Further Studies on the Soluble Amino Acid Incorporating System from Rat Liver*

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ABSTRACT: A system which incorporates [^{14}C]arginine, [^{14}C]glutamic acid, [^{14}C]glycine, [^{14}C]methionine, and [^{14}C]tryptophan into the protein fraction in the absence of ribosomes has been obtained from rat liver. The incorporation of [^{14}C]arginine was sensitive to puromycin, ribonuclease, and dependent upon transfer ribonucleic acid, adenosine triphosphate, and its generator system. Sucrose density gradient centrifugation of the system and the radioactive product revealed that both have a sedimentation coefficient of approximately 9–10 S. The incorporation represents a major portion of the transfer of amino acid from aminoacyl transfer ribonucleic acid to a preformed acceptor protein at the NH_2-terminal end.

In the preceding reports (Kaji *et al.*, 1965a,b, 1963a; Kaji and Kaji, 1964; Momose and Kaji, 1965, 1966) characteristics of the soluble amino acid incorporating system from *Escherichia coli* have been reported. It was found that certain amino acids such as phenylalanine or leucine are transferred to protein acceptors from aminoacyl-tRNA in the absence of ribosomes. In an attempt to discover the biological significance of this reaction, a search for a similar system has been extended to rat liver (Kaji *et al.*, 1963b). In this communication we report that rat liver contains a system which incorporates certain amino acids such as arginine, glutamic acid, methionine, tryptophan, and glycine. Since arginine is incorporated most actively, the nature of the product with [^{14}C]arginine was studied in detail. The reaction apparently represents NH_2-terminal addition of [^{14}C]-arginine to a preformed protein acceptor.

Materials and Methods

Preparation of the Soluble Amino Acid Incorporating System from Rat Liver. Male rats (Sprague–Dawley strain) weighing 250–300 g were decapitated and their livers were isolated quickly in cold (0–2°) 0.3 M sucrose. Liver (100 g) was minced by scissors in 15 ml of medium A consisting of 4 mM magnesium acetate, 12.5 mM KCl, 0.01 M β-mercaptoethanol, 0.05 M Tris-HCl (pH 7.8), and 0.3 M sucrose. Minced liver, 5 g at a time, was homogenized in 2.4 volumes of medium A in a glass homogenizer. The total volume of medium A added was 250 ml (2.5 times the tissue weight) and cellular debris was removed by centrifuging at 20,000*g* for 20 min. The top lipid layer was pipetted and discarded. The supernatant was recentrifuged at 32,000*g* for 20 min after dilution with 1.5 volumes of medium B (same as medium A but without Tris-HCl). The lipid layer was again aspirated and discarded. The supernatant (S-32) was centrifuged at 85,000*g* for 2.5 hr. The resulting supernatant, S-85, was diluted with medium A to a protein concentration of 12 mg/ml by medium A and 0.05 volume of freshly prepared 10% streptomycin sulfate was added. The resulting precipitate was centrifuged at 32,000*g* for 15 min and the precipitate was separated from the supernatant (streptomycin supernatant). To the streptomycin supernatant 0.05 volume of 1% protamine in medium A was added. The mixture was centrifuged at 32,000*g* for 15 min to obtain the supernatant (protamine supernatant) and precipitate (protamine precipitate). The latter was extracted with 50 ml of 0.3 M $(NH_4)_2SO_4$ in medium A, followed by centrifugation at 32,000*g* for 15 min to eliminate insoluble material. To the protamine eluate 23.5 ml of saturated $(NH_4)_2SO_4$ (pH 7.2) saturated at 25° was added and the precipitate (fraction I) was collected and dissolved in 5 ml of medium A. To the supernatant was added an additional 23.7 ml of the saturated $(NH_4)_2SO_4$; the precipitate was collected and dissolved in 10 ml of medium A (fraction II). The protein in the $(NH_4)_2SO_4$ supernatant left after removal of fraction II was precipitated by addition of 60 ml of the saturated ammonium sulfate solution and the collected precipitate was dissolved in 15 ml of medium A (fraction III). Fractions I–III were dialyzed against medium C (medium A without sucrose). The precipitate obtained after the addition of streptomycin was dissolved in 5 ml and dialyzed against medium C for 10 hr at 4°. In some cases streptomycin supernatant was added with $(NH_4)_2SO_4$ to 75% saturation; the precipitate was collected and suspended in 10 ml of medium C. The suspension was dialyzed against medium C for 10 hr at 4°. This fraction was called concentrated streptomycin supernatant fluid (conc SM-sup).

Preparation of Ribosomes and Supernatant Fluid for Incorporation. To part of the S-32 described in the previous section was added 0.083 volume of a 5% deoxycholate to obtain a final concentration of deoxycholate

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TABLE I: Incorporation of Various Amino Acids by Fraction II and by the Ribosomal System.[a]

Amino Acids	cpm/0.1 ml of Reaction Mixture at 40 min		mμmoles of Amino Acid Incorpd/mg of Protein	
	Fraction II	Ribosomal	Fraction II	Ribosomal
Glycine	5	431	2.4×10^{-4}	1.8×10^{-2}
Lysine	0	1225	0	1.6×10^{-2}
Glutamic acid	34	227	1.1×10^{-3}	6.3×10^{-3}
Valine	2	213	3.7×10^{-4}	3.5×10^{-3}
Serine	3	258	1.1×10^{-4}	8.2×10^{-3}
Isoleucine	13	736	2×10^{-4}	1.0×10^{-2}
Aspartic acid	10	538	4×10^{-4}	1.9×10^{-2}
Alanine	0	315	0	8.5×10^{-3}
Arginine	1255	450	3.6×10^{-2}	1.1×10^{-2}
Threonine	0	370	0	8.8×10^{-3}
Leucine	6	1323	2×10^{-4}	1.8×10^{-2}
Phenylalanine	24	513	6.5×10^{-4}	1.2×01^{-2}
Histidine	14	86	4×10^{-4}	2.2×01^{-3}
Tyrosine	8	180	2×10^{-4}	4×10^{-3}

[a] Components for the reaction mixture were identical with those of the complete system in Figure 1, except for amino acids. Each reaction mixture contained 0.025 μmole of each of 20 amino acids minus the particular amino acid whose incorporation was being tested. For the ribosomal system, 0.92 mg of S-85 and 0.58 mg of ribosomes were added and the amount of fraction II used was 1.25 mg. The incubation was carried out at 37° and 0.1 ml of the reaction mixture was taken for measurement.

of 0.65%. This was stirred for 1 min. The ribosomes were obtained as a pellet after centrifugation in the Spinco Model L for 2.5 hr at 85,000*g*. The resulting supernatant was discarded. The remaining S-32 was centrifuged for 2.5 hr at 85,000*g* without addition of deoxycholate. This supernatant fluid was the source of the aminoacyl-tRNA synthetases of the supernatant for ribosome-dependent incorporation.

Preparation of tRNA from Rat Liver and E. coli. Rat liver tRNA was prepared according to the method of Brunngraber (1962) and *E. coli* tRNA was prepared as described by Ofengand *et al.* (1961) or purchased from General Biochemical Corp.

Reaction Mixture for Incorporation of [^{14}C]Amino Acids. A typical complete reaction mixture contained in a total volume of 0.5 ml: 2.0 μmoles of ATP (potassium salt), 4.0 μmoles of phosphoenolpyruvate (sodium salt), 0.05 mg of pyruvate kinase, 50 μmoles of Tris buffer (pH 7.5), [^{14}C]amino acids, 4.8 μmoles of magnesium acetate, 1 μmole of $MnCl_2$, 2 μmoles of β-mercaptoethanol, 0.2 μmole of GTP (sodium salt), 0.075 μmole each of 20 L-amino acids minus the particular radioactive amino acid whose incorporation was to be tested, and 0.14 mg of tRNA. Aliquots of 0.1 ml were taken at the time intervals, and incorporated radioactivity was counted by the method of Mans and Novelli (1961). The incorporation of amino acids into the protein fraction means incorporation of amino acids into the fraction which is insoluble in hot (95°) 5% trichloroacetic acid, cold (5°) 5% trichloroacetic acid, ethanol–ether mixture (1:1, v/v), and ether.

Preparation of Labeled Product. For preparation of the [^{14}C]arginine-labeled product of the soluble system, the reaction mixture contained the following in μmoles/30 ml: Tris (pH 7.5), 3000; ATP-K, 120; phosphoenolpyruvate (Na), 2400; magnesium acetate, 144; manganese chloride, 60; β-mercaptoethanol, 120; KCl, 1500; and 50 μmoles each of 19 amino acids. In addition it contained 8.25 mg of rat liver tRNA, 3 mg of pyruvate kinase, 216 mg of "conc SM-sup," and 0.3 ml of [^{14}C]-arginine. After 60-min incubation at 37°, 30 ml of 10% trichloroacetic acid was added to the reaction mixture. Under these conditions, 3564 cpm/0.1 ml was incorporated into the protein fraction. The precipitate was washed three times with 30 ml of 5% trichloroacetic acid, suspended in 30 ml of 5% trichloroacetic acid, and heated at 90° for 30 min. The precipitate was then collected and the hot trichloroacetic acid treatment was repeated as above. The protein was suspended in 10 ml of ether–ethanol mixture (1:1, v/v) and incubated at 37° for 15 min. The precipitate was collected by centrifugation, treated again with ether–ethanol, and washed three times with ether. The white powder obtained after decantation of the ether was stored at −20° until further analyzed.

NH_2-Terminal Analysis. The [^{14}C]arginine-labeled product (5.25 mg) was prepared with "conc SM-sup." This was suspended in 1.0 ml of 0.6 M $(NH_4)_2CO_3$. Dinitrophenylation was started by the addition of 2 ml of 5% dinitrofluorobenzene in ethanol, and the mixture was shaken for 18–23 hr at room temperature (Fraenkel-Conrat *et al.*, 1955). After dinitrophenylation was completed, the pH was adjusted to around 2.0 by the addition of 12 N HCl and the mixture was treated three times

TABLE II: Incorporation of Various Amino Acids by "Conc SM-Sup" and by the Ribosomal System.[a]

Amino Acids	cpm/0.1 ml of Reaction Mixture at 40 min		mμmoles of Amino Acid Incorpd/mg of Protein	
	"Conc SM-sup"	Ribosomal	"Conc SM-sup"	Ribosomal
Glycine	506	607	1.4×10^{-2}	1.9×10^{-2}
Lysine	27	234	3.4×10^{-4}	2.4×10^{-3}
Glutamic acid	370	352	8.6×10^{-3}	7.4×10^{-3}
Valine	6	121	6.6×10^{-5}	1.2×10^{-3}
Serine	15	84	4×10^{-4}	2×10^{-3}
Isoleucine	4	233	4.4×10^{-5}	2.3×10^{-3}
Aspartic acid	1	125	2.9×10^{-5}	3.3×10^{-3}
Alanine	3	83	6.8×10^{-5}	1.7×10^{-3}
Arginine	829	1349	1.7×10^{-2}	2.4×10^{-2}
Threonine	21	126	4.2×10^{-4}	2.2×10^{-3}
Leucine	34	549	2.8×10^{-4}	5.6×10^{-3}
Phenylalanine	21	228	4.2×10^{-4}	4.1×10^{-3}
Histidine	11	60	2.3×10^{-4}	1.1×10^{-3}
Tyrosine	33	76	6.1×10^{-4}	1.3×10^{-3}
Methionine	46	90	3.3×10^{-3}	2.3×10^{-3}
Tryptophan	40	26	5.4×10^{-3}	7.5×10^{-3}
Proline	1	312	2.8×10^{-4}	7.8×10^{-3}

[a] Reaction components are identical with those of the complete system in Figure 1 and Table I except that 1.8 mg of "conc SM-sup" and 200 μg of ribosomes were added to 0.5 ml of reaction mixture for the ribosomal system. The amount of "conc SM-sup" for the soluble system used was 1.8 mg.

with 10 ml of ether to remove unreacted dinitrofluorobenzene. To the aqueous phase, an equal volume of 10% trichloroacetic acid, was added. The yellow precipitate was washed once with 10 ml of ethanol, twice with 10 ml ether, and dried at room temperature over paraffin shavings. The dried powder was suspended in 1.6 ml of 6 N HCl and sealed in a glass tube under an N_2 atmosphere. Hydrolysis was carried out for 12 hr at 105°. The hydrolysate was diluted six times with water and extracted five times with 5 ml of ether pretreated with $FeSO_4$ solution. The ether extract as well as the aqueous phase was dried under N_2 atmosphere. The residue was then dissolved in 0.2 ml of acetone. The acetone solution was applied on Whatman No. 1 filter paper and the paper chromatogram was developed with a solvent containing toluene, pyridine, and 2-chloroethanol (30:9:18, v/v), which had been equilibrated with 0.8 N NH_4OH for 4 hr at room temperature. The paper was dried at 40° for 1 hr. A 2.5-cm wide vertical strip was cut along the horizontal segments of 0.5 cm each. Each paper segment thus obtained was counted.

Results

Table I shows the incorporation of various [^{14}C]-amino acids into protein fraction by fraction II. It is noted from this table that the incorporation of arginine is far better than other amino acids. The specific incorporating activity of fraction II for arginine is almost three times higher than the conventional ribosome–supernatant fluid system for protein biosynthesis. In contrast, other amino acids such as phenylalanine and glutamic acids are incorporated by fraction II at a level of one-tenth of the conventional ribosomal system. The incorporating activity of the conventional ribosomal system for various amino acids is more or less of the same order of magnitude. In Table II a similar comparison is made between the incorporating activity of "conc SM-sup" fraction and the ribosomal system. It is noted that the "conc SM-sup" fraction, despite being devoid of most ribosomes, incorporated various amino acids such as glycine, glutamic acid, arginine, methionine, and tryptophan. Here again, arginine was most actively incorporated. The incorporating activity of the "conc SM-sup" fraction for various amino acids cannot be due to a simple contamination of ribosomes in this fraction. The activity of this fraction relative to that of the ribosomal system varied to a great extent, depending upon the amino acids. For example, incorporation of proline by the ribosomal system is far better than the soluble system, while almost the same efficiency was observed with glycine incorporation.

Distribution of Arginine-Incorporating Activity among Various Fractions. From the data in Tables I and II, it became clear that [^{14}C]arginine was most actively incorporated in a nonribosomal system. Our attention was thus centered on the incorporation of arginine. Table III shows the distribution of arginine-incorporating activity among various fractions, which are presumably devoid of ribosomes. The crude supernatant fluid

TABLE III: Distribution of Incorporating Activity among Various Fractions.[a]

Fractions	% Act.
85 S or streptomycin supernatant	100
Streptomycin precipitate	0.4
Protamine supernatant	90
Fraction I	1.4
Fraction II	6.0
Fraction III	0.14

[a] The reaction components were identical with those of the complete system in Figure 1, except for the fractions used for incorporation. Per cent activity was calculated from observed incorporated radioactivity/0.1 ml of the reaction mixture after 30-min incubation.

which contains very little, if any, ribosomes had considerable arginine-incorporating activity. The major portion of this incorporating activity is found in the "protamine sup" fraction. Fraction II had only 6% of the total activity, but this was higher than fractions I and III. In a fraction of *E. coli* soluble system, which corresponds to fraction II of the rat liver, about 50% of the total activity for incorporation of [^{14}C]leucine was found. In contrast to the *E. coli* system, treatment with protamine or streptomycin did not remove the major portion of the activity from S-85. Nevertheless, concentration of the activity was observed in fraction II and hence this fraction was used in some of the subsequent studies.

Nature of the Incorporation Reaction. Figure 1 represents the time course of incorporation of [^{14}C]arginine by fraction II. It can be seen that incorporation is almost complete at 30 min, and little or no increase of radioactivity in the protein fraction was observed after 30 min. Similar results were obtained when incorporation was measured using conventional ribosome–supernatant system. The incorporation was strictly dependent upon ATP and its generator, and tRNA from rat liver. In accordance with the requirement for tRNA, RNase (50 μg/ml) completely inhibited the incorporation. As in the case of the [^{14}C]leucine incorporation by the *E. coli* soluble system, no requirement of GTP was observed and elimination of GTP slightly stimulated the incorporation. The mixture of amino acids was not necessary for the major portion of the incorporation, but elimination caused premature decline of incorporation. Since the *E. coli* soluble amino acid incorporation system is sensitive to puromycin, the effect of this antibiotic was tested as shown in this figure. The incorporation at 15 min after the onset of reaction was inhibited only 30%, whereas incorporation at 60 min was inhibited more than 50% by 2×10^{-4} M puromycin. Thus, the initial rate of incorporation is less sensitive to puromycin than the incorporation which took place 15 min after the onset of the reaction. The reason for this remains obscure at present.

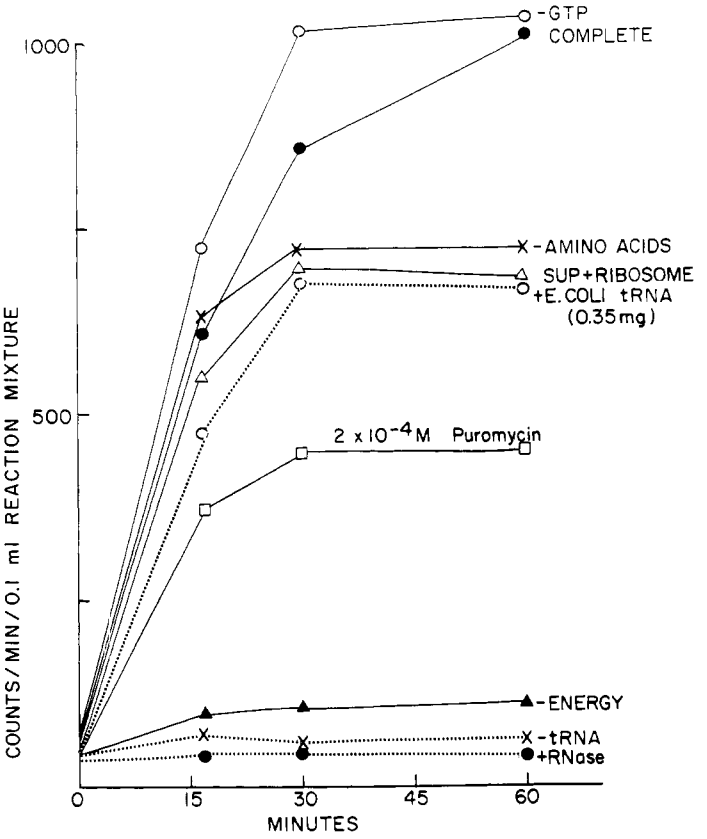

FIGURE 1: Time course of [^{14}C]arginine incorporation by fraction II, as well as by the ribosomal system. The reaction mixture contained, in addition to the components listed in the text, 1.3 mg of fraction II, or 2 mg of 105S fraction, 0.58 mg of rat liver ribosomes, and 231,000 cpm of [^{14}C]arginine.

Evidence for the Soluble Nature of the System. It was previously observed that high-speed centrifugation does not sediment the [^{14}C]arginine-incorporating activity. When either fraction II or the protamine supernatant fraction was subjected to centrifugation at 105,000*g* for 2 hr, the major portion of the incorporating activity remained in the supernatant (Kaji *et al.*, 1963b). Although this experiment suggests strongly that the incorporation observed is independent of ribosomes, further proof was necessary to establish this point. Unequivocal proof that the incorporating activity of the "conc SM-sup" does not depend upon the trace of 30S ribosomes was obtained from the sucrose gradient centrifugation experiment shown in Figure 2. It is noted in this figure that the peak of incorporating activity was found at the 24th tube, which corresponds to approximately 10 S.

No activity was found in the small amount of pellet obtained after the centrifugation. The addition of this pellet to the peak fraction did not result in the stimulation of incorporating activity. It should be noted that the peak of the ultraviolet-absorbing material matches the peak of the incorporating activity indicating that the fraction is mostly free of ribosomal subunits. Although data are not shown here when fraction II was analyzed with the Model E Spinco analytical ultracentrifuge, it was found that the most rapidly sedimenting particle present in fraction II was a trace of 27S component. This material represents less than 5% of the total.

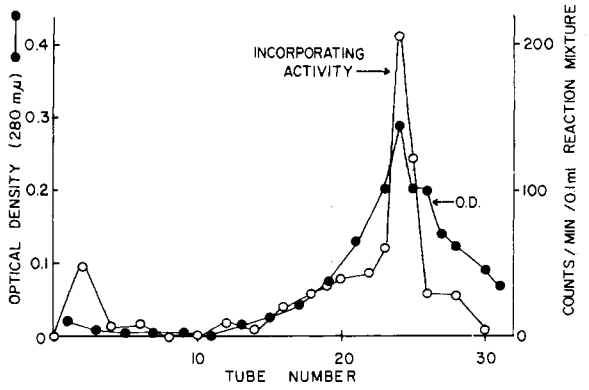

FIGURE 2: Sucrose density gradient centrifugation of incorporating activity of "conc SM-sup" fraction (19.8 mg/0.3 ml) was placed on 4.7 ml of 5–20% sucrose gradient containing 4 mM magnesium acetate, 12.5 mM KCl, 0.01 M β-mercaptoethanol, and 0.05 M Tris-HCl (pH 7.8). The tube was centrifuged for 3 hr and 45 min at 39,000 rpm using the Spinco SW-39L rotor at 0–4°. After the centrifugation 2-drop fractions were collected from the bottom of the tube. Each fraction (0.1 ml) was tested for its incorporating activity using [^{14}C]arginine. The reaction mixture for the incorporation was as described in the text except that the total volume was 0.4 ml. The 280-mμ absorption of each fraction was measured after 20-fold dilution of the fraction.

Presence of Aminoacyl-tRNA Synthetase in Fraction II. The complete dependence of arginine incorporation upon the addition of tRNA and sensitivity of the system to puromycin and RNase suggested that aminoacyl-tRNA is an intermediate in this soluble amino acid incorporating system. It was found that [^{14}C]arginine was incorporated into the fraction which is insoluble in cold trichloroacetic acid by fraction II. The formation of arginyl-tRNA was completed at 20 min and this is fast enough to support the incorporation into the protein fraction. These observations suggested that fraction II contains aminoacyl-tRNA synthetase and aminoacyl-tRNA is the most likely intermediate in this system. Direct proof of this concept is given in Table IV. In this experiment, [^{14}C]arginyl-tRNA was separately prepared and this was used as a donor of amino acid in the soluble system. Incorporation of radioactivity into the hot trichloroacetic acid insoluble fraction was observed and the amount incorporated was larger as the amount of [^{14}C]arginyl-tRNA increased. These data show that arginyl-tRNA is an intermediate in the soluble amino acid incorporation system.

TABLE IV: Incorporation of [^{14}C]Arginine from [^{14}C]-Arginyl-tRNA.[a]

Incubn Time (min)	[^{14}C]Arginine Incorpd (cpm)		
	Expt 1	Expt 2	Expt 3
5	364	471	541
15	392	500	553
30	530	620	820

[a] The reaction mixture (0.5 ml) was as described in the text except that ATP and the ATP generator system were omitted and 42,000 cpm (expt 1), 100,000 cpm (expt 2), and 200,000 cpm of [^{14}C]arginyl-tRNA (from *E. coli*) were added instead of [^{14}C]arginine.

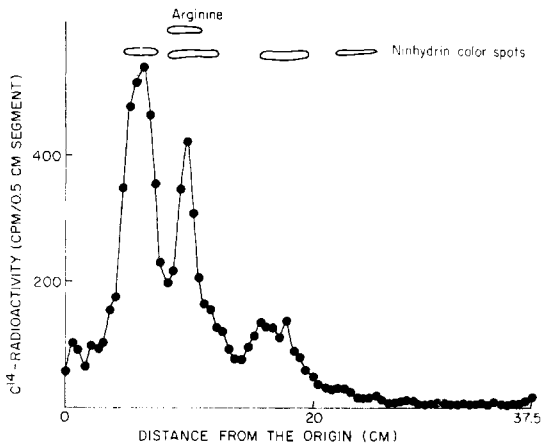

FIGURE 3: Paper chromatography of tryptic digest of [^{14}C]-arginine-labeled product. [^{14}C]Arginine-labeled product was prepared with concentrated streptomycin supernatant fraction as described in the text. The labeled material (5.4 mg) was suspended in 1 ml of 0.2 M NH_4HCO_3 at pH 8.5, and 25 μg of trypsin in 50 μl of 10^{-3} M $CaCl_2$ was added to this mixture. Digestion was carried out at 37° for 2.5 hr. After the incubation the insoluble material was removed by centrifugation and the supernatant fluid was lyophilized and stored at −20°. The powder was suspended in 0.1 ml of distilled water, and 40 μl of this solution containing 8300 cpm of the digests was streaked on Whatman No. 3MM paper. Descending chromatography with a solvent containing pyridine–1-butanol–acetic acid–H_2O (30:45:9:36, v/v) took 12 hr. The paper was dried at 70° for 10 min. A 2.5-cm. wide vertical strip was cut along the direction of solvent flow, and this strip was further cut into horizontal segments of 0.5 cm each. Each paper segment thus obtained was counted. The spots at the upper portion of the figure represent strong ninhydrin-positive material detected.

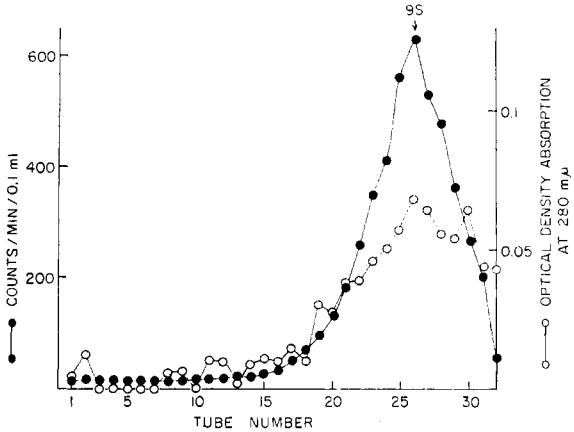

FIGURE 4: Sucrose density gradient centrifugation of [^{14}C]-arginine-labeled product made with "conc SM-sup" fraction. The reaction mixture for incorporation of [^{14}C]arginine was identical with that in the text. After 60-min incubation for the incorporation, 30% sucrose solution was added to make the final concentration 2%. Of this mixture 0.3 ml containing 10,200-cpm radioactive material was subjected to sucrose density gradient centrifugation as in Figure 2

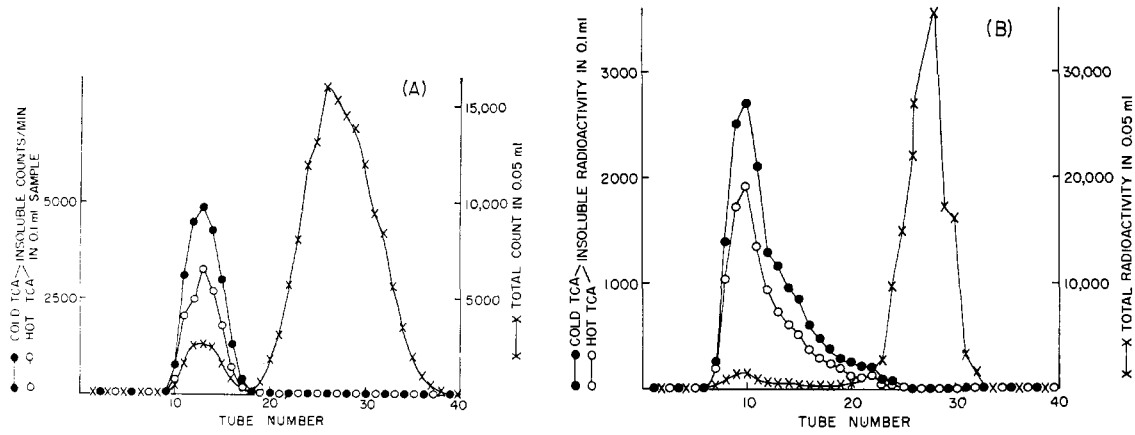

FIGURE 5: Sephadex treatment of incorporated product. The reaction mixture for [^{14}C]arginine incorporation (20 ml) was as described in the text except that it also contained 130 μg of "conc SM-sup" fraction. The mixture was incubated for 60 min and a 6-ml aliquot containing 232,920 cpm of labeled product was placed on a Sephadex G-25 (A) or G-100 (B) column (15.5 × 2.2 cm) and eluted with medium B. A total of 1.5 ml/tube was collected. (●—●) Radioactivity insoluble in cold trichloroacetic acid; (○—○) radioactivity insoluble in hot (90°) trichloroacetic acid/0.1 ml of each fraction; (×—×) total radioactivity/0.05 ml of each fraction.

Proteolytic Digestion of Incorporated Product. The site of incorporation of [^{14}C]amino acid into the protein fraction was first analyzed by studying the effect of proteolytic digestion. It was found that the incorporated radioactivity is readily rendered acid soluble by the action of various proteolytic enzymes. The incorporation of [^{14}C]arginine was allowed to proceed in the presence of fraction II for 60 min and proteolytic enzymes were added to the reaction mixture. In all cases a rapid loss of radioactivity in the protein fraction was observed. A separate experiment with RNase or DNase did not cause any appreciable loss of the radioactivity from the protein fraction. These observations suggest that incorporation is into protein and not into other macromolecules such as RNA and DNA.

Paper Chromatography of Tryptic Digests. The product labeled with [^{14}C]arginine was subjected to tryptic digestion and the digests were analyzed by paper chromatography. As shown in Figure 3, at least three distinct fractions were obtained by this procedure. The ninhydrin staining of the paper strip of the chromatogram shows the distribution of the major peptide formed in this mixture. Although the identification of the radioactive materials formed by the tryptic digestion has not been performed, the fact that one can obtain radioactive peptide-like materials by tryptic digestion gives further support to the notion that the radioactive amino acid is indeed linked to polypeptide through a covalent bond.

Solubility of Product in Various Solvents. In order to obtain some notion about the nature of the radioactive product made in the absence of ribosomes, the solubility of the product in various solvents was tested. The incorporated radioactivity was insoluble in pyridine, ethyl acetate, petroleum ether (bp 30–60), benzene, and *p*-dioxane. It was about 65% soluble in cold (5°) dichloroacetic acid and hot-water-saturated phenol. From these data one can perhaps exclude the possibility, with reasonable assurance, that the radioactivity is not incorporated into lipid or related compounds which would be soluble in the organic solvents used above.

Sucrose Density Gradient Centrifugation of the Product. The sedimentation coefficient of the product made by the "conc SM-sup" fraction was determined as follows (Figure 4). The incorporation of [^{14}C]arginine was allowed to proceed for 60 min and the reaction mixture was placed on top of the sucrose gradient. After centrifugation each fraction was treated with cold 10% trichloroacetic acid, hot 5% trichloracetic acid, and lipid solvents. The radioactivity insoluble in these solvents was counted. The peak of the radioactivity was found at the fraction corresponding approximately to 9 S. There was no appreciable radioactivity found at the bottom of the tube. Thus, we can conclude that the radioactive product made by the soluble system, though much smaller than ribosomes, is apparently a macromolecule.

Behavior of Labeled Product on Sephadex Column. In order to obtain further evidence that incorporated radioactivity is in the macromolecule fraction, the behavior of the product on Sephadex G-25 and G-100 was studied. In this experiment the incorporation of [^{14}C]-arginine was allowed to proceed for 60 min, and the reaction mixture was placed on the column. The eluates were tested for (1) total radioactivity, (2) radioactivity insoluble in trichloroacetic acid, and (3) radioactivity insoluble in hot trichloroacetic acid. As can be seen in Figure 5, two fractions were obtained representing macromolecular and small molecular fractions. The radioactivity which is insoluble in hot trichloroacetic acid was exclusively located in the macromolecular fraction. As is to be expected, radioactivity corresponding to [^{14}C]arginyl-tRNA present in the system was observed in the macromolecular fraction and detected by the difference between cold trichloroacetic acid insoluble radioactivity and that which is soluble in hot trichloroacetic acid.

COOH- and NH_2-Terminal Analysis of Product. From the analogy with the *E. coli* soluble amino acid incorporation system, it appeared possible that the [^{14}C]arginine incorporated had a free NH_2 group.

In order to establish whether the incorporated radio-

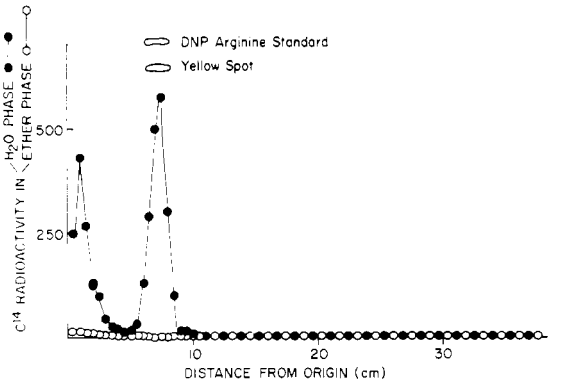

FIGURE 6: NH_2-terminal analysis of dinitrophenylated product. The experimental procedures are described in the text.

activity is at the COOH-terminal or NH_2-terminal position of the peptide, the radioactive products made by the "conc SM-sup" were treated with carboxypeptidase or subjected to NH_2-terminal analysis on the dinitrophenylated product. For the analysis of COOH-terminal position of the product, incorporation was allowed to proceed for 60 min and the reaction mixture was placed on Sephadex G-25 or G-100 column. The macromolecular fraction was pooled and treated with carboxypeptidase. Approximately 100 μg of 280-mμ-absorbing material was released from 1 mg of the radioactive product in a 2-hr treatment with 20 μg of carboxypeptidase. At the same time, there was, however, no detectable loss of trichloroacetic acid insoluble radioactivity noted.

For NH_2-terminal analysis, the radioactive product was dinitrophenylated and hydrolyzed as described previously. The hydrolyzed material was extracted into the ether and aqueous phase and 0.1 ml of each fraction was counted. Approximately 95% of the total radioactivity was found in the water phase. It was also chromatographed and 65% of the radioactivity was found in the position where dinitrophenylated arginine migrates on the paper strip (Figure 6). Although the omission of the mixture of unlabeled amino acids to the reaction system slightly depressed the amount of incorporating activity by this system, it would indicate that the radioactive product had a free amino group.

Discussion

Since the discovery of the soluble amino acid incorporating system, a number of similar systems have been reported in a wide variety of organisms. Thyroid cytoplasm which catalyzes the incorporation of arginine into protein fractions in the absence of ribosomes (Soffer and Mendelsohn, 1966) is one of the systems which has been found. Hird and his collaborators obtained a system which incorporates [^{14}C]leucine into NH_2-terminal position (Hird *et al.*, 1964). This incorporation was also dependent upon ATP but does not appear to have involved tRNA. In extracts from mammalian muscle (Florini, 1964) and also from heart mitochondria (Kalf and Simpson, 1959), incorporation of amino acids in the absence of conventional ribosomes has been reported. Despite the presence of soluble incorporating systems in such widely separated species as *E. coli* and the mammalian organs, the specific biological significance of the system still remains completely obscure. In this connection it is interesting to note that the acceptor proteins for leucine from leucyl-tRNA at their NH_2 terminals are found in chloramphenicol particles of *E. coli* (Otaka and Osawa, 1966). Since the chloramphenicol particle is regarded as a precursor to a mature *E. coli* ribosome (Nomura and Watson, 1959), these observations may suggest that the system is somehow involved in ribosome synthesis. On the other hand, the possibility that the system is involved in modifying preformed protein cannot be disregarded. The addition of arginine to a preformed protein at the NH_2-terminal end may change the biological activity of the protein. On the other hand, when an excess amount of [^{12}C]aminoacyl-tRNA was added to the reaction mixture the radioactivity incorporated into the protein fraction did not decrease, indicating that the reaction is not reversible. Further studies are in progress to assign a possible physiological role to this reaction.

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