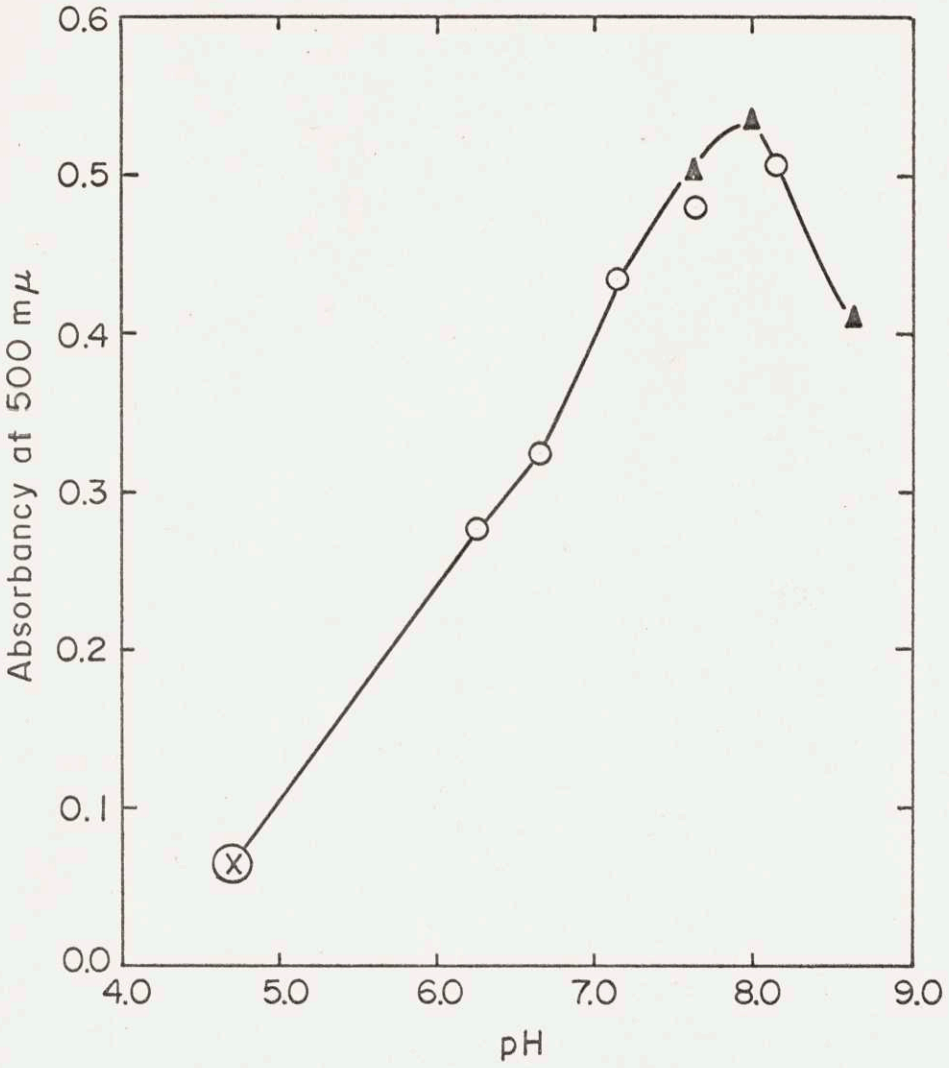
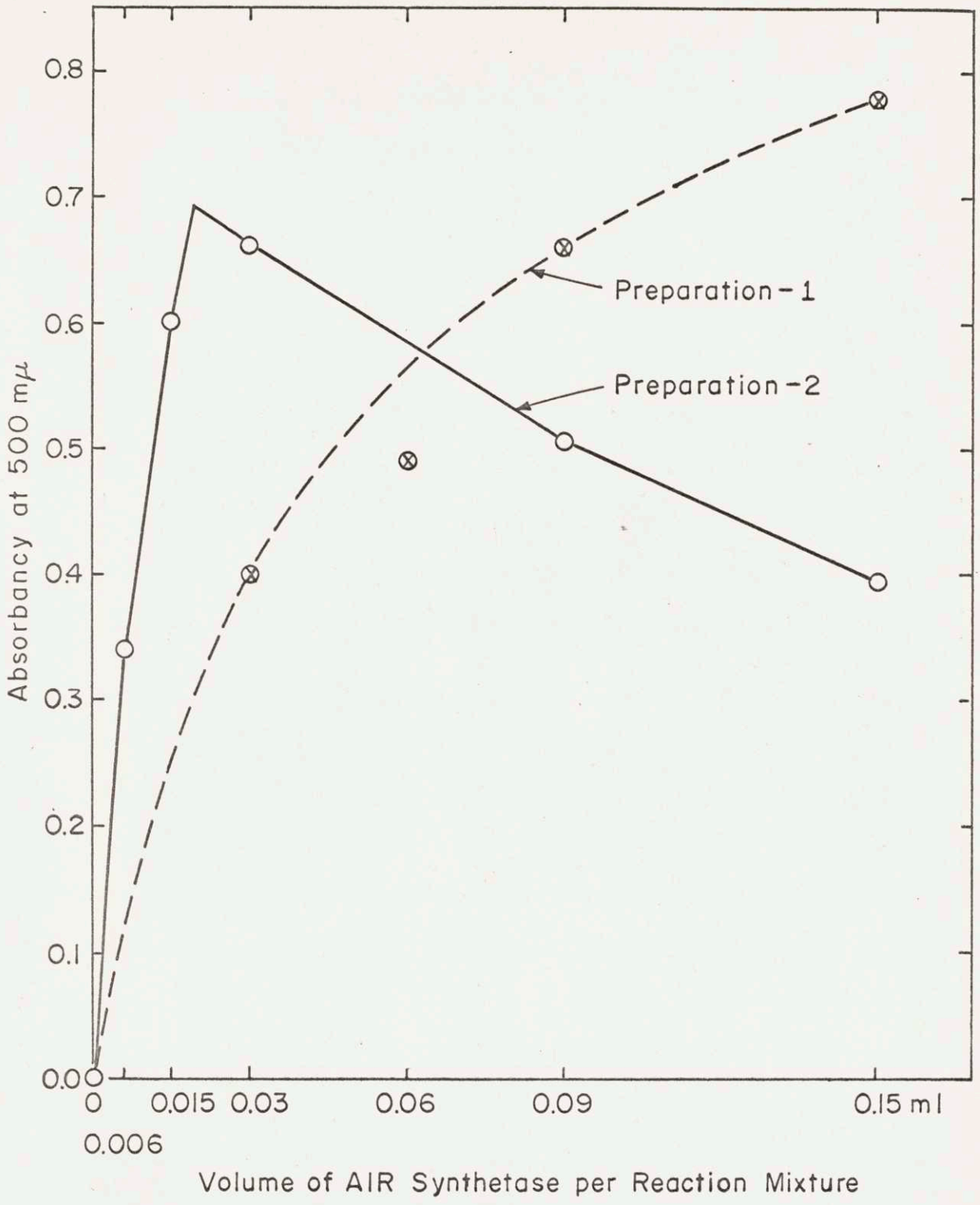

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbancy at 500 mμ
4.0
5.0
6.0
7.0
8.0
9.0
pH

Fig. 5. Effect of amounts of AIR synthetase on the assay system. Different preparations (1 and 2) of AIR synthetase from pigeon liver were used in the assay system described in the text.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbancy at 500 mμ
Preparation - 1
Preparation - 2
0
0.006
0.015
0.03
0.06
0.09
0.15 ml
Volume of AIR Synthetase per Reaction Mixture

specific activity is given as the units of enzyme per mg protein.

Protein concentration was determined by the biuret method (23), which was standarized against bovine serum albumin (*). With dilute solutions a method employing ultraviolet light absorption was used as a measure of protein concentration.

Purification of FGAR amidotransferase from chicken liver-

The purification of the enzyme was carried out in the cold room at approximately 5° C unless otherwise stated.

Step 1. Preparation of acetone powder of chicken liver. Fresh chicken liver (obtained from Merchants Poultry) was ground with a large meat grinder and mixed with acetone (c.p. grade) previously cooled at -20° C. Then the mixture was homogenized in a large waring blender for 30 seconds at a top speed. The homogenate was allowed to stand at -20° C for 60 minutes in order to remove heavy yellow materials. It was then poured into a large Buchner funnel at room temperature. The residue was washed with stirring on the funnel with cooled acetone until the filtrate became almost colorless. Then anhydrous ether was added to the residue on the funnel and the residue was sucked dry. During these operations, the temperature of the residue was maintained at less than 0° C.

* Dr. I. B. Dawid (cited in T. C. French Thesis) showed that a sample of azaserine-labeled FGAR amidotransferase, which was approximately 50 per cent pure, gave an absorbancy in the biuret assay that was 118 per cent of the value that was obtained with an equivalent amount of bovine serum albumin. However, in the present experiments, this correction factor was not used since the exact purity of the enzyme from chicken liver has not been determined accurately.

The dried residue was crumbled by hand and ether was removed over P_2O_5 by application of a vacuum pump for a few hours. Approximately 1,000 g of the powder were obtained from 10 pounds of chicken liver. The powder was sealed at -20° C until used. Under these conditions, FGAR amidotransferase was stable for at least 3 months.

Step-2. Extraction of acetone powder. Various conditions were tried for the extraction of the acetone powder from the view point of obtaining large quantities of the enzyme of high specific activity. Those included in the following procedure were found most suitable: 400 g of acetone powder were extracted with a total of 6,000 ml of 0.01 M potassium phosphate buffer, pH 7.2, containing 5×10^{-4} M L-glutamine and 2×10^{-3} M EDTA, adjusted to pH 6.5. The mixture was stirred mechanically for 40 minutes and centrifuged at about 5,000 x g (top speed of the "Stock" centrifuge) for 35 minutes. The supernatant fraction was used in the next step.

The extract from 400 g acetone powder usually contained about 250,000 to 320,000 units of the enzyme activity of specific activity 2 to 4. The variation of the total units was mainly a function of the freshness of chicken liver used.

Note added: At the beginning of experiments concerned with the purification of the enzyme the powder was extracted twice; for instance, 100 g of powder was first extracted with 1,500 ml of the buffer and then the precipitate from the first centrifugation was reextracted with 1,000 ml of the same buffer. This procedure yielded about 10 to 20 per cent more enzyme activity than that obtained by the single extraction. However, since double extraction took a long time, usually the former procedure was employed.

Step-3. Fractionation with ammonium sulfate. 1,650 g of solid ammonium sulfate were slowly added to 6,000 ml of extract with mechanical stirring over a period of 90 to 100 minutes (This ammonium sulfate concentration corresponded to 45% of saturation at the final point.). The solution was further stirred mechanically for 15 minutes and centrifuged at about 5000 x g in the "Stock" centrifuge for 35 minutes. The supernatant fraction was decanted and discarded. The precipitate, which contained usually 80 to 95% of the original activity, was dissolved in 2,000 ml of 0.01 M potassium phosphate buffer, pH 7.2, containing 5×10^{-4} M glutamine and 2×10^{-3} M EDTA.

Step-4. Heat treatment. The solution obtained in the preceding step was placed in a water bath of temperature 47° to 50° C, and stirred mechanically. After the temperature of the solution reached 45° C, it was maintained for 15 minutes. Then the solution was placed in an ice bath and cooled rapidly to 15° C. It was then centrifuged at 5,000 x g with the "Stock" centrifuge for 35 minutes. The supernatant fraction was used in the next step. The enzyme at this stage was stable at least for 24 hours at 3° C.

Step-5. Fractionation by pH and concentration with ammonium sulfate. The supernatant fraction in the preceding step (pH 6.7 $\pm$ 0.2) was carefully adjusted to pH 4.6 $\pm$ 0.1 with the addition of 1.0 M acetic acid-KOH, pH 3.0 (about 70 ml was required). The mixture was stirred mechanically for 5 minutes, during which time the solution became quite milky. It was centrifuged at 5,000 x g for 35 minutes. The supernatant solution, which was a little bit turbid, was collected and the protein was immediately

precipitated with solid ammonium sulfate at 40% of saturation in the same manner as that described in the Step-3. The precipitate was resuspended in a small volume of 0.01 M potassium phosphate buffer containing 5×10^{-4} M glutamine and 2×10^{-3} M EDTA.

Step-6. Treatment with calcium phosphate gel followed by concentration with ammonium sulfate. After the protein concentration of the resuspension in the Step-5 was adjusted to about 8.0 to 10.0 mg per ml with the buffer, calcium phosphate gel suspension (30 mg per ml) was added drop by drop to the fraction with stirring until the ratio of protein (mg) to gel (mg) reached 1.2. The mixture was stirred further for 5 minutes and centrifuged at 5,000 x g for 20 minutes. The precipitate was suspended twice in a small volume of 0.04 M potassium phosphate buffer, pH 7.2, and centrifuged. To the combined supernatant fractions 0.015 volume of 2.0 M potassium phosphate buffer, pH 7.2, 0.01 volume of 0.1 M of glutamine and 0.008 volume of 0.25 M EDTA, pH 6.5, were added and the mixture was concentrated with solid ammonium sulfate at 45% of saturation in the same way as that described in Step-5. After centrifugation the precipitate was collected and dissolved in a small volume of 0.03 M potassium phosphate buffer pH 6.5, containing 1×10^{-3} M glutamine and 1×10^{-3} M EDTA.

During the process of the gel treatment, no loss of enzyme activity was observed, but the recovery of the activity in the concentration step was usually 75 to 85%. The enzyme solution at this stage was completely clear and of dark brown color.

Step-7. First ("negative") DEAE-cellulose chromatography. The concentrated solution obtained in the Step-6 was dialyzed against 40 volumes of 0.01 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 1×10^{-3} M EDTA for about 10 to 12 hours. It was then applied to a DEAE-cellulose column (4 x 45 cm), which was packed with 3 p.s.i. and equilibrated with 0.01 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 1×10^{-3} M EDTA. If precipitation occurred during the dialysis, it was removed by centrifugation before application of the solution to the column. After the enzyme solution was absorbed into the column, elution was started with the same buffer at a flow rate of 2.0 ml per minute until the first protein peak (indicated by its green-brown color) had come off completely. Under these conditions, the enzyme did not stick to the column, and came off with the colored protein. (If further elution was tried with high ionic strength buffer, two more protein peaks were eluted from the column. However, these peaks did not contain the enzyme activity). The fractions with a specific activity of more than 40 were collected and pooled. After the addition of 0.02 volume of 1.0 M potassium phosphate buffer, pH 6.5, 0.01 volume of 0.1 M glutamine and 0.008 volume of 0.25 M EDTA, the pooled fractions were fractionated with solid ammonium sulfate first between 0 to 33% saturation and then between 33 to 50% saturation. The two fractions were then separately dissolved in a small volume of 0.03 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$. The yield of the activity in the chromatography was usually about 75% of the units from the Step-6.

The "33-50%" fraction contained approximately 90% and the "0-33%" fraction approximately 10% of the activity. Total recovery of the activity during the concentration was usually 80% of the units in the pooled fractions. The total recovery in Step-7 was thus about 60%. The enzyme solution was brown in color at this step.

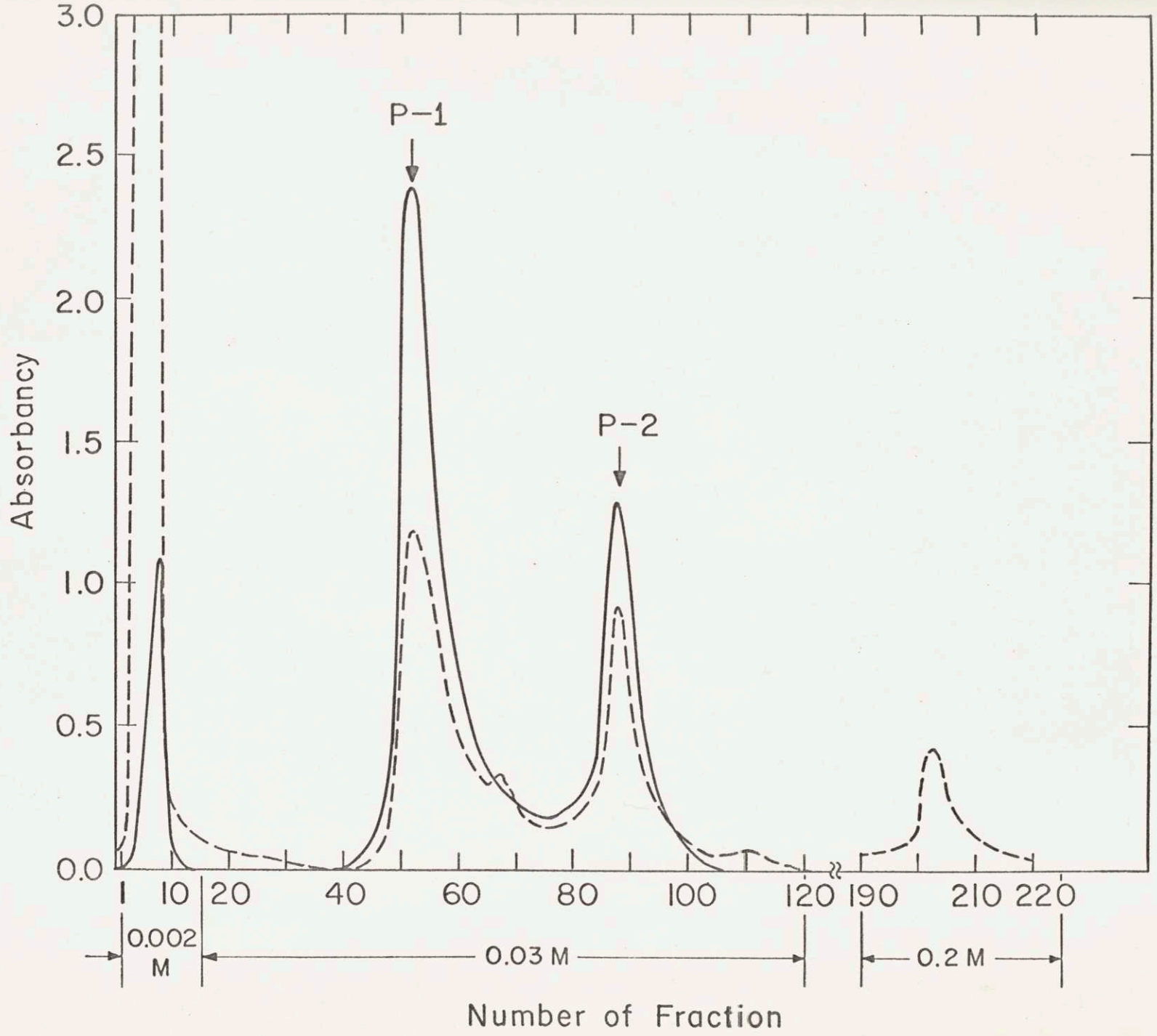
Step-8. 2nd ("positive") DEAE-cellulose chromatography. After the "33-50%" fraction was dialyzed against 40 volumes of 0.01 M potassium phosphate buffer, pH 6.5 containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$ for about 10 hours, the enzyme solution was applied at a flow rate of 1.0 ml per min. to a DEAE-cellulose column (4 x 35 cm), which was packed with 3 p.s.i. and equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$. After the absorption of the sample, the column was washed with 1.0 to 1.5 volumes (column size) of 0.002 M potassium phosphate buffer, pH 7.0 containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$. Then about 5 to 7 volumes of 0.03 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$ were applied to the column. In the first part of the chromatography about one third of the protein, which was dark brown in color, was eluted. Then two protein peaks with the enzyme activity were eluted separately during the second phase of the chromatography. Further elution of the column with high ionic strength buffers yielded one more peak which did not contain activity. The same chromatographic pattern was obtained, if instead, the proteins were eluted with potassium phosphate buffer, pH 6.5, applied as a linear gradient between 0.008 M and 0.04 M. The two enzyme peaks were pooled

separately and each was precipitated with solid ammonium sulfate at 50% of saturation. They were then dissolved in small volumes of 0.03 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$. The recovery of total activity in this step was again 60%. The concentrated protein solutions were light yellow. The chromatographic pattern is shown in Fig. 6.

Step-9. 3rd Chromatography on calcium phosphate gel-cellulose. The solutions from the preceding step were dialyzed against 100 volumes of 0.02 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$ and were then applied to calcium phosphate gel-cellulose columns (4 x 10 cm), which had been equilibrated with the same buffer. After absorption of the sample, the top of the columns were mixed with a glass rod to make a homogeneous distribution of the protein in the column. Then the columns were washed with 1.5 volumes of the same buffer. In this process, a small peak of protein without enzyme activity was first eluted. Then a linear gradient elution (with 500 ml of 0.02 M potassium phosphate buffer pH 6.5, containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$ in the mixer and 500 ml of 0.3 M potassium phosphate buffer pH 6.5 containing 1×10^{-3} M glutamine and 2×10^{-3} M $MgCl_2$ in the reservoir) was performed at a flow rate of 0.5 to 0.7 ml per minute. Under these conditions about 70 to 80% of the activity was recovered in this step and was separated from a yellow protein, which stuck tightly to the column and could not be eluted.

The tubes containing the activity were collected and pooled. Then the protein of the pooled fractions of each of the two chromatographic

Fig. 6. Second ("positive") DEAE-cellulose chromatography of FGAR amidotransferase. The solid line represents FGAR amidotransferase activity (per 0.01 ml) measured at 500 mμ, and the dashed line is absorbancy at 280 mμ. In this experiment, the enzyme was eluted to some extent at the first phase of the elution. This resulted from an overload of protein applied onto the column.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbancy
P-1
P-2
1
10
20
40
60
80
100
120
190
210
220
0.002 M
0.03 M
0.2 M
Number of Fraction

procedures (see Step-7) was precipitated with ammonium sulfate at 50% of saturation and was then dissolved in a small amount of 0.05 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 1×10^{-3} M EDTA. These concentrated solutions were dialyzed against the above buffer in order to remove ammonium sulfate. At this step, the enzyme solutions (about 5.0 mg per ml) were almost colorless.

The results of the above purification procedure are summarized in Tables I and II.

The enzyme was also highly purified by a modification of the procedure in which alcohol fractionation followed by heat treatment was used in place of second and third chromatographic steps. The pooled fraction of the first DEAE-cellulose step was further fractionated by addition of 90% ethylalcohol (previously cooled at -20° C) to a concentration of 20%. The temperature of the resulting aqueous alcohol solution was maintained at -10° C. The precipitate formed was removed by centrifugation at -10° C and was resuspended in 0.03 M potassium phosphate buffer, pH 6.5, containing 1×10^{-3} M glutamine and 1×10^{-3} M EDTA. It was immediately dialyzed against the same buffer for 5 to 10 hours. Then the dialysate was placed on a water bath at 45 to 47° C. After 5 to 7 minutes incubation in the bath, the precipitate formed was removed by centrifugation at 0° C. However, this procedure, especially the alcohol fractionation step, could not be easily reproduced even though in one instance enzyme of the highest specific activity (550-600) was obtained.

TABLE I

Purification of FGAR Amidotransferase from Chicken Liver

Fraction		Volume (ml)	Protein (mg)	Units	Specific Activity	% Recovery of Units	
Extract		6,000	81,000	312,000	3.85	(100)	
$(NH_4)_2SO_4$		2,000	21,200	261,000	12.4	83.7	
Heat		2,000	12,600	245,000	19.4	78.5	
Acidification		800	6,320	129,000	20.4*	41.3*	
Calcium phosphate gel		150	2,670	112,000	42.0	35.8	
1st (Negative) DEAE-cellulose		50	890	85,000	95.6	27.3	
2nd (Positive) DEAE-cellulose	P-1	20	138	21,400	155	6.8	10.0**
	P-2	20	80	10,000	125	3.2	
Calcium phosphate gel-cellulose	P-1	8	32.8	15,100	460	4.9	6.4
	P-2	6	12.0	4,800	400	1.5	

400 g of the acetone powder was used as starting material.

*Low activity and its recovery resulted from addition of excess amounts of KOH-acetate buffer.

**Low recovery of activity resulted from an overload of protein onto the column.

TABLE II

Purification of FGAR Amidotransferase from Chicken Liver Acetone Powder

Fraction		Volume (ml)	Protein (mg)	Units	Specific Activity	% Recovery of Units	
Extract		3,000	33,000	117,000	3.55	(100)	
$(NH_4)_2SO_4$		1,000	9,600	113,000	11.8	96.7	
Heat		1,000	6,500	97,600	15.0	83.5	
Acidification		500	3,840	87,000	22.6	74.4	
Calcium phosphate gel		100	1,720	71,200	41.4	61.0	
1st (Negative) DEAE-cellulose		40	600	41,600	69.5	35.5	
2nd (Positive) DEAE-cellulose	P-1	7.5	53.5	8,350	157	7.2	20.6
	P-2	17.4	100.0	15,600	156	13.4	

200 g of the acetone powder was used as starting material.

After the positive DEAE-cellulose, the samples were used for establishment of conditions for calcium phosphate gel-cellulose chromatography. Therefore, the final data is not available for this experiment.

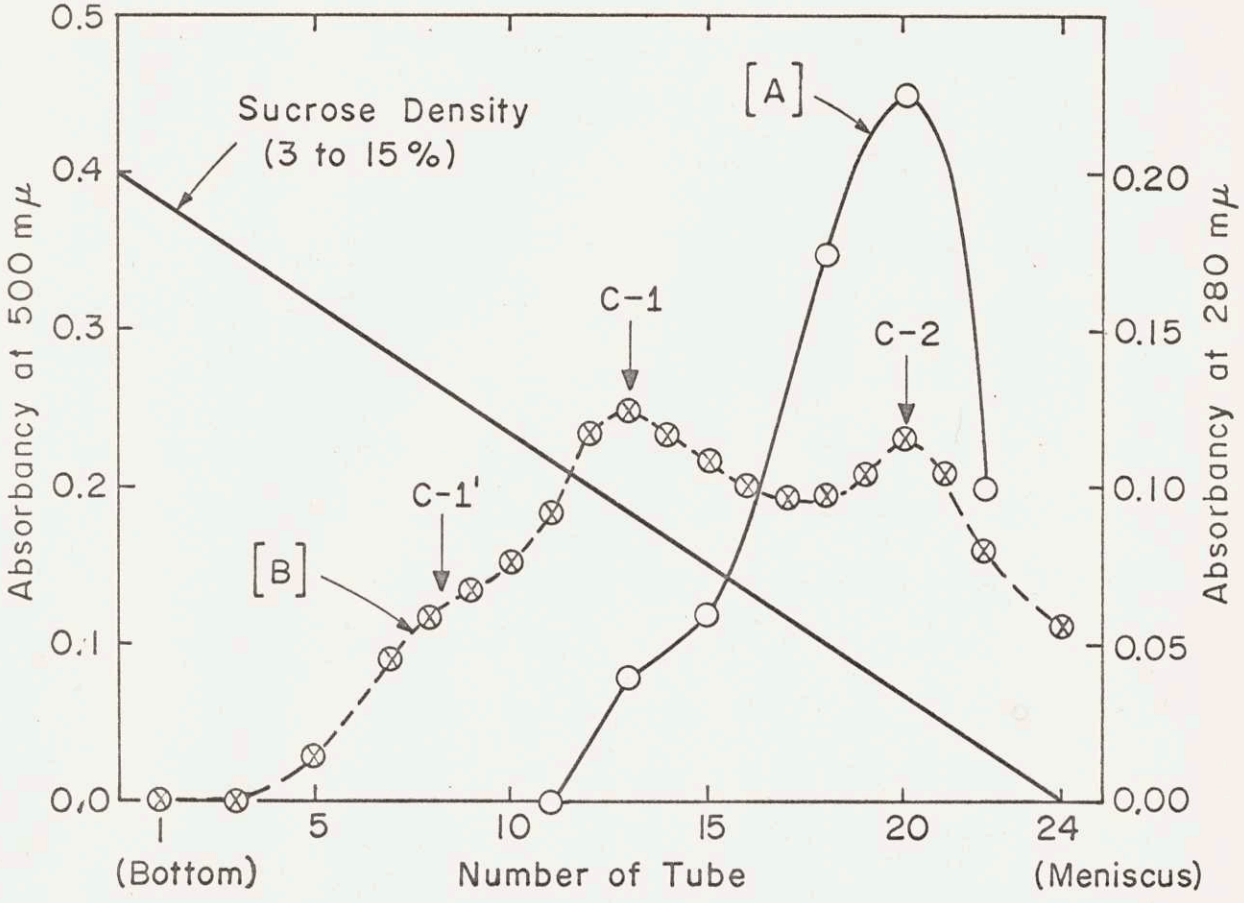
Criteria of the purity of FGAR amidotransferase.

A) Sucrose gradient centrifugation. Preliminary examinations on the determination of the purity of the enzyme preparation were carried out by the sucrose gradient centrifugation technique. The sample from Step-8 was used for the most part. Its specific activity was 169. The method employed was essentially that described by Martin and Ames (24). The 3 to 15 per cent sucrose gradient was made in a buffer consisting of 0.01 M potassium phosphate, pH 6.5, 5×10^{-4} M glutamine and 2×10^{-3} M EDTA. After centrifugation for 8.5 hours at 38,500 r.p.m. at 0° C, the bottom of the centrifuge tube was punctured with a needle and aliquots of five drops per tube were collected with weak suction. The protein content in each fraction was measured with a Gilford automatic recorder and the enzyme activity was measured by the standard assay system. The high concentrations of sucrose did not inhibit the assay. Fig. 7 shows the results of one such experiment. This preparation contained two main (C-1 and C-2) and one minor ($C\text{-}1^1$) protein components. The enzyme activity corresponded to the C-2 peak, although the pattern of the activity was slightly skewed toward the bottom. The amount of C-2 component was about 25 to 35 per cent to the total amount of protein applied, an indication that an enzyme preparation, in which the specific activity was about 500 to 680, might correspond to a pure preparation.

B) Starch gel electrophoresis. Starch gel electrophoresis was a more convenient means for the examination of the purity of enzyme samples. In two experiments the samples carried through the Step-9 (460 and 400 specific activity, respectively) were used. The gel was prepared in 0.05 M

Fig. 7. Sucrose gradient centrifugation of FGAR amidotransferase. The solid line (A) represents the activity of FGAR amidotransferase (per 0.01 ml) and the dashed line (B) is absorbancy at 280 mμ.

The sample employed in this experiment was obtained from the first DEAE-cellulose column chromatography. Its specific activity was 169.


Sucrose Density
(3 to 15%)
[A]
[B]
C-1
C-1'
C-2
Absorbancy at 500 mμ
Absorbancy at 280 mμ
0.5
0.4
0.3
0.2
0.1
0.0
0.20
0.15
0.10
0.05
0.00
1
(Bottom)
5
10
15
20
24
(Meniscus)
Number of Tube

potassium phosphate buffer, pH 7.6, according to the method of Smithies (25) (26) and was poured on a tray (0.7 x 11, 7 x 25.0 cm), the sample (5.0 mg protein per ml) was applied on the gel through a piece of Whatman No. 3 MM paper (0.6 x 2.0 cm) and was run for 36 hours at 3 to 7 milliamperes per cm^2 in the cold room. The results showed that each sample yielded only one band of protein that was detectable, although it had diffused to some extent. It migrated about 2.0 cm from the origin toward the anode under these conditions.

Stability of the enzyme.

In the course of the purification of the enzyme the stability of the enzyme against temperature and pH was examined.

The enzyme in a buffer consisting of 0.01 M potassium phosphate, pH 7.2 (or 0.03 M pH 6.5), 5×10^{-4} M glutamine and 2×10^{-3} M EDTA was heated to various temperatures (Table III).

The enzyme was stable at 45° C at least for 30 minutes, but was unstable at more than 50° C even for a short time.

Table IV shows that adjustment of pH to 5.0 (or 4.75) with acetic acid--KOH (1.0 M, pH 3.0) did not cause inactivation of the enzyme. However, the enzyme was rapidly denatured at pH 4.0 (within 10 min.). In Fig. 8 (a) is shown the results of experiments concerned with the stability of the enzyme at rather high pH. When FGAR amidotransferase was dissolved at 0° C in Tris-HCl buffer (even pH 7.2) much activity was lost. Fig. 8 (b) shows the extent of inactivation of the enzyme activity at pH 8.0 as a function of time. These data indicate that the enzyme is stable only within a narrow range of pH. Although the optimal activity in the assay system was at pH 8, the incubation of the enzyme at this pH in

TABLE III

Stability of the Enzyme Activity at Various Temperatures

Treatment	Absorbancy at 500 mμ	% Recovery of Activity
EXPT.-I		
no treatment (0° C)	0.300	(100)
45° C. 15 min.	0.300	100.0
48° C. 15 min.	0.290	96.7
51° C. 15 min.	0.205	68.4
54° C. 7 min.	0.116	38.7
57° C. 5 min.	0.057	19.0
60° C. 5 min.	0.051	17.0
EXPT. -II		
no treatment (0° C)	0.203	(100)
45° C. 7 min.	0.203	100
45° C. 15 min.	0.200	100
45° C. 22 min.	0.206	100
45° C. 30 min.	0.203	100

TABLE IV

Stability of the Enzyme at Various pH Values in the Acid Range

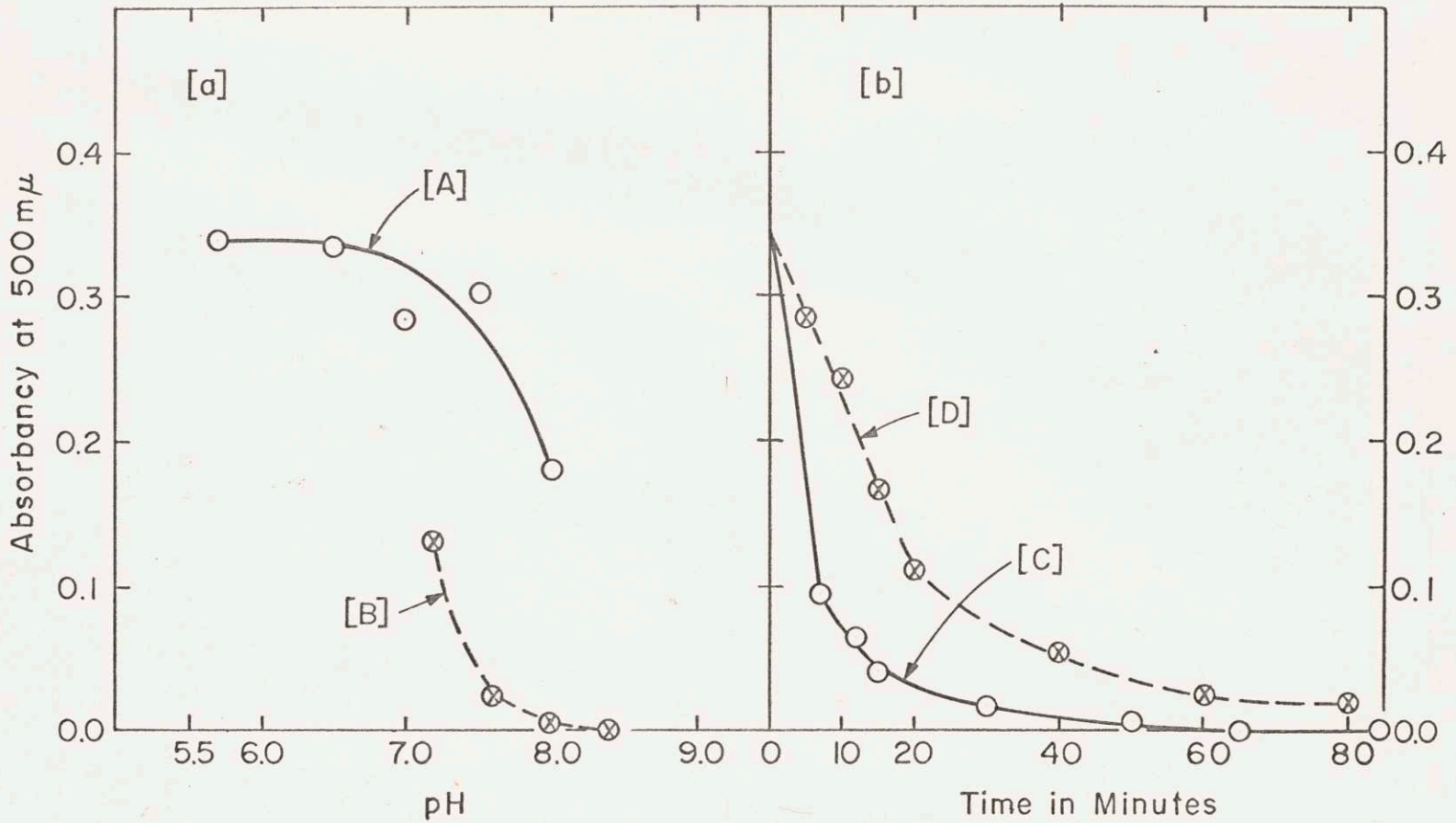
pH	Absorbancy at 500 mμ	% Recovery of Activity
6.7 (control)	0.227	(100)
5.0 (supernatant)	0.200	88.3
4.75 (supernatant)	0.235	103.
4.25 (supernatant)	0.192	84.8
4.0 (supernatant)	0.035	15.5
4.0 (precipitate*)	0.000	0

The pH values indicated were adjusted with the addition of 1.0 M acetic acid--KOH, pH 3.0. The sample employed in this experiment was obtained from the heat treatment (Step-4) .

*The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 6.5 and then the activity of the enzyme was measured.

Fig. 8. a) Stability of FGAR amidotransferase activity at various pH values.
Curve A: 0.05 M potassium phosphate buffer was used.
Curve B: 0.05 M Tris-HCl buffer was used.
The enzyme was incubated in a buffer at 0° C for 100 minutes, and then its activity was measured with the standard assay system.

b) Time course of the inactivation of FGAR amidotransferase at 0.05 M Tris-HCl buffer, pH 8.0.
Curve C represents the inactivation of the enzyme activity in the buffer alone.
Curve D showes the inactivation of the enzyme activity in the presence of the buffer and 2×10^{-3} M ATP and $MgCl_2$. All experiments were done at 0° C.


[a]
[b]
[A]
[B]
[C]
[D]
Absorbancy at 500 mμ
0.0
0.1
0.2
0.3
0.4
5.5
6.0
7.0
8.0
9.0
pH
0
10
20
40
60
80
Time in Minutes

the absence of cofactors and substrates resulted in loss of activity. Therefore, a more systematic study of the relationship of pH to enzyme activity was carried out to find a condition for the stabilization of the enzyme. In Table V and VI is shown the results of these experiments. The addition of FGAR (2×10^{-3} M), glutamine (2×10^{-3} M), ATP (2×10^{-3} M) and $MgCl_2$ (2×10^{-3} M) in the presence of 0.05 M Tris-HCl buffer, pH 8.0, resulted in almost no inactivation of the enzyme even during 100 minutes of incubation, whereas in the presence of buffer but in the absence of substrates and cofactors, about 95% of the activity was lost. The addition of one of the substrates showed that the inclusion of each substrate could protect the activity of the enzyme against the pH inactivation to some extent, for example, 60% of the activity was retained in the presence of FGAR alone, 30 to 40% in the presence of glutamine and 5% in the presence of Mg^{++} and ATP. The addition of either FGAR plus glutamine or FGAR plus ATP and Mg^{++} protected the activity to the same extent as that found in the complete substrate and cofactor system and the extent of the protection in the presence of both glutamine and Mg^{++}/ATP was also significantly higher than with glutamine alone.

In addition to the effects seen at high temperature and at lower or higher pH, dialysis against a buffer of relatively low ionic strength (less than 0.01 M potassium phosphate, for example) or against ammonium sulfate solution resulted in inactivation of the enzyme.

However, dialysis against solutions containing a high concentration of EDTA (i.e. 0.6%) and KCl (i.e. 0.9%) did not result in

TABLE V

Protection of the Enzyme Activity by Substrates and Cofactors Against pH 8.0 Tris-HCl Buffer (0.05 M)

Addition			EXPT. I		EXPT.-II		Average of
FGAR	glu-NH_2	ATP/Mg^{++}	Absorbancy at 500 mμ	% Recovery	Absorbancy at 500 mμ	% Recovery	EXPT.-I and II
+	+	+	0.300	98.0	0.307	93.2	95.6
-	+	+	0.210*	68.5			
			0.190	62.0	0.219	66.3	65.6
+	-	+	-	-	0.305	92.4	92.4
+	+	-	0.300	98.0	0.318	96.2	97.1
-	-	+	0.020*	6.5	0.024	7.3	7.5
			0.027	8.8			
-	+	-	0.140	45.7	0.140	35.2	40.5
+	-	-	-	-	0.220	66.6	66.6
-	-	-	0.010	3.2	0.024	7.3	5.3
no treatment (pH 6.5 , potassium phosphate buffer)			0.307	(100)	0.330	(100)	(100)

* 2×10^{-2} M ATP and $MgCl_2$ were used.

The experimental methods were described in the text. The sample employed in this experiment was obtained from that after second DEAE-cellulose step.

TABLE VI

Protection of the Enzyme Activity by Mg^{++} and Glutamine Against pH 8.0 Tris-HCl Buffer

Additions			
Mg^{++} (4×10^{-3} M)	Glu-NH_2 (2×10^{-3} M)	Absorbancy at 500 mμ	% Recovery
+	+	0.287	80.7
-	+	0.140	39.6
+	-	0.082	23.1
-	-	0.049	13.7
no treatment (pH 6.5, potassium phosphate buffer)		0.355	(100)

The experimental conditions were the same as those empolyed in Table V.

inactivation so far as has been tested.

Discussion

In previous attempts to purify FGAR amidotransferase, Herrmann (unpublished paper) has described a procedure that reportedly resulted in a purification of the chicken liver enzyme approximately 2000 fold. These results are possibly open to some question because of the difficulty experienced at that time with the linearity of the assay system. French et al. (13) have now developed a much improved and reliable assay system and have obtained enzyme from Salmonella typhimurium that is approximately 75 to 95% pure. However, this procedure was difficult to reproduce.

There are certain differences in the properties of the enzymes from the two different sources. The activity of the bacterial FGAR amidotransferase is lost upon treatment of the enzyme with calcium phosphate gel (French, personal communication) whereas the chicken liver enzyme is stable under the same conditions. Furthermore, the bacterial enzyme stuck tightly to a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer, pH 6.5, but the chicken enzyme did not under the same conditions. These facts presumably indicated that at least some of the physical characteristic of the two enzymes are different from each other.

The method of purification presented here, therefore, has been developed on the basis of the procedures previously reported by Herrmann and French et al. The method described here resulted in a purification of about 120 fold. Based on the activity in the extract the

yield was approximately 10 to 20%. Upon electrophoresis of this sample at pH 7.5 on starch gel only one band could be observed. Analysis of purity by sucrose gradient centrifugation suggested that this sample might be 70 to 90% pure. However, it should be said, at the present time, that the conclusive demonstration of the purity of this sample requires a further analysis.

The yield of the activity up to the first calcium phosphate gel step was usually 65% of the total activity extractable from the acetone powder. This value was mostly dependent on the success of the acidification step, since a long incubation at lower pH resulted in loss of the activity.

Besides DEAE-cellulose chromatography, chromatography on carboxymethyl cellulose, phosphocellulose, amberlite IRC 50, dextran gel Sephadex G100 and G200, and Pevikin zone electrophoresis were tested for their effectiveness in the purification of the enzyme. However, no satisfactory results were obtained with these materials. It should be noted that the enzyme migrated toward cathode at pH 6.5 during electrophoresis on Pevikon and toward anode at pH 7.5 during electrophoresis on starch gel. These results indicated that the isoelectric point of the chicken enzyme might be in the vicinity of pH 7. On the other hand the isoelectric point of the Salmonella enzyme was around pH 4.0 (16) The chicken enzyme was excluded by Sephadex G100 dextran gel but included on Sephadex G200, an indication that the molecular weight was less than 200,000.

It has been reported by Herrmann (unpublished) and French et al. (13) that the addition of glutamine to the buffer system during the purification of the enzyme was effective in the protection of the activity. This observation has been confirmed in the experiments concerned with the stability of the enzyme (see Tables V and VI). Furthermore, the addition of Mg^{++} plus glutamine was more effective than that of glutamine alone. The protection of the activity by the substrates against the pH inactivation, especially the differential protection by each substrate, was of interest. Not only did this observation suggest that the active site of glutamine on the enzyme might be different from that of FGAR, but also that the interaction of the enzyme with substrate gave rise to a more suitable conformation that resulted in the protection of its activity against denaturation.

In the purification of FGAR amidotransferase in Salmonella typhimurium, French, Dawid, Day and Buchanan (13) showed that the extract from the acetone powder gave three different enzyme peaks on chromatography with DEAE-cellulose when the sample was directly applied on the column without the heat treatment at 45° C in the presence of pancreatic ribonuclease. However, after the heat treatment, only one enzyme peak was detectable on the column. On the contrary, in chicken liver two peaks (P-1 and P-2) were obtained upon "positive" chromatography on DEAE-cellulose even after the heat treatment of the enzyme. Therefore, this phenomenon was further examined by chromatography on calcium phosphate gel-cellulose. When each peak was applied separately on the gel column, only one peak of activity was eluted

at a characteristic location on the chromatogram, wheras two peaks of activity were obtained upon application of the mixture of P-1 and P-2 to the gel column again. However, the analysis of these peaks on starch gel electrophoresis at pH 7.5 failed to reveal a difference in the two fractions, although P-2 seemed to migrate faster than P-1 toward the anode. Further study is required for a conclusive answer to this problem.

III

PART-2. AZASERINE REACTIVE SITE ON FGAR AMIDOTRANSFERASE

Azaserine reactive site on FGAR amidotransferase.

A preliminary experiment concerned with the azaserine reactive site of FGAR amidotransferase isolated from chicken liver was carried out with rather crude samples of the enzyme (12). French, Dawid, and Buchanan (14) (15) have extensively studied the same problem with the enzyme of Salmonella typhimurium, so that their experimental procedures could be directly applied to the chicken liver system with minor modifications. The object of the experiments was to compare the azaserine-reactive site of FGAR amidotransferase isolated from Salmonella and chicken liver.

Materials

Unlabeled azaserine, (o-diazoacetyl-L-serine) was generously provided by Drs. Alexander Moore and John Dice of Park, Davis and Company. C^{14}-L-Azaserine (o-diazoacetyl-2-C^{14}-L-serine) was prepared from glycine-2-C^{14} (New England Nuclear Corp.) as the starting material by the method of French, Dawid, Day and Buchanan (13). This procedure was based on the method of Nicolaides, Westland and Wittle (27). One mμmole of glycine-2-C^{14} (and consequently azaserine-C^{14} or radioactive peptides derived from the azaserine labeled-enzyme) was equivalent to 11,000 c.p.m. Radioactivity was measured with a Nuclear Chicago automatic counting system equipped with a D-47 Detector and a "Micromil" window. Radioactive or non-radioactive N-[2-{L-2-(L-Valyl) amino-2-carboxyethylthio} acetyl]-DL-serine (VCS) and N-{2-(L-2-amino-2-carboxyethylthio) acethyl}-DL-serine (CS) were generously prepared by Drs. T. French, I. Dawid and J. Buchanan.

The proteolytic enzymes, papain and pronase, were obtained from Worthington Biochemical Co. and California Corporation for Biochemical Research, respectively.

The volatile buffers employed in the paper electrophoresis are listed in Table VII. Triethylamine was purified according to the method of Sjöquist (28) and was adjusted to various pH values with acetic acid.

Enzymatic digestion of azaserine-C^{14} labeled FGAR amidotransferase and isolation of radioactive peptides.

48 ml of an enzyme sample, Step-4, prepared by the Herrmann's procedure, contained about 5,000 units of the enzyme with a specific activity of 20. This sample was dialyzed against 200 volumes of 0.005 M potassium phosphate buffer, pH 6.5, containing 5×10^{-4} M EDTA, pH 8.0 overnight in the cold room and was then reacted with 5 μmoles of radioactive azaserine (corresponding to 5.5×10^{7} cpm) at 38° C for 15 minutes. Under these conditions about 97 per cent of the total activity was lost by azaserine treatment. After the dialysis of the azaserine reacted-sample against 200 volumes of water with the use of a rocking dialyzer in the cold room it was concentrated to 21 ml with a stream of air at room temperature. The concentrated fraction was adjusted to pH 5.6 with the addition of 2.0 M NaCN, pH 7.0, which was prepared just before the use (1.05 ml was required). Then 30 mg of papain was added to the sample and the mixture was incubated at 40° C. After the 30 minutes, another 15 mg of papain was added with further incubation at 40° C for 30 minutes. During these operations, the pH of the mixture was maintained at 5.6 ± 0.2.

TABLE VII

Volatile Buffers

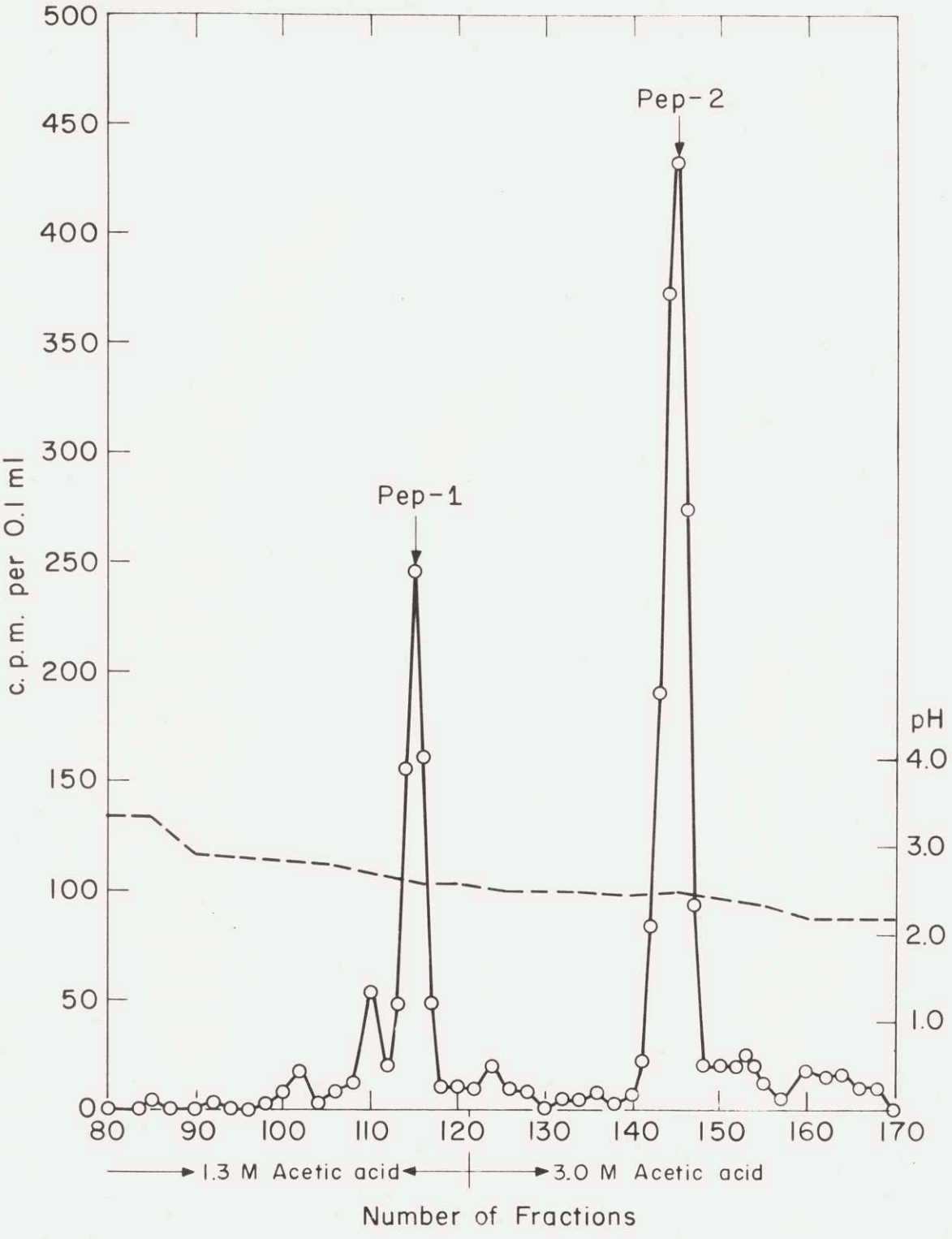
pH	composition
2.5	0.1 M chloroacetic acid adjusted with KOH
4.0	0.1 M triethylammonium acetate adjusted with acetic acid
6.0	0.1 M triethylammonium acetate adjusted with acetic acid
8.7	1% ammonium carbonate

The papain digested-sample was applied to a column (2 x 25 cm) of Sephadex dextran gel G-25 (Pharmacia), which was equilibrated with 0.01 M Triethylamine-acetic acid buffer, pH 6.0, and radioactive substances were fractionated by elution with the same buffer at a flow rate of 2.0 ml per minute. The fractions containing more than 20 c.p.m. per 0.01 ml were pooled and used in the next step. In this operation only one radioactive peak was detected.

After the pooled fractions were adjusted to pH 7.0 with 0.1 M KOH, 0.2 volume of 0.05 M potassium phosphate buffer, pH 7.0 (final concentration was 0.001 M) and 16 mg of pronase dissolved in 0.001 M potassium phosphate buffer, pH 7.0 were added and the mixture was incubated at 40° C. After 40 minutes, the pH of the mixture was adjusted to 7.5 with 0.1 M KOH and another 2.0 mg of pronase dissolved in 0.001 M potassium phosphate buffer, pH 7.5 were added. The mixture was further incubated at 40° C for 120 minutes and then, after adjustment of pH to 8.0, it was applied to a Dowex-1 X2 column (0.8 x 100 cm) equilibrated with 0.05 M pyridine-acetic acid buffer, pH 5.5. For the isolation of the radioactive substance(s) the following buffer systems were used: 1) a concave gradient elution of 600 ml of 0.05 M pyridine-acetic acid, pH 5.5 in the mixer and 600 ml of 0.5 M acetic acid in the reservoir; 2) then, 900 ml of 1.3 M acetic acid and 3) then 700 ml of 3.0 M acetic acid. During the elution, the temperature of the column and buffers was maintained at 35° C and a flow rate of the elution was kept at 0.3 to 0.5 ml per minute.

Fig. 9 shows the result of the fractionation of the radioactive substances on the Dowex-1 column. The Pep-1 and Pep-2 peaks were

Fig. 9. Isolation of radioactive peptides on Dowex-1 column chromatography. The solid line represents the radioactivity of the fractions and the dashed line is the indication of pH during the elution.


500
450
400
350
300
250
200
150
100
50
0
c.p.m. per 0.1 ml
Pep-1
Pep-2
pH
4.0
3.0
2.0
1.0
80
90
100
110
120
130
140
150
160
170
1.3 M Acetic acid
3.0 M Acetic acid
Number of Fractions

separately pooled and concentrated into about 2.0 ml with a rotary evaporator (Rinco). The concentrated fractions were dried with a vacuum pump over P_2O_5 and KOH and then were suspended in a small volume of water. These fractions were compared with the chemically synthesized compounds, VCS and CS, without further purification.

Table VIII showed the summary of the isolation of the radioactive substances.

Identification of the radioactive peptides.

1) High voltage electrophoresis. The apparatus that was routinely used for electrophoresis of peptides has been described by Ingram and Stretton (29). Samples dissolved in water were spotted on Whatman No. 1 paper (13.5 x 40 cm). The paper was wet with an appropriate buffer by the line-sharpening technique described by Naughton and Hagopian (30) and was placed on the cooled plate. The operating condition for electrophoresis was at 2 KV for 50 to 60 minutes in all experiments. Radioactive peptides were detected by radioautography with Ansco "non-screen" X-ray film and non-radioactive peptides were detected by the ninhydrin test.

In a preliminary experiment, fraction Pep-1 was compared to VCS as the reference compound. In order to determine whether Pep-1 could be hydrolyzed enzymatically to CS, it was treated with leucine aminopeptidase. 0.05 mg leucine aminopeptidase dissolved in 0.1 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.005 M $MgCl_2$ was added to the dried sample of Pep-1 and the mixture was incubated at 40° C for 120 minutes. After the mixture was dried completely, it was dissolved in water and subjected to electrophoresis.

TABLE VIII

Outline of the Isolation of Azaserine Labeled Peptides from FGAR Amidotransferase in Chicken Liver

Treatment	c.p.m.	% Recovery of the Radioactivity	
Step-1 Treatment of the enzyme with azaserine	5.5×10^7	-	
Step-2 Dialysis of the azaserine reacted-enzyme against water	4.5×10^5	(100)	
Step-3 Digestion with papain followed by the isolation of peptide on a Sephadex G25 column	4.0×10^5	90	
Step-4 Digestion with pronase followed by the isolation of peptides on a Dowex-1 X2 column	1.1×10^5 (Pep-1)	24.5	69.0
	2.0×10^5 (Pep-2)	44.5	

TABLE IX

Paper Electrophoretic Behaviors of the Isolated Peptides

compounds tested	mobility (cm) from origin			
	pH 2.5	pH 4.0	pH 6.0	pH 8.7
Glucose	0.7	0.2	1.1	- 1.0
Phosphoserine	7.5	9.2	14.7	13.5
Glycolic acid	2.1	12.5	18.6	16.5
Pep-1	- 1.5	4.6	7.3	9.7
VCS	- 1.5	4.7	7.3	(-)
Pep-1 + VCS	- 1.7	4.6	7.3	9.7
Pep-2	0.7	6.5	9.0	8.5
CS	0.7	6.5	9.1	8.5
Pep-2 + CS	0.7	6.5	9.0	8.6
Pep-1 (with LAP)	0.5	6.3	8.7	8.6
Pep-1 (with LAP) + CS	0.4	6.3	8.7	8.6

LAP: Treatment with leucine aminopeptidase.

TABLE X

Paper Chromatographic Behaviors of the Isolated Peptides

Compounds tested	I (MEK)	II (BAW)
Pep-1	0.200	0.588
Pep-1 + VCS	0.190	0.588
VCS	0.192	0.581
Pep-1 (sox)	0.113	0.288
Pep-1 (sox) + VCS (sox)	0.100	0.270
VCS (sox)	0.100	0.250
Pep-2	0.100	0.200
Pep-2 + CS	0.100	0.200
CS	0.103	0.200
Pep-1 (with LAP)	0.100	0.196
Pep-1 (LAP) + CS	0.093	0.192
Pep-2 (sox) + CS (sox)	0.040	0.092
Pep-1 {LAP) + sox) + CS (sox)	0.044	0.096
CS (sox)	0.044	0.100

MEK: methylethylketone: propionic acid: H_2O = 15:5:6

BAW: 2-Butanone: acetic acid: H_2O = 5:2:2

SOX: sulfoxide form of the peptides

LAP: treatment with leucine aminopeptidase.

Table IX shows the results of electrophoresis at various pH values. Glucose, phosphoserine and glycolic acid were used as indicators of the migration of the samples tested. Pep-1 was identical to VCS and the hydrolytic product of Pep-1 obtained after digestion with leucine aminopeptidase was the same as CS. Pep-2 was, on the other hand, identical to CS.

2) Paper chromatography. In order to identify fractions Pep-1 and Pep-2 by a method other than the electrophoretic technique, paper chromatography was employed with the following solvent systems: (1) methylethylketone-propionic acid-water, 15:5:6 (volume to volume and referred to as the MEK system) and (2) 2-butanone-acetic acid-water, 5:2:2 (referred as BAW system). These solvent systems were convenient for the detection of the products of sulfoxidation of both Pep-1 and Pep-2. For the sulfoxidation, the dried sample was treated with 1 per cent acetic acid solution and 1 per cent H_2O_2 solution at room temperature for 15 minutes. Then the sample was dried and subjected to paper chromatography.

Paper chromatography was performed at room temperature. Table X includes the R_f values of the samples tested. These data indicate that Pep-1 and Pep-2 correspond to VCS and CS, respectively.

Discussion

Two factors have permitted the study of the active site of FGAR amidotransferase. The first is the demonstration that azaserine reacts with enzyme as a competitive inhibitor of the natural substrate glutamine. Secondly, the inhibition is irreversible because of the

formation of a covalent bond between azaserine and the enzyme.

Because of the lack of the information about the determination of molecular weight and turnover number of chicken liver FGAR amidotransferase, it has not yet been possible to demonstrate stoichiometrically the amount of azaserine bound to the enzyme. However, the hydrolyses of the azaserine labeled enzyme with protelolytic enzymes, papain and pronase, and the isolation of the radioactive peptides indicated that azaserine reacted only with FGAR amidotransferase in the sample employed, although the enzyme was not purified extensively.

As was the case with the Salmonella enzyme, the chicken liver enzyme was effectively digested with papain. Only one species of the radioactive peptide could be detected upon gel filtration on a column of Sephadex G25. Digestion with pronase on the other hand yielded two species of the radioactive peptide (Pep-1 and Pep-2). Digestion of Pep-1 with leucine aminopeptidase to yield Pep-2 demonstrated that Pep-1 and Pep-2 are related to each other.

The electrophoretic and paper chromatographic experiments indicated that Pep-1 was identical to VCS and Pep-2 was the same as CS.

As has been discussed in the preceding section, several physical characteristics of the chicken enzyme are different from those of the Salmonella enzyme. However, the experiments on the azaserine reactive site of both enzymes indicate that they contain at least a common amino acid sequence around the azaserine reactive site, namely valyl-cysteine.

In the Salmonella system, a more extensive amino acid sequence at the point of binding of azaserine has been determined. A pentapeptide, ala-leu-gly-val-cysteine has been isolated and identified. Therefore, further studies of the amino acid sequence around the azaserine reactive site in both systems is of interest in order to compare the similarities and differences of the "active sites" of the two enzymes.

IV. REFERENCES

1. Melnick, I., and Buchanan, J., J. Biol. Chem., 225, 157, (1957).

2. Abrams, R., and Bentley, M., Arch. Biochem. Biophys., 79, 91 (1959).

3. Lagerkuist, U., J. Biol. Chem., 233, 143 (1958).

4. Preiss, J., and Handler, P., J. Biol. Chem., 233, 488 (1958).

5. Hartman, S., and Buchanan, J. M., J. Biol. Chem., 233, 451 (1958).

6. Goldthwait, D. A., J. Biol. Chem., 222, 1051, (1956).

7. Report of the Commission on Enzymes , I.U.B. Symposium Series Vol. 20, (1961).

8. Kornberg, A., J. Biol. Chem., 182, 779 (1950).

9. Remy, C., Remy, W., and Buchanan, J., J. Biol. Chem., 217, 885 (1955).

10. Levenberg, B., Melnick, I., and Buchanan, J., J. Biol. Chem., 225, 1636 (1957).

11. Koshland, D., in The Enzymes, Vol. I, P. 305 (1959) (Academic Press).

12. Buchanan, J., Hartman, S., Herrmann, R., and Day, R., J. Cell. and Comp. Physiol. 54 Supl. 139 (1959).

13. French, T., Dawid, I., Day, R., and Buchanan, J., J. Biol. Chem., 238, 2171 (1963).

14. Dawid, I., French, T., and Buchanan, J., J. Biol. Chem., 238, 2178 (1963).

15. French, T., Dawid, I., and Buchanan, J., J. Biol. Chem., 238, 2186 (1963).

16. French, T. Ph.D. Thesis (Massachusetts Institute of Technology 1963)

17. Hartman, S., Levenberg, B., and Buchanan, J., J. Biol. Chem., 221, 1057 (1956)

18. Levenberg, B., and Buchanan, J., J. Biol. Chem., 224, 1019 (1957).

19. Singer, T., and Kearney, E., Arch. Biochem. Biophy., 29, 190 (1950).

20. Deeb, S., and Hagar, L., J. Biol. Chem., 239, 1024 (1964).

21. Peterson, E. and Sorber, H., J. Am. Chem. Soc., 78, 751 (1956).

22. Peterson, E., and Sorber, H., in Methods of Enzymology (Ed. by Colowick and Kaplan) Vol. 5 P. 3 (1962) (Academic Press).

23. Mokrasch, L., and McGilvery, R., J. Biol. Chem., 221, 909 (1956).

24. Martin, R., and Ames, B., J. Biol. Chem., 236, 1372 (1961)

25. Smithies, O., Biochem. J., 61, 629 (1955).

26. Smithies, O., Biochem. J., 71, 585 (1959).

27. Nicolaides, E., Westland, R., and Wittle, E., J. Am. Chem. Soc., 76, 2887 (1954).

28. Sjöquist, J., Arkiv Kemi, 14, 323 (1959).

29. Ingram, V., and Stretton, A., Biochem. et Biophys. Acta, 62, 456, (1962).

30. Naughton, M., and Hagopian, H., Anal. Biochem., 3, 276 (1962).