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Separation of Specific Glutamate- and Glutamine-activating Enzymes from *Escherichia coli*

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Two enzyme preparations have been obtained from *Escherichia coli* that exhibit specificity for esterifying glutamate and glutamine, respectively, to s-RNA. Each s-RNA species has been shown to be specific for the individual amino acid by determinations of capacity for acceptance of glutamate and glutamine alone and in combination, and by separation on columns of methylated albumin of the two species of s-RNA charged with the respective amino acids.

Recent investigations have given convincing evidence that the activation of amino acids as the enzyme-bound amino acid adenylate and the subsequent transfer to a soluble RNA molecule form a highly specific process in which the identity of the amino acid is conserved at each step. Thus, with few exceptions (Bergmann *et al.*, 1961; Sharon and Lipmann, 1957; Davie *et al.*, 1956), purified amino acid–activating enzymes catalyze a pyrophosphate-ATP exchange that is dependent upon a particular amino acid. Similarly, the transfer of the activated amino acid to soluble RNA is an equally specific process in which only certain s-RNA molecules can accept a particular amino acid. Chapeville *et al.* (1962) and Von Ehrenstein *et al.* (1963) have shown that the s-RNA moiety rather than the attached amino acid serves as the identifying character in the final step, the amino acid polymerization at the ribosomal sites.

Recently it has been proposed that glutamate, like hydroxyproline in collagen, is not directly incorporated into protein but arises there from the deamidation of glutamine (Zubay, 1962). This proposal was based on kinetic evidence that showed that glutamine was incorporated in the s-RNA fraction more rapidly than glutamate and the product of glutamate incorporation was glutaminyl-s-RNA. However, this proposal is not supported by the reports from the laboratories of Nirenberg (Matthaei *et al.*, 1962) and Ochoa (Wahba *et al.*, 1963), which show that the coding units for glutamine and glutamate are different. More direct evidence for the independence of glutamate and glutamine incorporation recently has been presented by Coles (Coles *et al.*, 1962; Coles and Meister, 1962). These data show that glutamate can be incorporated into yeast s-RNA without passing through a free glutamine intermediate, and that the product of glutamate incorporation is α-glutamyl-s-RNA.

In the course of our work on the activation and transfer of amino acids in protein synthesis we have separated and purified the activating enzymes for glutamate and glutamine from *Escherichia coli* and have obtained evidence for separate s-RNA's for each amino acid. The evidence presented in this communication strengthens and extends the argument (Coles and Meister, 1962) that glutamine and glutamate are incorporated into s-RNA independently.

Materials and Methods

Tritium and carbon-14-labeled glutamic acid were obtained from Nuclear Chicago Corp. and New England Nuclear Corp., respectively. Carbon-14-labeled glutamine was obtained from Schwartz Bioresearch, Inc. *E. coli* s-RNA was purchased from General Biochemicals. Protamine sulfate and ATP were obtained from Sigma Chemical Co. Diethylaminoethyl-cellulose was purchased from the Brown Co. Columns of hydroxylapatite and methylated albumin-kieselguhr were prepared as described by Levin (1962) and Mandell and Hershey (1960), respectively.

Estimation of Enzyme Activity.—Glutamyl- and glutaminyl-RNA synthetases were estimated by their ability to catalyze the incorporation of ^{14}C-labeled amino acid into a trichloroacetic acid–precipitable material. The assay mixture contained in 0.2 ml: 25 μmoles potassium cacodylate, *p*H 7.0; 0.2 μmole ATP; 1 μmole $MgCl_2$; 0.25 μmole mercaptoethanol; 0.5 mg s-RNA; 0.01 μmole ^{14}C-labeled amino acid (about 10 μc/μmole); and enzyme. The reactions were initiated by the addition of the enzyme and terminated after 10 minutes' incubation at 35° by the addition of 1 ml of 10% trichloroacetic acid. The resulting suspensions were chilled for 10 minutes in an ice bath, and filtered through a 25-mm Millipore filter. The precipitated material was washed with 50 ml of 5% trichloroacetic acid. The precipitates, together with the Millipore filters, were then neutralized with 1 ml 1.5 N NH_4OH, dissolved in 10 ml of the liquid scintillator described by Bray (1960), and assayed for radioactivity in a Packard Tricarb liquid scintillation spectrometer.

One unit of glutaminyl- or glutamyl-RNA synthetase is defined as the amount of enzyme that incorporates 1 mμmole of amino acid into RNA under the conditions of the standard assay.

Glutaminase was estimated by ammonia liberation from glutamine. The assay mixture contained in 1 ml: 100 μmoles of potassium cacodylate, *p*H 5.0, 100 μmoles of glutamine, and enzyme. The reaction was allowed to proceed for 15 minutes at 35°. The ammonia was isolated by microdiffusion and estimated by nesslerization.

The products of glutamate and glutamine incorporation into s-RNA were determined by hydrolysis of the amino acids from the charged RNA and identification of the amino acids by paper electrophoresis, paper chromatography, or ion-exchange chromatography on Dowex-1 (formate). For these purposes 200-mg quantities of RNA were enzymatically charged with [^{14}C]-glutamate or glutamine. The charged RNA was separated from the majority of the free amino acid by precipitation with one-half volume of 5 M NaCl and three volumes of ethanol. The precipitated RNA, dissolved in a minimal amount of H_2O, was further purified by passage through a Sephadex G-25 column. The desalted RNA solution was reduced to 3 ml in a rotary evaporator, adjusted to a final concentration of 1.0 N NH_4OH, and incubated for 2.5 hours at 37°. The RNA was precipitated from the ammoniacal solutions by the addition of two volumes of 2-propanol and one volume of ethanol. After 1 hour's storage at −20° the suspension was centrifuged and the supernatant liquor was taken to dryness in a rotary evaporator at 45°. The residues were first analyzed by paper electrophoresis with a diethyl barbiturate buffer at *p*H 8.5 (Coles *et al.*, 1962) and with pyridine acetate at *p*H 6.4 (Ingram, 1956). In these systems glutamine migrates only 1 cm while glutamate and pyrrolidonecarboxylic acid migrate about 15 cm in 2 hours at 26 volts/cm. When the presence of glutamine was indicated by electrophoresis, the identity of the amino acid was confirmed by paper chromatography in butanol–acetic acid–H_2O, 4:1:1 (Krishnaswamy *et al.*, 1960). Similarly, when indicated by electrophoresis, the presence of glutamate was confirmed by ion-exchange chromatography on Dowex-1 (formate). For this analysis the hydrolysate, diluted to 5 ml with water, was adsorbed on a 0.8 × 6-cm column of Dowex-1 (formate). Unadsorbed material was removed from the column with 20 ml of water. Glutamate and pyrrolidinecarboxylic acid were eluted with a linear gradient formed between 75 ml of H_2O and 75 ml of 0.1 M formic acid. Five-ml fractions were collected during the gradient elution. Under these conditions glutamate appears in fractions 6 through 8 and pyrrolidonecarboxylic acid in the fractions 19 through 24. Glutamine is not adsorbed under these conditions and appears in the forerun and initial water wash.

Results

Purification of Glutamyl-RNA Synthetase.—Two purification procedures were developed in the course of our work. The advantage of the first method (A) is that a preparation of glutaminyl-RNA synthetase, which is free of glutamyl-RNA synthetase, is a by-product. The second method (B), however, yields a more purified preparation of glutamyl-RNA synthetase that is free of the glutamine activating enzyme. Since enzymes purified by both methods were employed in this study, both methods are briefly outlined.

Method A.—A crude extract of *E. coli* B was prepared by blending, at 2/3 line voltage in a Waring Blendor, 50 g of frozen *E. coli* cells, 100 ml of 0.025 M Tris, *p*H 8.0, and 150 g of Superbrite glass beads. The mixture was blended until the temperature rose to 8°. It was then cooled in an acetone–water–dry ice slurry until ice formed in the extract. The blending-cooling cycle was repeated until the extract had been blended for a total of 15 minutes. Seventy-five ml of 0.025 M Tris, *p*H 8.0, was then added and the diluted suspension was blended for an additional 2 minutes. The sediment was allowed to settle and the supernatant fluid was decanted. The sediment was further extracted with 150-ml aliquots of Tris buffer and the combined extracts were clarified by centrifugation for 90 minutes at 11,000 × *g*. A warm (40°) protamine sulfate solution (10 mg/ml) was added slowly to the rapidly stirred crude extracts until 10 mg of protamine had been added for every 100 mg of bacterial protein. The precipitate was allowed to coagulate for 15 minutes at ice-bath temperature and was then removed by centrifugation. The slightly turbid supernatant fluid was adjusted to *p*H 7.0 with 1 M KH_2PO_4 and applied to a DEAE-cellulose column (3 × 30 cm) previously equilibrated with 0.02 M potassium phosphate, *p*H 7.0, containing 3×10^{-3} M mercaptoethanol. The column was washed with 0.02 M potassium phosphate, *p*H 7.0, containing 10^{-3} M mercaptoethanol, until the E_{280} of the effluent fell below 0.1. The activities were then eluted with a linear gradient formed between 750 ml 0.02 M potassium phosphate, *p*H 7.0, and 750 ml 0.33 M potassium phosphate, *p*H 7.0, both containing 3×10^{-3} M mercaptoethanol. The elution pattern is shown in Figure 2. Fractions 29 through 41, inclusive, were combined and saved as glutaminyl-RNA synthetase. Fractions 62 to 74, inclusive, were combined, desalted by passage through a column of Sephadex G-25 equilibrated with 0.01 M potassium phosphate (*p*H 6.7), and adsorbed on a hydroxylapatite column (70-ml vol). The column was eluted sequentially with 150-ml aliquots of 0.02 M, 0.05 M, and 0.08 M potassium phosphate, *p*H 6.7. The glutamyl-RNA synthetase was then eluted with 300 ml 0.1 M and 150 ml 0.15 M potassium phosphate, *p*H 6.7. After dialysis against 10 liters of 0.005 M potassium phosphate, *p*H 7.0, the combined eluates were concentrated and further purified by absorption on a small DEAE-cellulose column (1 × 5 cm). The column was first developed with 50-ml aliquots of 0.02 M, 0.07 M, and 0.1 M phosphate, *p*H 7.0. The activity was then eluted with 50 ml 0.13 M and 25 ml 0.17 M phosphate, *p*H 7.0.

Method B.—A crude extract of 100 g of *E. coli* was prepared, treated with protamine sulfate, and centrifuged as described in method A. The glutamyl-RNA synthetase was precipitated from the protamine supernatant fluid by the addition of an amount of protamine sulfate equal to the first addition. The precipitate was collected by centrifugation, and the glutamyl-RNA synthetase was extracted by suspending the precipitate in 50 ml of 0.05 M $(NH_4)_2SO_4$ with the aid of a glass homogenizer and allowing the suspension to stand overnight at 4°. The suspension was then centrifuged at 32,000 × *g* for 20 minutes to remove the inactive protein. The extract was treated with an equal volume of calcium phosphate gel (14 mg/ml) and after 10 minutes the gel was removed by centrifugation. The gel supernatant protein was then adsorbed on a column of hydroxylapatite (65 ml vol.) and the column was sequentially developed with 100-ml aliquots of 0.05 M, 0.07 M, and 0.10 M potassium phosphate, *p*H 6.7. The glutamyl-RNA synthetase was eluted with 150 ml of 0.13 M phosphate, *p*H 6.7. The buffer concentration of the active fraction was adjusted to 0.01 M phosphate, *p*H 6.7, by passing through a Sephadex G-25 column previously equilibrated with that buffer. The enzyme was then readsorbed on a small column of hydroxylapatite (7 ml vol). After the column was developed with 10-ml aliquots of 0.04 M, 0.07 M, and 0.1 M phos-

TABLE I
SUMMARY OF PURIFICATION OF GLUTAMYL-RNA SYNTHETASE

Fraction	Volume (ml)	Activity (units)	Protein (mg)	Specific Activity (units/mg)	Recovery (%)
Method A					
Crude extract	230	83,000	9,300	8.9	
Protamine supernatant	285	74,000	2,480	29.9	89
Combined DEAE fractions	175	45,400	193	235	54
Hydroxylapatite eluate	470	30,200	52	586	37
2nd DEAE eluate	75	26,200	16	1640	32
Method B					
Crude extract	420	68,600	14,700	4.7	
Protamine eluate	50	31,000	1,070	29.0	46
$Ca_3(PO_4)_2$ gel supernatant	84	30,200	400	75.5	45
1st Hydroxylapatite eluate	142	16,700	25.5	656	25
2nd Hydroxylapatite eluate	20	16,800	10.2	1650	25

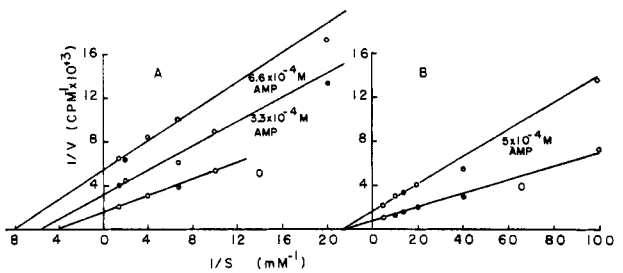

FIG. 1.—The effect of 5′-AMP on glutamate incorporation as a function of ATP and glutamate concentrations. (A) Reciprocal plot of velocity against ATP concentration in the presence and absence of 5′-AMP. (B) Reciprocal plot of velocity against glutamate concentration in the presence and absence of 5′-AMP. The reaction conditions were those of the standard assay except for the departures indicated in the figures.

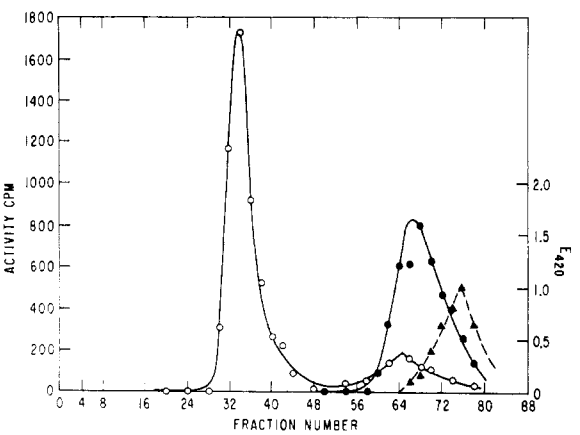

FIG. 2.—Elution pattern from DEAE-cellulose under a gradient of increasing phosphate concentration. The open and solid circles represent activity obtained with glutamine and glutamate, respectively, expressed as cpm incorporated by 1 μl of eluate. Triangles represent the results of glutaminase assays expressed as E_{420}. The specific activities of the [^{14}C]glutamate and [^{14}C]glutamine were 2500 and 8200 cpm/mμmole, respectively.

phate, *p*H 6.7, the enzyme was eluted with 20 ml of 0.13 M phosphate, *p*H 6.7.

Summaries of the two methods of purification are presented in Table I. As can be seen, both methods yield a purification of 200 to 300-fold with a recovery of 25–30%.

The incorporation of glutamate and glutamine into RNA exhibited an absolute requirement for ATP, enzyme, and RNA. Under the standard reaction conditions the incorporation of the amino acids was proportional to time and enzyme concentration during the first 25% of the reaction. Purified preparations of glutamyl-RNA synthetase exhibited maximal activity between *p*H values of 6.7 and 7.2 when Tris-HCl, imidazole-HCl, or potassium cacodylate was used as the buffering agent. In 0.05 M imidazole-HCl 28% and 48% of maximal activity were observed at *p*H 6.0 and *p*H 8.0, respectively.

The incorporation of glutamate into RNA is strongly inhibited by 5′-AMP. The rate of incorporation is 50% inhibited by 5×10^{-4} M 5′-AMP. Surprisingly, the inhibition is not a simple competitive inhibition between the mononucleotide and ATP (Fig. 1A). This inhibition appears to be quite specific; IMP, UMP, GMP, ADP, and IDP were without effect on the rate of glutamate incorporation when tested at 10^{-3} M.

The responses of the glutamate-activating enzyme to increasing concentration of glutamate and ATP are shown in Figure 1 in the Lineweaver-Burk representation. The Michaelis constant for glutamate is approximately 1×10^{-4} M and is unaffected by the presence of 5′-AMP, while that for ATP is 2.5×10^{-4} M and decreases in the presence of AMP.

The molecular weight of the glutamate-activating enzyme was estimated to be approximately 50,000 by sucrose-gradient centrifugation (Martin and Ames, 1961).

The elution patterns of glutaminyl- and glutamyl-RNA-synthetase activities (Fig. 2) provide evidence that glutamate is activated by an enzyme different from that activating glutamine. A variable amount of apparent glutamine-activating enzyme elutes much later (fractions 52–78) than the greater part of that activity. This second peak yields, upon rechromatography, two peaks of activity corresponding in position to those found in the original elution pattern. Although the appearance of the original second peak of glutaminyl-RNA synthetase in part is due to a chromatography artifact, some of the activity is due to the presence of enzymes catalyzing the incorporation of glutamine into glutamyl-RNA.

The independence of glutamine and glutamate incorporation into RNA is illustrated further in Table II, where the ratios of the two activities in different fractions are compared. Glutaminyl-RNA synthetase

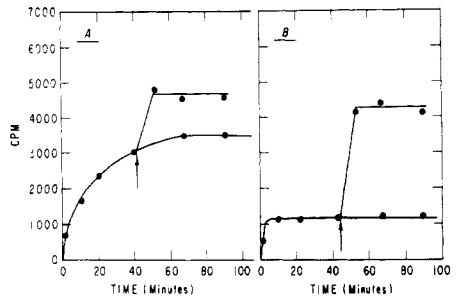

FIG. 3.—Sequential incorporation of glutamate and glutamine into s-RNA. (A) The incorporation of glutamine into s-RNA charged with glutamate. Reaction mixture contained in 2 ml: 250 μmoles potassium cacodylate, *p*H 7.0; 2 μmoles ATP; 2 μmoles mercaptoethanol; 10 μmoles $MgCl_2$; 0.1 μmole [^{14}C]glutamate (13,000 cpm/mμmole); 5.0 mg RNA; and 30 units of glutamyl-RNA synthetase (sp act 1640 units/mg). At the time indicated by the arrow 1.4 ml of the reaction mixture was mixed with 0.1 μmole [^{14}C]glutamine (8200 cpm/mμmole) and 16 units of glutaminyl-RNA synthetase. The balance of the reaction mixture was unaltered and served as a control. The experimental points represent the trichloroacetic acid—precipitable radioactivity per 0.1 ml of reaction mixture. (B) The incorporation of glutamate into s-RNA charged with glutamine. The reaction mixture and conditions were the same as in (A) except that glutamine and glutaminyl-RNA synthetase were present at zero time and glutamate and glutamyl-RNA synthetase were added at the time indicated by the arrow.

TABLE II
GLUTAMYL- AND GLUTAMINYL-RNA-SYNTHETASE LEVELS IN VARIOUS FRACTIONS

Fraction	Glutamyl-RNA Synthetase (units/ml)	Glutaminyl-RNA Synthetase (units/ml)	Ratio
Crude	360	130	2.8
Combined DEAE fractions 29-41 (method A)	0.3	320	<0.001
2nd Hydroxylapatite eluate (method B)	900	14.3	70

eluted from DEAE-cellulose is virtually free of contaminating glutamyl-RNA synthetase. However, the the most purified fractions of glutamyl-RNA synthetase still contain apparent glutaminyl-RNA synthetase activity. That this activity was in fact due to hydrolysis of glutamine to glutamate and incorporation of the latter is shown by the fact that the amino acid obtained upon mild hydrolysis of the product was glutamate, not glutamine (*vide infra*), and that prior incorporation of glutamate into the RNA eliminated subsequent activity with glutamine. This was established by adding [^{14}C]glutamine to an incubation mixture in which the RNA had been fully charged with [^{14}C]-glutamate. The specific activity of the glutamine and the amount of enzyme present were sufficient to allow the incorporation of 4000 cpm/mg RNA (maximal incorporation) in 15 minutes in the absence of glutamate. The maximum increase over the 2200 cpm/mg RNA incorporated as glutamate observed during the next 2 hours was 400 cpm. This increase is much less than the 3200 cpm/mg RNA, incorporated when glutamine is maximally charged into RNA by glutaminyl-RNA synthetase.

The Product of Glutamine and Glutamate Incorporation into RNA.—An analysis of the amino acid esterified to the RNA when glutamine and glutamate are employed as substrates was undertaken to determine whether the amino acids maintained their identity throughout the course of activation and esterification to the s-RNA. For this purpose three aliquots of s-RNA were maximally charged with a labeled amino acid. The first was charged with [^{14}C]glutamine using glutaminyl-RNA synthetase, the second with [^{14}C]glutamine using glutamyl-RNA synthetase, and the third with [^{14}C]glutamate using glutamyl-RNA synthetase. The product of each reaction was purified and hydrolyzed, and the amino acid was freed of the nucleic acid as described under Materials and Methods. Approximately 95% of the radioactivity recovered from the product of glutamine incorporation by the glutaminyl-RNA synthetase was shown to be glutamine by paper electrophoresis and paper chromatography. However, the products of glutamine and glutamate incorporation by the glutamyl-RNA synthetase were found to consist mainly of glutamate. The hydrolyzed product of glutamate incorporation migrated as a single peak at *p*H 6.4 and 8.5, while that of glutamine was resolved into two components, one in the glutamine region representing approximately 10% of the total activity, the other in the glutamate region. Analysis of these hydrolysates by ion-exchange chromatography on Dowex-1 (formate) confirmed the identification of glutamate as the major radioactive material (Table III).

TABLE III
CHROMATOGRAPHIC ANALYSIS OF THE HYDROLYZED PRODUCTS OF GLUTAMYL-RNA SYNTHETASE-CATALYZED REACTIONS

[^{14}C]-Amino Acid Substrate	Radioactivity Applied to Column	Radioactivity Recovered: Forerun	Glutamate	Pyrrolidonecarboxylic Acid
Glutamine	108,000	13,000	75,000	<100
Glutamate	34,000	2,160	29,230	<100

The Independence of Glutamate and Glutamine Incorporation into RNA.—Independent incorporation of glutamate and glutamine into protein requires that each amino acid have a specific s-RNA. That glutamate and glutamine are charged onto separate and distinct s-RNA molecules is indicated by the data shown in Figure 3. The extent to which glutamate can be incorporated into the s-RNA fraction is unaffected by glutamine incorporation. Similarly the incorporation of glutamine is not diminished by prior incorporation of glutamate. Corroborating evidence for separate s-RNA molecules is provided by chromatographic separation of glutamine- and glutamate-charged s-RNA. For this experiment s-RNA was sequentially charged with ^{3}H-labeled glutamate and ^{14}C-labeled glutamine, separated from the free amino acids by passage through a Sephadex G-25 column, and chromatographed on a column of methylated albumin-kieselguhr according to the method of Sueoka and Yamane (1962). The elution pattern (Fig. 4) exhibits separate peaks of tritium and radiocarbon activity, indicating a separation of the two species of s-RNA.

DISCUSSION

The hypothesis advanced by Zubay (1962) requires that glutamine but not glutamate be directly incorporated into s-RNA and that apparent charging of s-

RNA with glutamate is the result of sequential reactions in which glutamate is first converted to the amide and then incorporated. Thus this hypothesis predicts the absence of both a glutamyl-RNA synthetase and a separate species of s-RNA specific for glutamate. The data presented in this communication demonstrate that glutamate and glutamine are activated by different enzymes and incorporated into different species of s-RNA. Furthermore, the results of the amino acid analysis show that glutamyl-s-RNA and glutaminyl-s-RNA were the products of glutamyl- and glutaminyl-RNA-synthetase reactions. Although the most purified preparation of glutamyl-RNA synthetase showed some activity with glutamine, it is unlikely that this small amount of activity could affect the specificity of amino acid incorporation for several reasons. First, the activity of the enzyme is 70-fold greater with glutamate and the incorporation of glutamate into RNA eliminates the activity with glutamine. This latter observation suggests that the amino acid is esterified to the RNA specific for glutamate. Second, the major product of the incorporation is glutamyl-RNA as shown by the analysis of the hydrolyzed product. The glutamine recovered in the hydrolysate probably represents a contamination of the product with some of the [^{14}C]-glutamine originally present in the reaction mixture. A contamination of 0.2% of the original amino acid would account for the level of glutamine found.

Experiments in which glutamine and glutamate were sequentially incorporated into the same preparation of s-RNA provide direct evidence for the existence of separate acceptor sites for these amino acids. Maximal incorporation of one amino acid was not diminished by prior incorporation of the other. Furthermore, the chromatographic elution pattern of RNA preparations that have been maximally charged with [^{3}H]glutamate and [^{14}C]glutamine exhibit separate peaks of tritium and carbon-14 activity, indicating that separate species of RNA are involved in the acceptance of glutamine and glutamate.

These results were foreshadowed by reports that the incorporation of [^{14}C]glutamate into s-RNA was only marginally affected by the presence of free glutamine (Fraser *et al.*, 1959; Coles and Meister, 1962) and that the product of glutamate incorporation was α-glutamyl-RNA (Coles *et al.*, 1962; Fraser, 1962). More recently, Alford *et al.* (1963) have presented additional evidence for the discrete activation of glutamate and glutamine by enzymes in rat liver and mouse ascites-tumor cells. However, the purification procedures reported here separate glutaminase, glutamyl-RNA synthetase, and glutaminyl-RNA synthetase, allowing a clear demonstration that glutamate can be independently incorporated into s-RNA. Thus, from the point of view of activation for incorporation into proteins, glutamate and glutamine are two distinct amino acids that are handled specifically by the same mechanisms as other amino acids. This is consistent with the implication that separate s-RNA molecules must carry these amino acids to account for the differential response to synthetic messengers in ribosomal transfer systems (Matthaei *et al.*, 1962; Wahba *et al.*, 1963).

While this manuscript was in preparation, similar conclusions were published in a preliminary report by Ravel *et al.* (1964).

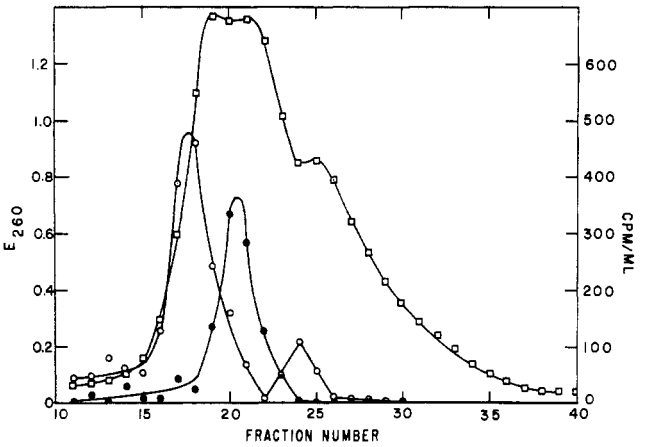

FIG. 4.—Elution pattern of s-RNA from a column of methylated albumin-kieselguhr. Four mg of s-RNA that had been maximally charged with [^{3}H]glutamate (7120 cpm/mμmole) and [^{14}C]glutamine (8200 cpm/mμmole) was chromatographed on methylated albumin-kieselguhr according to the method of Sueoka and Yamane (1962). Five-ml fractions were collected. Squares represent the absorbance at 260 mμ of the fractions, open and solid circles represent, respectively, the tritium and radiocarbon activity per ml of the fractions.

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