Rat Liver Glutamyl Ribonucleic Acid Synthetase

I. PURIFICATION AND EVIDENCE FOR SEPARATE ENZYMES FOR GLUTAMIC ACID AND GLUTAMINE*

MURRAY P. DEUTSCHER

From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461

SUMMARY

Glutamyl ribonucleic acid synthetase was purified approximately 150-fold from homogenates of rat liver to a specific activity of 60 mμmoles of glutamyl-RNA formed per min per mg of protein. The enzyme at this stage of purification was judged to be about 40% pure, but in a state of aggregation. Although the enzyme was quite stable to storage, it was very heat labile, and lost activity during its assay. The purified enzyme required Mg++, adenosine triphosphate, l-glutamate, and soluble RNA for activity. Evidence is presented for the existence of distinct enzymes for glutamyl-RNA and glutaminyl-RNA formation. A rapid method for obtaining pure soluble RNA from liver is also described.

Considerable evidence has accumulated during the past decade which indicates that before amino acids can be incorporated into proteins they must first be activated and then transferred to a low molecular weight species of ribonucleic acid, known as soluble ribonucleic acid. Both the activation of the amino acid and its subsequent transfer to sRNA are catalyzed by the same aminoacyl-RNA synthetase.

Despite the extensive work devoted to the study of these enzymes, relatively few investigations have been concerned with the synthetases specific for the dicarboxylic amino acids and their amides (1-14). Conflicting reports have also appeared concerning the existence of separate synthetases for glutamic acid and glutamine (1-3). Zubay (1) showed that in crude Escherichia coli extracts unlabeled glutamate had no effect on the incorporation of 14C-glutamine into sRNA, but that unlabeled glutamine inhibited the incorporation of 14C-glutamate. He suggested that glutamic acid in proteins may arise from the hydrolysis of protein-bound glutamine residues. However, Coles and Meister (2) and Coles, Bukenberger, and Meister (3) presented evidence that glutamate was incorporated into sRNA without prior conversion to glutamine. Fraser, Shimizu, and Gutfreund (7), Fraser (8), and Alford et al. (9) showed glutamyl-RNA and glutaminyl-RNA formation by pH 5 fractions from guinea pig mammary gland, rat liver, and Ehrlich ascites cells, and also partially purified the glutamyl-RNA synthetase. They also presented some evidence for the separate activation of glutamate and glutamine. Very recently, Lazzarini and Mehler (11) and Ravel et al. (12) reported the separation of the glutamyl- and glutaminyl-RNA synthetases from Escherichia coli R and E. coli W, respectively.

The present communication describes the purification of rat liver glutamyl-RNA synthetase which after storage is essentially free of activity for glutamine. Further evidence is also presented for the existence of separate enzymes for glutamic acid and glutamine activation in mammalian tissues. In addition, several of the properties of the purified glutamyl-RNA synthetase are presented in this report and the accompanying communication (15).

EXPERIMENTAL PROCEDURE

Materials—14C-Amino acids were purchased from New England Nuclear, except l-glutamine which was a product of Schwarz BioResearch and l-arginine, l-tyrosine, l-isoleucine, l-alanine, and l-methionine which were obtained from Nuclear-Chicago. Paper electrophoresis of the 14C-glutamic acid and 14C-glutamine in pyridine-acetic acid-H₂O (8:15:977), pH 4.3, indicated that these amino acids were at least 98% pure, and they were used without further purification. All unlabeled amino acids used for diluting the radioactive compounds were obtained from Sigma, except monosodium-l-glutamate which was a product of Calbiochem. This latter compound was pure when examined by paper electrophoresis.

ATP, alumina Cy, and calcium phosphate gel were purchased from Sigma. DEAE-cellulose (type 20) was a product of Brown Company and hydroxylapatite was purchased from Bio-Rad. Fresh frozen rabbit livers were obtained from Pel-Freez Biologicals, Rogers, Arkansas.

Standard Assay for Aminoacyl-RNA Synthetase Activity—
Aminoacyl-RNA synthetase activity was measured by determining the incorporation of \( ^{14} \text{C} \)-labeled amino acid into trichloroacetic acid-precipitable material. The standard reaction mixture for assay of glutamyl-RNA synthetase activity contained in 100 \( \mu \)l: 25 \( \mu \) moles of Tris-acetate buffer, pH 7.0; 0.5 \( \mu \) mole of ATP adjusted to pH 7.0 with NaOH; 0.5 \( \mu \) mole of MgCl\(_2\); 25 \( \mu \) moles of \( ^{14} \text{C} \)-labeled glutamic acid (10 \( \mu \) Ci per \( \mu \) mole); 6 to 10 \( A_{260} \) units of rabbit liver sRNA (depending on the sRNA preparation); and a limiting amount of enzyme. The reaction was initiated by the addition of enzyme and terminated after 3 min of incubation at 37\(^\circ\) by the addition of 1.5 ml of cold 10\% trichloroacetic acid containing 1 \( \mu \) mole glutamate. After standing for about 5 min in ice, the precipitate was collected on a 0.45-\( \mu \) Millipore filter and washed with 20 ml of 5\% trichloroacetic acid containing 0.5 \( \mu \) mole glutamate. The filter was dried by suction and assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter in 10 ml of toluene: scintillation fluid (5 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis(2-(5-phenyloxazoyl)]benzene in 1 liter of toluene). The efficiency of counting \( ^{14} \text{C} \) on the filters was 70\%. Activity for other aminoacyl-RNA synthetases was assayed in an identical manner except that the appropriate amino acid replaced glutamic acid. Other modifications of the standard assay are described in the legends to the tables and figures.

One unit of glutamyl-RNA synthetase activity is the amount of enzyme that incorporates 1 \( \mu \) mole of glutamic acid into sRNA per min. Specific activity is expressed as units per mg of protein.

Other Assays—Protein concentrations were measured either by the method of Lowry et al. (16) with serum albumin as a standard or by ultraviolet absorption according to Warburg and Christian (17). These two methods gave almost identical results with purified enzyme fractions, so that the ultraviolet absorption method was used for rapid protein analyses. The concentration of RNA was measured spectrophotometrically in distilled water at 260 \( \text{nm} \) in a cell of 1-cm light path. One \( A_{260} \) unit is defined as that amount of RNA giving an absorbance of 1 when measured under these conditions. One milligram of sRNA was assumed to have an absorbance of 21.4 \( A_{260} \) (18).

Sucrose Gradient Centrifugation—Purity of RNA preparations was determined by centrifugation in a sucrose gradient for 16 hours at 25,000 rpm in an SW 25 rotor. The gradient was made from 3 to 20\% sucrose containing 10 \( \mu \) mole sodium acetate (pH 5.1), 50 \( \mu \) mole NaCl, and 0.1 \( \mu \) mole MgCl\(_2\). Fractions were collected by pumping out solutions from the bottom of the centrifuge tube. The optical density profile was determined with a flow cell attachment to the Gilford recording spectrophotometer.

Analytical Ultracentrifugation—Sedimentation velocity of the purified protein was carried out at a speed of 59,780 rpm in a Spinco model E ultracentrifuge according to the method of Schachman (19). The purified enzyme preparation (specific activity, 60) was studied at a concentration of 0.1\% in 0.1 \( \mu \) mole sodium phosphate buffer, pH 7.0, containing 10\% ammonium sulfate.

Gel Electrophoresis—Electrophoresis studies of the purified protein preparation were performed on a Canalco unit with 5\% polyacrylamide gel according to the methods of David (20) and Williams and Reisfeld (21) for the pH 8.3 and pH 7.0 systems, respectively. Sodium dodecyl sulfate-treated protein was run in gel containing sodium dodecyl sulfate according to Maizel (22). In all cases staining was done according to Maizel (22) with 20\% sulfoisalicylic acid as the fixative and 0.25\% Coomassie brilliant blue R250 as the stain.

Preparation of Liver sRNA—During the course of this work it was necessary to develop a method for obtaining pure soluble RNA. The preparation of pure sRNA was very important since, sRNA prepared by certain methods contained an inhibitor of glutamyl-RNA formation. The best preparations of sRNA (based on purity, yield, and acceptor activity) were obtained by a combination of several purification procedures. The essential steps in the preparation of the sRNA included the following: a phenol homogenization step, which removed most of the non-nucleic acid material; a DEAE-cellulose chromatography step, which separated the sRNA from the glycogen and much of the high molecular weight RNA; and an isopropanol fractionation step which was found to separate the sRNA from DNA and an RNA fraction inactive as amino acid acceptor. All procedures except the isopropanol fractionation were performed at 0-\( ^{-20} \)\(^\circ\).

In a typical preparation, 500 g of frozen rabbit liver were homogenized in a blender for 90 sec with 500 ml of 0.14 \( \mu \) mole NaCl, 0.01 \( \mu \) mole sodium phosphate, pH 7.0, 500 ml of aqueous phenol, and 10 ml of 1\% EDTA. The actual homogenizations were performed with 100-g portions of liver. The homogenate was centrifuged and the aqueous layer was decanted. The aqueous layer was then re-extracted with another equal volume of phenol. Nucleic acids and glycogen in the final aqueous layer were precipitated with 3 volumes of 95\% ethanol. After 2 hours at \( -20^\circ \), to ensure complete precipitation, the precipitate was collected by centrifugation and dissolved in 250 ml of 0.1 \( \mu \) mole Tris-acetate buffer, pH 7.5. The sRNA was further purified on a DEAE-cellulose column by a modification of the method of Brunngraber (23).

The sRNA at this stage of purification was capable of accepting glutamic acid, but did not give normal saturation kinetics, suggesting the possible presence of an inhibitor. The sRNA could be purified further by application of the isopropanol fractionation method of Zubay (24). The nucleic acid solution was adjusted to 0.3 \( \mu \) mole sodium acetate, pH 7.0, by the addition of 3 \( \mu \) mole sodium acetate and a small amount of dilute acetic acid. Isopropanol, 0.54 volume, was added to this solution at room temperature with constant stirring, and the precipitate was immediately removed by centrifugation. Isopropanol was then added to the supernatant solution to a final concentration of 0.98 volume and the sRNA precipitate was collected by centrifugation. The precipitate was dried in a stream of air and dissolved in distilled water to give a final concentration of 150 to 200 \( A_{260} \) per ml. sRNA prepared by this method was stable in the frozen state for at least 6 months. Recent experiments have indicated that the DEAE-cellulose step can be eliminated, so that mammalian sRNA of a high degree of purity may be isolated in just two rapid steps; 30 to 50 mg of sRNA per 100 g of liver with \( A_{260}:A_{230} \) ratios of 1.85 to 2.00 were routinely isolated by this method. The sRNA prepared by this method was homogeneous as judged by sucrose gradient centrifugation.

RESULTS

Purification of Glutamyl-RNA Synthetase—Fresh livers were obtained from 170- to 250-g Sprague-Dawley rats that had been decapitated. The livers were excised and immediately

\(^2\) M. P. Deutscher, manuscript in preparation.
placed in ice-cold 0.35 M sucrose. All subsequent steps in the fractionation procedure were carried out at 4°.

Preparation of Homogenate—Rat liver, 35 g, was blotted dry with filter paper and minced in 65 ml of a medium containing 0.35 M sucrose, 0.06 M KCl, 0.01 M MgCl₂, and 0.1 M Tris-acetate (pH 7.5). The mixture was then homogenized in a motor-driven Teflon homogenizer in two portions to give 98 ml of homogenate. Similar yields of enzyme with the same specific activities were obtained if the livers were homogenized in 0.35 M sucrose alone or in a similar medium containing potassium phosphate instead of Tris-acetate buffer.

Differential Centrifugation—Cellular debris and mitochondria were removed by centrifugation of the homogenate for 15 min at 27,000 × g in a Sorvall refrigerated centrifuge. The supernatant fluid (64 ml) was decanted through glass wool to remove fatty material and diluted with an equal volume of the homogenizing medium. This mixture was then centrifuged at 105,000 × g for 90 min in a Spinco model L preparative ultracentrifuge to remove the microsomal fraction. The 105,000 × g supernatant solution (106 ml) was again filtered through glass wool. Approximately 25% of the activity of the homogenate was lost during the second centrifugation step. Presumably the enzyme is attached to some particulate matter or is quite large itself since a 7-hour centrifugation at 105,000 × g resulted in sedimentation of 75% of the activity but only 30% of the protein. This activity could be recovered from the pellet and bottom 1 ml of material in the centrifuge tube. Centrifugation in a similar medium containing potassium phosphate buffer, or in 0.35 M sucrose, or in 0.05 M KCl did not alter the rate of sedimentation of activity or protein during the 7-hour period.

Alumina Cy treatment—An alumina Cy suspension (38.5 mg per ml), 20 ml, was then added to the 105,000 × g supernatant solution and the mixture was stirred for 40 min. The precipitate was collected by centrifugation, suspended with 100 ml of 0.1 M sodium phosphate, pH 7.4, containing 2% ammonium sulfate, and stirred for 60 min. The mixture was centrifuged and the eluate was discarded. The precipitate was then stirred for 75 min with 100 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 10% ammonium sulfate. In order to ensure efficient washing and elution, the precipitates were suspended initially by homogenization with the eluting solvent. In addition to purification, this procedure served to remove all of the pigmented material present in the 105,000 × g supernatant solution.

Ammonium Sulfate Fractionation—Solid ammonium sulfate (11.8 g) was then added to the clear 10% ammonium sulfate eluate until 30% saturation, followed by stirring for 30 min to allow complete precipitation. The material was centrifuged for 15 min at 27,000 × g and the precipitate was discarded. Solid ammonium sulfate (9.4 g) was added to the supernatant fraction until 45% saturation and, after stirring for 30 min, the precipitate was collected by centrifugation and dissolved in 10 ml of 0.01 M sodium phosphate buffer, pH 7.4. Appreciable material absorbing at 260 μg remained in the 45% supernatant solution and was removed during this fractionation step. Occasionally, a 10 to 15% loss of activity in the 30 to 45% fraction, with the formation of an inactive precipitate, was observed when this fraction was stored overnight. In these cases the material was clarified by centrifugation before the next step.

Calcium Phosphate Gel Treatment—The 30 to 45% (NH₄)₂SO₄ fraction was adjusted to pH 6.3 with dilute acetic acid and then stirred for 40 min with sufficient calcium phosphate gel to adsorb all the activity. This required 2.5 mg of gel per mg of protein. (Before use, the gel suspension (35 mg per ml) was centrifuged to remove the water present in order to avoid dilution of the protein.) The gel was stirred with 10 ml of 0.1 M sodium phosphate (pH 7.4) for 45 min, with 10 ml of 0.15 M sodium phosphate (pH 7.6) for 75 min, and finally with 10 ml of 0.1 M sodium phosphate (pH 7.4) containing 10% (NH₄)₂SO₄ for 75 min. The final eluate was then brought to 45% saturation with solid ammonium sulfate and stirred for 20 min, and the precipitate was collected by centrifugation for 15 min at 27,000 × g. The precipitate was dissolved in 4 ml of 0.1 M sodium phosphate, pH 7.4. The (NH₄)₂SO₄ precipitation served to concentrate the protein in the gel eluate and also to change buffers for the next step. It was found convenient, at this stage, to combine several preparations for chromatography. The enzyme at this stage was stable to storage at 0-5°C for at least 10 days.

Hydroxylapatite Chromatography—In the purification scheme summarized here, three preparations were combined (12 ml), and were then adsorbed on a column of hydroxylapatite (10-ml volume). The column had been packed under pressure with a pump and equilibrated with 0.1 M sodium phosphate, pH 7.4. The column was eluted successively under pressure with 20-mI aliquots of 0.2 M potassium phosphate (pH 6.9), 0.25 M potassium phosphate (pH 6.9), and 0.3 M potassium phosphate (pH 6.9), and most of the enzyme was eluted with 0.1 M sodium phosphate, pH 7.4, containing 10% ammonium sulfate. The tubes with the highest specific activities were combined. Approximately 65% of the activity put on the column was recovered in the 10% (NH₄)₂SO₄ eluate, while another 20% was present in the 0.3 M phosphate eluate. The 10% (NH₄)₂SO₄ eluate was used directly to study the properties of the enzyme that are described below.

Occasionally, the enzyme was also prepared from the 105,000 × g supernatant fraction by precipitation at pH 5.5, negative adsorption with alumina Cy, and (NH₄)₂SO₄ precipitation of the supernatant protein between 30 and 45% saturation. The enzyme purified by the second method had a specific activity of approximately 20 units per mg.

As judged by all the parameters examined, the enzymes purified by these two methods were identical. Except where noted, the studies reported here were done with the enzyme purified by the first method.

A summary of a typical preparation obtained by the first method of purification is presented in Table I. The enzyme was purified about 150-fold from a rat liver homogenate with a recovery of 0% and a final specific activity of 60 units per mg.

Preparations with specific activities as high as 90 units per mg have also been obtained by this procedure. This purification represents a minimum value since, as discussed below, the purified enzyme lost about half of its activity during the course of the standard assay.

Further attempts to purify the enzyme by the techniques of ion exchange, gel filtration, sucrose density centrifugation, and partition in two phase systems were all unsuccessful. As discussed below, the enzyme at this stage of purity seemed to be an aggregate of several proteins and a small amount of nucleic acid. This aggregation probably contributed to the inability to achieve further purification.

Purity of Enzyme—The purified enzyme had a $A_{260}/A_{300}$ absorbance ratio of 1.24 which, according to Warburg and Christian
(17), indicates the presence of 1.5% nucleic acid. This nucleic acid material was not essential for activity since fractions were obtained from DEAE-Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.

Several experimental observations indicated that the purified preparation was a nucleoprotein aggregate of large size: (a) all of the protein and enzymatic activity were eluted from Sephadex columns with ratios of 1.6 to 1.8 and with specific activities of 20 units per mg. However, removal of the nucleic acid increased the lability of the enzyme. Since the enzyme was probably tightly bound to nucleic acid, the various adsorption steps utilized in the purification of the enzyme were effective in removing contaminating proteins. In addition, since very high ionic strengths were necessary to elute the enzyme from the different adsorbents used, proteins not bound to nucleic acid would probably not be present in the purified enzyme preparation.
purified enzyme lost half of its activity when stored at 0° for 1
month, and that 10^-4 M EDTA and bovine serum albumin (2.5
mg per ml) afforded some protection against this inactivation.
Sulfhydryl compounds, such as glutathione or mercaptoethanol,
completely inactivated the enzyme. In contrast to the results
obtained at 0°, storage of the enzyme in the frozen state at -20°
for 1 month resulted in only a 20% loss of activity. EDTA, 10^-4
M, had no effect on the frozen enzyme and serum albumin offered
some protection. Mercaptoethanol, 10^-3 M, also completely
inactivated the frozen enzyme, but glutathione did not have as
great an effect when the enzyme was frozen. In addition, the
enzyme still retained 66% of its activity after storage with serum
albumin for 5 months at -20°, while at 0° most of the activity
was lost during this period. These results indicate that purified
glutamyl-RNA synthetase is quite stable to storage.

Requirements of Reaction—The incorporation of glutamate
into sRNA exhibited a requirement for ATP, Mg++, sRNA, and
enzyme (Table IV). The small amount of incorporation in the
absence of Mg++ or of ATP was a consistent finding, and may be
due to the presence of small amounts of these substances in the
sRNA or enzyme preparations. The specificity of the enzyme
with respect to these various requirements for activity is discussed
in more detail in the accompanying paper (15).

The incorporation of glutamate into trichloroacetic acid-insol-
uble material was not due to incorporation into protein. This
was shown by the marked alkaline lability of the product and the
malleability of puromycin to inhibit the reaction. Thus, addi-
tion of 0.25 ml of 1 M NaOH for 1 min to a completed reaction
mixture entirely eliminated the incorporation of glutamate into
acid-precipitable material, even with a crude 105,000 X g super-
natant fraction. Similarly, puromycin, at concentrations as
high as 1 mM, had no effect on the incorporation of glutamate
catalyzed by the 30 to 45% (NH_4)_2SO_4 fraction. These results
indicate that all of the activity being measured is due to the
incorporation of glutamate into sRNA.

Effect of Enzyme Concentration—The results shown in Fig. 1
show that the amount of glutamyl-RNA formed in 3 min is
linearly related to the amount of purified enzyme added over a
range of approximately 0.1 to 1 µg of protein, with the formation
of up to 0.13 µmole of product. Under the standard conditions
for measurement of the rate of glutamyl RNA formation, an
amount of enzyme was added which resulted in the utilization of
less than 20% of the available sRNA sites.

Time Course of Reaction—Both the rat liver homogenate and
the 105,000 X g supernatant fraction gave linear rates of glu-
tamate incorporation for at least 10 and 5 min, respectively (Fig.
2). In contrast to these results, the purified enzyme did not

Table IV

<table>
<thead>
<tr>
<th>Omissions from complete reaction mixture</th>
<th>Glutamyl-RNA formed (µmoles/3 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>123.3</td>
</tr>
<tr>
<td>sRNA</td>
<td>0</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>8.5</td>
</tr>
<tr>
<td>ATP</td>
<td>4.7</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of protein concentration on the rate of glutamyl-RNA formation. Incubations were performed for 3 min according to the standard assay conditions with the addition of the indicated amounts of enzyme. Before assay the enzyme was diluted to the appropriate concentrations with a solution of bovine serum albumin (5 mg per ml) in potassium phosphate, pH 7.4.

Fig. 2. Time course of glutamyl-RNA formation by the homog-
enate and 105,000 X g supernatant fraction. Incubations were
performed according to the standard assay conditions for the
times indicated. Either 20 µg of protein from the homogenate
[●] or 37 µg of protein from the 105,000 X g supernatant
fraction (■) were present during the assays.
Rat Liver Glutamyl Ribonucleic Acid Synthetase. I

Vol. 242, No. 6

FIG. 3. Time course of glutamyl-RNA formation by the purified enzyme in the presence of serum albumin or EDTA. Incubations were performed according to the standard assay conditions for the times indicated. Each reaction mixture contained 0.28 µg of purified enzyme. The reactions were carried out in the presence of either 100 µg of bovine serum albumin (■), 200 µg of bovine serum albumin (O—O), or 2 × 10⁻⁴ M EDTA (□—□) or without any additions (●—●).

FIG. 4. Selwyn plots of the time course of glutamyl-RNA formation. Incubations were performed according to the standard assay conditions with the addition of 0.28 µg (O—O) or 0.56 µg (■—■) of purified enzyme. The units on the abscissa are microliters of enzyme times minutes of incubation.

catalyze a linear rate of incorporation, but yielded a continuous die-away curve (Fig. 3). Assuming a linear reaction rate for the first 1-min interval, the incorporation at 3 min was only 65% of that expected. Therefore, as measured in the standard assay procedure, the specific activity of the purified enzyme was only maximally 65% of its true value and the relative purification compared to the crude homogenate may have been in error by almost a factor of 2. The actual purification achieved, considering the difference in linearity of the rates for the crude homogenate and the purified preparation, may be as high as 300-fold.

The data presented in Fig. 3 also show that EDTA and bovine serum albumin increased markedly the incorporation of glutamate at all periods beyond 1 min. However, increasing the amount of serum albumin present from 0.1 to 0.2 mg did not increase the incorporation further. Since EDTA and bovine serum albumin were also the two substances that protected the purified enzyme against inactivation during storage (Table IV), these results suggest that the reason for the lack of linear kinetics may have been caused by an inactivation of the enzyme during the assay.

In order to examine this possibility, the time course of the reaction was followed at two different enzyme concentrations, and the data were plotted according to the method of Selwyn (26). Fig. 4 reveals that when the incorporation of glutamate was plotted against enzyme concentration times time (instead of time) the results fell on two different curves, indicating that the nonlinear results were due to an inactivation of the enzyme during the assay. In addition, since the incorporation was proportional to the enzyme added during a 3-min assay, as shown in Fig. 1, the inactivation of the enzyme must have been a first order process (i.e. the fraction of enzyme inactivated is constant during every time period irrespective of the amount of enzyme present). These results indicate that, although the incorporation was nonlinear with time, accurate kinetic measurements could be made, since at a time such as 3 min, all the rates would be in error by the same constant factor. This assumes, of course, that different conditions did not change the first order character of the inactivation. A possible cause for the enzyme inactivation is discussed below.

Heat Lability of Enzyme—One possible reason for the inactivation of the enzyme during the assay was the temperature factor. Therefore, in order to examine this possibility, the effect of heating the enzyme prior to the assay was studied. As shown in Fig. 5A, the purified enzyme was extremely heat labile, losing over 50% of its activity in 2 min at 45° and all its activity in 2 min at 55°. Even at 37°, the temperature of the standard assay, the enzyme lost 30% of its activity after heating for 10 min; 10⁻⁴ M EDTA completely overcame this heat inactivation at 37°.

Fig. 5B shows similar results for the enzyme purified by the second method. In this case the enzyme was even more labile, losing 60% of its activity during a 10-min preincubation at 37°. This greater lability of the enzyme prepared by the second method may have been due to the conditions of the heat treatment, since the latter enzyme was heated in Tris buffer instead of phosphate buffer. It is also seen that 25 or 50% glycerol completely protected the enzyme against heat inactivation at 37°. The presence of 1 mM glutamate, 10 mM Mg⁺⁺, or 1 mM ATP did not afford any protection against the rapid rate of enzyme inactivation at 45° or 50°.
These results suggest that the most probable reason for the inactivation of the enzyme during the standard assay was its extreme heat lability, even at 37°C. The quantitative differences between the inactivation during preincubation and during the assay were probably due to the much lower protein concentration present during the assay (10 µg per ml with respect to 1 mg per ml). This is seen from the partial protection afforded by serum albumin during the standard assay. In addition, storage of the enzyme at 0°C at low protein concentrations (0.1 mg per ml) resulted in the loss of half of the activity overnight. This is in contrast to the results obtained at higher protein concentrations (Table III).

Evidence of Separate Activities for Glutamic Acid and Glutamine
—As shown in Table II, the purified glutamyl-RNA synthetase still contained some activity for glutamine. However, during the purification, the ratio of glutamate activity to glutamine activity increased from 4:1 in the 105,000 x g supernatant fraction to 7:1 in the hydroxylapatite eluate. In addition, after storage of the purified enzyme at 0°C for 2 weeks, this ratio changed to 22:1; after storage at -20°C for 5 months the ratio increased to 60:1. These results suggest that two distinct enzymes probably exist for catalysis of the incorporation of glutamate and glutamine into sRNA, and that the glutamine enzyme is much more labile to storage.

In addition, Table V shows that a 10-fold excess of unlabeled glutamine had no effect on the incorporation of glutamate, while a similar excess of unlabeled glutamate resulted in an 85% decrease in activity. Similar results were obtained with a 100-fold excess of each unlabeled amino acid. The small decrease in the incorporation with a 100-fold excess of unlabeled glutamine was probably due to a very small contamination of the glutamine with glutamate. This contamination would only have to amount to 0.25% to account for the observed decrease. Similarly, a large excess of unlabeled glutamate did not affect 14C-glutamine incorporation into sRNA.

These results show that glutamic acid and glutamine do not compete with each other for incorporation into sRNA, and also suggest the existence of a separate enzyme for each amino acid. However, absolute proof for the presence of two distinct enzymes in mammalian systems for the activation of glutamate and glutamine will come only with the complete separation and purification of each of these activities.

**DISCUSSION**

The preparation of glutamyl-RNA synthetase described in the studies presented here resulted in a 150-fold purification of the enzyme from a rat liver homogenate and a final specific activity of 60 to 90 µmoles of glutamyl-RNA formed per min per mg of protein. The relative purification and the final specific activity were both minimum values since the purified preparation, in contrast to crude fractions, did not give linear rates of incorporation during the 3-min period of the assay. The actual specific activity may have been as high as 200 units per mg. In addition, it was previously shown by Fraser (8) and Alford et al. (9) that crude liver enzyme fractions contain an active glutamine

---

**TABLE V**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glutamyl-RNA formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles/3 min</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>95.2</td>
<td>100</td>
</tr>
<tr>
<td>0.25 µmoles of 14C-glutamate</td>
<td>13.4</td>
<td>14</td>
</tr>
<tr>
<td>0.25 µmoles of 14C-glutamine</td>
<td>97.6</td>
<td>103</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>94.2</td>
<td>100</td>
</tr>
<tr>
<td>2.5 µmoles of 14C-glutamate</td>
<td>4.9</td>
<td>5</td>
</tr>
<tr>
<td>2.5 µmoles of 14C-glutamine</td>
<td>74.2</td>
<td>79</td>
</tr>
</tbody>
</table>
synthetase which converts the added 14C-glutamate to 14C-glutamine. The 14C-glutamate is also transferred to sRNA and results in an overestimation of the activity for glutamate in the crude fractions. Fraser (8) eliminated the interfering interconversion of glutamate and glutamine by the addition of methionine sulfoxime. However, in our studies, the presence of this inhibitor sometimes led to a stimulation of incorporation, depending on the protein concentration. Alford et al. (9) observed similar results with their partially purified enzyme. For this reason, any activity in the crude fraction due to 14C glutamine incorporation was ignored in the present study. With the pure enzyme, this interconversion of the two amino acids did not interfere, since the addition of a large excess of unlabelled glutamate did not alter the incorporation of 14C-glutamate into sRNA.

The specific activity of the purified rat liver glutamyl-RNA synthetase reported here is considerably higher (60 with respect to 1 unit per mg) than the enzyme prepared from the same source by Alford et al. (9), although the relative purification of the two enzymes did not differ to that degree. The difference in results is probably due to the presence of an inhibitory RNA in the heated pH 5 fraction that Alford et al. (9) used as a source of sRNA. The existence of this inhibitor in pH 5 fractions will be discussed elsewhere. In addition, the presence of methionine sulfoxime, and other differences in the assay system (9), may also have affected the activity of their preparation. The preparation described here has approximately the same specific activity as the glutamyl-RNA synthetase purified from E. coli B by Lazzarini and Mehter (11). The specific activity is also in the range of those reported for several other homogeneous aminocetyl-RNA synthetases (27–31).

In addition to activity for glutamic acid, the purified enzyme also contained essentially the same activity for lysyl-RNA formation, as well as small amounts of activity for several other amino acids. However, efforts directed toward further purification of the enzyme were unsuccessful. This was probably due to the presence of about 1.5% nucleic acid which led to aggregation of all the proteins present.

Polyacrylamide gel electrophoresis, after treatment of the enzyme with 1% sodium dodecyl sulfate, revealed the presence of two major protein components and several minor ones. Since there are only two major synthetase activities present, and the purified enzyme has a specific activity in the range of other highly purified preparations, it is possible that one of the two major protein components is the glutamyl-RNA synthetase. In addition, proteins that are not bound to nucleic acid would probably have been removed during the purification procedure. If these assumptions are correct, the enzyme appears to be about 40% pure.

In contrast to the preparation of Alford et al. (9), the enzyme described here was quite stable to storage. The reason for the inactivation of the enzyme in the presence of sulfhydryl compounds is not known, but it may have been due to disulfide formation or interconversion of groups on the enzyme. The heat lability of the purified enzyme was somewhat greater than that of a pH 5 fraction from rat liver studied by Alford et al. (9), which lost about half its activity in 10 min at 45°C. A glutamyl-RNA synthetase purified from E. coli W by Ravel et al. (12) was quite stable to heat, losing only about 5% of its activity in 10 min at 55°C. Some of these differences, however, may be due to different conditions of heating.

Several types of evidence indicate that glutamic acid and glutamine are activated by different enzymes in liver. During the purification and storage of glutamyl-RNA synthetase, the ratio of glutamate activity to that for glutamine changed. In addition, a large excess of unlabelled glutamine did not affect 14C-glutamate incorporation into sRNA. Similarly, the presence of unlabelled glutamine did not alter 14C-glutamine incorporation. Alford et al. (9) have reported different rates of heat inactivation for the two activities, and have shown that the ATP·PP' exchange activities dependent on these two amino acids were additive. Although all these results do not unequivocally prove the existence of two enzymes, they suggest that, as in E. coli (11, 12), mammals also have separate routes for the incorporation of glutamate and glutamine into proteins. Final proof for this idea will come only after the separation of the two mammalian enzymes, as has been achieved for the corresponding enzymes in E. coli (11, 12).

Acknowledgments—I would like to express my appreciation to Drs. Sasha Englard, Bertram Lowy, and Julius Marmor for their advice and encouragement throughout this work. I also wish to thank Dr. Abraham White and Dr. Sasha Englard for their helpful criticisms of this manuscript. The assistance of Miss Sharon Pifko and Dr. Arabinda Guha in some of the studies reported here is gratefully acknowledged.

REFERENCES

17. Wadburg, O., and Christman, W., Biochem. Z., 310, 384 (1941).
Rat Liver Glutamyl Ribonucleic Acid Synthetase: I. PURIFICATION AND EVIDENCE FOR SEPARATE ENZYMES FOR GLUTAMIC ACID AND GLUTAMINE
Murray P. Deutscher


Access the most updated version of this article at http://www.jbc.org/content/242/6/1123

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/242/6/1123.full.html#ref-list-1