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Properties of Transfer Ribonucleic Acid and Aminoacyl Transfer Ribonucleic Acid Synthetases from an Extremely Halophilic Bacterium*

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ABSTRACT: Aminoacyl transfer ribonucleic acid synthetases have been prepared by precipitation at pH 5, from cell-free extracts of *Halobacterium cutirubrum* in 3.4 M KCl, 0.1 M magnesium acetate, and 0.01 M Tris-HCl buffer (pH 7.6); tRNA was isolated from the pH 5 supernatant by phenol extraction after first reducing the salt concentration. Aminoacylation of transfer ribonucleic acid was found to depend upon adenosine triphosphate, Mg^{2+}, and aminoacyl transfer ribonucleic acid synthetases; it is inhibited by pancreatic ribonuclease and *p*-mercuribenzoate. In the presence of guanosine triphosphate, a supernatant fraction containing transfer enzymes, ribosomes, NH_4^+ and Mg^{2+} ions, the amino acid is transferred from the aminoacyl transfer ribonucleic acid into polypeptide; puromycin is inhibitory. Thus, both reactions are similar to those in nonhalophilic systems except for their ionic requirements: aminoacylation of transfer ribonucleic acid requires specifically 3.8 M KCl and the transfer reaction 3.8 M KCl, 1 M NaCl, and 0.4 M NH_4Cl for maximum activity. Kinetic studies on the formation of aminoacyl transfer ribonucleic acid show that replacing K^+ with Na^+ does not affect the apparent K_m for the amino acid but causes some reduction in the apparent V_{max}. In contrast, replacing K^+ with NH_4^+ does not affect the apparent V_{max}, but considerably increases the apparent K_m for the amino acid. The kinetic data are consistent with the NH_4^+ ion being a competitive inhibitor of the enzyme with respect to the amino acid.

It is suggested that binding of the amino acid to the aminoacyl transfer ribonucleic acid synthetase involves ionic interaction between the α-amino group of the amino acid and an anionic site on the enzyme. The results also indicate that in extremely halophilic bacteria ionic interactions are specific as they must be insensitive to the high ionic strength.

The first enzymic step in the biosynthesis of protein is the activation of the amino acid and its esterification to tRNA. The enzymes responsible are particularly interesting for their ability to recognize individual tRNAs and amino acids. The nature of this specificity is not known. However, Loftfield and Eigner (1967) have suggested that since the aminoacylation of tRNA in *Escherichia coli* is very sensitive to salt concentration,

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there must be a large contribution from electrostatic forces involved in binding tRNA to aminoacyl-tRNA synthetases.

Within extremely halophilic bacteria, the concentration of salt is close to saturation (Christian and Waltho, 1962; Larsen, 1967). Thus in these bacteria, ionic interactions between proteins, nucleic acids, and other components required for protein synthesis should be considerably reduced. A study of the mechanism of protein synthesis in halophilic bacteria may therefore lead to a better understanding of the effects of ionic environment on the macromolecular components and on the molecular interactions involved.

Recently a cell-free amino acid incorporating system from the extremely halophilic bacterium, *Halobacterium cutirubrum*, was described (Bayley and Griffiths, 1968a). This system is extremely halophilic in requiring 3.8 M KCl, 1 M NaCl, and 0.4 M NH_4Cl for full activity, but in all other essential features it closely resembles protein synthesizing systems from nonhalophilic bacteria. In the present paper, the isolation of tRNA and aminoacyl-tRNA synthetases from this bacterium is reported and some of the properties of the reactions involving aminoacyl-tRNA formation and the transfer of amino acid from aminoacyl-tRNA into polypeptide are described.

Materials and Methods

Cells of *H. cutirubrum* were grown, harvested, and washed as described previously (Bayley and Griffiths, 1968a). The subsequent procedures described below were carried out at 0–5°, except where indicated.

Preparation of Aminoacyl-tRNA Synthetases and tRNA. Washed cells were ground in a mortar for 5–10 min with an amount equal to their wet weight of acid-washed alumina. A volume of 3.4 M KCl, 0.1 M magnesium acetate, and 0.01 M Tris-HCl buffer (pH 7.6) (solution D, Bayley and Griffiths, 1968a) corresponding to 1–1.5 times the wet weight of cells, together with 0.015 ml of β-mercaptoethanol and 1 mg of DNase per 30 ml of solution D, was added and the mixture was ground for a few more minutes. The extract was centrifuged three times at low speed to remove alumina and cell debris, and once at 150,000g for 3 hr to remove ribosomal particles. The pH of the final S150 supernatant was then adjusted to 5 by adding 1 M acetic acid. The precipitate formed was sedimented, resuspended in the desired volume of solution D, containing β-mercaptoethanol at the same concentration as before, and dialyzed overnight against the same solution. After clarification, it was frozen in vials in isopentane cooled by liquid nitrogen and stored under liquid nitrogen. This preparation was used as the source of aminoacyl-tRNA synthetases.

Unlike the situation in nonhalophilic systems, the tRNA did not precipitate with the synthetases at pH 5. To prepare the tRNA from the pH 5 supernatant the pH was first readjusted to 7.6 with 1 M KOH and the salt concentration reduced by dialyzing overnight against a large volume of a solution containing 0.005 M KCl, 0.005 M magnesium acetate, and 0.005 M Tris-HCl buffer (pH 7.6). After dialysis, 0.01 volume of sodium dodecyl sulfate (0.125 g/ml) was added and the mixture was stirred for 20 min. tRNA was then isolated by the usual phenol extraction procedure and precipitated by acid potassium acetate (pH 5, 20% w/v) and ethanol (Kirby, 1956; von Ehrenstein and Lipmann 1961; Moldave, 1963). The precipitated tRNA was redissolved in 0.005 M KCl, 0.005 M magnesium acetate, and 0.005 M Tris-HCl buffer (pH 7.6), and reprecipitated with potassium acetate and ethanol as before. The final precipitate was washed with ethanol–H_2O (2:1) and either (1) dissolved in the required volume of 0.01 M KCl, 0.01 M magnesium acetate, and 0.01 M Tris-HCl buffer (pH 7.6) and dialyzed for 6 hr against solution D, clarified, frozen, and stored under liquid nitrogen; or (2) washed successively with ether–ethanol (1:2, 1:1, and 2:1) and finally anhydrous ether, dried under vacuum, and stored as a dry powder at −20°. When the latter preparations were to be used, the required amount was dissolved in solution D, in which it was completely soluble.

Solutions of aminoacyl-tRNA synthetases and tRNA were prepared free of Mg^{2+} ions by first dialyzing them for 2 hr against 3.4 M KCl and 0.01 M Tris-HCl buffer (pH 7.6) containing β-mercaptoethanol at the same concentration as before, and then against the same solution containing 0.005 M Na_2EDTA for 10 hr. Finally, preparations were dialyzed again against 3.4 M KCl and 0.01 M Tris-HCl buffer (pH 7.6) containing β-mercaptoethanol for 10 hr. Samples so treated were assayed for activity immediately.

Measurement of Aminoacyl-tRNA Formation. The ability of the aminoacyl-tRNA synthetase preparation to catalyze the formation of aminoacyl-tRNA and of the tRNA to accept amino acids was measured by determining the incorporation of ^{14}C-labeled amino acid into cold trichloroacetic acid precipitable material.

The standard reaction mixture for [^{14}C]aminoacyl-tRNA formation contained: sodium adenosine triphosphate (ATP, 0.3 μmole), Tris-HCl buffer (pH 8) (0.03 M), magnesium acetate (0.04 M), KCl (3.8 M), [^{14}C]amino acid (0.5–1 μCi), aminoacyl-tRNA synthetase preparation (typically 0.2–0.4 mg of protein), and tRNA (typically 0.1–0.3 mg of RNA). The total volume of solutions added was always 0.225 ml.

As in the whole cell-free amino acid incorporating system (Bayley and Griffiths, 1968a), the concentration of 3.8 M KCl was attainable only by adding dry salt; since no allowance was made for the volume of this salt, the final concentrations were slightly lower than the values given here. The exact composition of the mixture is detailed in Table I (mixture A). All the KCl was dissolved with stirring and the mixture was then incubated at 37° for 15–20 min. The reaction was terminated by adding in order: 2 ml of an aqueous solution at 0° containing a 1000-fold excess of unlabeled amino acid corresponding to the [^{14}C]amino acid used; 0.1 ml of a solution of serum albumin (5 mg/ml), to act as carrier; and 2 ml of cold aqueous 10% trichloroacetic acid.

When short incubations (2 min) were carried out for the determination of the rate of aminoacylation of tRNA, all the components of the system, except the enzyme, were mixed and then cooled in ice. The reaction was initiated by adding the enzyme and transferring the samples to a water bath at 37°. The reaction was terminated after 2-min incubation by cooling to 0° and adding cold unlabeled amino acid solution, serum albumin, and 10% trichloroacetic acid solution as described above.

After standing for about 5 min in ice the precipitated material was washed three times with 4 ml of cold 5% trichloroacetic acid with centrifuging and collected on a 0.45-μ Millipore filter. The filter was then washed with about 3 ml of 5% trichloroacetic acid, dried in an oven at 60° for 30 min, and assayed for radioactivity in a liquid scintillation counter as described by Bayley and Griffiths (1968a).

Modifications of the standard assay are described in legends to tables and figures.

Preparation of [^{14}C]*Aminoacyl-tRNA*

[^{14}C]Aminoacyl-tRNA was prepared by acylating tRNA with one [^{14}C]amino acid and 19 [^{12}C]amino acids in a large scale incubation mixture, increased proportionally from that described above. After incubation at 37° for 20 min, the reaction was stopped by adding one-tenth volume potassium acetate (20% w/v, pH 5) and the mixture dialyzed overnight against 0.005 M KCl, 0.005 M magnesium acetate, and 0.01 M potassium acetate–acetic acid buffer (pH 5). A procedure similar to that described for the isolation of tRNA was then followed, namely phenol extraction and the precipitation of the charged tRNA with potassium acetate and ethanol. The precipitated aminoacyl-tRNA was washed with cold ethanol–H_2O mixture (2:1), redissolved in the required volume of 0.01 M KCl, 0.01 M magnesium acetate, and 0.01 M potassium acetate–acetic acid buffer (pH 5), and dialyzed for 4 hr against a solution of 3.4 M KCl, 0.1 M magnesium acetate, and 0.005 M potassium acetate–acetic acid buffer (pH 5.6). Such preparations of labeled aminoacyl-tRNA were used immediately. For digestion with pancreatic RNase the [^{14}C]aminoacyl-tRNA was dissolved in water.

Preparation of Ribosomes and Transfer Enzymes. Ribosomes and transfer enzymes were obtained from dialyzed extracts prepared from cells homogenized in a glass–Teflon homogenizer with solution D containing β-mercaptoethanol, as described by Bayley and Griffiths (1968a). The ribosomes were sedimented at 150,000 *g* for 2.5 hr, resuspended in solution D containing β-mercaptoethanol, sedimented again, and finally resuspended in the required amount of the same solution. In all experiments, the 150,000*g* supernatant, S150, was used as the source of transfer enzymes without further purification. Ribosomal suspensions and S150 supernatants were stored under liquid nitrogen.

Transfer of [^{14}C]*Amino Acid from* [^{14}C]*Aminoacyl-tRNA into Polypeptide.* The reaction mixture for the transfer of amino acids from aminoacyl-tRNA into hot trichloroacetic acid insoluble residue contained: lithium or sodium guanosine triphosphate (0.18 μmole), Tris-HCl buffer (pH 8) (0.03 M), NH_4Cl (0.4 M), KCl (3.8 M), NaCl (1 M), magnesium acetate (0.04 M), S150 supernatant (0.1–0.2 mg of protein), washed ribosomes (0.6 mg), charged tRNA (0.1–0.2 mg, containing a [^{14}C]amino acid and 19 [^{12}C]amino acids), and a mixture of 19 unlabeled amino acids (0.008 μmole of each). Unlabeled amino acid (0.9 μmole), corresponding to the radioactive one in the charged tRNA, was also present in the reaction mixture to dilute any label which became discharged during the incubation procedure and prevent it from being recycled. Again, as in the reaction mixture for forming aminoacyl-tRNA, concentrations were lowered slightly by the volume of dry salt added. The exact composition of the transfer reaction mixture, mixture B, is therefore given in Table I.

TABLE I: Composition of Reaction Mixtures.[d]

Solution (M)	Mixture A[a] (μl)	Mixture B[b] (μl)
NaATP (0.03)	10	
Na- or LiGTP (0.012)		15
Tris-HCl buffer (pH 8) (0.29)	20	20
NH_4Cl (2.25)		40
Solid KCl	41[e]	41[e]
Solid NaCl		13.2[e]
Unlabeled amino acid corresponding to the [^{14}C]-amino acid in aminoacyl-tRNA (0.46)		20
[^{14}C]Amino acid, neutralized	10	
Solution D[c]	20	
Water	95	30
[f]Cold amino acid mixture less labeled amino acid (8×10^{-4} each)	(10)	10
tRNA in solution D	50	
Aminoacyl-tRNA synthetases in solution D	20	
Washed ribosomes in solution D		30
Charged tRNA in solution D		50
S150 supernatant		10
Total volume of solutions	225	225

[a] Mixture A = mixture for aminoacyl-tRNA formation. [b] Mixture B = mixture for transfer of amino acids from aminoacyl-tRNA into polypeptide. [c] Solution D = 3.4 M KCl–0.1 M magnesium acetate–0.01 M Tris-HCl buffer (pH 7.6). [d] Alterations in the concentrations of the mixtures were made so as to keep the volumes and all the remainder of the components the same. Additional compounds were added in aqueous solution in place of water or solution D as required. [e] In milligrams. [f] This was also added to mixture A when required.

Sample tubes were incubated at 37° for 40 min and the hot (90°) trichloroacetic aicd residue was precipitated, washed, and counted as described by Bayley and Griffiths (1968a).

Preparation and Assay of Aminoacyl-tRNA Synthetases from E. coli. E. coli B were kindly provided by Dr.

A. T. Matheson, National Research Council, Ottawa.

A cell-free extract was obtained by grinding the washed cells with acid-washed alumina as described above for *H. cutirubrum*, except that solution D was replaced by 0.04 M Tris-HCl buffer (pH 8), 0.01 M magnesium acetate, 0.03 M KCl, and 0.08 M β-mercaptoethanol (*E. coli* basic reaction solution). From this extract, an S150 supernatant was prepared as before. After dialysis against basic reaction solution for 5 hr, this S150 supernatant was used as a source of *E. coli* aminoacyl tRNA synthetases without further purification. It was stored under liquid nitrogen. Stripped *E. coli* tRNA was purchased from General Biochemicals.

The reaction mixture for the incorporation of [^{14}C]-amino acids into cold trichloroacetic acid insoluble material in the *E. coli* system was that normally used by other workers (Nirenberg, 1963).

Analyses. The concentration of ribosomes suspended in solution D was estimated from the absorbance at 258 mμ using $E_{1\,\text{cm}}^{1\%}$ 158 (Bayley and Kushner, 1964). Protein was estimated by the method of Lowry *et al.* (1951) using plasma albumin as standard.

The RNA content of samples was assumed to be 11 times their phosphorus content, which was determined by the method of Allen (1940), after digestion with perchloric acid.

Paper Electrophoresis and Autoradiography. Paper electrophoresis was carried out between water-cooled plates using Whatman No. 3MM chromatography paper and 5% acetic acid, adjusted to pH 3.5 with ammonia solution, as solvent. A potential of 18 V/cm was applied for 8 hr. After air drying, the papers were autoradiographed for 4–6 days to locate the radioactive material.

Source of Materials. Inorganic salts and trichloroacetic acid were reagent grade from Anachemia Chemicals, Ltd., Montreal; Tris from Sigma Chemicals Co.; β-mercaptoethanol from Eastman Organic Chemicals; crystalline pancreatic RNase and electrophoretically purified DNase from Worthington Biochemical Corp.; sodium dodecyl sulfate from Matheson Coleman and Bell Co.; alumina (A-301) from Alcoa Chemicals; disodium adenosine 5′-triphosphate, sodium cytidine 5′-triphosphate, sodium guanosine 5′-triphosphate, and sodium uridine 5′-triphosphate from P-L Biochemicals, Inc.; tetralithium guanosine 5′-triphosphate from Schwartz Bioresearch, Inc.; trisodium 2-phosphoenolpyruvate and sodium *p*-mercuribenzoate from Calbiochemical Corp.; serum albumin and puromycin dihydrochloride from Nutritional Biochemicals; unlabeled L-amino acids from Schwarz Bioresearch, Inc., and Mann Research Laboratories, Inc.; EDTA (disodium salt) and phenol from Fisher Scientific Co., Ltd.; singly or uniformly ^{14}C-labeled L-amino acids were obtained from New England Nuclear Corp. or the Radiochemical Centre, Amersham, as specified below.

Results

In all the experiments, samples were run in duplicate with similar results; only the means of these are quoted below.

Formation of Aminoacyl-tRNAs; Characteristics of the Reaction. Table II shows the response of the system to various omissions and additions. The incorporation of amino acids into cold trichloroacetic acid insoluble residue required ATP, tRNA, and enzyme preparation. The addition of RNase to the reaction mixture completely inhibited the system; *p*-mercuribenzoate was also inhibitory.

TABLE II: Incorporation of [^{14}C]Leucine[a] into Cold Trichloroacetic Acid Residue.

	Mean cpm	%
Complete	5172	100
Complete − tRNA	139	3
Complete − aminoacyl-tRNA synthetases	65	1
Complete − ATP	46	1
Complete + RNase (20 μg)	67	1
Complete + *p*-mercuribenzoate (10^{-4} M)	1087	21
Complete + NH_4Cl (1.1 M)	5005	97

[a] L-[1-^{14}C]Leucine, 34.1 mCi/mm (New England).

Independent experiments showed that for leucyl-tRNA formation the concentration of ATP used was optimal for the amounts of other components present. Unlike the whole cell-free amino acid incorporating system (Bayley and Griffiths, 1968a), the addition of phosphoenolpyruvate had no effect, indicating that an endogenous ATP-generating system was not present. ATP could not be replaced to any significant extent by either CTP, GTP, or UTP. Incorporation was not notably affected by pH over the range 7.5–8.

The incorporation of [^{14}C]amino acids into cold trichloroacetic acid precipitable material was not due to incorporation into protein or to general adsorption. This was shown by the complete decomposition of the product when heated at 90° in 5% trichloroacetic acid and by the inability of puromycin (50 μg) to inhibit the reaction. That the radioactivity measured was indeed due to the formation of aminoacyl-tRNA was also indicated by the alkaline lability of the product. When the labeled product was dialyzed overnight against solution D, pH 7.6, at 4°, 98% of the label was lost; when dialyzed against a similar solution at pH 6, only about 6% of the label was lost. The presence or absence of concentrated KCl did not affect the stability of the labeled product; thus it was stable to dialysis against 0.005 M KCl–0.005 M magnesium acetate–0.01 M potassium acetate–acetic acid buffer (pH 5).

Further evidence of true aminoacyl-tRNA formation was obtained by isolating [^{14}C]aminoacyl-tRNA from the halophile reaction mixture (see Materials and Methods). Digestion of this [^{14}C]aminoacyl-tRNA with pancreatic RNase for 10 min at room temperature released ^{14}C label from the RNA. Examination of the digestion products by paper electrophoresis followed by autoradiography showed that RNase liberated a compound, basic at pH 3.5, carrying practically all of the radioactivity and migrating much faster than free [^{14}C]-amino acid. When the labeled compound was eluted from the paper and incubated with dilute ammonia at pH 10.5 for 1 hr at 37°, it was converted into free [^{14}C]-amino acid, as identified by electrophoresis and autoradiography. These results are consistent with the known alkaline lability of the aminoacyl-tRNA ester linkage and suggest that the product obtained by digesting the aminoacyl-tRNA with RNase is an aminoacyl nucleoside ester, as in other systems (Zachau *et al.*, 1958; Marcker and Sanger, 1964). The nature of the nucleoside has not been determined.

The time course of aminoacyl-tRNA formation, under the assay conditions described, is similar to that in nonhalophilic systems, increasing linearly with time for 5–10 min and being complete in 10–20 min (Allen *et al.*, 1960; Berg *et al.*, 1961; Lindahl *et al.* 1967). There was no apparent loss of [^{14}C]aminoacyl-tRNA for a period of 40 min after maximal labeling had been reached.

The amount of [^{14}C]amino acid incorporated into the cold trichloroacetic acid insoluble residue was proportional to the tRNA concentration in the mixture (Figure 1). A requirement for Mg^{2+} ions for the formation of aminoacyl-tRNA in the halophile system was demonstrated by using preparations of aminoacyl-tRNA synthetases and tRNA which had previously been dialyzed against EDTA (Figure 2). Mn^{2+} was found to be about 75% as efficient as Mg^{2+} in this role. The small amount of incorporation occurring in the absence of added Mg^{2+} may have been due to its incomplete removal from the preparations, a situation similar to that reported by Deutscher (1967), or to some compensation by the very large monovalent cation concentration present. It was noticed that when no Mg^{2+} was added to the system, the presence of 1 M NaCl, in addition to the 3.8 M KCl already present, caused a threefold increase in aminoacyl-tRNA formation. The addition of 1 M NaCl in the presence of 0.04 M Mg^{2+} caused only a very slight increase in aminoacyl-tRNA formation.

Sixteen amino acids were tested in the standard assay for aminoacyl-tRNA formation (Table III). Comparable incorporation into tRNA was obtained with every amino acid except asparagine and glutamine; the amount of asparaginyl-tRNA and glutaminyl-tRNA formed was extremely small. The additivity of incorporation with more than one [^{14}C]amino acid present was demonstrated by using [^{14}C]leucine and [^{14}C]arginine in the standard assay (Table IV). As expected, the addition of other nonradioactive amino acids did not inhibit [^{14}C]-leucine incorporation (Table IV).

Monovalent Cation Requirement for Aminoacyl-tRNA Formation. Aminoacyl-tRNA formation dropped markedly at KCl concentrations below 3.8 M as shown in Figure 3, and was only 10% of normal even at the comparatively high concentration of 1 M. NaCl could partially compensate for reductions in the KCl concentration (Figure 3), but when KCl was completely replaced by NaCl, the activity was only about 30% of that in 3.8 M KCl. Table V shows that results obtained by par-

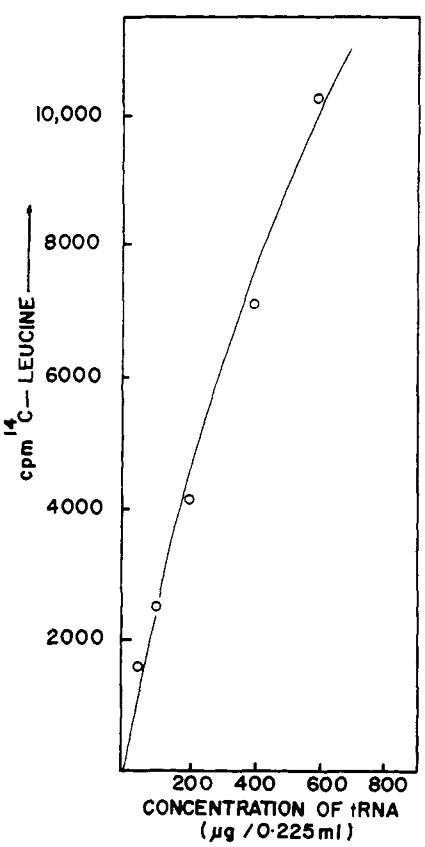

FIGURE 1: Incorporation of [^{14}C]leucine (34.1 mCi/mm, New England) as a function of tRNA concentration.

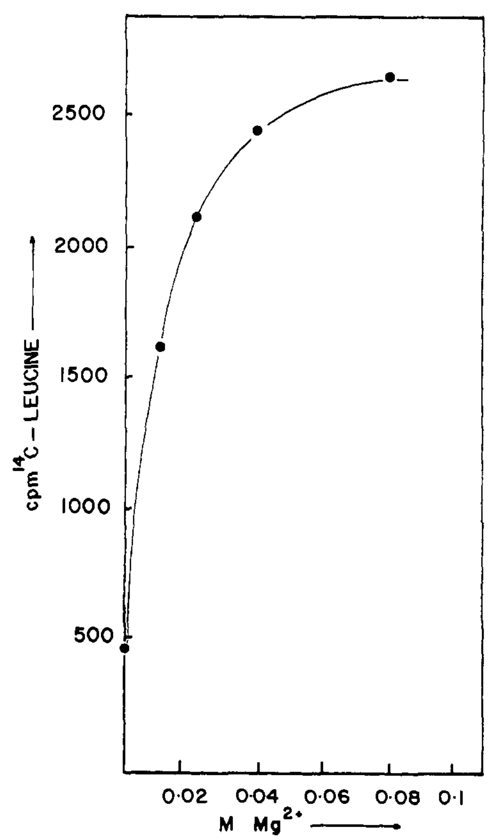

FIGURE 2: The effect of Mg^{2+} concentration on the formation of [^{14}C]leucyl-tRNA by aminoacyl-tRNA synthetases and tRNA treated with EDTA to remove Mg^{2+} ([^{14}C]-leucine, 34.1 mCi/mm, New England).

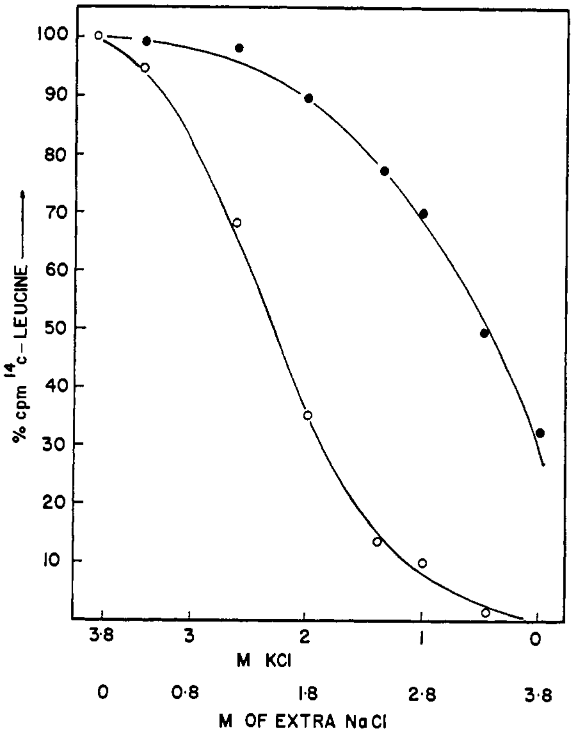

FIGURE 3: Percentage [^{14}C]leucyl-tRNA formed as a function of the monovalent cation concentration. Changes in the concentration were achieved by altering the amount of dry salt added. Dry NaCl replaced KCl where indicated. KCl concentration lowered with no compensating addition of other cations (—O—); KCl concentration lowered and compensated with added NaCl (—●—). [^{14}C]Leucine (34.1 mCi/mm) was from New England.

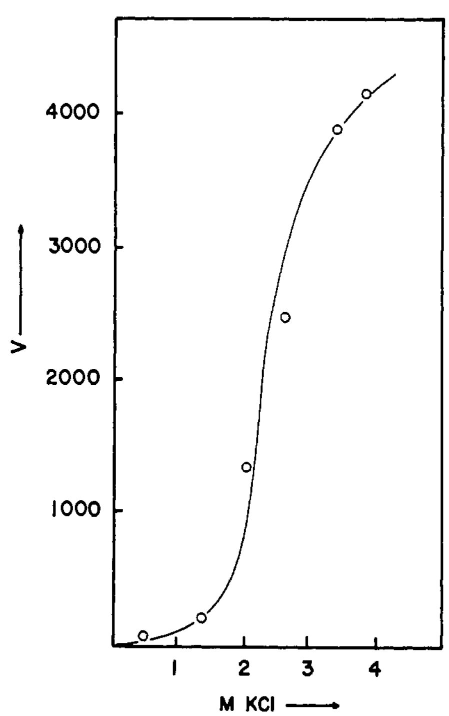

FIGURE 4: The dependence of the rate of isoleucyl-tRNA formation on the concentration of KCl. The reaction velocity V is expressed as counts per minute of [^{14}C]isoleucine (262 mCi/mm, New England) incorporated in a reaction time of 2 min.

tially replacing KCl with NH_4Cl varied with the concentration of labeled amino acid in the incubation mixture. This effect, which is discussed in detail below, does not appear to be a result of a lowered pH caused by the addition of solid NH_4Cl. In the presence of 2.44 M NH_4Cl and 1.36 M KCl, the activity was reduced to 25% of that at 3.8 M KCl (Table V). Independent measurements suggest that, in the presence of Tris-HCl buffer (0.029 M), this concentration of NH_4Cl might have reduced the pH to about 7.1. Other experiments in the presence of

TABLE III: Formation of Aminoacyl-tRNAs.[a]

	mμmole/mg of tRNA
Alanine	0.7
Arginine	1.4
Asparagine	0.02
Aspartic acid	0.6
Glutamic acid	0.6
Glutamine	0.005
Glycine	0.8
Isoleucine	0.3
Leucine	0.4
Lysine	0.3
Methionine	0.2
Phenylalanine	0.3
Proline	0.2
Serine	0.3
Threonine	0.4
Valine	0.5

[a] Net incorporation of amino acid into tRNA = experiment − control with added RNase expressed as millimicromoles per milligram of tRNA. [^{14}C]Amino acids used were (in millicuries per millimole): DL-alanine, 3.48; DL-arginine, 3.3; DL-aspartic acid, 2.47; DL-glutamic acid, 1.97; glycine, 2.0; L-phenylalanine, 16.5; DL-proline, 4.0; DL-serine, 5.0; DL-valine, 2.54; L-leucine, 248; L-lysine, 237; L-threonine, 167; L-methionine, 187; L-isoleucine, 234 (New England); L-asparagine, 102; and L-glutamine, 38.8 (Amersham).

TABLE IV: The Additive Effect of Amino Acid Incorporation into tRNA.

Radioactive Amino Acid Added	Mean cpm 1	Mean cpm 2
Leucine[a]	5556	5956
Leucine[a] + 19 unlabeled amino acids		5912
Arginine[b]	2490	
Arginine[b] + leucine[a]	7925	
Sum of counts per minute from the separate incorporations of leucine and arginine	8046	

[a] L-Leucine, 34.1 mCi/mm. [b] DL-Arginine, 3.3 mCi/mm (New England).

TABLE V: The Effect of Replacing KCl with NH_4Cl on the Incorporation of [^{14}C]Leucine into Cold Trichloroacetic Acid Residue.

Concn of L-[^{14}C]Leucine in the Incubn Mixture	% cpm Incorpd 0.015 μmole[a]	% cpm Incorpd 0.001 μmole[b]
3.8 M KCl at pH 8.05	100	100
1.36 M KCl + 2.44 M NH_4Cl	95	25
3.8 M KCl at pH 7.7		96
3.8 M KCl at pH 6.95		83

[a] L-[^{14}C]Leucine, 34.1 mCi/mm (New England). [b] L-[^{14}C]Leucine, 251 mCi/mm (New England).

3.8 M KCl showed, however, that the activity was not particularly sensitive to a lowering of the pH within this range, and at pH 6.95 the activity was still 83% of the value at pH 8.05 (Table V).

Bayley and Griffiths (1968a) demonstrated that the whole cell-free amino acid incorporating system required Na^+ and NH_4^+ ions for full activity. However, NH_4^+ ions are unnecessary for aminoacyl-tRNA formation (Table II) while 1 M NaCl added to the system gave only slight improvement over 3.8 M KCl alone and therefore was not included in the normal reaction mixture for charging tRNA.

Effect of Monovalent Cations on the Kinetics of Aminoacylation of tRNA. In view of the conflicting results obtained when KCl was partially replaced by NH_4Cl (Table V), the effects of ionic concentration, cation species, and concentration of amino acid on the initial velocity of formation of aminoacyl-tRNA were examined.

Figure 4 shows the effect of the KCl concentration on the rate of aminoacylation of tRNA. Note the sigmoidal character of the curve, indicating cooperative multiple binding sites, and the extreme dependence of the rate upon the ionic concentration in the region of 2–3 M KCl.

The effects of varying the leucine concentration in a reaction mixture containing 3.8 M KCl and in one containing 1.36 M KCl plus 2.44 M NH_4Cl are shown in the form of Lineweaver–Burk (1934) plots in Figure 5. Clearly the apparent K_m for leucine was increased considerably in 1.36 M KCl plus 2.44 M NH_4Cl, while the apparent V_{max} remained the same as in 3.8 M KCl. At very high leucine concentrations, therefore, the inhibitory effect of partially replacing K^+ with NH^+_4 was abolished. The kinetics are the same as those for competitive inhibition of the enzyme (Dixon and Webb, 1964). Figure 5 also shows the effect of adding 1 M NH_4Cl to a reaction mixture containing 3.8 M KCl. Here again NH_4Cl appears to inhibit the enzyme competitively with respect to leucine. The inhibitory effects of concentrations of NH_4Cl above 1 M in the presence of 3.8 M KCl could not be studied since the higher concentrations caused precipitation of salts. Results similar to these obtained with leucine were also obtained with arginine and isoleucine; other amino acids were not tested.

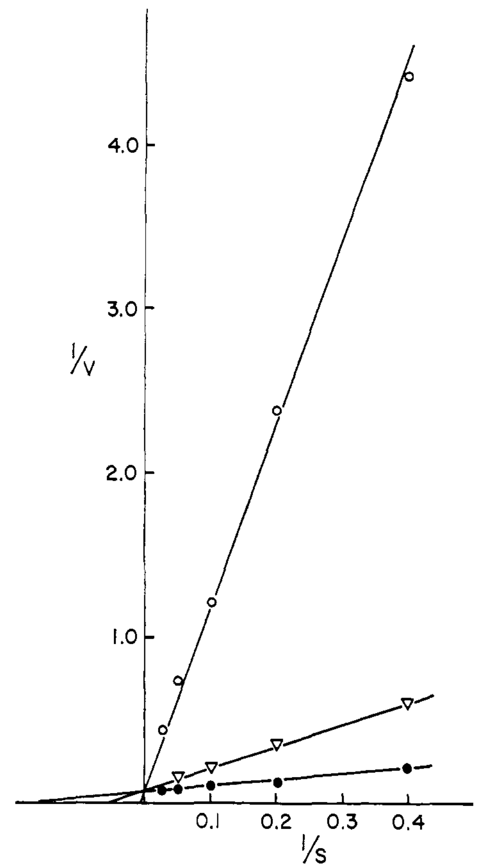

FIGURE 5: The effect of varying the leucine concentration on the rate of leucyl-tRNA formation in 3.8 M KCl (—●—), in 3.8 M KCl plus 1 M NH_4Cl (—▽—), and in 1.36 M KCl plus 2.44 M NH_4Cl (—○—). The reaction velocity, V, is expressed as (cpm incorporated/2-min reaction time) $\times 10^{-3}$; substrate concentration (S) is expressed as the volume (microliters) of neutralized amino acid solution added to the incubation mixture (1 μl = 0.00016 μmole); tRNA = 0.18 mg, ATP = 0.3 μmole. [^{14}C]Leucine (251 mCi/mm) was from New England.

A plot of the reciprocal of the initial velocity ($1/V$) against [NH_4^+] replacing [K^+], obtained at a nonsaturating concentration of leucine, is shown in Figure 6. The inhibitory effect of replacing KCl with NH_4Cl increases rapidly as more than 1.4 M KCl is replaced with NH_4Cl and it is clear that at NH_4Cl concentrations above 2.5 M the reaction would be completely inhibited.

Figure 7 shows the results, in the form of Lineweaver–Burk plots, of partially replacing KCl with the same molarity of NaCl. In contrast to the situation found with NH_4Cl, replacing K^+ with Na^+ affected the apparent V_{max} but not the K_m for leucine. Thus the reduction in the rate of reaction could not be overcome by increasing the amino acid concentration.

In order to differentiate between the effects of partially replacing KCl by NaCl or NH_4Cl and those due to lowering the KCl alone, the apparent kinetic parameters for leucine were determined in 3.8 M KCl, 2 M KCl, 2 M KCl plus 1.8 M NaCl, and in 2 M KCl plus 1.8 M NH_4Cl (Figure 8). Lowering the KCl concentration alone from 3.8 to 2 M increased the apparent K_m and reduced the apparent V_{max} for leucine. Addition of 1.8 M NaCl to

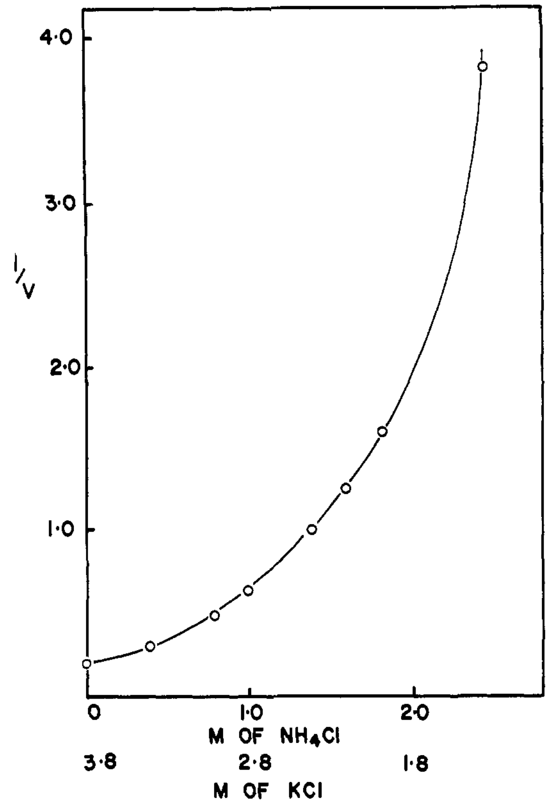

FIGURE 6: The effect of replacing KCl to different extents by the same molarity of NH_4Cl on the rate of leucyl-tRNA formation at a nonsaturating leucine concentration ([^{14}C]-leucine, 0.001 μmole, 273 mCi/mm, New England). tRNA = 0.18 mg; ATP = 0.3 μmole. Reaction velocity, V, is expressed as (cpm incorporated/2-min reaction time) $\times$ 10^{-3}. Changes in the concentrations of the salts were achieved by altering the amounts of each dry salt added.

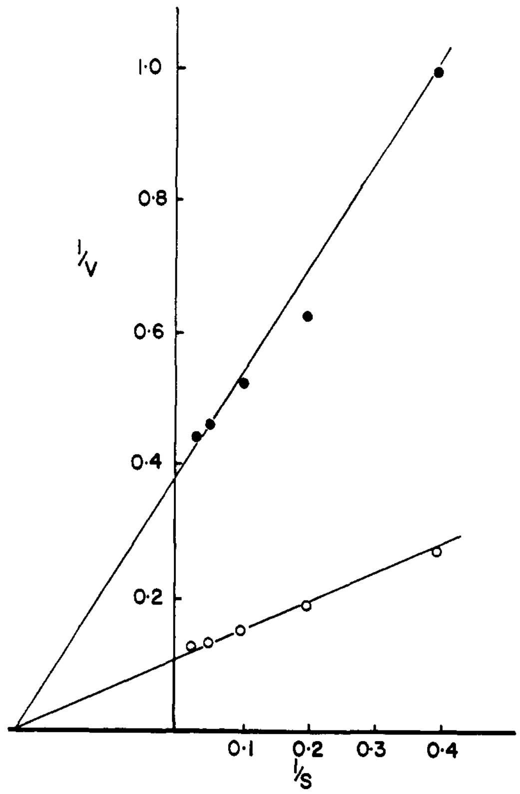

FIGURE 7: The effect of varying the concentration of [^{14}C]-leucine (251 mCi/mm, New England) on the rate of leucyl-tRNA formation in 3.8 M KCl (—○—) and in 1.36 M KCl plus 2.44 M NaCl (—●—). The reaction velocity, V, is expressed as (cpm incorporated/2-min reaction time) $\times$ 10^{-3}; S is expressed as the volume (microliters) of neutralized amino acid solution added to the incubation mixture (1 μl = 0.0002 μmole); tRNA = 0.18 mg; ATP = 0.3 μmole.

the 2 M KCl returned the apparent K_m to the original value at 3.8 M KCl and also increased the apparent V_{max}, although the original value at 3.8 M KCl was not attained. On the other hand, addition of 1.8 M NH_4Cl to the 2 M KCl restored the apparent V_{max} to the value found at 3.8 M KCl, but in addition it increased still further the apparent K_m for leucine. In the presence of 2 M KCl, therefore, NH_4^+ ions have two conflicting effects on the system: they restore the apparent V_{max} for the amino acid to normal, but at the same time, they increase tremendously the apparent K_m for the amino acid. Thus NH_4^+ ions inhibit the system whereas Na^+ ions improve it both by restoring the apparent K_m for the amino acid to the original value and by increasing the apparent V_{max}.

The apparent K_m for leucine in the formation of leucyl-tRNA at 3.8 M KCl is 4.2 $\times$ 10^{-6} M; this is similar to the value of 5 $\times$ 10^{-6} M obtained by Rouget and Chapeville (1968a) from an *E. coli* B system.

Properties of the Aminoacyl-tRNA Synthetases and tRNA Preparations. The aminoacyl-tRNA synthetases showed no reduction in activity after being stored under liquid nitrogen for at least 6 months; however, when the KCl was removed by dialysis for 15 hr against 0.1 M magnesium acetate and 0.01 M Tris-HCl buffer (pH 7.6) containing β-mercaptoethanol, the enzymes were irreversibly inactivated. Clearly halophile tRNA was not irreversibly inactivated by very low salt concentrations, since such conditions prevailed during its isolation. The halophile tRNA was found to be completely soluble in solution D and in distilled water. In solution D it had an ultraviolet absorption spectrum with λ_{max} 258 mμ and λ_{min} 228 mμ; optical density 258 mμ/228 mμ = 2.1–2.26; preparations contained between 1 and 3% protein.

Cases of complete interaction, partial interaction, and strict specificity between tRNAs and aminoacyl-tRNA synthetases from different sources have been reported in the literature (reviewed by Novelli, 1967). Table VI shows the results obtained for [^{14}C]proline, using tRNA and aminoacyl-tRNA synthetases from *H. cutirubrum* and *E. coli;* similar results were obtained with leucine. In one case *E. coli* tRNA replaced the halophile tRNA in the standard halophilic incubation mixture; in the other, halophile tRNA replaced *E. coli* tRNA in the basic reaction mixture for aminoacylation in the *E. coli* system. In neither case was there any appreciable interaction; the halophile tRNA in the *E. coli* system did not increase the incorporation above the endogenous level due to the small amount of tRNA present in the enzyme preparation.

TABLE VI: Species Specificity of tRNAs and Aminoacyl-tRNA Synthetases.

	cpm[a]
Halophile tRNA + halophile aminoacyl-tRNA synthetases[b]	8,670
E. coli tRNA + halophile aminoacyl-tRNA synthetases[b]	394
E. coli tRNA + *E. coli* enzymes[c]	23,684
Halophile tRNA + *E. coli* enzymes[c]	925

[a] [^{14}C] Proline (209 mCi/mm, New England) incorporated into tRNA. [b] In standard halophile reaction mixture (3.8 M KCl). [c] In standard *E. coli* basic reaction solution (0.03 M KCl).

TABLE VII: Transfer of [^{14}C]Leucine from [^{14}C]Leucyl-tRNA[a] into Hot Trichloroacetic Acid Insoluble Residue.

	Mean cpm	%
Complete mixture	3436	100
Complete mixture − GTP	233	7
Complete mixture − S150 enzymes	720	21
Complete mixture + puromycin	74	2
Complete mixture − NH_4Cl (0.4 M)	1825	53
Complete mixture − NaCl (1 M)	2567	75

[a] Counts per minute added as [^{14}C]-Leucyl-tRNA, 13,910. [^{14}C]Leucine, 248 mCi/mm (New England).

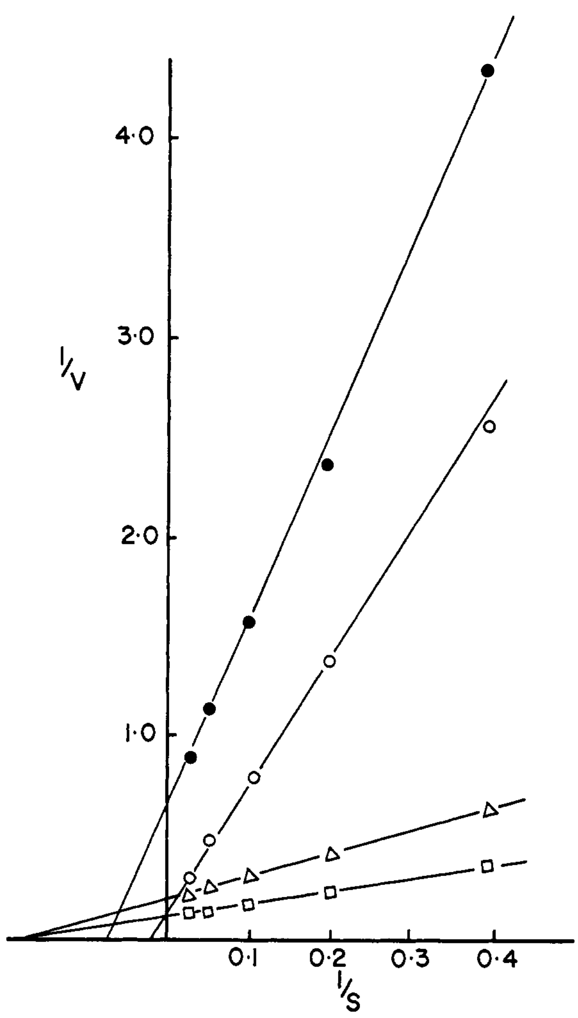

FIGURE 8: The effect of varying the concentration of [^{14}C]-leucine (273 mCi/mm, New England) on the rate of leucyl-tRNA formation under four different monovalent cation conditions; 3.8 M KCl (—□—); 2 M KCl (—●—); 2 M KCl plus 1.8 M NaCl (—▽—); 2 M KCl plus 1.8 M NH_4Cl (—○—). V is expressed as (cpm incorporated/2-min reaction time) × 10^{-3} and S as the volume (microliters) of neutralized amino acid solution added (1 μl = 0.0002 μmole); tRNA = 0.18 mg; ATP = 0.3 μmole.

Transfer of Amino Acids from Aminoacyl-tRNA into Polypeptide. To demonstrate that aminoacyl-tRNA produced in the halophilic system was capable of transferring the amino acid into polypeptide in the presence of ribosomes and transfer enzymes, [^{14}C]leucyl-tRNA was isolated from a large-scale incubation mixture and used as the source of [^{14}C]amino acid in a subsequent incubation. Dialyzed S150 supernatant was used as the source of transfer enzymes in the incubation mixture for the transfer reaction (see Materials and Methods and Table I). The charged tRNA contained, in addition to the [^{14}C]leucine, a mixture of 19 other [^{12}C]amino acids. Excess of unlabeled leucine was present in the reaction mixture to dilute any labeled leucine which might have been discharged during the incubation.

Table VII shows that approximately 25% of the [^{14}C]-leucine added as [^{14}C]leucyl-tRNA was transferred into polypeptide. The results of Table VII also show that GTP and enzymes are essential for transfer. The small amount of transfer occurring in the absence of the S150 supernatant may have been due to traces of enzymes still present in the washed ribosomes preparation. Puromycin completely inhibited the reaction.

The ionic composition of this reaction mixture is the same as that for amino acid incorporation in the whole cell-free extract (Bayley and Griffiths, 1968a). Although the system contained 3.8 M KCl and 1 M NaCl, omission of 0.4 M NH_4Cl reduced the amount of leucine transferred into polypeptide by nearly 50%. The omission of 1 M NaCl, on the other hand, had a less drastic effect.

It was not possible to examine the cationic requirements of the transfer enzymes themselves as this activity depends upon the integrity of the ribosome structure which is stable only at high concentrations of KCl (Bayley and Kushner, 1964).

Discussion

The mechanism of protein synthesis in *H. cutirubrum* has previously been shown to be generally similar to that in nonhalophilic systems (Bayley and Griffiths, 1968a,b). The present work shows this more clearly for specific reactions involving formation of aminoacyl-tRNA and the subsequent transfer of amino acids into polypeptide. Thus, the aminoacylation of tRNA is dependent upon ATP, Mg^{2+}, and halophile aminoacyl-tRNA synthetases and is inhibited by RNase and *p*-mercuribenzoate; the aminoacyl-tRNAs formed have properties similar to those from nonhalophilic sources; transfer of amino acid from aminoacyl-tRNA into polypeptide requires GTP, transfer enzymes, ribosomes, NH_4^+ and Mg^{2+} ions and is completely inhibited by puromycin.

The ionic environment in which these reactions occur, however, is totally different from that in nonhalophilic systems. The reactions are truly halophilic in requiring extremely high concentrations of monovalent cations for activity. Furthermore, this activity depends not only upon total ion concentration but also on the species of ions present (Bayley and Griffiths, 1968a). Studies on the aminoacyl-tRNA synthetases show that only in 3.8 M KCl do they function normally. Reducing the KCl concentration or partially replacing KCl with the same molarity of either NaCl or NH_4Cl reduces the activity of the system. Sodium ions can completely replace K^+ but not without lowering the activity. Halophile aminoacyl-tRNA synthetases, therefore, have a preference, but not an absolute requirement, for high concentrations of K^+ ions.

These results differ from those with the complete cell-free system which requires 3.8 M KCl specifically but is further improved in efficiency of incorporation (Bayley and Griffiths, 1968a) and in fidelity of translation (Bayley and Griffiths, 1968b) by the addition of 1 M NaCl. It seems probable that this heightened requirement for salt occurs during transfer of the amino acid from tRNA into peptide and the results of Table VII support this. Since the ribosomes of *H. cutirubrum* require high concentrations of K^+ ions to preserve their structural integrity (Bayley and Kushner, 1964), it has not been possible to determine whether these particularly stringent salt requirements are due to the enzymes involved at the ribosomal level, to the ribosomes themselves, or to the multimolecular interactions involved on the ribosomes.

Previous authors have found that the activities of other halophilic enzymes depend upon high concentrations of monovalent cations (Baxter and Gibbons, 1956; Baxter, 1959; Holmes and Halvorson, 1965). Although all these authors found that the activity of their halophilic enzymes was greatest with KCl at high concentrations, and quite significantly so in some cases, most of the investigations to date have been done in the presence of NaCl (Holmes and Halvorson, 1963; Holmes *et al.*, 1965; Dundas and Halvorson, 1966). The present study shows that halophile aminoacyl-tRNA synthetases have a much lower activity in a medium of NaCl alone. These facts and also the knowledge that the major cation within these cells is K^+ (Christian and Waltho, 1962) suggest that halophile enzymes should first be characterized in a medium of high KCl concentration rather than NaCl.

Kinetic studies on the formation of aminoacyl-tRNA showed that replacing K^+ with NH_4^+ leads to effects quite different from those obtained by replacing K^+ with Na^+. Na^+ ions cause some reduction in the apparent V_{max}, but NH_4^+ ions have no effect. Whether the effect of Na^+ is on the enzyme itself or on the binding of ATP or tRNA is not known. It is clear, however, that whereas K^+ and NH_4^+ have similar ionic and hydrated ionic radii (Kachmar and Boyer, 1953), Na^+ is quite different. Thus if, as seems likely, the enzyme possesses binding sites for monovalent cations and the detailed conformation of the enzyme depends upon the size of cations bound, K^+ and NH_4^+ may be equally effective in preserving the most active configuration, whereas Na^+, being of a different size, is not.

In contrast to the effects on the apparent V_{max}, the apparent K_m for the amino acid is unaffected by Na^+ ions but is increased considerably by NH_4^+ ions. There is no indication that this effect is due to any changes in the pH of the incubation mixture caused by the addition of solid NH_4Cl. However, a thorough kinetic analysis of the system with a single enzyme species and taking into consideration the other substrates, ATP and tRNA, is clearly necessary to explain fully the specific inhibitory effect of NH_4^+ ions. The evidence available at present suggests that NH_4^+ ions are true competitive inhibitors of the enzyme with respect to the amino acid (Dixon and Webb, 1964). This being so, a possible explanation is that NH_4^+ ions compete with the amino acid for an anionic site on the enzyme, or as the results of Rouget and Chapeville (1968a,b) suggest, on an enzyme–ATP complex. This site, to which K^+ and Na^+ cannot bind or bind only weakly, would be distinct from those discussed in the previous paragraph. The protonated α-amino group of the amino acid, while not contributing to the recognition of different amino acids, would therefore be involved in binding the amino acid to the enzyme. Further binding and the actual recognition of an amino acid by the enzyme could come from a variety of interactions such as hydrogen bonding and van der Waals and hydrophobic forces.

The importance of the protonated α-amino group is suggested by the results of Calendar and Berg (1966). These authors studied the effect of analogs of L-tyrosine activation as measured by the PP_i–ATP exchange. Analogs in which the α-amino group of L-tyrosine was modified or replaced were neither substrates nor competitive inhibitors, whereas analogs involving the carboxyl group, although inactive substrates, were good competitive inhibitors of L-tyrosine activation. Since binding is presumably necessary for competitive inhibition, these results also suggest that the α-amino group is necessary for binding the amino acid to the enzyme.[1] That the carboxyl group is not necessary for binding was also shown by Cassio *et al.* (1967) and by Rouget and Chapeville (1968a,b).

Loftfield and Eigner (1967) found that in an *E. coli* system, esterification of tRNA with valine dropped markedly as the ionic strength was raised, the ionic species being unimportant. These authors concluded

[1] While this paper was in preparation, Bruton and Hartley (1968) made a similar suggestion from results with amino acid derivatives.

that ionic interactions are important in binding tRNA to aminoacyl-tRNA synthetases. Since the aminoacyl-tRNA synthetases from *H. cutirubrum* function only in concentrated salt, ionic interactions are either unimportant in binding tRNA, or they are much more specific than in *E. coli* as they must be insensitive to high ionic strength. That specific ionic interactions do occur in the halophile system is suggested by the dependence of the protein-synthesizing system upon comparatively low concentrations of NH_4Cl (0.4 M), even in the presence of 3.8 M KCl and 1 M NaCl, and by the particular effect of NH_4^+ ions in increasing the apparent K_m for the amino acid.

In the whole cell-free system, asparagine and glutamine were poorly incorporated into polypeptide (Bayley and Griffiths, 1968a). In the present work, the poor incorporation of these amino acids into tRNA was even more marked. It is possible that these results reflect an inefficient system in *H. cutirubrum* for incorporating these acid amides into protein. However, since optimal conditions for each aminoacyl-tRNA synthetase can vary by several orders of magnitude (Novelli, 1967), the possibility cannot be excluded that these results merely reflect nonoptimal concentrations of amino acids, ATP, or Mg^{2+} in the incubation mixture used here. Wilcox and Nirenberg (1968) have reported that, in *Bacillus megaterium*, one species of glutamyl-tRNA can be converted into glutaminyl-tRNA by an enzyme system present in the cell-free extracts. Whether dicarboxylic acid amides find their way into the protein of these halophilic organisms by a similar route, that is by conversion of part of aspartyl- and glutamyl-tRNAs into asparaginyl- and glutaminyl-tRNA, remains to be determined.

Acknowledgments

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