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Ribosomal proteins and effect of Na^+ and K^+ on the protein-synthesizing system in a slightly halophilic marine bacterium, *Vibrio* sp. strain ABE–1

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A slightly halophilic marine bacterium, *Vibrio* sp. strain ABE-1 requires 0.5 M NaCl for the optimum growth, and the active transport system for L-leucine and *in vivo* protein synthesis also required Na^+ but not K^+. More than 60% of the ribosomes were found to exist as polysomes at the concentration range of Na^+ and K^+ between 0.03 and 0.5 M, but the protein-synthesizing system itself did not require such the salts and, on the contrary, inhibited markedly by 0.5 M Na^+ or K^+. Ribosomal proteins of *V.* ABE-1 were not definitely different from those of non-halophilic bacteria, *Escherichia coli* Q 13 and *Pseudomonas aeruginosa*, in respect of acidity and content of the acidic proteins.

In an extremely halophilic bacterium, *Halobacterium cutirubrum*, which can grow in a medium containing saturated NaCl, the ribosome was stable in the presence of 4 M KCl (BAYLEY and KUSHNER, 1964), and the cell-free protein-synthesizing system required nearly saturated salts for the maximum activity (BAYLEY and GRIFFITHS, 1968). The majority of ribosomal proteins of *H. cutirubrum* was acidic ones, suggesting the adaptation of the protein-synthesizing system to the high salt environments (BAYLEY, 1966; FALKENBERG *et al.*, 1976; VISENTIN *et al.*, 1972).

In contrast with the extremely halophilic bacteria, moderately halophilic bacteria did not show these unusual properties in the protein-synthesizing system. *Vibrio costicola*, a moderately halophilic bacterium which can grow over a wide range of NaCl concentrations between 0.5 and 3.5 M (FORSYTH and KUSHNER, 1970) showed the maximum activity of the cell-free protein synthesis at as low concentration as about 0.2 M of Na^+ or K^+ (WYDRO *et al.*, 1977). Furthermore, it was reported that the composition of ribosomal proteins of certain moderately halophilic bacterium resembled that of non-halophilic *E. coli* rather than that of *H. cutirubrum* (FALKENBERG *et al.*, 1976).

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On the other hand, the Na^+-requirement for the growth is less in slightly halophilic bacteria than in moderately and extremely halophilic bacteria (LARSEN, 1962).

Therefore, the protein-synthesizing system in slightly halophilic bacteria may have other adaptive mechanism to salts than those of moderately and extremely halophilic bacteria. However, as nonhalophilic *E. coli* K-12 also could grow in a medium containing NaCl (5.3%) as the same as slightly halophiles (NAKAMURA, 1977), the protein-synthesizing systems of slightly halophilic bacteria may be similar to those of nonhalophilic bacteria. Nevertheless, as for the protein-synthesizing system of slightly halophilic bacteria, only the reports with *Vibrio parahaemolyticus* have been available (MORISHITA, 1974, 1980).

Recently, TAKADA *et al.* (1979) isolated a slightly halophilic marine bacterium, *Vibrio* sp. strain ABE-1 (*V.* ABE-1), which can grow in the range of NaCl from 0.2 to 1.2 M, and showed the optimum growth at about 0.5 M NaCl. This paper reports the electrophoretic properties of ribosomal proteins of *V.* ABE-1, and the effect of Na^+ and K^+ on the polysome pattern and the protein-synthesizing activities in the cell-free system and the intact whole cells.

Materials and Methods

Bacteria and growth conditions

Vibrio sp. strain ABE-1 (*V.* ABE-1) (TAKADA *et al.*, 1979) was cultivated at 10°C by vigorous shaking in a medium consisting of 1% each of peptone and meat extract, and 3% NaCl. *Escherichia coli* Q13 and a laboratory strain of *Pseudomonas aeruginosa* grown under a similar condition except for omission of NaCl and at 20°C were used as nonhalophilic bacteria.

Isolation of ribosomal proteins

Unless otherwise stated, all procedures were carried out at 0-4°C. Cells of *V.* ABE-1, *E. coli* Q13, and *P. aeruginosa* in the late log phase were harvested and washed with Buffer I consisting of 20 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol. In the case of *V.* ABE-1, Buffer I containing 0.5 M NaCl was used through the preparation of S-30 fraction. Washed cells were disrupted by grinding with sea sand. The paste was diluted with Buffer I, and centrifuged at 20,000×g for 20 min. The supernatant was recentrifuged at 30,000×g for 30 min, and the supernatant was used as S-30 fraction. The S-30 fraction layered on 0.4 and 1.2 M (w/v) sucrose bilayers in Buffer I was centrifuged

at 50,000 rpm for 4 hr in a Spinco #65 rotor. The ribosomal pellet was suspended in 20 mM Tris-HCl buffer, pH 7.6, containing 1.0 M NH_4Cl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol. After homogenization for 10 to 12 hr by stirring, the homogenate was centrifuged at 12,000×g for 15 min, and the supernatant was centrifuged at 50,000 rpm for 3 hr in a Spinco #65 rotor. The pellet was suspended in 10 mM Tris-HCl buffer, pH 8.0, containing 3 mM disodium succinate and 10 mM $MgCl_2$, and dialyzed overnight against the same buffer, and then ribosomal proteins were extracted with 67% acetic acid as described by HARDY *et al.* (1969). The RNA precipitate was removed by centrifugation at 12,000×g for 10 min, and ribosomal proteins in the supernatant were precipitated by cold acetone (80%). The precipitate was dialyzed overnight against distilled water and lyophilized.

Polyacrylamide gel electrophoresis

100 μg of ribosomal proteins were fractionated by 7.5% polyacrylamide slab gel electrophoresis at pH 4.5 and 8.7 (VISENTIN *et al.*, 1972) at 24 mA/gel for 3 to 4 hr at 4°C. In other experiments, 400 μg of ribosomal proteins were separated by two-dimentional gel electrophoresis developed by KALTSCHMIDT and WITTMANN (1970). The first and second dimension gel electrophoresis were carried out for 7.5 to 9 hr at 90 V, and for 18 to 22 hr at 106 V, respectively, at room temperature. The proteins were stained by 0.2% Coomassie brilliant blue.

Polysome analysis

Polysome pattern was analyzed by the sucrose density gradient centrifugation. After chloramphenicol was added to the culture (250 μg/ml) in an early log phase, the cells were harvested and resuspended in the cold 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, and various concentrations of NaCl or KCl between 0.03 and 0.5 M. Polysomes were prepared and analyzed as described previously (SARUYAMA *et al.*, 1980) with the exception that the sucrose gradient was made up with the 10 mM Tris-HCl buffer, pH 7.4, containing the same components as mentioned above.

Assay of in vitro protein-synthesizing activity

The S-30 fraction prepared as mentioned above was incubated for 30 min at the desired temperature (10°C for *V.* ABE-1, and 24°C for *E. coli* Q-13 and *P. aeruginosa*), dialyzed against Buffer I, and used for the assay. Protein-synthesizing activities of *V.* ABE-1 at 25°C, and *E. coli* Q13 and *P. aeruginosa* at 35°C were measured by polyuridylic acid (poly(U))-directed polyphenylalanine synthesis as reported previously (OSHIMA *et al.*, 1980)

except that the reaction mixture contained various concentration of NaCl or KCl between 0 and 0.5 M. Each of three standard assay systems contained in a volume of 0.2 m*l*: (A) 100 mM Tris-HCl, pH 7.8; 12.5 mM magnesium acetate; energy-generation system (which consisted of 1 mM adenosine triphosphate (ATP), 0.2 mM guanosine triphosphate (GTP), 13.2 mM creatine phosphate, and 50 μg creatine kinase); 50 μg poly(U); 0.8045 nmol ^{14}C-phenylalanine; and 6 mM 2-mercaptoethanol for assay with the S-30 fraction of *V.* ABE-1. (B) 50 mM Tris-HCl, pH 7.8; 10 mM magnesium acetate; the energy-generation system; 50 μg poly(U); 0.8045 nmol ^{14}C-phenylalanine; and 6 mM 2-mercaptoethanol for assay with the S-30 fraction of *E. coli* Q13. (C) 100 mM Tris-HCl, pH 7.8; 25 mM magnesium acetate; the energy-generation system; 50 μg poly(U); 0.8045 nmol ^{14}C-phenylalanine; and 6 mM 2-mercaptoethanol for assay with the S-30 fraction of *P. aeruginosa*. Protein in the S-30 fraction was estimated by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard.

Assay of leucine uptake and protein-synthesizing activity in intact cells

Total uptake of ^{3}H-leucine and incorporation to hot trichloroacetic acid (TCA)-insoluble fraction in whole cells of *V.* ABE-1 were measured at 25°C as previously reported (SARUYAMA *et al.*, 1979) except that the reaction mixture contained various concentrations of NaCl or KCl between 0 and 0.5 M.

Chemicals

Chloramphenicol, poly(U), ATP, GTP, creatine phosphate, and creatine kinase were obtained from Boehringer, Mannheim. L-Phenylalanine (U-^{14}C) (531 mCi/mmol) and L-leucine (4, 5-^{3}H) (68 Ci/mmol) were purchased from the Radio-chemical Centre, England.

Results

The electrophoretic properties of ribosomal proteins

The ribosomal proteins of *V.* ABE-1 were compared with those of *E. coli* Q13 and *P. aeruginosa* by polyacrylamide slab gel electrophoresis at pH 4.5 and 8.7. In pH 4.5 gels, a large number of ribosomal proteins of *V.* ABE-1 moved toward the cathode likewise those of *E. coli* Q13 and *P. aeruginosa* (Fig. 1 a). The profiles of acidic proteins of these three bacteria are shown in pH 8.7 gels (Fig. 1 b). As the most acidic protein of *V.* ABE-1 was more acidic than that of *E. coli* Q13 but not so acidic as that of *P. aeruginosa*, it was to be said that unusually high acidic ribosomal proteins were not present in *V.* ABE-1.

The individual ribosomal proteins were separated by two-dimentional gel

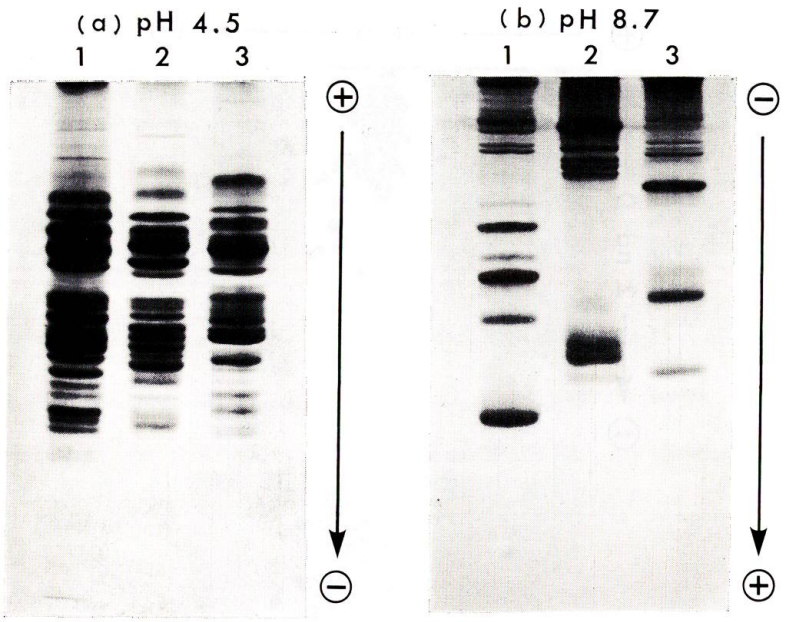

Fig. 1. Ribosomal proteins of *V.* ABE-1 (1), *E. coli* Q13 (2), and *P. aeruginosa* (3) separated by polyacrylamide slab gel electrophoresis at pH 4.5 (a) and 8.7 (b).

electrophoresis. A majority of ribosomal proteins of *V.* ABE-1 were basic ones, and the number of acidic proteins did not very differ from those of *E. coli* Q13 and *P. aeruginosa* (Fig. 2).

Effect of Na^+ and K^+ on polysome integrity

As the ribosomal proteins of *V.* ABE-1 was actually similar to those of *E. coli* Q13 and *P. aeruginosa*, salt-resistance of the ribosomes might resemble those of the nonhalophilic bacteria. Then the effect of the salt on polysome pattern was examined at the concentration between 0.03 and 0.5 M. The pattern of polysomes and the relative amounts of polysomes, monosomes, and subunits are shown in Figs. 3 and 4. Polysome pattern was changed a little by different concentrations of Na^+ and K^+, but more than 60% of ribosomes were present as polysomes at all the concentrations tested.

Effect of Na^+ and K^+ on the cell-free protein-synthesiszing activity

As seen in Fig. 4, polysomes were scarcely degraded into monosomes and subunits even in a presence of high concentration (0.5 M) of NaCl and KCl. However, the activity of protein-synthesizing system of *V.* ABE-1 decreased at above 0.2 M NaCl and 0.06 M KCl, and at 0.5 M NaCl and KCl,

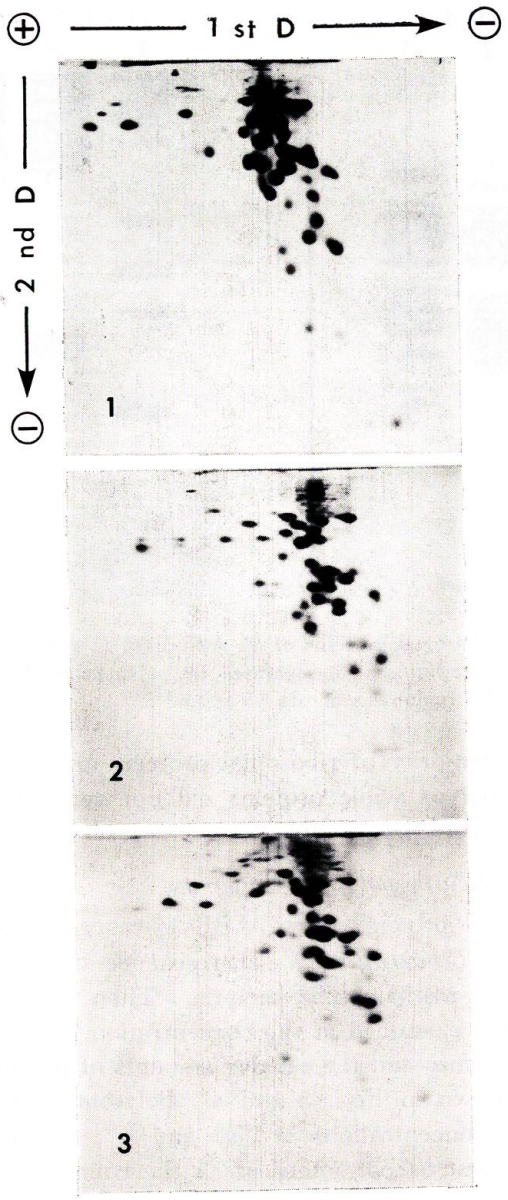

Fig. 2. Two-dimentional gel electrophoresis of ribosomal proteins of *V.* ABE-1 (1), *E. coli* Q13 (2), and *P. aeruginosa* (3).

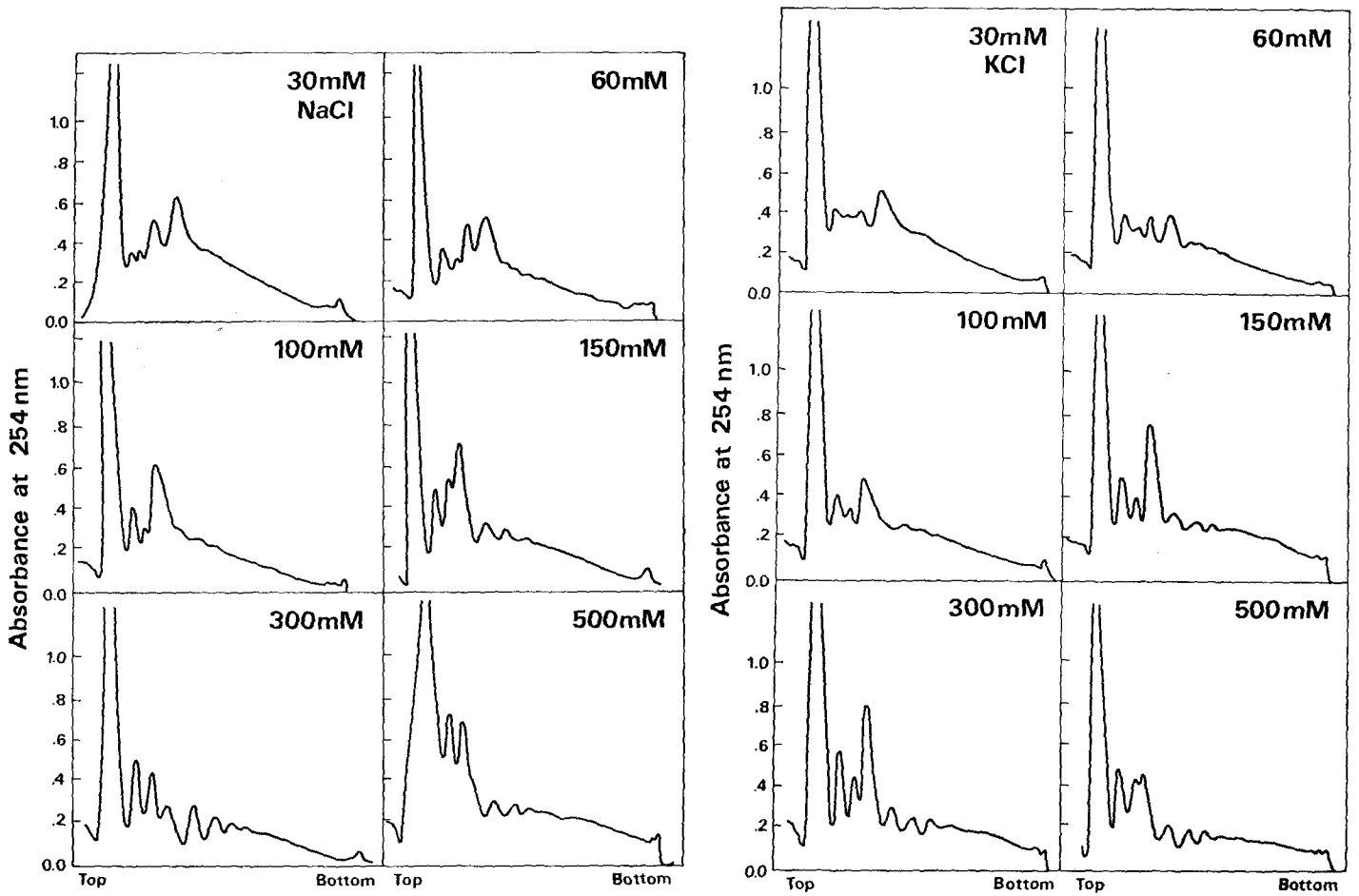

Fig. 3. Profiles of polysomes of *V.* ABE-1 at various concentrations of NaCl (a) and KCl (b).

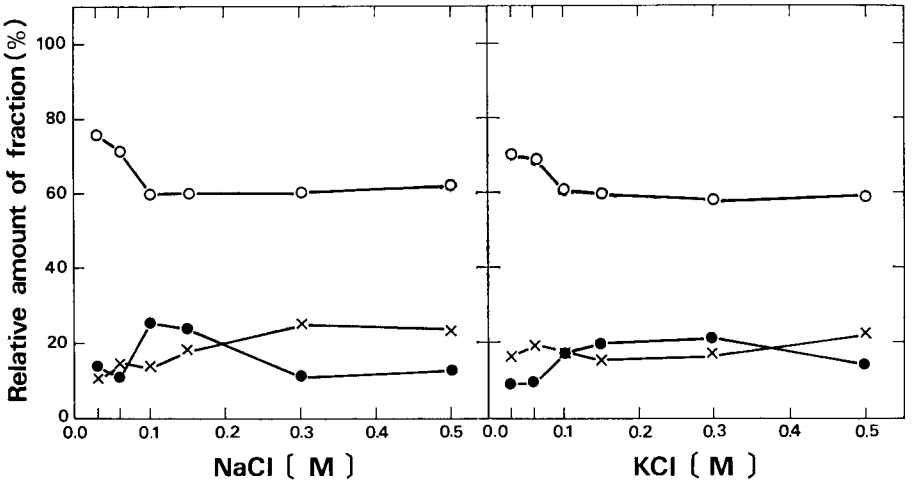

Fig. 4. Relative amounts of ribosomal fractions calculated from Fig. 3. polysomes (○); monosomes (●); and subunits (×).

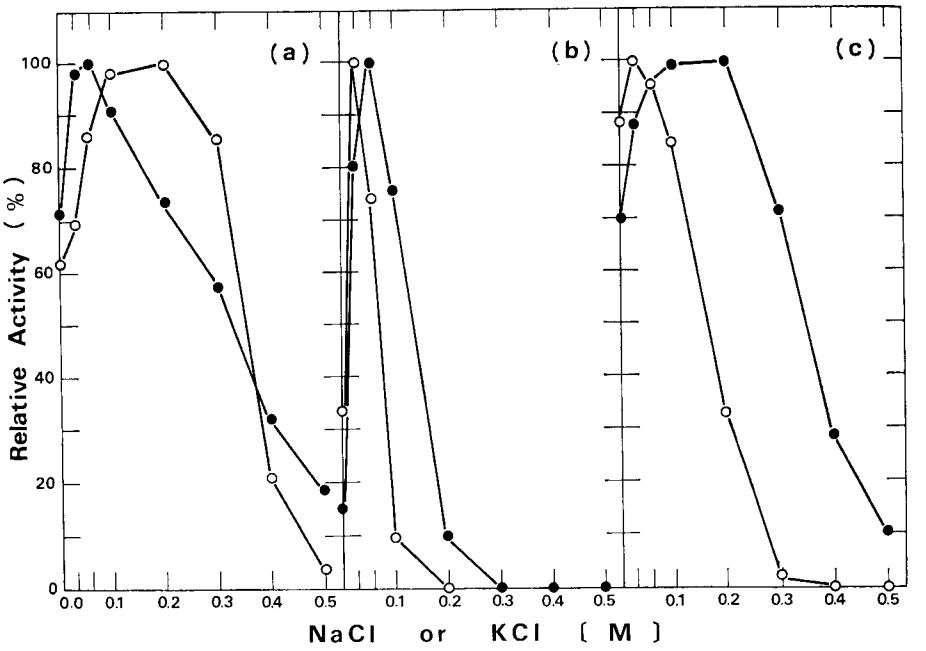

Fig. 5. Effect of Na^+ (○) and K^+ (●) on the cell-free protein-synthesizing activities of *V.* ABE-1 (a), *E. coli* Q 13 (b) and *P. aeruginosa* (c).

about 95 and 80% of the maximum activities, respectively, were inhibited (Fig. 5 a). These inhibitions were also observed in *E. coli* Q13 at more than 0.03 M NaCl and 0.06 M KCl, and in *P. aeruginosa* at more than 0.03 M NaCl and 0.2 M KCl (Figs. 5 b and 5 c). Furthermore, in spite of the requirement of NaCl for the growth of *V.* ABE-1, considerable high activities of the *in vitro* portein synthesis were preserved even when Na^+ and K^+ were omitted from the reaction mixture (Fig. 5 a).

Requirement of Na^+ on the leucine uptake and protein-synthesizing activity in whole cells

As described above, Na^+ was not essential for the protein synthesis in the *in vitro* system of *V.* ABE-1. However, as shown in Fig. 6, L-leucine uptake and incorporation to hot TCA-insoluble fraction in whole cells diminished with the decrease of NaCl concentration, and these activities lost completely at 0.03 M. Such requirement of Na^+ in the whole cells may be related to the active amino acid transport system at least for L-leucine, since 8 mM KCN stopped the uptake of L-leucine completely (data not shown). In the case of K^+, the activity of L-leucine uptake was remarkably low even at

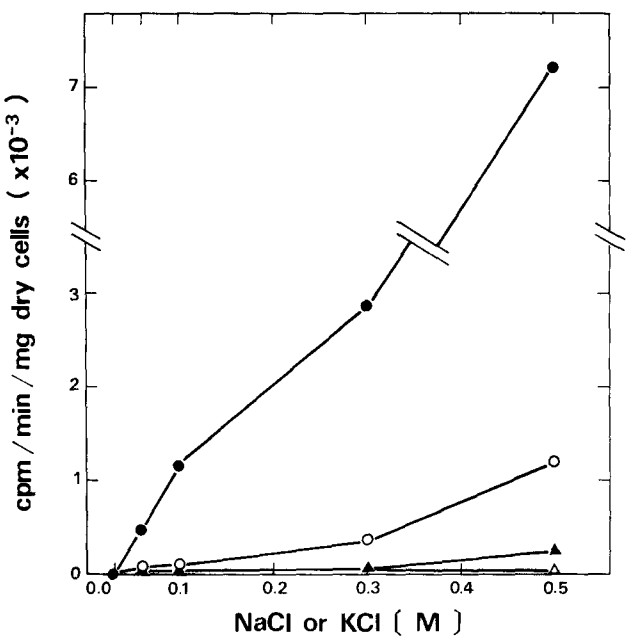

Fig. 6. Effect of Na^+ (●) and K^+ (▲) on the total uptake of leucine, and Na+ (○) and K^+ (△) on the incorporation to hot TCA-insoluble fractions.

high concentration of KCl as 0.5 M, and no protein synthesis was observed at that concentration. These results indicate that Na^+ is probably essential for the active transport system of L-leucine, and Na^+ cannot be replaced with K^+.

Discussion

A large number of highly acidic ribosomal proteins in an extremely halophilic bacterium, *H. cutirubrum*, has been presumed to be one of the strategies for adaptation to the high intracellular concentration of K^+ (BAYLEY 1966, 1976; FALKENBERG *et al.*, 1976; VISENTIN *et al.*, 1972; MATHESON *et al.*, 1978). However, these unusual properties have not been found in moderate halophiles. The content and acidity of acidic ribosomal proteins of an unidentified moderate halophile, NRCC 11227, were rather similar to those of *E. coli* (FALKENBERG *et al.*, 1976; MATHESON *et al.*, 1978). The homology of amino acid sequence was reported in the most acidic ribosomal proteins of moderate halophile, *V. costicola* and NRCC 11227, and nonhalophile, *E. coli* (MATHESON *et al.*, 1978). This time, we found that in a slightly halophilic marine bacterium, *V. ABE*-1, the number of acidic ribosomal proteins was similar to those of *E. coli* Q13 and *P. aeruginosa*, and that there was no definite difference in acidity of the robosomal proteins between *V.* ABE-1 and the nonhalophilic bacteria (Figs. 1 and 2). Therefore, the extensive modification of the ribosomal proteins is not the case in a slightly halophile, *V.* ABE-1.

The inhibition of the *in vitro* protein-synthesizing activities by the high concentration of the salts was observed in all bacteria tested (Fig. 5). In the case of *V.* ABE-1, it seemed unlikely that the inhibition was arised by the degradation of polysomes into monosomes and subunits, since more than 60% of the ribosomes existed as polysomes in the range of concentration of salt examined (Fig. 3 and 4). In this connection, it was reported in *E. coli* that polysomes were not degraded even when the concentration of KCl was increased to 0.6 M (PHILLIPS *et al.*, 1969). However, it was also reported in *E. coli* that both the sedimentation behavior of the ribosome and the content of ribosomal proteins were changed by the salt (0.5 M or higher) (SPITNIK-ELSON and ATSMON, 1969). Accordingly, it may possibly be presumed that the change of the structure of ribosome by the salt is one of the causes of the inhibition in *V. ABE*-1.

Another possibility arose from the salt-inhibition of the *in vitro* protein-synthesizing system of *V.* ABE-1 (Fig. 5 a) is that only a small part of intracellular salts was free form as was reported by WYDRO *et al.* (1977)

with *V. costicola*. MASUI and WADA (1973) actually found that a large amount of Na^+ was bound to the membrane in a moderately halophilic pseudomonad. TAKADA *et al.* (1981) found that the activity of cytoplasmic NADH dehydrogenase of *V.* ABE-1 was inhibited by NaCl and KCl, but that of membrane bound enzyme was not. These observations support the presumption proposed by NAKAMURA (1979) that the concentration of salts in the cytoplasm of halophilic bacteria may be low, consequentially salt-sensitive enzymes and enzyme systems could be functioning in the cells even under high salt environment.

In spite of Na^+ requirement of the *in vivo* protein-synthesizing activity of *V.* ABE-1 (Fig. 6), no Na^+ nor K^+ was necessary for the *in vitro* protein-synthesizing activity (Fig. 5 a). Moreover, we confirmed that phenylalanyl-tRNA synthetase from *V.* ABE-1 functioned at low concentration of KCl as 20 mM (OSHIMA *et al.*, 1980). On the other hand, as the active transport system for amino acid was extremely dependent on Na^+ (Fig. 6), depression of the amino acid-transport at lower concentration of Na^+ seems to limit the *in vivo* protein synthesis. In addition, we found that at least 0.1 M of NaCl was necessary to prevent the damage of the membrane, since some proteins and RNAs leaked out below such the concentration, and K^+ could not prevent the leakage (data not shown). MORISHITA (1980) suggested the correlation between the alteration of the membrane structure and the degradation of rRNA in *V. parahaemolyticus*. Thus, we presumed that such the damage of the membrane structure at low NaCl concentration must be another cause of the decrease of the *in vivo* protein synthesis of *V.* ABE-1.

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