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A Cell-Free Amino Acid Incorporating System from an Extremely Halophilic Bacterium*

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ABSTRACT: A cell-free system has been developed from *Halobacterium cutirubrum* that will incorporate 10–100 $\mu\mu$moles/mg of ribosomes of each of 17 amino acids into hot trichloroacetic acid insoluble material. The system requires adenosine triphosphate, phosphoenolpyruvate, guanosine triphosphate, and ribosomes, is sensitive to pancreatic ribonuclease and puromycin, and will incorporate increased amounts of [^{14}C]phenylalanine in response to added polyuridylic acid. This evidence together with the nature of the labeled product suggest that incorporation of amino acids represents true polypeptide synthesis.

The optimum Mg^{2+} concentration for incorporation is 0.02–0.04 M, but the system is extremely halophilic in requiring nearly saturated salt and specifically 3.8 M KCl, 1 M NaCl, and 0.4 M NH_4Cl for maximum activity. The possible significance of this specificity is briefly discussed.

The synthesis of a protein depends on a series of molecular interactions which, by and large, are inadequately understood. One feature of these interactions that has been recognized for some time is the importance of cations. Binding of mRNA to ribosomes (Okamoto and Takanami, 1963a,b), the structural integrity of ribosomes (see, *e.g.*, Petermann, 1964; Gesteland, 1966) and of tRNA (Nishimura and Novelli, 1963; Lindahl *et al.*, 1966), and the activity of transfer enzymes (Gordon and Lipmann, 1967) all involve Mg^{2+} ions, which, in some instances, can be replaced by Ca^{2+} (Gordon and Lipmann, 1967). Binding of tRNA to ribosomes depends on NH_4^+ or K^+ (Spyrides, 1964).

Within extremely halophilic bacteria, the concentration of salt is very high (Christian and Waltho, 1962) so that ionic interactions between protein and nucleic acid components of the protein synthesizing system should be correspondingly reduced. Study of the mechanism of protein synthesis in these bacteria may therefore shed new light on the nature of the molecular interactions involved. Interesting observations have already been made on the ribosomes of one of these organisms, *viz.*, *Halobacterium cutirubrum* (Bayley and Kushner, 1964; Bayley, 1966a,b). To extend this work, a cell-free amino acid incorporating system from this bacterium was required. This paper describes such a system.

Materials and Methods

Preparation of Cell-free Extracts. Cells were grown at 37° under continuous aeration, with added oxygen, in a 150-l. stainless-steel fermentor in the medium described by Sehgal and Gibbons (1960), except that 10 ppm of Fe^{2+} (as $FeSO_4$) was added and the final pH was adjusted to 6.2. The concentrations of the principal inorganic salts in this medium were 4.3 M NaCl, 0.027 M KCl, and 0.08 M $MgSO_4$. Sterilized medium (100 l.), which had been centrifuged to remove the precipitate, was inoculated with a 5-l., 30-hr culture. After 19–20 hr the culture was cooled and the cells, in early log phase, were harvested in the cold. The cells were washed at 0° with centrifuging, once in a solution containing 4.3 M

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NaCl, 0.03 M KCl, 0.08 M $MgSO_4$, and once in 3.4 M KCl, 0.1 M magnesium acetate, and 0.01 M Tris (pH 7.6) (solution D; this corresponds to solution D used previously (Bayley and Kushner, 1964) except that the concentration of KCl has been reduced to the saturation amount at 0° and $MgCl_2$ has been replaced by magnesium acetate). The yield of cells was 0.5–0.6 g wet weight/l. of culture.

The procedure for preparing and assaying cell-free extracts is based on that developed by Nirenberg and Matthaei (1961; see also Nirenberg, 1964) for *Escherichia coli*. Extracts were prepared at 0–4°. Harvested cells of *H. cutirubrum* were homogenized in a glass Teflon Potter-Elvehjem homogenizer with a volume of solution D corresponding to about 1.5 times their wet weight, together with 0.015 ml of β-mercaptoethanol and 1 mg of electrophoretically purified DNase per 30 ml of solution D. An S-60 supernatant extract was obtained from the homogenate by centrifuging once at 40,000*g* for 20 min and twice at 60,000*g* for 30 min; in each case only the upper, clear part of the supernatant was retained. The S-60 extract was dialyzed for 4 hr against three changes of solution D containing β-mercaptoethanol at the same concentration as before and was then frozen in vials in isopentane at liquid nitrogen temperature and stored under liquid nitrogen.

Incubation and Assay Conditions. The reaction mixture was evolved gradually but the final mixtures with which most of the work reported here was carried out were as follows. Reaction mixture I typically contained the following components in 3.8 M KCl, 1.1 M NH_4^+ (as $(NH_4)_2SO_4$ and NH_4Cl; see below), 0.04 M magnesium acetate, and 0.03 M Tris-HCl buffer (pH 8): NaATP, 0.3 μmole; NaPEP,[1] 1.2 μmoles; Na- or LiGTP, 0.18 μmole; mixture of 19 amino acids, omitting the ^{14}C-labeled amino acid, 0.008 μmole of each (this mixture lacked glutamine but included both cysteine and cystine); [^{14}C]amino acid, 0.5 μCi; and S-60 fraction, 1.3 mg of protein (0.25 mg of ribosomes). The total volume of solutions added in this mixture was 0.225 ml.

Mixture I was subsequently modified to give the final reaction mixture II, containing half the amounts of the same components as mixture I in 3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl, 0.04 M magnesium acetate, and 0.03 M Tris-HCl buffer (pH 8). Here the total volume of solutions added was 0.125 ml.

To obtain a concentration of 3.8 M KCl in these mixtures, it was necessary to add dry salt. Since no allowance was made for the volume of this salt, the final concentrations of all salts in the mixtures were in fact slightly lower than the values just given. The exact compositions of reaction mixtures I and II are therefore detailed in Table I. As much as possible of the salt was dissolved with stirring and the mixture was then incubated as 37° usually for 40 min. During this time, the remaining salt dissolved.

At the end of the incubation, the sample tube was cooled to 0° and to it were added 2 ml of an aqueous solution at 0° containing 1000-fold excess of unlabeled amino acid corresponding to the [^{14}C]amino acid used, followed by 2 ml of cold aqueous 10% TCA. The precipitate was then washed twice in 5% TCA with centrifuging, heated at 90° for 15 min in 5% TCA (Siekevitz, 1952), cooled at 0° for 10–20 min, collected on a 0.45-μ Millipore filter, and washed with about 3 ml of cold 5% TCA (Nirenberg, 1964). The filters were dried for 30 min at 60° and counted in a Packard or Beckman scintillation counter using a scintillation solution containing 5 g of PPO and 0.3 g of POPOP in 1 l. of toluene. The counting efficiencies were measured as 70% in the Packard counter and 82% in the Beckman.

Enzymatic Digestion of the Labeled Reaction Product. The reaction product, insoluble in TCA, was digested with samples of carboxypeptidases A and B and of leucine aminopeptidase which had been treated by the supplier with DFP to destroy trypsin and chymotrypsin activity. Immediately before use, a small volume of the carboxypeptidase A suspension as supplied was diluted with water and centrifuged and the sedimented enzyme was dissolved in 10% LiCl. The leucine aminopeptidase was activated by incubating in the presence of $MnCl_2$ as recommended by the suppliers. During activation, the enzyme solution was dialyzed against excess $MnCl_2$ buffer solution to remove ammonium sulfate which would interfere with amino acid determinations later. The activated enzyme was tested against leucylglycine at

TABLE I: Composition of Reaction Mixtures.[a]

Solution (M)	Mixture I (ml)	Mixture II (ml)
NaATP (0.03)	0.010	0.005
NaPEP (0.12)	0.010	0.005
NaGTP or LiGTP (0.012)	0.015	0.010
Cold amino acid mixture less labeled amino acid (each 8×10^{-4})	0.010	0.005
KCl (3.4), magnesium acetate (0.07), and Tris (0.1), pH 8.05	0.070	
KCl (3.4), magnesium acetate (0.12), and Tris (0.15), pH 8.05		0.025
NH_4Cl (5) and $(NH_4)_2SO_4$ (0.6)	0.040	
NH_4Cl (1.25) and NaCl (3.125)		0.040
Solid KCl	36[b]	24[b]
[^{14}C]Amino acid (neutralized)	0.010	0.005
S-60 fraction	0.040	0.020
Water	0.020	0.010
Total volume of solutions	0.225	0.125

[a] Alterations in the concentrations of components of the mixtures were made so as to keep the volumes and all the remainder of the components the same. Most additional compounds were added in aqueous solution in place of water. [b] In milligrams.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PEP, phosphoenolpyruvate; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

25° in a mixture containing 5 μg of enzyme and 10 μmoles of leucylglycine in 0.005 M $MgCl_2$–0.02 M Tris (pH 8.5).

To prepare material for digestion, several times the normal volume of reaction mixture I was incubated and precipitated and the precipitate was washed and heated as before. The precipitate was freed of last traces of contaminating [^{14}C]amino acids by redissolving it in water containing dilute KOH to neutralize the TCA and reprecipitating with TCA. For digestion, the precipitate was again redissolved in water with KOH.

With carboxypeptidases A and B, the digestion mixture contained about 12 mg of protein in the redissolved ^{14}C-labeled precipitate (this included ribosomal protein as well as product), 1.5 mg of carboxypeptidase A, and 0.6 mg of carboxypeptidase B in a final volume of 2 ml, made 0.025 M with respect to Tris buffer (pH 8.05). The mixture was incubated at 37°, and at appropriate time intervals two aliquots were removed, *viz.*, 0.100 ml for determining free amino acids by the ninhydrin method (Matheson and Tattrie, 1964) and 0.200 ml for radioactivity measurements. To the latter, an equal volume of 10% TCA was added and the whole was centrifuged. A 0.200-ml sample of the supernatant was removed and added to a 13-ml scintillation mixture containing 6 g of PPO and 100 g of naphthalene in 1 l. of dioxane for counting. The precipitate was collected and washed on Millipore filters for counting as described above.

For digestion with leucine aminopeptidase, the redissolved ^{14}C-labeled product was first dialyzed in the cold for 2–3 hr against several changes of 0.007 M $MgCl_2$–0.03 M Tris buffer (pH 8.5) to remove traces of TCA which might have interfered with the enzyme. The digestion mixture contained about 4 mg of protein in the ^{14}C-labeled product and 0.2–0.3 mg of activated enzyme in 3.1 ml of 0.005 M $MgCl_2$ and 0.02 M Tris buffer (pH 8.5). The mixture was incubated at 25°, and at appropriate time intervals two aliquots were removed, 0.100 ml for amino acid determinations as before and 0.300 ml for radioactivity measurements. To the latter, an equal volume of 10% TCA was added and the whole was centrifuged. The radioactivities of the supernatant, in duplicate, and of the precipitate were measured as above.

Other Analyses. The concentration of ribosomes in an S-60 fraction was estimated by sedimenting the ribosomes at 150,000*g* for 2 hr, resuspending them in solution D, and calculating their concentration from the absorbance at 258 mμ using $E^{1\%}_{1\,cm}$ 158 (Bayley and Kushner, 1964).

Protein was estimated by the method of Lowry *et al.* (1951) using plasma albumin as a standard.

Source of Materials. Inorganic salts and TCA were reagent grade from Anachemia Chemicals Ltd., Montreal; Tris from Sigma Chemicals Co.; β-mercaptoethanol from Eastman Organic Chemicals; crystalline pancreatic RNase, electrophoretically purified DNase, and DFP-treated carboxypeptidases A and B and leucine aminopeptidase from Worthington Biochemical Corp.; disodium adenosine 5-triphosphate and sodium guanosine 5′-triphosphate from P-L Biochemicals Inc.; tetralithium guanosine 5′-triphosphate from Schwarz BioResearch Inc.; trisodium 2-phosphoenolpyruvate from Calbiochem Corp.; unlabeled L-amino acids from Schwarz BioResearch and Mann Research Laboratories Inc.; polyuridylic acid from Miles Chemicals Corp.; puromycin dihydrochloride from Nutritional Biochemical Corp.; and POPOP and PPO from Packard Instrument Co. Chloramphenicol was a gift for which we thank Parke Davis and Co.

Singly or uniformly ^{14}C-labeled L-amino acids were obtained from New England Nuclear Corp., Schwarz BioResearch Inc., or the Radiochemical Centre, Amersham, as specified below.

Results

Unless otherwise specified, experiments were carried out with samples of [1-^{14}C]leucine from New England Nuclear Corp. with specific activities of 30–34 mCi/mmole. In all experiments except those in Figure 3, samples were run in duplicate with similar results; only the means of these are quoted below.

General Properties of the Incorporating System. The response of the system to the omissions and addition shown in Table II demonstrates that it depends on ATP, PEP,[2] and GTP, and is sensitive to puromycin. Independent experiments showed that the concentrations of ATP, PEP, and GTP used in reaction mixtures I and II were optimal. No stimulation of incorporating activity was obtained by adding cytidine, uridine, or inosine triphosphate. When S-60 extract was used in the reaction mixture, no dependence on a mixture of unlabeled amino acids was evident, but we shall return to this point below.

TABLE II: Incorporation of [^{14}C]Leucine into Hot TCA Residue.

	Mean cpm	%
Complete[a]	1150	100
Complete − ATP, PEP	192	17
Complete − GTP	601	52
Complete − amino acid mixture	1119	97
Complete + puromycin (25 μg)	50	4

[a] Mixture I except that in this experiment, which was carried out before the optimum Mg^{2+} concentration had been accurately defined, the final Mg^{2+} concentration was 0.09 M.

The dependence of the incorporation on ribosomes

[2] In other experiments, ATP and PEP together were found to be superior to ATP alone. However the addition of rabbit muscle pyruvate kinase had no effect, so that the system probably depends on endogenous enzyme.

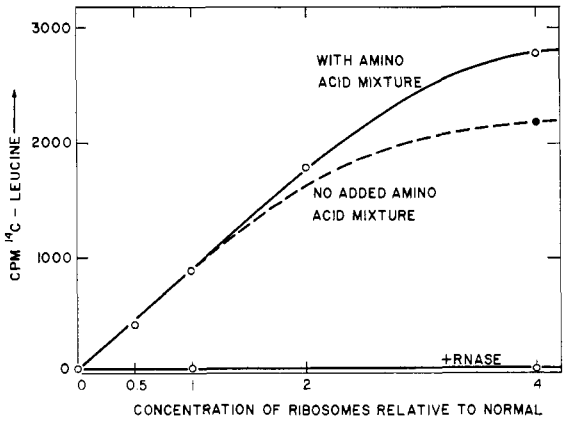

FIGURE 1: Incorporation of [^{14}C]leucine as a function of ribosomal concentration. Reaction mixture I was used with twice the normal concentration of unlabeled amino acids and with a ribosomal suspension and S-150 fraction replacing the S-60 fraction. Experiments with RNase contained 20 μg of the enzyme.

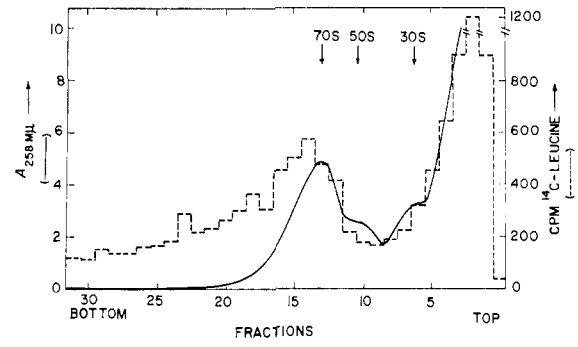

FIGURE 2: Density gradient analysis of an incubated mixture. Reaction mixture I (0.9 ml), containing twice the normal amounts of S-60 fraction and of [^{14}C]leucine, was incubated in the usual way, then dialyzed in the cold for 2 hr against two changes of 3.0 M KCl, 0.2 M magnesium acetate, and 0.01 M Tris buffer (pH 8) to adjust the density to the correct value and to remove free [^{14}C]leucine. The sample was then layered onto 30 ml of a 10–21% (w/v) sucrose gradient containing 2.5 M KCl, 0.4 M magnesium acetate, and 0.01 M Tris buffer and centrifuged at 37,000g for 15 hr. $A_{258\,m\mu}$ was monitored continuously as 1-ml fractions were collected from the top of the gradient. The fractions were precipitated with equal volumes of cold 10% TCA, and the precipitates were washed, mounted on Millipore filters, and counted. Similar results were obtained when hot TCA precipitates were prepared from fractions.

was examined by centrifuging S-60 extract for 2 hr at 150,000g to yield a ribosomal pellet and an S-150 supernatant fraction. The ribosomes were resuspended in a small volume of the supernatant and this suspension and more of the supernatant were combined in various proportions in the reaction mixture in place of the S-60 extract. The results, shown in Figure 1, clearly demonstrate a requirement for ribosomes. They also show that the system is sensitive to the action of pancreatic RNase. The system is of course insensitive to DNase as this was present during cell homogenization and fractionation.

It is probable that the dialyzed S-60 extract contained amounts of amino acyl-tRNAs, which, under conditions of moderate incorporation, were adequate for polypeptide synthesis. Thus an added mixture of cold amino acids had no effect. However, it was found in two independent experiments that when incorporation was increased by adding excess ribosomes, omission of the amino acid mixture reduced incorporation by an average of 25% (Figure 1).

The time course of incorporation is similar to that in nonhalophilic systems, *e.g.*, *E. coli* (Matthaei and Nirenberg, 1961). From a background level at zero time, incorporated counts rise during the first 40-min incubation, the period usually employed in this work, but increase negligibly thereafter. Incorporation is not significantly affected by pH at least over the range 7.6–8.0. The system is insensitive to chloramphenicol as a concentration of 0.7 mM reduced incorporation by only 7%.

The incorporations of 20 amino acids are listed in Table III. The first 14 were compared in two separate experiments using samples of identical specific activities in mixture I with the same S-60 fraction. In these experiments, the incorporating activity was only about half that obtained with later preparations of S-60 fraction in mixture II, but the results serve to demonstrate that, except for histidine, incorporations of these amino acids are comparable in order of magnitude. The results for the last six amino acids were obtained with later, more active S-60 fractions in mixture II and with amino acid samples of different specific activities. Again the incorporations are comparable except for asparagine and glutamine which were consistently low.

After being stored in liquid nitrogen for 5, 10, and 12 weeks, respectively, three different preparations of S-60 extracts showed no reduction in incorporating ability.

Nature of the Labeled Incubation Product. The sedimentation properties of components labeled during incubation were examined on sucrose gradients containing concentrated salt (W. E. Rauser and S. T. Bayley, in preparation). The results of a typical experiment are shown in Figure 2. (We are grateful to Dr. W. E. Rauser for running this gradient.) It is clear that, apart from slowly sedimenting material at the top of the gradient, the label is associated with slightly heavier 70S ribosomes and with larger components, possibly polyribosomes, but not with ribosomal subunits. In a separate study in which the sedimentation properties of the label associated with the more rapidly sedimenting components were examined under a variety of ionic conditions, it was found that this labeled material behaved as [^{14}C]peptidyl-tRNA (W. E. Rauser and S. T. Bayley, in preparation).

An attempt was made to examine the distribution of ^{14}C label within the polypeptide product by enzymatic digestion. In the experiments described here, most of the incorporation should represent extension or completion of preexisting nascent polypeptide chains. The labeled portions of the chains should therefore be digestible with carboxypeptidases but not with aminopeptidases.

The hot TCA-insoluble residue, which contained ribosomal proteins in addition to synthesized material, was digested with a mixture of carboxypeptidases A and B as described under Materials and Methods and the release of radioactivity and of free amino acids was

TABLE III: Incorporation of 20 Amino Acids.[d]

Amino Acid	Expt 1[b] (Mean)	Expt 2[b] (Mean)
Alanine	106	103
Arginine	115	100
Aspartic acid	34	34
Glutamic acid	33	27
Glycine	49	54
Histidine	8	8
Isoleucine[a]	50	46
Leucine[a]	97	127
Lysine	18	18
Phenylalanine[a]	19	17
Proline	45	54
Serine	38	
Threonine	75	82
Valine[a]	105	106
	Expt 3[c] (Mean)	Expt 4[c] (Mean)
Asparagine	2	3
Cystine[a]	14	15
Glutamine	5	10
Methionine	32	29
Tryptophan	21	21
Tyrosine[a]	32	29

[a] Amino acids for which the RNase controls gave counts several times background. These high control counts were obtained only with uniformly and not with singly labeled compounds and were serious only in the case of phenylalanine where they represented half the experimental counts. [b] Experiments 1 and 2 used the same S-60 fraction and uniformly ^{14}C-labeled amino acids with specific activities of 50 mCi/mmole from Schwarz BioResearch Inc. in reaction mixture I with the cold amino acid mixture omitted. [c] Experiments 3 and 4 used reaction mixture II (with the cold amino acid mixture present) and different preparations of S-60 fraction. [^{14}C]Amino acids (all uniformly labeled except tryptophan ([^{14}C]methylene)) were asparagine, 102 mCi/mmole (Amersham); cystine, 218 mCi/mmole (New England); glutamine, 38.8 mCi/mmole (Amersham); methionine, 187 mCi/mmole (New England); tryptophan, 52 mCi/mmole (Amersham); tyrosine, 50 mCi/mmole (Schwarz). [d] Net incorporation = experiment − control with added RNase expressed as micromicromoles per milligram of ribosomes.

followed as a function of time. The results of a typical experiment with [^{14}C]leucine are shown in Figure 3. In the first 3 hr, release of label exactly parallels release of amino acids, although it lags at later times. Identical results were obtained when a mixture of eight ^{14}C-labeled amino acids (Ala, Arg, Asp, Glu, Gly, Lys, Pro, and Thr) was used in place of leucine. In all of these experiments, the fraction of radioactivity released after

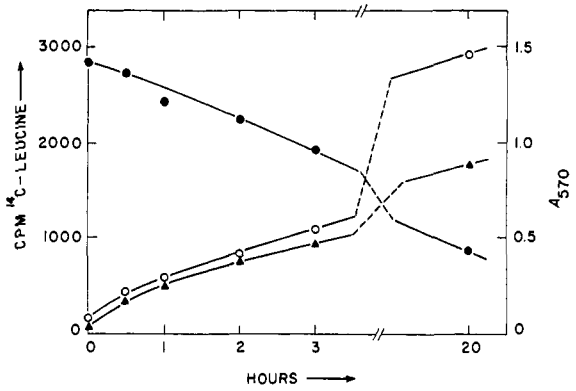

FIGURE 3: Release of radioactivity and of amino acids from reaction product labeled with [^{14}C]leucine as a function of time of digestion with carboxypeptidases A and B. Cpm in TCA-insoluble fraction (—●—) and in TCA-soluble fraction (—▲—); A_{570} (—○—) is quantitatively proportional to the concentration of free amino acids (Matheson and Tattrie, 1964), at least up to 3-hr digestion.

3-hr digestion ranged from 23 to 32% and after 20 hr, from 63 to 70%.

Unfortunately, experiments with leucine aminopeptidase were unsatisfactory. Although the enzyme preparation actively digested leucylglycine, it had no detectable effect on the labeled residue as measured by the release of amino acids or of radioactivity.

Cationic Requirements. A. MONOVALENT CATIONS. Incorporation of [^{14}C]leucine by the system dropped markedly at concentrations of KCl below 3.8 M as shown in Figure 4. The results of replacing KCl to different extents by the same molarity of NaCl or NH_4Cl are also plotted in Figure 4. These clearly show that the KCl can be replaced only to a small extent by NaCl, and not at all by NH_4Cl, without serious loss of incorporating abil-

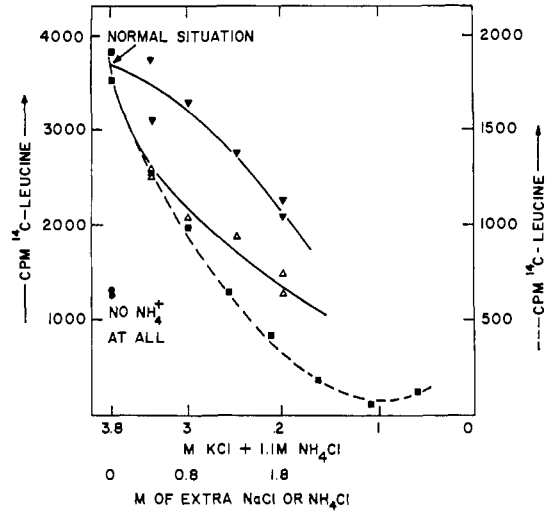

FIGURE 4: Incorporation of [^{14}C]leucine by mixture I as a function of monovalent cation concentration. Changes in concentration were achieved by altering the amount of dry salt added. KCl concentration lowered with no compensating addition of other cations (—■—); KCl concentration lowered and compensated with added NaCl (—▼—) or NH_4Cl (—△—).

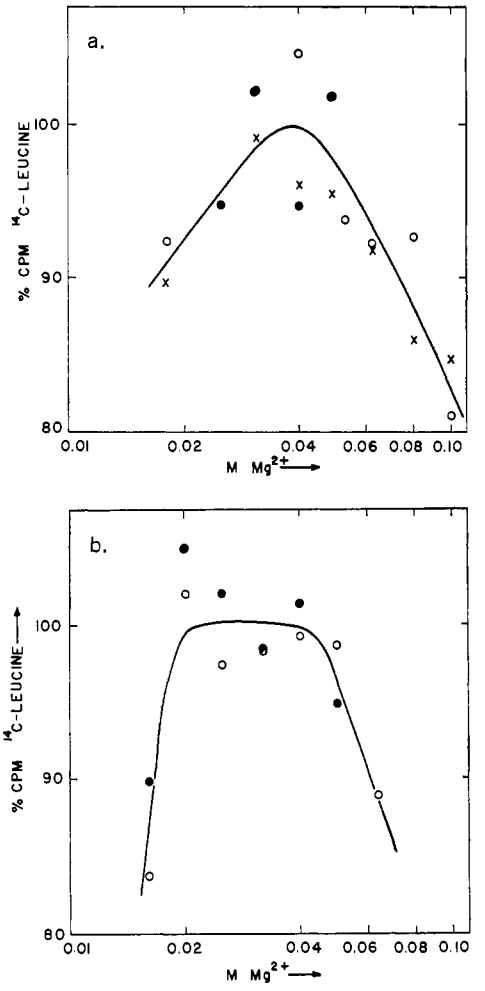

FIGURE 5: Percentage incorporation of [^{14}C]leucine at different concentrations of Mg^{2+}. (a) Mixture I in three separate experiments with the same S-60 fraction. (b) Mixture II in two separate experiments with different S-60 fractions.

ity. The system therefore has a specific requirement for a nearly saturated concentration of KCl.

Throughout these experiments, 1.1 M ammonium was present as NH_4Cl and $(NH_4)_2SO_4$ (reaction mixture I). When NH_4^+ ions were omitted from the normal mixture containing 3.8 M KCl, incorporation fell to about one-third, as is also shown in Figure 4. Thus there is a need for monovalent cations in addition to the high levels of K^+ already present. It was then found that incorporation could be improved further by replacing much of the ammonium salts in mixture I by NaCl (Table IV) as is done in mixture II.

B. DIVALENT CATIONS. Figure 5a shows the incorporation of [^{14}C]leucine by mixture I measured at different Mg^{2+} concentrations in three separate experiments. Although there is some scatter, the optimum Mg^{2+} concentration is clearly about 0.04 M. Similar experiments with mixture II, *i.e.*, in the presence of Na^+ and NH_4^+ rather than NH_4^+ alone, gave the results depicted in Figure 5b. The effect of improving the monovalent cationic environment has been to extend the optimal range of Mg^{2+} concentrations toward lower values.

Table V shows that Ca^{2+} and Mn^{2+} cannot replace Mg^{2+} in the system without loss of incorporating ability and are probably inhibitory.

Addition of cobaltous, manganous, or zinc chloride to the reaction mixture did not increase incorporation, except for a spurious effect of $CoCl_2$ which has been reported before (Fraser, 1963).

Response to Added Polyuridylic Acid. The effect on [^{14}C]phenylalanine incorporation of adding poly U to the reaction mixture is shown in Table VI. In later work, it was found that the cell-free system, after preincuba-

TABLE IV: The Effect of NH_4Cl and NaCl on the Incorporation of [^{14}C]Leucine[a] by Mixture I.[b]

NH_4Cl (M)	NaCl (M)	Mean cpm
1.4		9,570
	1.4	13,532
0.4	1.0	18,193

[a] Uniformly labeled; specific activity 275 mCi/mmole (New England). [b] In these experiments, $(NH_4)_2SO_4$ was omitted.

TABLE V: Effect on Incorporation of [^{14}C]Leucine by Mixture I of Partially Replacing Mg^{2+} by Ca^{2+} or Mn^{2+}.

Solution (M)	Mean % cpm
Magnesium acetate (0.04)	100
Magnesium acetate (0.018) + $CaCl_2$ (0.022)[a]	71
Magnesium acetate (0.018) + $MnCl_2$ (0.022)[a]	48
Magnesium acetate (0.018)	90

[a] $CaCl_2$ and $MnCl_2$ were added in place of magnesium acetate in the concentrated salt solution.

TABLE VI: Effect on [^{14}C]Phenylalanine Incorporation of Adding Poly U.

	Mean cpm
Normal[a]	375
Normal[a] + 25 μg of poly U	1139
Normal[a] + 25 μg of poly U + 20 μg of RNase	76

[a] Reaction mixture I at a Mg^{2+} concentration of 0.09 M and 1-C^{14}-phenylalanine from New England Nuclear Corp. with a specific activity of 16.5 mCi/mmole.

tion to destroy endogenous mRNA activity, will respond to a variety of synthetic messengers (Bayley and Griffiths, 1968).

Discussion

Because of the unusual ionic environment in which the cell-free system of this extremely halophilic bacterium operates, we have been concerned in this work to demonstrate so far as possible that the incorporation of amino acids represents true peptide synthesis, and not nonspecific absorption. On the basis of the currently accepted model for protein synthesis, the evidence can be summarized as follows. The system requires ATP, PEP, and GTP for energy, and there is evidence of the need for an amino acid supply. Ribosomes are essential for incorporation, and in a later paper (E. Griffiths and S. T. Bayley, in preparation) it will be shown that activation and transfer of amino acids depend on distinct synthetase and transferase enzymes and on tRNA. Incorporation is time dependent. It is sensitive to the action of pancreatic RNase but not of DNase, and is inhibited by puromycin. There is evidence that synthesis is associated with polyribosome-like material and that many of the labeled amino acids are incorporated at the C-terminal ends of growing chains. Perhaps the strongest evidence for polypeptide synthesis taking place under the direction of mRNA comes from experiments to be described in a subsequent publication (Bayley and Griffiths, 1968), in which the cell-free system, after preincubation to destroy endogenous messenger activity, responds to added synthetic polyribonucleotides.

The properties of the system, together with the observation that most of the 20 amino acids are incorporated to comparable extents, further suggest that incorporation is not due to N-terminal addition of the type described by Kaji *et al.* (1965) for leucine and phenylalanine in an *E. coli* system. The low incorporations of asparagine and glutamine are an interesting feature of the system that will be discussed elsewhere (E. Griffiths and S. T. Bayley, in preparation).

The cell-free system is truly halophilic in requiring extremely high concentrations of salts, containing particularly monovalent cations, for activity. The salt concentrations in the final reaction mixture II, which are very similar to the internal salt concentrations of *H. salinarium* estimated by Christian and Waltho (1962), are close to saturation at 37°. Indeed the trend of the present results suggests that the system functions best only in the absence of free, unbound water.

Ionic strength or total ion activity is not the only consideration in halophilic systems however, since the species of ions are also important. Thus the incorporating system has a specific requirement for nearly saturating concentrations of KCl. This requirement appears to be more specific than that of the amino acyl synthetases of *H. cutirubrum* (E. Griffiths and S. T. Bayley, in preparation) or of other halophilic enzymes (see, *e.g.*, Baxter and Gibbons, 1956; Holmes and Halvorson, 1965). On the basis of previous studies on halophilic ribosomes (Bayley and Kushner, 1964; Bayley, 1966a,b) therefore, it seems likely that K^+ ions are primarily required to preserve the integrity of the ribosomes. Because of the limited solubility of KCl, however, NaCl or less effectively NH_4Cl may also be necessary in the system to lower water activity to the level at which the ribosomes and enzymes function properly.

Addition of NaCl to the system not only improved incorporation but it broadened the optimum range of Mg^{2+} concentrations to lower values as well. Recently, studies with synthetic messengers have shown that Na^+ also affects fidelity of translation (Bayley and Griffiths, 1968). Whether these effects are due entirely to the more effective lowering of water activity by Na^+ compared to NH_4^+ or whether they demonstrate further specificity of Na^+ in the system is not clear.

In view of the extremely high concentrations of potassium and sodium salts that are necessary, the system is remarkable in two respects. One is the close similarity in all essential features to protein synthetic systems from nonhalophilic organisms. The other is that the system still depends to some degree on comparatively low concentrations of other cations. Thus the incorporation is enhanced by low concentrations of NH_4^+ and it depends specifically on a relatively very low concentration of Mg^{2+}, between 0.02 and 0.04 M, which is not greatly different from that required by nonhalophilic systems. The various functions that Mg^{2+} and NH_4^+ ions serve in the system will be considered in more detail elsewhere (W. E. Rauser and S. T. Bayley, in preparation), but it is clear that to serve these functions, these relatively scarce ions must be bound by some cooperative mechanism since they cannot be in dynamic equilibrium with their surroundings.

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Inhibition of Calf Thymus Deoxyribonucleic Acid Polymerase by Histones*

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ABSTRACT: The previously reported inhibition of deoxyribonucleic acid (DNA) synthesis by histones cannot be attributed solely to precipitation of primer DNA as a nucleohistone complex since present data demonstrate that an even greater inhibition occurs with the calf thymus DNA polymerase system under conditions (low Mg^{2+} concentration and low ionic strength) in which the complex of histones and heat-denatured DNA is soluble. Under both conditions the F1 fraction of histones is the most effective and the F3 fraction is the least effective inhibitor of DNA synthesis. Sonication of the insoluble nucleohistone complex (under conditions of higher Mg^{2+} concentration and ionic strength) disperses the gel but does not relieve the inhibition. At very low histone:DNA ratios there is a small "stimulation" of incorporation of [^{3}H]deoxyadenosine triphosphate into DNA in comparison with histone-free controls. There appears to be no specificity of the inhibition with respect to DNA bases since the incorporation of [^{3}H]deoxycytidine triphosphate by unfractionated histones (or fractions F1 and F3) is inhibited to the same extent as the incorporation of [^{3}H]thymidine triphosphate. At a Mg^{2+} concentration of 3.75 mM, the optimum concentration of potassium phosphate buffer is 30–40 mM for the DNA polymerase reaction with or without histones. At low concentrations (5 mM) of phosphate buffer (pH 7.4) the optimum concentration of Mg^{2+} is 3 mM for the DNA polymerase reaction in the absence of histones and 1–1.5 mM in the presence of histone fraction F1, with progressively increasing inhibition as the concentration is raised from 1.5 to 6 mM.

These data suggest the possibility of a cooperative role of histones and Mg^{2+} in one aspect of the control of DNA synthesis.

Studies in this and other laboratories (Bazill and Philpot, 1963; Gurley *et al.*, 1964; Billen and Hnilica, 1964; Schwimmer, 1965; Schwimmer and Bonner, 1965) have shown that histones inhibit various DNA polymerase systems *in vitro*, but the role of histones in the regulation of DNA replication *in vivo* is uncertain. Although the DNA polymerase of *Escherichia coli* provides a useful model system for investigation of effects of histones (Billen and Hnilica, 1964; Schwimmer, 1965; Schwimmer and Bonner, 1965), it is difficult to extrapolate these data and interpretations with confidence to the synthesis of DNA in animal systems in view of considerable differences, such as requirements for DNA primer, between the two enzyme systems. This problem is not entirely resolved by the use of DNA polymerases isolated from animal sources since several of these enzyme systems have been described with differing properties and primer requirements, and it is still uncertain whether these enzymes are primarily concerned with DNA replication or with DNA repair. A purified DNA polymerase from calf thymus (Bollum, 1960; Yoneda and Bollum, 1965) has an absolute requirement for single-stranded or de-

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