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An NH_4^+-Dependent Protein Synthesis Cell-Free System for Halobacteria†

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ABSTRACT: An efficient poly(U)-dependent polyphenylalanine system for extreme halophilic archaebacteria is described. The system was tested with eight different species including the six genera described to date: *Halobacterium*, *Haloferax*, *Haloarcula*, *Halococcus*, *Natronobacterium*, and *Natronococcus*. In all of them the optimal ionic conditions are similar. The system is extremely dependent on NH_4^+, with the optimal concentration being 1.5 M $(NH_4)_2SO_4$, and has virtually no dependence on K^+. The internal concentration of NH_4^+ and K^+ of three halobacteria with different degrees of halophilia was measured. A close correlation between the internal concentration of K^+ and the external concentration of cations, as well as a relatively low concentration of NH_4^+, was obtained in all cases. The possible importance of all the variables of the system is discussed.

Halobacteria are a large group of archaebacterial microorganisms that concentrate high levels of K^+ within the cell to overcome the extreme external osmotic pressure. They colonize very diverse habitats such as solar salterns, desertic terminal lakes like the Great Salt Lake or the Dead Sea, soda lakes with very alkaline pHs like the Wadi-Natrum and the Magadi, or man-made substrates like salt fish, sausage, and tanned leather.

All the reported cell-free protein synthesis systems for this type of archaebacteria have been performed with phylogenetically and taxonomically similar halobacteria, belonging to the *Halobacterium* genus (Bayley & Griffiths, 1968; Kessel & Klink, 1981; Saruyama & Nierhaus, 1985). Recently, Torreblanca et al. (1986) proposed a new taxonomic classification for the nonalkalophilic halobacteria based on numerical taxonomy and the lipid composition of the membrane. They describe two new genera, *Haloarcula* and *Haloferax*, including some of the species previously ascribed to the *Halobacterium* genus.

The differences in the ionic conditions of the habitats in which the three genera of nonalkalophilic halobacterial bacillus

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(genera *Halobacterium*, *Haloferax*, and *Haloarcula*), the nonalkalophilic cocci (genus *Halococcus*), and the alkalophilic bacteria (genera *Natronobacterium* and *Natronococcus*; Ross & Grant, 1984) grow allow us to postulate different optimal ionic conditions for each of their cell-free protein synthesis systems.

In spite of intracellular differences exhibited by different halobacteria, an efficient in vitro protein synthesis system is described that can be used in all the genera described in the halophilic archaebacterial family.

Materials and Methods

Microorganisms and Their Growth Conditions. Halobacterium halobium NCMB 777, *Halobacterium salinarium* CCM 2148, *Haloferax gibbonsii* ATCC 33959 (Ma 2.38), *Haloarcula californiae* ATCC 33799, *Halobacterium marismortui*, *Haloferax mediterranei* ATCC 33500, *Haloarcula sinaiiensis* ATCC 33800, *Halobacterium saccharovorum* ATCC 29252, *Halococcus morrhuae*, *Natronobacterium pharaonis* NCMP 2191 (sp-1), and *Natronococcus occultus* NCMB 2192 (sp-4) were kindly provided by F. Rodriguez-Valera, A. Ventosa, and H. Ross.

The nonalkalophilic halobacteria were grown in SW25 media as described by Rodriguez-Valera et al. (1980). The $CaCl_2$ was eliminated in order to avoid precipitation during sterilization. The alkalophilic halobacteria were grown in media described by Tindall et al. (1980), eliminating Fe^{2+} and Mn^{2+} in order to avoid precipitation in the alkaline pH (10.5) of the media. All the incubations were performed at 40 °C.

Preparation of Ribosomes and S-100 Fraction. Cells were collected in the middle of the exponential growth phase (OD_{660} = 0.6–0.8), disrupted with alumina in a proportion of 2 g/g of wet weight of cells. When the paste acquired a thick consistency due to the liberation of DNA into the media, 1 mL of buffer D (30 mM Tris-HCl, pH 7.6; 2 M $(NH_4)_2SO_4$; 0.4 M NH_4Cl; 60 mM $Mg(OAc)_2$; and 5 mM β-mercaptoethanol) per gram of cells was added together with 2 μg/mL final volume of DNase grade I.

The suspension was centrifuged at 12000 rpm for 10 min to separate alumina, intact cells, and cell debris. The supernatant was further clarified by centrifugation at 60000*g* for 30 min. Part of the supernatant (S60) was stored at −70 °C, while the rest was centrifuged at 90000*g* overnight in order to pellet the ribosomes. The pellet was resuspended in buffer D, the ribosomal concentration was determined by measuring its absorption at 260 nm, and the solution was stored at −70 °C. The supernatant S100 was dialyzed against buffer D with 5% glycerol and stored at −70 °C.

Poly(U)-Directed Poly(Phe) Synthesis. The system described by Amils et al. (1979) was used for *Escherichia coli*. For the halobacteria the following reaction mixture was used: 30 mM Tris-HCl, pH 7.6, 30 mM $Mg(OAc)_2$, 1 M KCl, 400 mM NH_4Cl, 1.5 M $(NH_4)_2SO_4$, 7 mM β-mercaptoethanol, 2 mM ATP, 0.5 mM GTP, 5 mM PEP, 36 μM Phe ([^{3}H]Phe 320 dpm/pmol), 5 μg of pyruvate kinase, 80 μg of poly(U), and 100 μg of yeast tRNA in a 100-μL volume. Normally 20–35 pmol of 70S ribosomes was used per test tube, and the amount of S100 was optimized for each ribosome preparation. When the effect of one component was tested, the rest were maintained constant.

The samples were incubated at 40 °C for 1 h. The reaction was stopped by adding 2 mL of 5% cold TCA. The tubes were heated at 90 °C for 15 min and filtrated after cooling at 4 °C. Each filtrate was washed three times with 5% TCA and 2 mL of ethanol. The radioactivity incorporated was measured in a liquid scintillation counter.

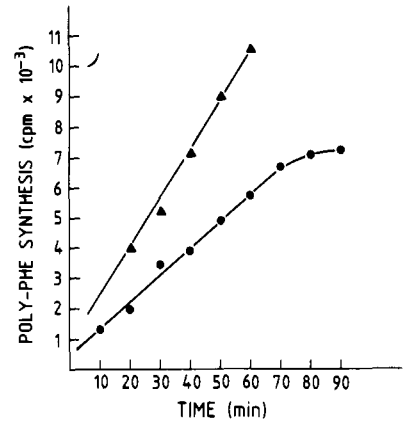

FIGURE 1: Kinetics of [^{3}H]polyphenylalanine synthesis directed by poly(U) in *H. mediterranei* (▲) and *N. occultus* (●). Assays were carried out as described under Materials and Methods.

Intracellular Concentration of K^+ and NH_4^+. Intracellular volume was determined according to the method of Sadler et al. (1980), using [^{3}H]H_2O and [^{14}C]dextran to measure the total volume and the extracellular space, respectively.

The K^+ concentration was measured by emission spectroscopy, using a Perkin-Elmer 4000 attomic absorption spectrophotometer at a wavelength of 760 nm. The NH_4^+ concentration was determined by using a Technicon autoanalyzer and comparing the results with those obtained by the colorimetric method described by Solorizano (1969).

Results and Discussion

In all cases the cells were harvested in the logarithmic phase of growth. The ammonium buffer used for ribosome preparation gives more active ribosomes and stable S100 fractions than the potassium buffers described in the literature (Bayley, 1968; Saruyama, 1985). However, the optimal ionic conditions of the poly(U)-dependent poly(Phe) synthesis are not dependent on the K^+ or NH_4^+ buffers used to prepare the ribosomes. The S100 dialysis described under Materials and Methods does not improve the polymermization activity but does improve the stability of the soluble factors fraction over long periods of time. Using dialyzed S100 as a source of soluble factors allows a more accurate calculation of the specific radioactivity of phenylalanine.

When this project began, the protocols described in the introduction were followed, but many variations have been introduced over time as we tried to optimize protein synthesis activity. The results obtained during this optimization are discussed in the following section, in which the influence of the variation of one component was tested while the rest remained constant.

Kinetics. The elongation rate was constant over a period of 60 min. This incubation period was maintained for the rest of the experiments (Figure 1).

Temperature. The optimal temperature was 40 °C, corresponding to the optimal temperature for cell growth.

Poly(U). All the in vitro systems tested depended on the addition of external messenger. The concentration dependence for *H. mediterranei* is shown in Figure 2.

tRNA. Most of the in vitro halobacterial protein synthesis systems are able to function without exogenous tRNA, albeit with moderate efficiency. The addition of *E. coli* tRNA does not improve the rate of synthesis, but the addition of yeast tRNA does improve polymerization efficiency by 100% in a concentration of 100 μg/tube. Higher concentrations do not improve the rate of polymerization. In more recent experiments yeast tRNA was replaced by *Sulfolobus solfataricus*

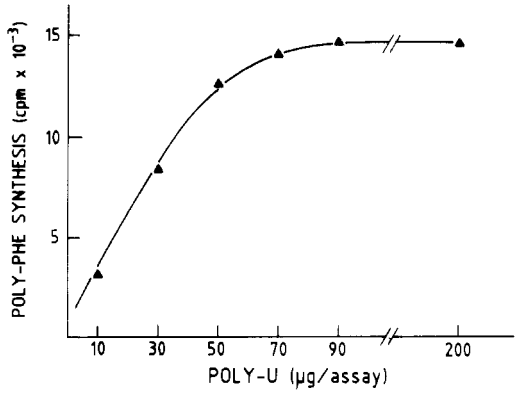

FIGURE 2: Dependence on exogenous messenger of the poly(U)-dependent poly(Phe) synthesis in *H. mediterranei*.

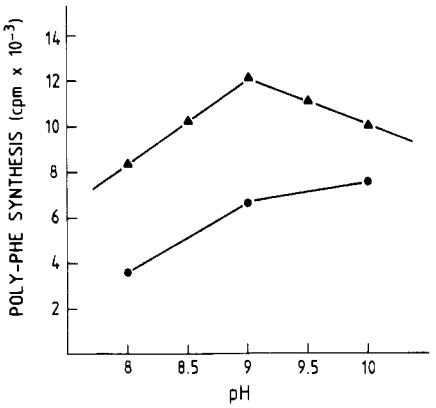

FIGURE 3: Effect of pH on the poly(U)-dependent poly(Phe) synthesis in *H. mediterranei* (▲) and *N. occultus* (●).

tRNA, which allows higher synthesizing efficiencies. These experiments suggest that the halobacterial aminoacyl tRNA synthetases are capable of recognizing and charging archaebacterial and eukaryotic tRNAs. Furthermore, nonhalophilic archaebacterial and eukaryotic tRNAs can interact with halobacterial elongation factors and ribosomes in extreme ionic conditions.

pH. The effect of performing the protein synthesis in the presence of 30 mM Tris-HCl at a pH between 8 and 10 is presented in Figure 3. An optimal elongation efficiency is observed at pH 9, although when the final pH of the reaction mixture is measured in the presence of high ionic monovalent cations, a value of 7.5 is obtained.

Mg^{2+}. In all the systems assayed the efficiency of polymerization is not strictly dependent on a specific concentration of Mg^{2+} (Figure 4) which contrasts with the results obtained in other archaebacterial, eubacterial, and eukaryotic systems in which small variations in the Mg^{2+} concentration dramatically affect the rate of protein synthesis (Londei, 1986). The lack of inhibition exhibited at high Mg^{2+} concentrations must be related to the competition produced by the necessarily high monovalent cationic concentration. The lack of effect of polyamines in the halobacterial systems agrees with the report that they are not present in most of the halobacteria tested so far (Kneifel et al., 1986).

Monovalent Cations. In contrast to previous reports, none of the systems assayed in this work exhibit dependence on K^+ in concentrations lower than 1 M. Higher concentrations produce inhibitions (Figure 5), probably due to precipitation of the NH_4^+ required for an efficient in vitro synthesis of proteins.

The same is true for Na^+, which has been used in previous systems. The Na^+ dependency reported for halobacterial

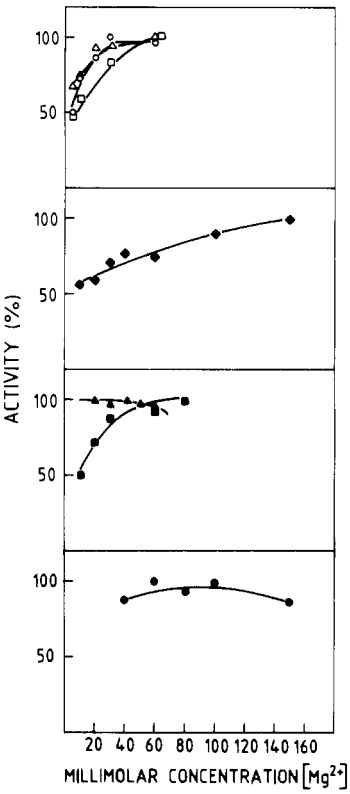

FIGURE 4: Effect of Mg^{2+} concentration on the poly(U)-dependent poly(Phe) synthesis systems from different halobacteria. The NH_4^+ and K^+ concentrations were maintained constant at 1.5 and 1 M $(NH_4)_2SO_4$ and KCl, respectively. Assays were carried out as described under Materials and Methods. For each system, *N. occultus* (○), *N. pharaonis* (△), *H. morrhuae* (□), *H. halobium* (◆), *H. mediterranei* (▲), *H. gibbonsii* (■), and *H. sinaiiensis* (●), the maximum incorporation obtained in the range of Mg^{2+} concentration studied was considered 100% activity.

protein synthesis is not clear. Bayley and Griffiths (1968) suggested a Na^+ effect related to translation fidelity. In our case we have eliminated this cation due to its inefficiency and in order to prevent precipitation problems with other cations needed for efficient protein synthesis and to facilitate the calculation of the real concentrations of cations present in the mixture. In any case, it is important to emphasize that the optimal ionic concentrations obtained in this paper are quite different from the ones described for *Halobacterium cutirubrum.*

The NH_4^+ cation plays a fundamental role in all the halobacterial systems studied so far, suggesting that it has a regulatory effect that becomes optimal at concentrations of approximately 1.5 M of $(NH_4)_2SO_4$ (Figure 6). As we will discuss later, optimal polymerization is dependent not only on the class and concentration of cations but on those of the anions, too.

The lack of dependence on K^+ in protein synthesis and the absolute need for NH_4^+ in a cell-free system for *H. mediterranei* is clearly shown in Figure 7.

It should be noted that although K^+ is the compatible solute found in halobacteria, none of the protein synthesis cell-free systems assayed so far require K^+, but they are, nevertheless, dependent on NH_4^+. It could be argued that since K^+ is responsible for the regulation of the external concentration of Na^+, its cytosol concentration must depend on the exterior osmotic pressure (Perez-Fillol & Rodriguez-Valera, 1986) and that the enzymatic systems should, in theory, not depend on the variation of this cation but be regulated by the concentration of another effector, which, in the case of ribosomes,

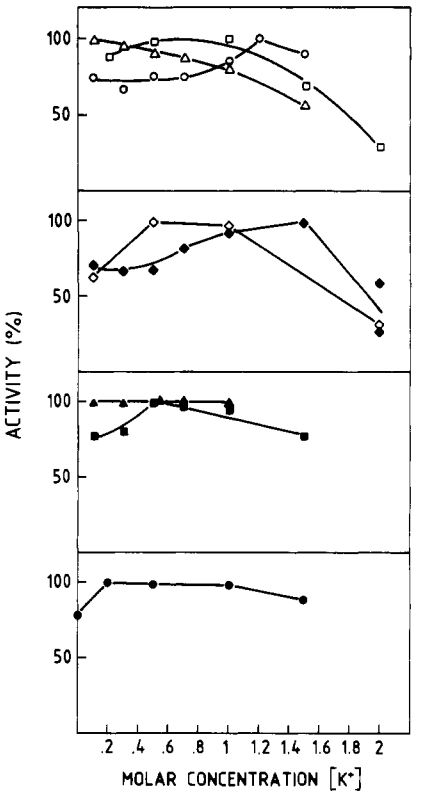

FIGURE 5: Effect of K^+ concentrations on the poly(U)-dependent poly(Phe) synthesis system from different halobacteria. The Mg^{2+} and NH_4^+ concentrations were maintained constant at 30 mM and 1.5 M $Mg(OAc)_2$ and $(NH_4)_2SO_4$, respectively. Assays were carried out as described under Materials and Methods. For each system, *N. occultus* (○), *N. pharaonis* (△), *H. morrhuae* (□), *H. saccharovorum* (◇), *H. halobium* (◆), *H. mediterranei* (▲), *H. gibbonsii* (■), and *H. sinaiiensis* (●), the maximum incorporation obtained in the range of concentration studied was considered 100% activity.

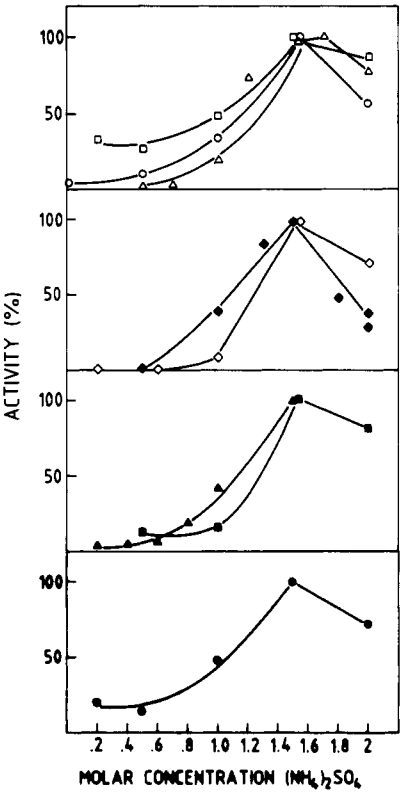

FIGURE 6: Effect of NH_4^+ concentrations on the poly(U)-dependent poly(Phe) synthesis system from different halobacteria. The Mg^{2+} and K^+ concentrations were maintained constant at 30 mM and 1 M $Mg(OAc)_2$ and KCl, respectively. Assays were carried out as described under Materials and Methods. For each system, *N. occultus* (○), *N. pharaonis* (△), *H. morrhuae* (□), *H. saccharovorum* (◇), *H. halobium* (◆), *H. mediterranei* (▲), *H. gibbonsii* (■), and *H. sinaiiensis* (●), the maximum incorporation obtained in the range of NH_4^+ concentration studied was considered 100% activity.

could be NH_4^+, as is shown in Figures 6 and 7.

In addition, this effect is not limited to halobacteria. *Vibrio costicola*, a halotolerant eubacteria, is able to grow in a wide range of NaCl concentrations (Forsyth & Kushner 1970). The cell-free protein synthesis system for *V. costicola* grown in the presence of 1 M NaCl (in which the internal concentrations of Na^+, K^+, and NH_4^+ are 0.6, 0.7, and 0.4 M, respectively) shows optimal activity at concentrations of 150 mM of these cations and is completely inhibited at concentrations of NaCl, KCl, or NH_4Cl higher than 400 mM (Wydro et al., 1977). Wydro et al. (1977) suggest that the cations are not free in the cytoplasm but are somehow trapped in the internal structure of the cell. As will be discussed later, this effect is not only dependent on the cation concentration but is related to the quality and concentration of the correspondent anions, too, making the analysis of the effect and the interpretation much more complicated (Kamekura & Kushner, 1984).

In order to test the possible regulatory effect of NH_4^+ and the apparent lack of effect of K^+ in halobacterial protein synthesis, intracellular concentrations of both cations were measured for *H. halobium*, *H. mediterranei*, and *N. pharaonis*. The results obtained agreed partially with the hypothesis but posed new questions. The internal K^+ concentrations found in these halobacteria (3.8 M for *H. halobium*, 1.8 M for *H. mediterranei*, and 2.6 M for *N. pharaonis*) are in accordance with the ionic strength of the habitats in which they develop naturally (Juez, 1987).

Because the differences in internal K^+ concentration found in these halobacteria do not influence the optimal ionic conditions of the respective protein synthesis cell-free systems, it

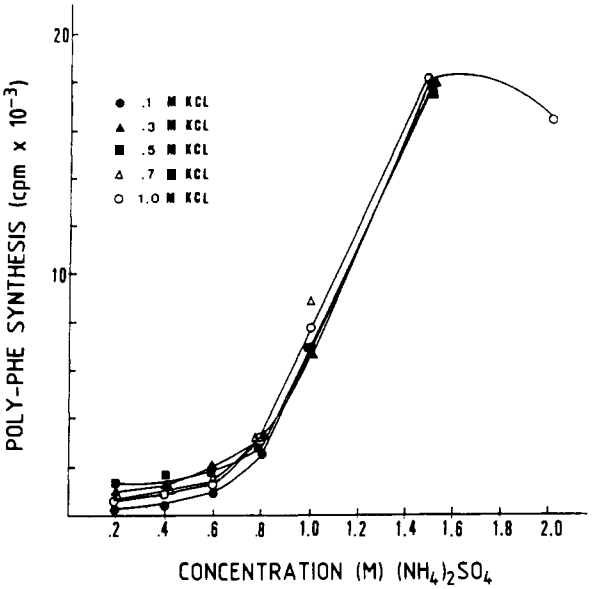

FIGURE 7: Effect of KCl concentration on the poly(U)-dependent poly(Phe) synthesis of *H. mediterranei* at different concentrations of $(NH_4)_2SO_4$. KCl concentrations used: 0.1 M (●); 0.3 M (▲); 0.5 M (■); 0.7 M (△); 1.0 M (○). Assays were carried out as described under Materials and Methods.

is reasonable to conclude that the translational apparatus of the halobacteria are not regulated by the compatible solute and are insensitive to its variations. However, the ammonium concentration is extremely low in all the measured systems (10–30 mM), which makes it difficult to explain the consistent

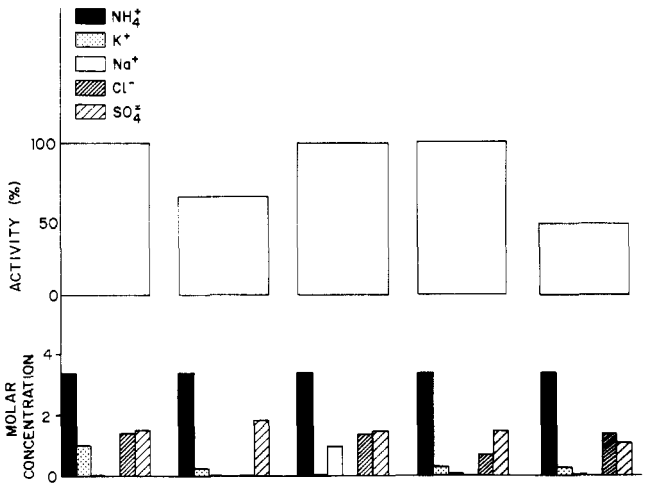

FIGURE 8: Comparative effect of K^+, NH_4^+, Na^+, Cl^-, and SO_4^{2-} concentrations on the poly(U)-dependent poly(Phe) synthesis of *H. mediterranei*. Assays were carried out as described under Materials and Methods. Different concentrations of variable components were achieved by using different concentrations of KCl, NH_4Cl, NaCl, and $(NH_4)_2SO_4$; the rest of the components were kept constant. The molar concentrations of the different components are correlated with the relative efficiency of polymerization obtained by using the mixture of 1.5 M $(NH_4)_2SO_4$, 0.4 M NH_4Cl, and 1 M KCl as 100% activity.

dependence on this cation found in all the halobacteria protein synthesis systems analyzed so far.

Bayley and Griffiths (1968), in their now classic synthesis of polyphenylalanine directed by poly(U) from *H. cutirubrum* ribosomes, described optimal efficiency at 3.8 M KCl concentrations, with 1 M NaCl and 0.4 M NH_4Cl and the Na^+ and NH_4^+ concentrations being much higher than the physiological ones. The modifications introduced by Kessel and Klink (1981) in this system were essentially the elimination of Na^+, a cation that is not, in fact, physiological, and the addition of moderate concentrations of $(NH_4)_2SO_4$, without eliminating the NH_4Cl (80–350 mM). This modified system is possibly the most physiological, but like the former it showed very low polymerization efficiency in our hands.

Saruyama and Nierhaus (1985) have recently described a poly(Phe) poly(U)-directed synthesis for *H. halobium* [which can probably be considered the same species as *H. cutirubrum*, (Larsen 1981)] that introduced important modifications, especially an increase in the $(NH_4)_2SO_4$ concentration, a decrease in KCl to 2 M, and the use of yeast $tRNA^{Phe}$ as an external source of tRNA. The high concentration of monovalent cations used in this system produced a partial precipitation of the salts, impeding the calculation of the real concentration of the cations present in the mixture. The system described in this paper has an optimum of NH_4^+ much lower than the one described by Saruyama and is not dependent on K^+ concentration.

Anions. As stated before, another interesting variable in the system is the quality and concentration of the necessary anions bound to the required cations. Some preliminary results have been obtained regarding the presence of different anions in the protein synthesis assay. The presence of Cl^- alone in the reaction mixture does not allow protein synthesis to proceed, probably due to an analogous mechanism previously described for other eubacterial and eukaryotic systems (Weber, 1977).

Of all the anions tested, SO_4^{2-} produces the best results. Figure 8 shows that optimal polymerization is obtained when Cl^- and SO_4^{2-} are combined and a higher molar concentration of SO_4^{2-} than Cl^- is used. Total replacement of Cl^- by SO_4^{2-} gives a suboptimal value. Unfortunately, the complete set of cation and anion replacements cannot be achieved due to the lack of suitable salts that could give all the possible combinations at the high ionic concentrations needed for the halobacterial systems.

Recent data obtained by Kamekura and Kushner (1984) showed that Cl^- also has a strong inhibitory effect on the *V. costicola* protein synthesis system. In this case the cell-free system allows protein synthesis at high ionic concentration, using Na^+ salts from organic acids like glutamate, etc.

In the halobacterial ribosomes the anion requirement cannot be fulfilled by organic anions, which implies that anions and cations have separate effects on protein synthesis, making the analysis of the optimal conditions very difficult. We have studied partial Cl^- (KCl) or total Cl^- (KCl + NH_4Cl) substitution in *H. saccharovorum*. The substitution of KCl by sodium or potassium glutamate, formate, or aspartate does not have a significant effect on protein synthesis when the optimal $(NH_4)_2SO_4$ concentration is maintained (1.5 M). The replacement of NH_4Cl by $(NH_4)_2SO_4$ (total NH_4^+ concentration constant) and of KCl by potassium glutamate, formate, and aspartate, with the subsequent total elimination of Cl^-, produces a small but consistent decrease in the efficiency of protein synthesis (data not shown).

The effect of organic anions has also been tested in the *H. halobium* cell-free system. The total substitution of $(NH_4)_2SO_4$ by NH_4Cl, NH_4OAc, or ammonium formate does not allow the protein synthesis to occur, demonstrating the importance of the anions in this process. Ammonium glutamate is capable of partially replacing $(NH_4)_2SO_4$, obtaining polymerization activities that are 50% of those obtained with the optimal SO_4^{2-}/Cl^- concentrations.

In conclusion, the in vitro protein synthesis systems studied in different halobacterial species show an extreme dependence on NH_4^+, with similar optimal concentration in all systems. The independence from other monovalent cations, Na^+ and K^+, and the absence of a critical concentration of Mg^{2+} are important factors that are common to all the halobacteria systems studied. These studies do not concur with the physiological ionic conditions measured for several halobacteria; although currently we do not have an adequate explanation, the cell-free system described in this paper seems to be reliable in all the halobacteria studied so far. The system is extremely complex, because the anions have an additional effect that should be considered. Further understanding of the interactions of the different charged elements present in the system, nucleic acids, proteins, anions, and cations, and of the effect of their relative concentrations will require many more comparative physiological studies.

ACKNOWLEDGMENTS

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Registry No. NH_4^+, 14798-03-9; K, 7440-09-7; Na, 7440-23-5; Cl^-, 16887-00-6; SO_4^{2-}, 14808-79-8; Mg, 7439-96-5; polyphenylalanine, 25191-15-5; polyphenylalanine, SRU, 25248-59-3.

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Germination of *Dictyostelium discoideum* Spores. A ^{31}P NMR Analysis†

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ABSTRACT: Perchloric acid extracts of *Dictyostelium* spores have been investigated by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. This analysis has allowed the assignment of all the ^{31}P resonances observed in vivo to specific compounds. Dormant spores have been found to contain as prominent phosphorylated metabolites two phosphomonoesters, phosphoethanolamine and inositol hexakis(phosphate), two phosphodiesters, glycerophosphocholine and glycerophosphoethanolamine, as well as nucleoside triphosphates and polyphosphates. The very large amounts of glycerophosphocholine, glycerophosphoethanolamine, and phosphoethanolamine in spores were the most remarkable differences from *Dictyostelium* amoebae. In vivo ^{31}P NMR has shown that the peak of nucleoside triphosphates in dormant spores was maintained metabolically since it disappeared completely upon anaerobiosis. The pH-sensitive ^{31}P NMR signal of phosphoethanolamine was used to determine internal pH, and a value of pH 6.5 was found in aerobic *Dictyostelium* dormant spores. Spore germination, induced by activation with heat shock treatment, was monitored noninvasively by ^{31}P NMR. No change in phosphorylated components was observed to have occurred during the activation step. The major modifications in phosphorylated metabolites observed upon germination of the activated spores were the progressive disappearance of the two phosphodiesters glycerophosphocholine and glycerophosphoethanolamine.

The differentiation program of the cellular slime mold *Dictyostelium discoideum* is engaged upon starvation and leads to the formation of a fruiting body composed of a mass of spores supported by a thin cellular stalk (Bonner, 1967; Loomis, 1975; Raper, 1984). Experimental parameters governing the germination process have revealed a complex and highly controlled mechanism (Cotter, 1981; Cotter & Raper, 1966, 1968a,b).

In this work, we have examined the phosphorylated metabolites of *Dictyostelium* spores in the dormant stage and during the whole germination process using the noninvasive ^{31}P NMR spectroscopy technique. We report that *Dictyostelium* spores contain very large amounts of two phosphodiesters: glycerophosphocholine (GPC)[1] and glycerophosphoethanolamine (GPE). These two compounds progressively disappeared during the germination of activated spores.

Experimental Procedures

Chemicals. Methylenediphosphonic acid, GPE, GPC, phosphoethanolamine, and phosphocholine were purchased from Sigma. Poly(propylene glycol) 2000 was from Baker Chemical.

Culture Conditions. Dictyostelium discoideum, strain NC4H (ATCC 34071), was grown at 22 ± 1 °C in association

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[1] Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; IP3 *myo*-inositol 1,4,5-tris(phosphate); IP4, *myo*-inositol 1,3,4,5-tetrakis(phosphate); IP6, *myo*-inositol hexakis(phosphate) (phytic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; P_i, inorganic phosphate.

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