Ribosomes of Moderately Halophilic Bacteria

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CURRICULUM STUDIORUM

Robert M. Wydro was born March 5, 1949, in Washington, D.C. He received the Bachelor of Arts degree in Biology from Lycoming College, Williamsport, Pennsylvania, in 1971. He received the Master of Science degree in Biology from Indiana University of Pennsylvania, Indiana, Pennsylvania, in 1974. The title of his thesis was Studies on a Moderately Halophilic Isocitrate Dehydrogenase.

Abstract

The ribosomes of Vibrio costicola were stable over a wide range of salt concentrations. Sucrose gradient profiles of ribosomes from cells grown in different salt concentrations and isolated in various salt concentrations were similar. The sedimentation coefficients of the ribosomal particles were 64, 48, and 28 S for the monosome, large subunit, and small subunit, respectively. These values were not altered by the NaCl concentration of the growth medium nor by the salt concentration of the isolation buffer. There was, however, slight evidence that higher salt concentrations were removing some of the ribosomal proteins.

The total ribosomal proteins were found to be more acidic (B/A = 1.08) than the total ribosomal proteins of E. coli (B/A = 1.58) but less acidic than the total ribosomal proteins of H. cutirubrum (B/A = 0.69). The relative acidity of the total ribosomal proteins was not changed by the NaCl concentration of the growth medium. Two ribosomal proteins equivalent to the E. coli S1 and L7/12 were purified and the partial amino acid sequence of the latter was determined. The V. costicola protein was 79% homologous with the E. coli protein and 74% homologous with an equivalent protein from the unclassified moderate halophile, H.x. The average hydrophobicity of the V. costicola total and purified ribosomal proteins were not significantly different than those of E. coli.

An in vitro protein synthesizing system was developed for V. costicola. The in vitro system was energy dependent and sensitive to inhibitors of protein synthesis. The poly-

uridylic acid dependent system incorporated phenylalanine into oligophenylalanines. The in vitro system directed by poly-U had a salt optimum of 0.15 M with greater activity in NH_4Cl than KCl which had greater activity than NaCl. The magnesium optimum was approximately 18 mM. The in vitro system directed by "endogenous" messenger had similar salt response as the poly-U system except that NaCl was inhibitory at all concentrations. Increasing the salt concentration above the optimum increased the misreading of the poly-U message. The in vitro protein synthesizing system was stable for at least 8 hours in 1.0 M NaCl, KCl, or NH_4Cl.

The ribosomes and protein synthesizing system demonstrated considerable salt stability. The low salt optimum of the protein synthesis system and previously studied V. costicola enzymes and the effects of salts on misreading of poly-U suggest that the distribution of ions measured as intracellular may not be uniform throughout the cell. The envelope of the cell may bind large amounts of these ions leaving the cytoplasm with a relatively low "free" ion concentration.

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List of Abbreviations

ATA - aurin tricarboxylic acid

ATP - adenosine-5'-triphosphate

B/A - basic/acidic amino acid ratio

C - degrees centigrade

DEAE - diethylaminoethyl

GTP - guanosine-5'-triphosphate

H - average hydrophobicity

η - viscosity

PAGE - polyacrylamide gel electrophoresis

phe - phenylalanine

poly-U - polyuridylic acid

ρ - density

RNA - ribonucleic acid

rRNA - ribosomal ribonucleic acid

SDS - sodium dodecyl sulfate

$S_{20,w}$ - standard sedimentation coefficient in Svedberg units

Tris - tris(hydroxymethyl)aminoethane

$\bar{v}$ - partial specific volume

Chapter I

Adaptation to an Ionic Environment

Extreme halophiles

In all living organisms, water serves as the solvent for the molecular interactions necessary in cellular processes. The solutes dissolved in water control all the metabolic processes in the cell either directly or by affecting the structure of water. Small changes in solute concentration or in water activity can result in disruption of the normal functioning of the cell's enzyme activity and regulation and of the structure and functioning of cellular structures and organelles. This is of particular importance to unicellular organisms, such as bacteria, which are in direct contact with the environment. Each microbial cell must come to terms with the external milieu. Bacteria growing in very dilute environments have to devise ways of concentrating solutes, while bacteria growing in saturated brines have to deal with high solute concentrations. The problems of ionic adaptation stem from two sources. On one hand, there is the basic osmotic problem concerning the overall ionic concentration of the cell and its environment. Then, there is the problem of qualitative differences in the ionic composition of the cell and the external environment.

In nature, there are two basic strategies of ionic adaptation. The organism may regulate the ions qualitatively but not quantitatively. Thus the osmolarity of the intracellular fluid would remain equal to that of the surrounding water, but

the ionic composition would differ from that of the environment. In the second strategy, the organism maintains both the osmotic content and the qualitative ionic composition of the fluids at low concentrations and within narrow limits. These limits are relatively independent of the external milieu.

Originally, the halophilic (GR. salt-loving) bacteria were thought to use the last mentioned strategy of adaptation (Robinson et al, 1952). However, studies by Christian and Ingram (1959) revealed that the intracellular water activity of these organisms, as well as those of non-halophilic[1] bacteria, were not greater than the surrounding growth medium. Christian and Waltho (1962) went a step further and measured the intracellular ion concentrations of Halobacterium salinarium, grown to stationary phase of growth. They found that, when grown in 4.0 M NaCl, this extreme halophile[2] had intracellular concentrations of: 1.37 M Na^+, 4.57 M K^+, and 3.61 M Cl^-. Ginzberg et al (1970) found that internal ion concentrations were dependent on the age of the culture. Working with a Halobacterium sp isolated from the Dead Sea, these authors showed that intracellular ion concentrations of logarithmically growing cells were: 5.5 M K^+, 1.0-2.3 M Na^+, and 2.3-3.7 M Cl^-.

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[1] Non-halophiles are those bacteria that grow best in 0.2 M salt or less.

[2] Extreme halophiles grow best in 3.0-5.2 M NaCl.

The exceptionally high concentration of K^+ ions, even in cells that are not biologically active, indicates that there may be internal binding sites for this ion. Ginzberg et al (1971) tried to measure the proportion of bound K^+ ions by the use of an ion-specific electrode. Although they could not detect any bound K^+, they did show, however, that K^+ could be released from the cells by treatment with anions such as trinitrocresolate. This they interpreted as being due to a modification of K^+ binding loci, thus indicating that ions could be bound to cellular components and not retained by active transport. The ability of extreme halophiles to concentrate K^+ions by some other means besides active transport was also studied by Lanyi and Hilliker (1976), who found that cell envelope vesicles of H. halobium loaded with 3 M KCl and suspended in 3 M NaCl, lost very little K^+. They concluded that the extremely halophilic membrane is relatively impermeable to K^+ and thus can retain large amounts without expenditure of energy.

Research into the physiology and biochemistry of the extreme halophiles has revealed that their basic life processes are similar to those of non-halophilic bacteria except for a salt requirement (Flannery, 1956; Brown, 1964, 1976; Larsen, 1967; Kushner, 1968, 1971; Lanyi, 1974). This salt requirement means, however, that the cellular components have differences in structure and ionic interactions which enable them to function in a salt milieu which would denature analogous structures from non-halophiles. The aspartate transcarbamylase (ATCase) from H. cutirubrum, for example, requires approximately 4.0 M NaCl or KCl for maximum

activity (Liebl et al, 1969), but the analogous enzyme from the non-halophile, E. coli, is inhibited by low concentrations of NaCl and KCl (Bethell and Jones, 1969). The H. cutirubrum enzyme also requires high salt concentrations for stability. When placed in 1.0 M NaCl, the enzyme is readily inactivated (half-life of 15 sec), but in 3.5 M NaCl it remains active for hours (half-life of 6.5 hours) (Norberg et al, 1973). This is not to say, however, that all extremely halophilic enzymes respond to salt in the same manner. The NADP-specific isocitrate dehydrogenase shows maximum activity in only 0.5-1.5 M NaCl and is inhibited by higher salt concentrations (Hubbard and Miller, 1969). There are also exceptions to the generalization that all extremely halophilic enzymes require salt for activity. The fatty acid synthetase studied by Pugh et al (1971) requires no salt for maximum activity and is inhibited by higher salt concentrations. Enzymes associated with the cell membrane of extreme halophiles all seem to have salt optima around 4.0 M NaCl or KCl. Soluble enzymes, like those involved in intermediary metabolism (ie. isocitrate dehydrogenase), have a wider range of salt optima, but many have optima around 1.0 M NaCl or KCl (Brown, 1976).

Perhaps a better illustration of the extent of biochemical adaptation to high ionic concentrations is the protein synthesis system. Such a system is composed of an organelle, the ribosome, and a multitude of enzymes, co-factors, and RNA molecules. The effects of salts on the proteins is important as well as the effects on protein-protein, protein-RNA, and RNA-RNA interactions.

The basic structure and functioning of the halophilic protein synthesis system closely resembles that of the non-halo-

philes (Bayley, 1976). The complete ribosome is a 70 S particle consisting of a 50 S subunit and a 30 S subunit (Bayley and Kushner, 1964). The 50 S subunit contains a 23 S and a 5S RNA molecules and the 30 S subunit contains a 16 S molecule (Visentin et al, 1972). The ribosome is composed of 60% RNA and 40% protein, and has a Mg^{2+}/phosphorous molar ratio of 0.3. The number of proteins in each subunit is also the same as the non-halophile, E. coli, with 34 proteins in the 50 S subunit and 21 proteins in the 30 S subunit (Strøm and Visentin, 1973).

The difference between halophilic and non-halophilic ribosomes was first shown by Bayley and Kushner's (1964) demonstration that the halophilic ribosomes required high (3-4 M) KCl concentrations to remain in the associated (monomer) form. In 2.0 M NaCl or KCl, the ribosomes of non-halophiles lose not only their monomer association but also their structure (Atsmon et al, 1969). The ribosomes of the extreme halophiles lose their structure when placed under conditions which favor the stability of non-halophilic ribosomes (Bayley and Kushner, 1964; Bayley, 1966a, 1966b; Visentin et al, 1972). Bayley and Griffiths (1968a) showed that the entire system necessary for in vitro protein synthesis required 3.8 M KCl, 1.0 M NaCl, 0.4 M NH_4Cl, and 0.04 M $MgCl_2$ for optimum incorporation of amino acids. A similar system from the non-halophile, E. coli, has an optimum salt concentration of 0.16 M KCl or NH_4Cl and 7-15 mM $MgCl_2$ (Conway, 1964). The amino acyl synthetases of the halophile require 3.8 M KCl for activity (Griffiths and Bayley, 1969).

Since the difference between the halophilic and non-halophilic protein synthesizing system is not at the gross structural level, nor is it in the mechanism in which the protein synthesis occurs (Bayley, 1976), the difference probably occurs in the protein and RNA components of the system. The ribosomal RNA (rRNA) of the halophilic ribosomes showed no unusual properties. The C+G content is only slightly higher than that of the non-halophile, E. coli (Visentin et al, 1972). However, distinct differences in the ribosomal proteins of halophiles and non-halophiles were shown. Bayley and Kushner (1964) observed that the ribosomal proteins released from the ribosomes suspended in low K^+ concentrations were acidic. These proteins could also be reassociated on the ribosomes by restoring the KCl concentration (Bayley, 1966b). Bayley (1966a) investigated this further and found that most of the ribosomal proteins were acidic, having isoelectric points around 3.9. The base/acid amino acid ratio of total ribosomal proteins was 0.49, a value far below the 0.99 of E. coli ribosomal proteins (Bayley, 1966a). Visentin et al (1972) selectively removed the ribosomal proteins from H. cutirubrum ribosomes with different salt washings and found that the more basic proteins were more difficult to remove. The ribosomal proteins first removed by low K^+ presumably contain the "external" proteins, those on the surface, which encounter the high ionic environment. This protein fraction is also the most acidic.

Strøm and Visentin (1973) developed a procedure for the simultaneous separation and identification of the halophilic ribo-

somal proteins. Using this procedure, Strøm et al (1975) studied effects of ions on protein-RNA interactions of the 50 S subunit. They postulated that the ribosomal proteins of the extreme halophiles are significantly different from those of non-halophiles, but retain certain conserved portions that may be important for their functions in protein synthesis and/or in the interaction with the rRNA. Studies on the sequence homologies between purified H. cutirubrum ribosomal proteins and equivalent ribosomal proteins from other species of bacteria have been performed (Chow et al, 1972; Yaguchi et al, 1973; Visentin et al, 1974; Oda et al, 1974; Duggleby et al, 1975; Matheson et al, 1975).

The problems caused by high ionic concentrations may be due to the effects of the ions on proteins, or on the water structure. The former effects may involve what Lanyi (1974) terms "long-range electrostatic interactions" and "residue interactions". Long-range electrostatic effects arise from the difference in electrostatic free-energy between the folded and the unfolded states of a protein. The distribution and charges of groups in a protein molecule will interact so as to help the protein fold into a particular conformation of a lower electrostatic free-energy. Increasing the ionic concentration surrounding the protein would start to cancel out these charged groups, preventing their interaction within the protein molecule. As the protein starts to unfold, these ions can penetrate into the protein interior and thus increase the possibility of charge screening. This makes the unfolded state of the protein more thermodynamically stable than the folded state in high ionic concentrations.

Interactions between specific residues can affect the overall structure of the protein molecule. These interactions include mainly: disulfide bridges, hydrogen bonding, ionic bonds, and hydrophobic interactions. The decrease in free energy on the folding on the protein molecule contributes to the stability of the structure. Ions in the proper concentration can aid this effect by forming complexes with the protein molecule. As the ion concentration increases, however, it can prevent or even disrupt some of these interactions.

The effect of ions on water structure is at least as important as the effect on proteins. Any ion dissolved in water has the tendency to attract the water molecules, thereby reducing their freedom. The specific effect of the ion on water structure is dependent on the properties of the particular ion. Some ions, from "salting-out" type salts (HPO_4^{-2}, SO_4^{-2}), encourage the formation of water aggregates, thereby increasing the water structure Other ions, from "salting-in" type salts (ClO_4^-, SCN^-, NO_3^-), interrupt the formation of water aggregates. This can affect hydrophobic interactions that help maintain conformation within proteins. Hydrophobic side chains increase the free energy when suspended in water by increasing the water structure. In a protein molecule, these apolar groups have the tendency to sequester themselves towards the interior so that their solvation is decreased, thus resulting in a decreased free energy. Salting-in salts tend to disaggregate water so that apolar groups can mix more easily with it; this makes hydrophobic interactions within the protei less favorable. Conversely, the tendency of salting-out salts to

increase the water aggregates favor these hydrophobic interactions. These kinds of ionic and water structuring effects must be considered in understanding the biochemical adaptation of the halophiles to the high ionic content of the internal milieu.

The acidic groups on these halophilic proteins are supposedly canceled by the monvalent cations by charge screening. It is postulated that high salt concentrations are required to cancel these acidic groups so that the protein can fold into its active conformation (Brown, 1964). Lanyi (1974) reports, however, that the effects of salts on charge shielding of acidic residues is complete at approximately 0.2 M. The requirement for additional salt concentrations are effects due to the structuring of water.

As previously discussed, the main effect that the water structure has on the protein molecule is the effect on the hydrophobic interactions. Lanyi (1974) showed, using the method of Bigelow (1967), that the average hydrophobicity of the extremely halophilic proteins was significantly lower (less than 1.0 kcal/residue) than that of non-halophiles (greater than 1.0 kcal/residue). Lanyi then theorized that because the halophilic proteins are less hydrophobic they require a high ionic strength environment of "salting-out" salts such as chlorides, to increase the water structure and make the fewer hydrophobic interactions more stable. Lanyi (1974) also points out that several enzymes from the extreme halophiles, the cytochrome oxidase, the menadione reductase, and the threonine deaminase require high concentration of salting-out salts for stability.

Involvement of hydrophobic interactions are also implicated by the cold lability of halophilic enzymes (Norberg et al, 1973;

Lanyi, 1974) and the characteristic of halophilic proteins to be thermostable (Keradjoupolos and Wulff, 1974) in high salt concentrations. Lowering the temperature of water to around 4 C increases the size of the water "clusters", decreasing the water activity. Hydrophobic interactions within the protein become less favorable and in the case of halophilic proteins become disrupted, resulting in protein denaturation. Hydrophobic interactions may not be strong enough to withstand thermal energy (of water or the protein molecule) and may be broken at high temperatures (Hochachka and Somero, 1973). Keradjopoulos and Wulff (1974) found that a number of enzymes (alanine deaminase, ethanol dehydrogenase, alkaline phosphatase, NADH-oxidase, malate dehydrogenase, isocitrate dehydrogenase, and glutamate dehydrogenase) of H. cutirubrum were thermostable. It might be possible that the high salt concentrations required by halophilic proteins are so efficient in stabilizing the hydrophobic interactions that the proteins are also thermostable. Many proteins are more thermostable in high solute concentrations, possibly because of the decrease in water activity (Mahler and Cordes, 1966).

Thus, the biochemical adaptation of the extreme halophiles may occur in at least two ways. The increased number of acidic residues in the proteins are stabilized by a charge screening mechanism at low salt concentations and the decreased number of hydrophobic residues are stabilized by the increased water structure at the higher salt concentrations. The importance of each biochemical adaptation and whether these are the only adaptations remain uncertain.

Moderately halophilic bacteria

The extreme halophiles have attracted the attention of researchers by virtue of their very high salt requirement. A survey by Forsyth et al (1971) showed, however, that most of the micro-organisms isolated in a coastal region that can grow in low salt concentations can also grow at high salt concentrations. This implies that most marine bacteria have the capability of growing in salt concentrations much higher than those in their natural oceanic environment. These micro-organisms exhibit the ability to grow over a range of salt concentrations and are, thus, considered moderate halophiles. More precisely defined, the moderate halophiles are those bacteria that grow best in 0.5-2.5 M NaCl. The ability to grow over a range of salt concentrations poses some questions about the mechanism of adaptation. The extreme halophiles do not exclude salts but have adopted biochemical characteristics which permit functioning only under such high ionic conditions. Do the moderately halophilic bacteria also adapt in the same manner when grown in high salt concentrations? If the same bacteria were then grown in low salt concentrations, would they then adapt to the lower salt?

Vibrio costicola is a moderate halophile having the ability to grow over a range of 0.4-3.5 M NaCl (Shindler, 1976). It was originally isolated from pork ribs marinated in salt brine (Smith, 1938). Smith named the organism Vibrio costicolus because of its comma shape and the Latin words: "costa" meaning rib and "cola" meaning dweller. Although Smith's original strain appears to be lost, a similar organism was isolated by Robinson (1950) from bacon curing brines in Canada. This culture was

submitted to the culture collection of the National Research Council of Canada and supposedly is the strain used for this thesis. There is some question as to the origin of the strain because of discrepancies between the biochemical characteristics of the National Research Council strain (NRC 37001) and those of the strain originally isolated by Smith (1938) and Robinson (1950). Shindler (1976) discusses the differences of these strains in some detail, but concludes that they are probably the same organism. The name was changed from V. costicolus to V. costicola in the eighth edition of Bergey's Manual (Buchanan and Gibbons, 1974) to conform with the rules of etymology.

The growth of V. costicola was first described by Smith (1938), who found the optimum concentration for growth was 0.7-1.0 M NaCl, but growth could occur in as low as 0.3 M and as high as 3.9 M NaCl. Flannery et al (1952) found that Na^+ resulted in a better growth response than the other cations tested and Cl^- as the anion was not essential. With small amounts of NaCl present (0.2 M), other salts could support growth as well as comparable amounts of NaCl. Forsyth and Kushner (1970) eliminated the possibility that different salt concentrations selected different populations of V. costicola by showing that the cells able to grow at one salt concentration were also able to grow at all other salt concentrations. They developed a glucose-mineral salts medium for V. costicola, which did not necessitate the addition of amino acids as previously reported (Flannery and Kennedy, 1952), and were able to show that the range of salt concentrations for growth was dependent on the complexity of the growth medium used. When grown in the glucose

minimal medium, V. costicola could grow from 0.4-2.5 M NaCl, but when grown in a complex medium, the range was extended from 0.2-3.5 M NaCl (Forsyth and Kushner, 1970; Shindler, 1976).

Robinson et al (1952) studied the lactate dehydrogenase and nitratase activity of a similar moderate halophile, Micrococcus halodenitrificans (now called Paracoccus halodenitrificans), and concluded, from the fact that enzymes were inhibited by salt concentrations greater than 0.15 M, that the intracellular ion concentrations were significantly lower than the ion concentration of the medium. They stated that the mechanism of adaptation of moderate halophiles was to exclude the salts from the cells at a tremendous energy expense. Christian and Ingram (1959a) disproved this idea by measuring the freezing points of cell pastes of both V. costicola and M. halodenitrificans and showed that the water activity inside the cells was lower than or equal to that of the medium in which the cells were grown. They also showed (Christian and Ingram, 1959b) that V. costicola would lyse when suspended in salt concentrations approximately 1/3 that of the concentration in which the cells were grown. Cells in the stationary phase of growth were not as susceptible to lysis as cells in the logarithmic phase of growth. From a series of lysis experiments in different salts and salt concentrations these authors concluded that small ions such as K^+ penetrate the plasma membrane rapidly when the cell is subjected to ionic stress, suggesting that this ion may be of major importance in osmotic regulation.

Christian and Waltho (1962) measured the intracellular ion concentrations from stationary cells of V. costicola grown in 1.0 M NaCl. The values obtained were 0.68 M Na^+, 0.22 M K^+, and 0.14 M Cl^-. A more detailed study of the intracellular ions was performed by Shindler and Wydro (Shindler, 1976; Shindler et al, 1977, see Appendix) in which the effects of growth in different salt concentrations, metabolic state of the cells, and complexity of the growth medium were tested. The intracellular ion concentrations of logarithmically growing, metabolically active V. costicola grown in 1.0 M NaCl were: 0.39 M Na^+, 1.02 M K^+, and approximately 0.04 M Mg^{2+}. The values were affected by the salt concentration of the growth medium, being slightly lower when the cells were grown in 0.5 M NaCl and higher when grown in 3.0 M NaCl (Tables I-1 and I-2). The K^+ level in the cells is high, anywhere from 60-100 times the concentration found in the growth medium, supporting Christian and Ingram's theory that K^+ is the osmotically active ion. However, contrary to their theory, the K^+ level remains constant as compared to the changes in Na^+ levels when the cells are grown in different salt concentrations. It should be pointed out, however, that it is the state of the ions in the cell (ie. whether free or bound) that is of major importance in determining osmotic activity.

The strategy of adaptation to the high salt environment of the moderate halophiles is not as straightforward as in the extreme halophiles. The intracellular ion concentration, when an organism like V. costicola is grown at its optimum salt concentration, is at least as high as that of the environment.

Table I-1

Cell-associated ions of V. costicola grown in glucose minimal media at various NaCl concentrations.[1]

Medium NaCl (M)	Molal Na^+	Molal K^+	Molal K^+/Na^+	Molal $Na^+ + K^+$	Cellular $Na^+ + K^+$ / Medium $Na^+ + K^+$
0.6	0.77	0.51	0.66	1.27	1.69
	0.76	0.52	0.69	1.28	1.71
1.0	0.90	0.62	0.68	1.52	1.32
	0.99	0.69	0.69	1.68	1.46
1.6	1.68	0.50	0.30	2.18	1.25
	1.74	0.53	0.30	2.27	1.30

[1] Data from Shindler (1976) and Shindler et al (1977)

Table I-2

Cell-associated ions of two moderate halophiles in complex medium at various NaCl concentrations.

Bacterium and medium NaCl	Molal Na^+	Molal K^+	Molal Mg^{2+}	Molal $Na^+ + K^+$
V. costicola[1]				
0.5 M NaCl	0.65	0.39	0.046	1.04
1.0 M NaCl	0.39	1.02	0.021	1.49
3.0 M NaCl	1.78	0.37	0.028	2.15
H.x.[2]				
0.63 M NaCl	0.05	0.34	0.16	0.39
4.35 M NaCl	0.62	0.58	0.18	1.20

[1] Data from Shindler (1976) and Shindler *et al* (1977).

[2] Data from Matheson *et al* (1976).

When V. costicola is grown in salt concentrations approaching those required by the extreme halophiles, the total intracellular ions are significantly lower than the external medium. The same is true for the unclassified moderate halophile (Table I-2) studied by Matheson et al (1976).

The cell envelopes of moderate halophiles are not as unusual as those of the extreme halophiles which contain unique glycerol di-ether isopropenoid phopholipids (Kates et al, 1966). The cell walls of extreme halophiles are characterized by small amounts of sugars, hexosamines, and nucleic acids, and by the absence of muramic and diaminopimelic acid (Kushner, 1968). Smithies et al (1955) studied the chemical composition of cell walls from two moderate halophiles and found that like those of the extreme halophiles they contained little carbohydrate. Forsyth (1971), however, found that V. costicola did contain muramic acid. Kates et al (1966) in studying the lipid composition of a variety of halophilic and non-halophilic bacteria found that the moderate halophiles lacked any glycerol diether derived lipids and had fatty acid ester-derived components, mainly phosphatidylglycerol, which are found in non-halophiles. One unusual feature of moderate halophiles is that several contain a DNA slime layer (Smithies et al, 1955; Smithies and Gibbons, 1955). Perhaps this slime layer plays some role in the moderately halophilic mode of existence. Changes in this slime layer have been noted when the organisms are grown in different salt concentrations (Smithies and Gibbons, 1955).

Baxter and Gibbons (1954) studied the glycerol dehydrogenase (GDH) of V. costicola, grown at 1.0 M NaCl, and found that the salt optimum for activity was 0.5 M with KCl giving twice the activity of NaCl. The salt optimum was not changed when the cells were grown in 0.7 or 2.6 M NaCl. Antisera produced against the GDH from V. costicola and E. coli inhibited the respective enzymes but the enzymes failed to react with the heterologous antisera indicating that the two enzymes have no antigenic determinant sites in common.

The NADP-specific isocitrate dehydrogenase (IDH) (Wydro, 1974) and the pyruvate kinase (PK) (de Medicis and Rossignol, in press, Can. J. Biochem.) of V. costicola both have optimum salt concentrations around 0.1 M. NaCl or KCl. These two enzymes are the only moderately halophilic enzymes which have been purified to any appreciable degree. The IDH was purified approximately 56 fold, while the PK was purified to homogeneity. Salt (NaCl and KCl) had similar effects on the kinetics of these two enzymes. Concentrations above 0.1 M affected the apparent K_m but not the V_{max}, an effect typical of competitive inhibitors. The enzymes could be stabilized for days at room temperature in solutions of reduced water activity. The IDH was stable in 0.5 M NaCl, while the PK could be stabilized by 25% glycerol. Both enzymes were cold sensitive, losing activity if placed at 4 C. This could be overcome, however, by the addition of higher salt concentrations (1.0 M NaCl or KCl). This cold lability suggests the interaction of hydrophobic groups which may be contributing to the stability of the enzymes.

Shindler (1976) partially purified (1.5-3.0 fold) the threonine deaminase (TDase) from V. costicola. This enzyme showed a salt response curve similar to that observed in the TDase of E. coli in which the maximum activity occured in the absence of salt (although salts were required for stability). Both these enzymes were inhibited as the NaCl concentration of the reaction mixture was increased. The TDase from V. costicola leveled off at about 30% maximum activity at 1.0 M NaCl and remained at that activity up to 3.0 M NaCl. The TDase from E. coli continued to be inhibited resulting in only 10% of maximum activity at 2.0 M NaCl. Shindler studied the effects of salt on the kinetics of the TDase from V. costicola and found, as with the IDH and PK, that NaCl concentrations did not affect the V_{max} of the reaction, but increased the apparent K_m. Feedback inhibition of the V. costicola TDase by isoleucine was high and remained high over the whole range of salt concentrations contrary to the E. coli TDase in which isoleucine inhibition was reduced by higher salts. The V. costicola enzyme, then, can function fairly well over a range of salt concentrations with regards to activity and regulation. The salt response of the enzyme was the same regardless of what concentration of NaCl the cells were grown.

Like the enzymes from the extreme halophiles, each enzyme of the moderate halophile, V. costicola, has its own requirements for salts and there is no set pattern as to the maximum salt required for optimum activity. Shindler (1976) studied the aspartate transcarbamylase (ATCase) in crude extracts of V. costicola and found that both activity and stability were optimum in about 2.0 M NaCl. Studies on the regulation of this enzyme were not possible

since, unlike the ATCases from other bacteria (including the extreme halophiles), the enzymes was not susceptible to feedback inhibition by UTP and CTP. This enzyme does illustrate that enzymes in this moderate halophile do not all have salt optima below that which is measured as intracellular.

The salt concentrations which support optimum activity are not necessarily those salt concentrations at which the enzyme is operating *in situ*. More information can be gained from the effects of salts on the enzyme-substrate and enzyme-modulator interactions (Hochachka and Somero, 1973) which will be reflected in the metabolism of the cell. More detailed studies of this kind would give a greater insight into the moderately halophilic mechanism of adaptation. It was thought that a better understanding of the phenomenon of moderately halophilic adaptation would be gained by studying the ribosomes and protein synthesizing system of *V. costicola*.

Goals of thesis

The goal of this thesis is to present work on the ribosomes and protein synthesizing system of the moderately halophilic bacterium, *Vibrio costicola*.

Chapter II deals with the effects of salts on the physical properties of the ribosomes as determined by analytical ultracentrifugation and sucrose gradient analysis. The amino acid composition of the total ribosomal proteins is described in Chapter III along with studies of two purified ribosomal proteins. Chapter IV describes the development and study of the *in vitro* protein synthesizing system of *V. costicola* with special attention

to the effects of salts on the amino acid incorporation. A general discussion of the results of all the studies is provided in Chapter V. Indications of a possible mechanism of adaptation to a high ionic environment are also discussed. It is hoped that the present study will contribute to a better understanding of the moderately halophilic mechanism of adaptation and be helpful to other investigators studying biochemical adaptation.

CHAPTER II

Physical Properties of Vibrio costicola Ribosomes

Introduction

Ribosomes are very complex structures and are sensitive to the ionic conditions in which they are isolated. Escherichia coli ribosomes will alter in sedimentation behavior if placed in an ionic environment of 0.15 M NaCl or higher (Atsmon et al, 1969), while Halobacterium cutirubrum ribosomes will change in their behavior if the ionic concentration of potassium is lower than 2.0 M (Bayley and Kushner, 1964; Bayley, 1966). Most studies of this nature use sucrose gradient profile analysis or analytical ultracentrifugation. These ribosomes are then analyzed for the presence of polysomes, 70 S monomers, and subunits or their respective 'S' values.

Vibrio costicola can grow over a range of salt concentrations (0.4-3.5 M NaCl) with increases in the cell-associated ions (Shindler et al, 1977). When grown in 0.5 M NaCl the Na^++ K^+ of the cells is 1.04 M, while growth at 3.0 M results in Na^++K^+ concentrations of 2.15 M. Assuming that the measured cell-associated ions indicate the intracellular salt levels, the ribosomes of this organism can be exposed to quite a range of salt concentrations. What, then, would be the effects of salt concentrations on the behavior of the ribosomes? Are the ribosomes able to 'tolerate' a wide range of salts, or are different ribosomes produced when the cells are grown at different NaCl concentrations? A further alternative could be that the rib-

somes are sensitive to salt but are protected from high salt exposure inside the cell.

In this section, some of the physical properties of the ribosomes and their subunits from V. costicola will be examined as well as the effect of salts on these physical properties.

Experimental Methods

Growth conditions

Vibrio costicola (NRC 37001) cells used for analytical ultracentrifugation and sucrose gradients were grown in liquid culture medium containing: 10.86 g Na_2HPO_4, 1.12 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 2.0 g NH_4Cl, 2.0 g $(NH_4)_2SO_4$, 5.0 g vitamin-free casamino acids (DIFCO), 10.0 g glucose (autoclaved separately), NaCl depending on the desired final concentration, and distilled-deionized water to a volume of one liter. The pH was adjusted to 8.0-8.5 which resulted in a final pH of 7.2-7.5 after autoclaving. Media were distributed into Fernbach flasks in volumes of one-fifth of that of the flask. This helped insure proper aeration of culture during incubation.

The growth of this organism is unpredictable when inoculated directly from a solid slant to a liquid medium. Two 100 ml pre-cultures were generally used in succession to provide reproducible growth curves in the experimental culture flask. Cultures grown in this manner (1.0 M NaCl) had a generation time of 1.36 hours. Cells were harvested in late logarithmic phase of growth at an optical density of 0.8 at 660 nm. This resulted in approximately 1.2 g wet weight or 0.4 g dry weight of cells per liter of culture.

Preparation of extracts and ribosomes

At the appropriate optical density, cells were rapidly cooled by immersion of the culture flask in a frozen CO_2/ethanol bath. Cells were then harvested by centrifugation 10,000 xg

for 10 min at 4 C and then washed once in buffer containing: 60 mM KCl, 10 mM $MgCl_2$, 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, and the same NaCl concentration as the growth medium. Cells were broken in one of two ways. The first method was osmotic lysis modelled after Godson and Sinsheimer (1967). The only difference was that the NaCl concentration was maintained at the level at which the cells were grown. The second method involved breakage with the French Pressure Cell (American Instrument Co.). This procedure required at least 1.0 g of cells. The washed cells were suspended in washing buffer at a ratio of 3.33 ml of buffer to 1.0 g wet weight of cells. This cell suspension was placed in a pre-chilled french pressure cell and put under 9,000 psi. The ball valve was released slowly and the extract collected. This procedure was performed twice to break the highly viscous cell suspension.

The cell extract, broken by either method, was treated with DNase (Worthington; final concentration of 3 μg/ml) and incubated on ice for 10 min. Whole cells and debris were removed by centrifugation at 35,000 xg for 20 min at 4 C. A purified ribosomal preparation was made by centrifuging this extract at 150,000 xg for 3 hours at 4 C. The ribosomal pellet was suspended in the desired buffer and clarified by centrifugation at 35,000 xg for 20 min at 4 C. This clarification removes some of the contaminating membranous material and very large ribosomal aggregates. Generally, 1-2 A_{260} units (the absorbance at 260 nm with a 1.0 cm light path) of the cell extract or 0.5 A_{260}

units of the purified ribosomes were placed on top of a sucrose gradient or 1.0 A_{260} units used in the analytical ultracentrifuge.

Sucrose gradient centrifugation

Twelve ml 5-20% (w/v) linear sucrose gradients were prepared using a two chamber density gradient mixer (Buchler Instruments). The sucrose (grade 1, SIGMA) solutions were made up in 10 mM Tris, pH 7.4, containing the desired amounts of magnesium, sodium, and potassium chlorides. After application of the sample, the gradients were spun in a Beckman SW 41T rotor at 208,000 xg for 3 hours, when the salt concentration was 0.5 M or less, and 4 hours, when the salt concentration was above 0.5 M.

Gradients were fractionated with an ISCO Model 640 Fractionator equipped with a Model UA-5 Analyzer monitoring the absorbance at 254 nm. Generally, 0.4 ml fractions were collected and, depending upon the experiment, either analyzed for protein or RNA and protein. RNA was determined by first precipitating the ribosomes with an equal volume of 95% ethanol and then using the method of Schneider (1957). Protein was determined by the method of Lowry et al (1951).

Analytical ultracentifugation

Sedimentation analyses were carried out in a Spinco Model E Analytical Ultracentrifuge (Beckman) equipped with an AN-D rotor and a 12 mm double sector cell. Sedimentation values were determined at 20 C by taking cell scans every 4 min with an Ultraviolet Photoelectric Scanner (Beckman)

set at 265 nm. At the 36,000 rpm used, this allowed 10-12 scans of each sample before the monomer pelleted on the bottom of the cell. These scans were measured for the sedimentation of each particle species by the method outlined by Schachman (1957) and Chervenka (1973). Observed sedimentation values (S_{obs}) were evaluated from the slope of a log x (distance of boundary from the axis of rotation) versus time plot (Schachman, 1957).

In order to determine sedimentation under standard conditions ($S_{20,w}$), it was necessary to make corrections for the density and viscosity of the buffers. Density measurements were made in a 25 ml Gay-Lussac pycnometer. Due to the evaporation of some of the buffer, weight measurements were taken at one minute intervals for five minutes and extrapolated back to zero time. Each buffer was done in triplicate. Viscosity measurements were made using an Ostwald-Fenske viscometer (0.5 ml) immersed in a 20 C water bath (Schachman, 1957). The buffer was allowed to equilibrate to the bath temperature for a least 30 minutes before the measurements were made. Measurements were repeated until a constant flow rate was obtained.

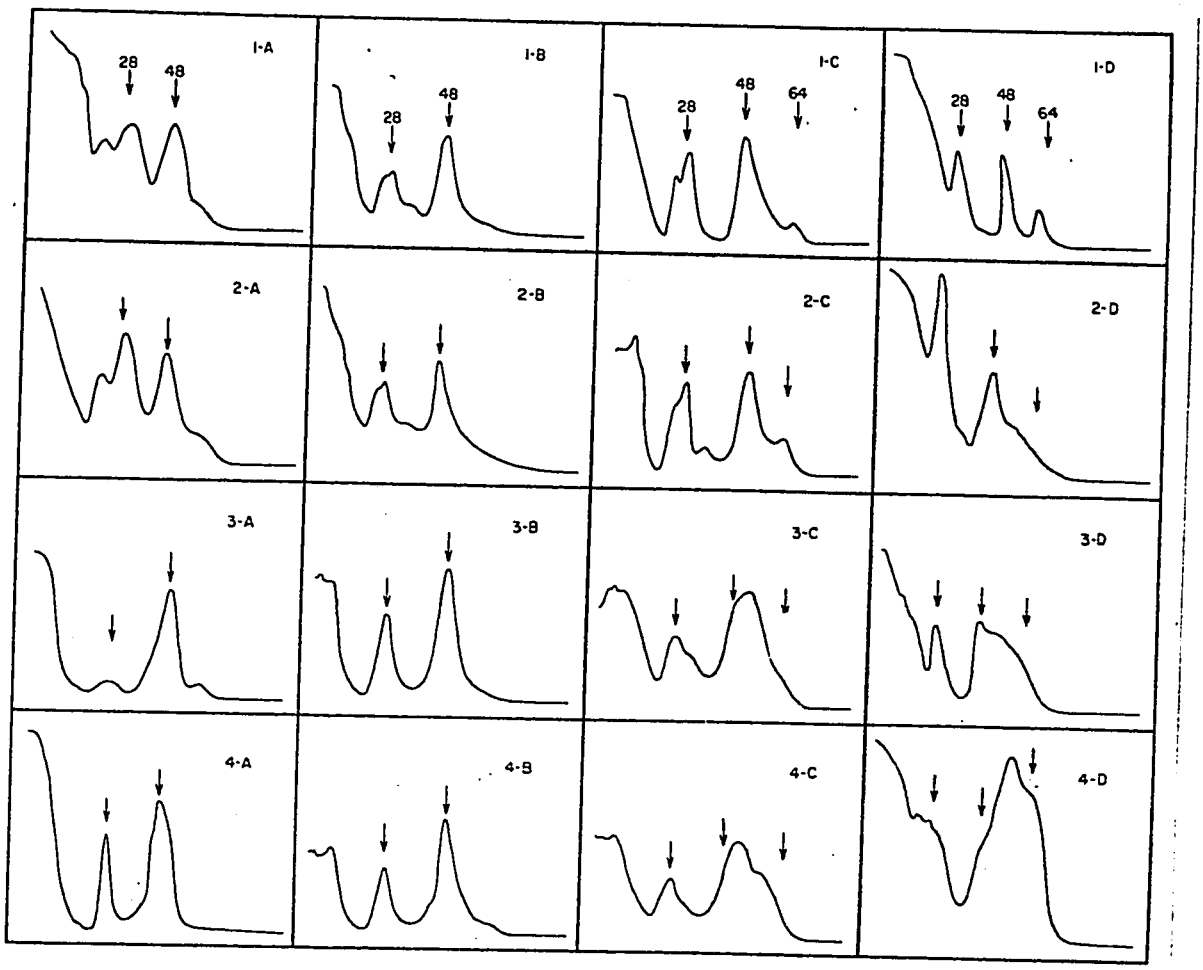
Results

Sucrose gradients

At the time these studies were started, only preliminary data was available on the intracellular salt concentration of V. costicola. The preliminary data indicated that when cells of V. costicola were grown in 1.0 M NaCl, the intracellular Na^+ concentration was approximately 1.0 M, the K^+ was approximately 0.5 M, and the Mg^{2+} was approximately 40-50 mM. This, then, was considered the starting point for determining the salt concentrations used to separate the ribosomes in sucrose gradients. There was also data to suggest that the intracellular salt concentration changed with the NaCl of the growth medium. V. costicola cells were grown in 1.0 M NaCl and the ribosomes separated in sucrose gradients containing multiples (0.5X, 1.0X, 1.5X, and 2.0X) of the salts indicated as intracellular. For comparison, V. costicola was also grown in 0.5, 1.5, and 2.0 M NaCl and the ribosomes centrifuged in sucrose gradients containing the different salt concentrations. The resulting ribosomal profiles are shown in Figure II-1. Generally, taking into account that the concentration of the salts affects the viscosity of the gradients and thus the separation of the ribosomal peaks, all of the gradients are similar. In all profiles, the predominant peaks were of the subunits. Some minor differences can be noted: if the cells were grown in low salt and the ribosomes prepared in low salt, some of the intermediate material between the large and small subunits appears. Rib-

Figure II-1

Sucrose density gradients of ribosomes from V. costicola. Cells grown in the presence of: (1) 0.5 M NaCl; (2) 1.0 M NaCl; (3) 1.5 M NaCl; (4) 2.0 M NaCl. Ribosomes prepared and centrifuged in gradients containing 10 mM Tris, pH 7.5, plus: (A) 3 mM Na-succinate, 10 mM $MgCl_2$; (B) 0.5 M NaCl, 0.25 M KCl, 25 mM $MgCl_2$; (C) 1.0 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$; (D) 2.0 M NaCl, 1.0 M KCl, 100 mM $MgCl_2$. Gradients (5-20% sucrose) were run in 12.4 ml tubes at 208,000 xg; 3 hours for (A) and (B); 4 hours for (C) and (D). In each run a marker tube with 48 S V. costicola subunits (and a small amount of 28 and 64 S material) was included; positions of the markers are indicated. The results are normalized so that all 48 S peaks have the same height (A_{260} usually 0.4). The tops of gradients appear on the left of each graph.


1-A
28
48
1-B
28
48
1-C
28
48
64
1-D
28
48
64
2-A
2-B
2-C
2-D
3-A
3-B
3-C
3-D
4-A
4-B
4-C
4-D

osomal preparations from cells grown in higher NaCl concentrations did not show this intermediate material when centrifuged in low salts, but showed a greater proportion of monomers.

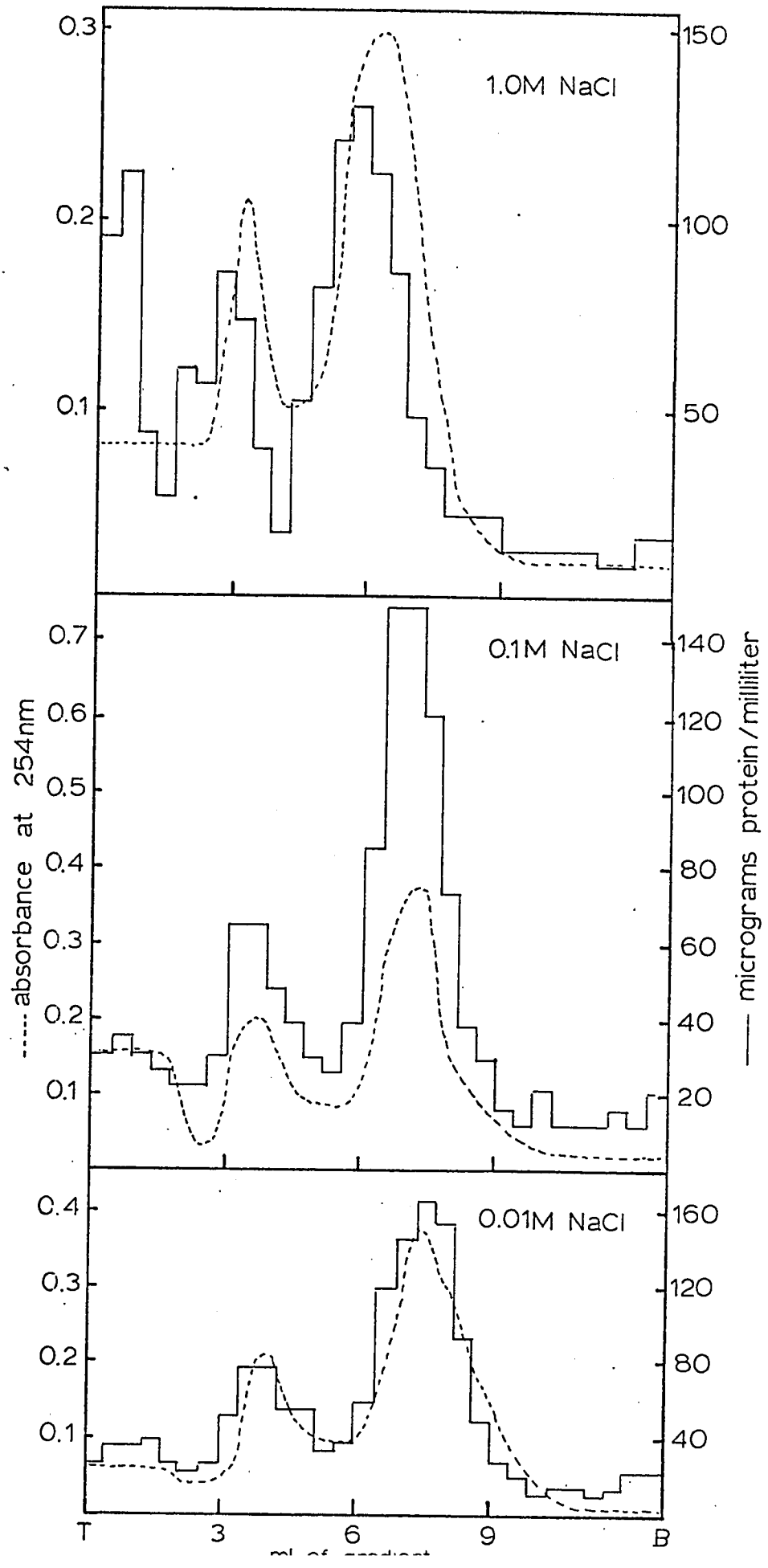
One of the effects salts have on the ribosomes of E. coli is the removal of ribosomal proteins (Atsmon et al, 1969). Depending on the amount of protein removed, this does not necessarily result in a concomitant decrease in the sedimentation behavior. The first sucrose gradients were run with cell extracts of V. costicola which resulted in a tremendous peak of material at the top of the gradient. Any ribosomal proteins removed by the salt would be obscured by this material. To see if the salts were removing ribosomal proteins, purified ribosomes were run on three sucrose gradients containing different NaCl concentrations and the gradients analyzed for U.V.-absorbing material and protein (Figure II-2). Correlation of the absorption at 254 nm and the protein content of each of the gradients show striking differences. As the NaCl content of the gradients increase, the amount of protein at the top of the gradients increases, indicating that protein was released from the ribosomes.*

Since the evaluation of the intracellular salts were proceeding concurrently with these studies, revised values were constantly available. A more accurate estimation of the intracellular salts indicated that Na^+ and K^+ were present in equimolar amounts, the sum of which approximated the molar concentration of the NaCl in the growth medium. To see what effect these salt concentrations had on the ribosomes, V. costicola was grown

*Since the ribosomes were prepared in 1.0 M NaCl, all of the gradients should have demonstrated removal of proteins from the ribosome. It has been shown (A.T. Matheson, personal communication) that the effect of salts on ribosomes is dependent upon the ribosomal concentration. The higher the ribosome concentration, the less effect salts have on the removal of ribosomal proteins. It is possible that during preparation the ribosomal concentration was high enough to prevent the removal of the ribosomal proteins by the salt. It was only after the ribosomes were diluted into the sucrose gradient that the ribosomal proteins were removed. Subsequent experiments on ribosomes isolated in low salt concentrations (see Chapter III) showed that salt concentrations as low as 0.5 M will remove some of the ribosomal proteins.

Figure II-2

Sucrose gradients of ribosomal particles of V. costicola in different NaCl concentrations. The sucrose solutions contained: 0.5 M KCl, 0.1 M $MgCl_2$, and 10 mM Tris, pH 7.4, in addition tc the NaCl indicated. Gradients were centrifuged at 30,000 rpm for 6 hours. 0.4 ml fractions were collected and analyzed for absorbance at 254 nm and protein content. Purified ribosomes were from cells grown in 1.0 M NaCl and prepared in 1.0 M NaCl, 0.5 M KCl, 0.1 M $MgCl_2$, and 10 mM Tris, pH 7.4.


1.0M NaCl
0.1M NaCl
0.01M NaCl
absorbance at 254nm
micrograms protein/milliliter
T
B
ml of gradient

in 1.0 M NaCl and the ribosomes purified in 0.5 M NaCl and 0.5 M KCl (plus 10 mM Tris, pH 7.4 and 40 mM $MgCl_2$). The ribosomes were placed on a sucrose gradient containing the same salt concentrations and the gradients analyzed for RNA and protein (Figure II-3). As in the previous gradients, there was a substantial amount of protein remaining at the top of the gradient. The area under each of the peaks was determined for both the RNA and protein profiles and the ratio of the concentrations determined. The purified ribosomes applied to the top of the gradient had a RNA to protein ratio of 1.50. The large and the small subunits had ratios of 1.63 and 1.50, respectively. This indicates that the protein released could be from the large subunit.

Recent studies by Algranati *et al* (1975) have demonstrated the importance of polyamines in ribosomal integrity. They postulate that intracellular concentrations of polyamines substitute (in part) for the *in vitro* magnesium requirement of the *E. coli* ribosome. To test whether polyamines could also play a role in the stability of *V. costicola* ribosomes, sucrose gradients were run in high and low magnesium and in the presence and absence of 3 mM spermine (Figure II-4). Data from the *in vitro* protein synthesis of *V. costicola* indicated a low requirement for salt (Chapter IV) so gradients were run in 100 mM KCl. The gradient run in 20 mM $MgCl_2$ shows that the majority of ribosomal particles are in the monomer form, while those in 1 mM $MgCl_2$ are only in the subunit form. At 20 mM $MgCl_2$, the presence of spermine does not greatly affect the ribosomal profile.

Figure II-3

Sucrose gradient of V. costicola ribosomes analyzed for RNA and protein content. The sucrose gradient contained: 0.5 M NaCl, 0.5 M KCl, 40 mM $MgCl_2$, and 10 mM Tris, pH 7.4. Purified ribosomes were from cells grown in 1.0 M NaCl and prepared in the above buffer. The gradient was centrifuged for 5 hours at 30,000 rpm and 0.4 ml fractions analyzed for RNA and protein content.

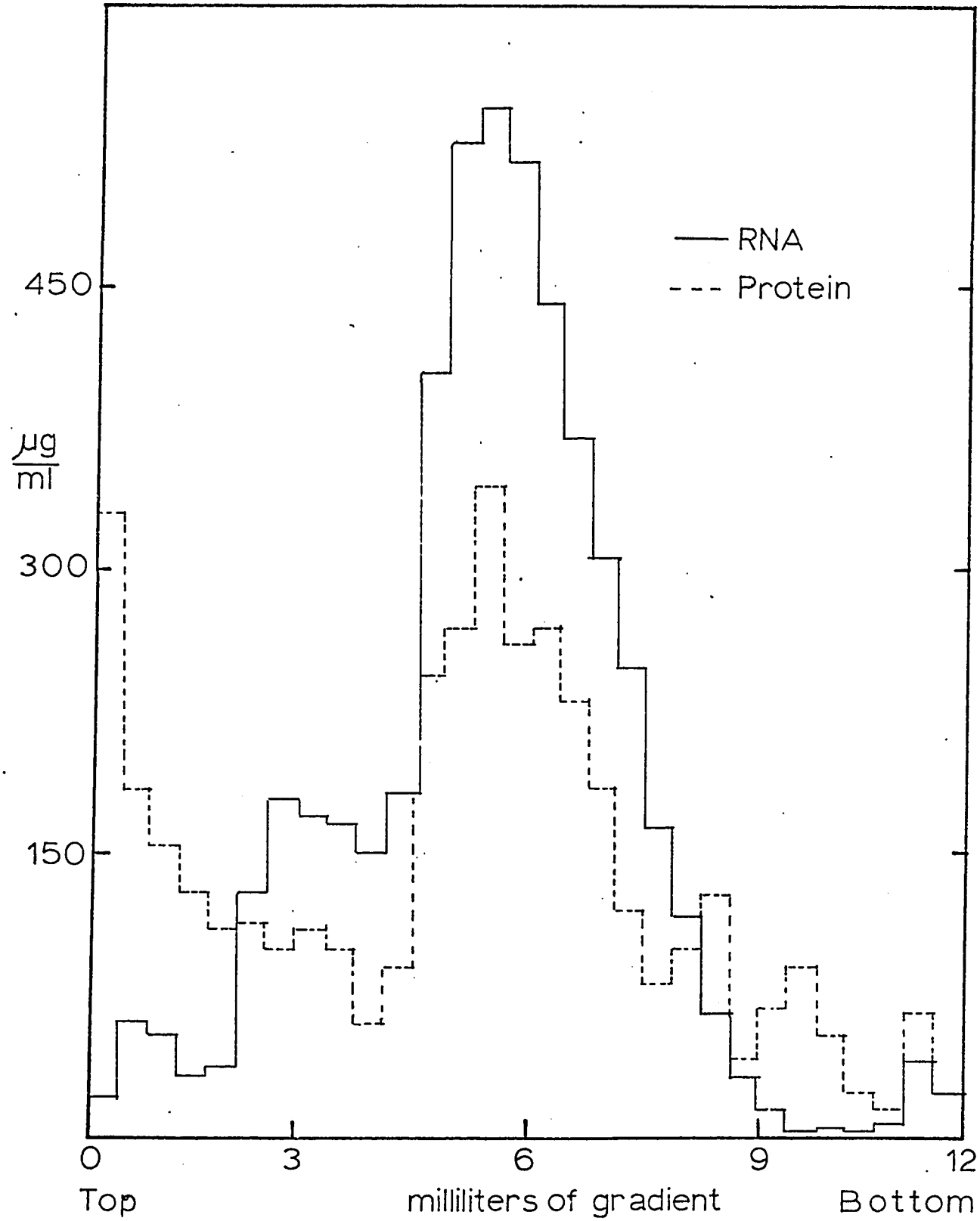
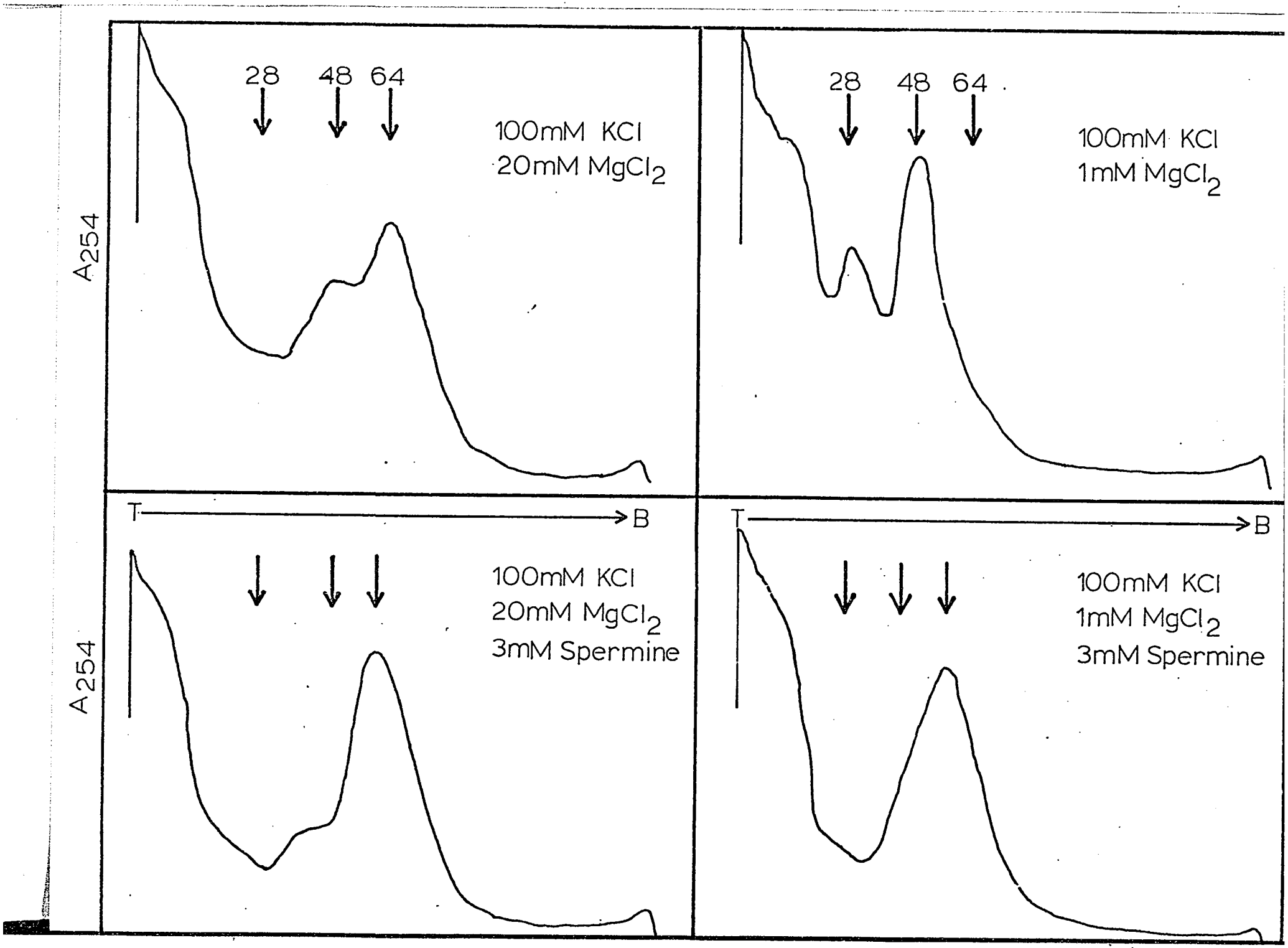

RNA
Protein
450
300
150
µg/ml
0
3
6
9
12
Top
milliliters of gradient
Bottom

Figure II-4

The effect of magnesium and spermine on the ribosomal profiles of V. costicola. Cells were grown in 1.0 M NaCl and the extract prepared in 100 mM KCl, 20 mM $MgCl_2$, and 10 mM Tris, pH 7.4. The gradients contained the above salt concentrations and were centrifuged at 208,000 xg for 2 hours, then fractionated, monitoring the absorbance at 254nm continually.


28
48
64
100mM KCl
20mM MgCl2
A254
28
48
64
100mM KCl
1mM MgCl2
T
B
100mM KCl
20mM MgCl2
3mM Spermine
A254
T
B
100mM KCl
1mM MgCl2
3mM Spermine

However, lower $MgCl_2$ with the presence of spermine results in all of the ribosomes in the monomer form. A slight large subunit shoulder on the front of the monomer peak is present.

Attempts were made to standardize the sucrose gradients so that the sedimentation rates of the ribosomal particles in various salt concentrations could be evaluated and compared. E. coli ribosomes run in dilute buffer served as an adequate marker, but in sucrose gradients containing higher salt concentrations, the ribosomes formed non-discrete sedimenting particles and aggregates (sucrose gradients are not shown, but see Figure II-6 for sedimentation in the analytical ultracentrifuge). Purified E. coli specific R17 bacteriophage (78 S) also failed as an adequate marker. Preliminary results suggested that it interacted with V. costicola ribosomes at higher salt concentrations (D.J. Kushner, personal communication). There were also literature reports of high salt concentrations removing the coat proteins of bacteriophages, indicating that their sedimentation behavior in high salt might be unpredictable (Gesteland and Boedtker, 1964). Analytical ultracentrifugation was then considered necessary in order to evaluate the effects of salts on the sedimentation behavior of V. costicola ribosomes. It was only after analytical ultracentifugation had shown that the V. costicola ribosomal subunits retained the same 'S' values over a wide range of salt concentrations (see below) that these could be used as markers.

Analytical ultracentrifugation

A more quantitative investigation of the effects of salts on V. costicola ribosomes was carried out on an analytical ultracentrifuge equipped with ultraviolet optics. U.V. optics were used because they allowed the use of much lower concentrations of ribosomes than those required for Schlieren optics. With such low concentrations, particle-particle interactions could be ignored and the effects of particle concentration eliminated (Schachman and Edelstein, 1966; Chevenka, 1973).

In order to correct the sedimentation values to standard conditions in which the sedimentation of the particle is evaluated in pure water at 20 C, the density and viscosity of each of the buffers used had to be determined (Table II-1). Another important value in the determination of the $S_{20,w}$ is a correction involving the partial specific volume of each of the sedimenting particles. To measure the partial specific volume directly requires large amounts of each species to be purified. The large subunit was the only species for which sufficient amounts of pure material was obtained to make such measurements. The procedure involves measuring the density of the buffer in which the particle is suspended (in this case 1.0 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$, and 10 mM, Tris, pH 7.4). A known concentration of ribosomes suspended in this buffer is then placed in the pycnometer and weighed. The difference between the theoretical increase in the weight of the solution and that actually measured, represents the amount of buffer displaced by the presence of the ribosomes. Dividing the volume

Table II-1

Viscosity and density of buffers used in analytical ultracentrifugation.

Buffer salts	Density, ρ [1]	Viscosity, η [2]
3 mM Na-succinate, 10 mM $MgCl_2$	1.002	0.9982
0.5 M NaCl, 0.25 M KCl, 25 mM $MgCl_2$	1.0321	1.0510
0.5 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$	1.0435	1.0672
1.0 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$	1.0618	1.1102
1.5 M NaCl, 0.75 M KCl, 75 mM $MgCl_2$	1.0914	1.1686
2.0 M NaCl, 1.0 M KCl, 100 mM $MgCl_2$	1.1317	1.3032

[1] Density values have a standard deviation of ± 0.0011 g/ml.

[2] Viscosity values have a standard deviation of ± 0.0020 centipoise.

of the displaced buffer by the weight of the added ribosomes gives the partial specific volume of the ribosomal particle, in this case 0.516 ml/g of large subunit. An indirect method for determining the partial specific volume was performed by plotting the product of the observed sedimentation values (S_{obs}) and the viscosity of each salt solution versus the density of each solution (Schachman and Lauffer, 1949; Hill and Cox, 1965) as done by Bayley and Kushner (1964), Figure II-5. In this plot the points in salt solutions more concentrated than 0.5 M extrapolated to, or near, the values observed in the most dilute buffer, whose density and viscosity were indistinguishable from those of water. Using all the points, including those obtained in dilute buffer, the intercepts of the abcissa and the ordinate were determined by linear regression, using the method of least squares. Intercepts on the ordinate gave the $S_{20,w}$ values: 64, 48, and 28 S, corresponding to "standard" values for procaryotic ribosomes (ie. 70, 50, and 30 for E. coli; Hill et al, 1969). However, the $S_{20,w}$ for the monosome was slightly but significantly lower than 70 S. The intercept of the abcissa gave the density at which there is no sedimentation of the particle, hence, the density of the material itself. Results for the 64 and 48 S material are quite close; those for the 28 S material somewhat lower. Partial specific volumes of 0.595, 0.581, and 0.713 ml/g were calculated as the inverse of the densities for the 64, 48, and 28 S material, respectively. E. coli monosomes and subunits have partial specific volumes about 0.60 (Hill et al, 1969) while those of H. cutirubrum are about 0.71.

Figure II-5

Plot of the relative viscosity of solutions (η) times the sedimentation coefficients observed (S_{obs}) of ribosomal particles against the solution density. Different symbols indicate different experiments. Cells were grown in media containing 1.0 M NaCl.

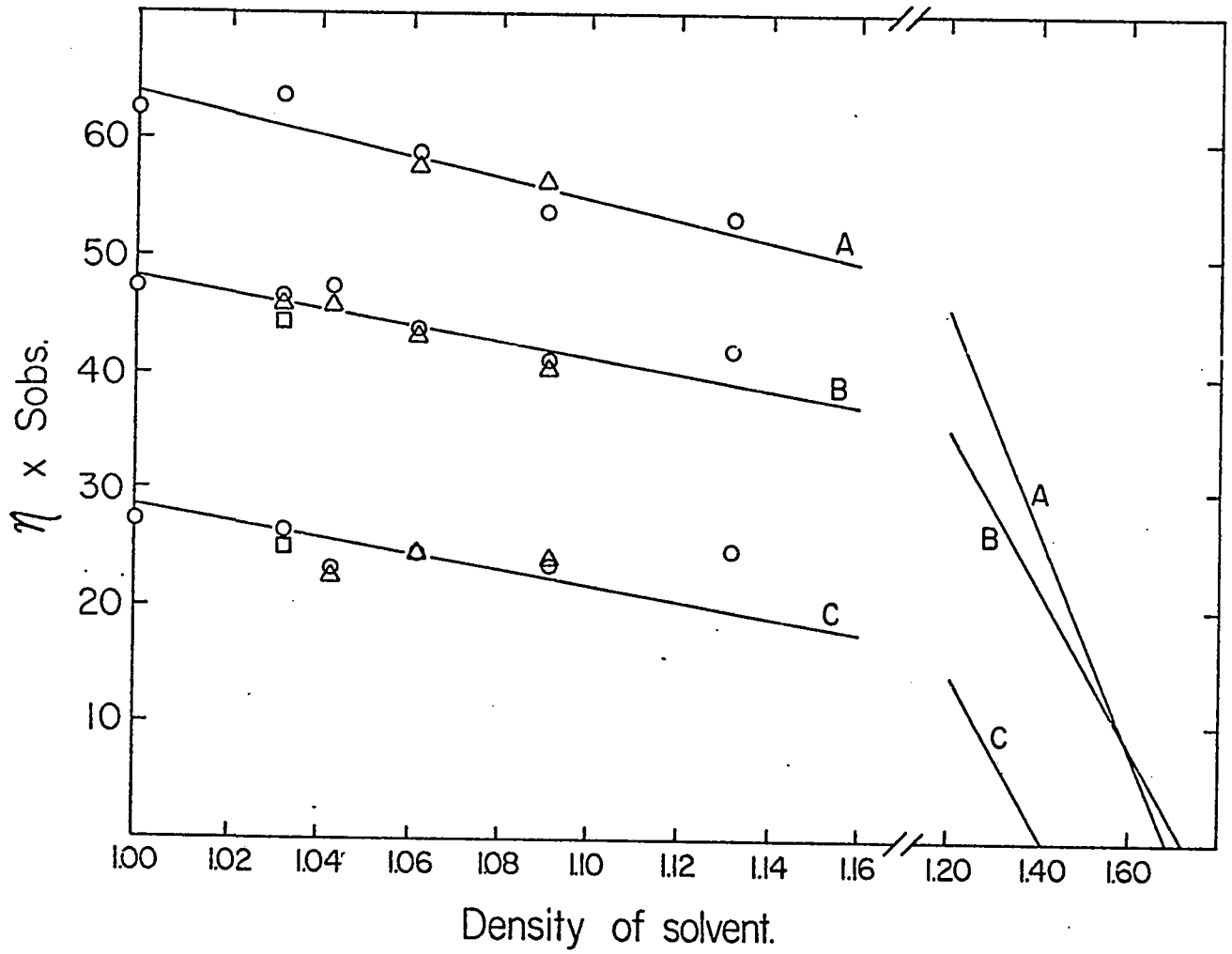

60
50
40
30
20
10
η x Sobs.
A
B
C
1.00
1.02
1.04
1.06
1.08
1.10
1.12
1.14
1.16
1.20
1.40
1.60
Density of solvent.

Table II-2

Effects of NaCl concentration in growth media, and composition of suspending media on sedimentation coefficients of V. costicola ribosomes.

NaCl in growth media	Composition of Suspending media	$S_{20,w}$ values[1] monosomes	large subunit	small subunit
0.5 M	0.5M NaCl,0.25M KCl, 25mM $MgCl_2$		48.2 ± 0.9(2)	28.3 ± 0.5 (2)
	0.5M NaCl,0.5M KCl,50mM $MgCl_2$		48.5 ± 0.7(2)	24.6 ± 1.2 (2)
1.0M	3mM Na-succinate, 10mM $MgCl_2$	62.5(1)	46.9 ± 1.7(2)	27.4 (1)
	0.5M NaCl,0.25M KCl,25mM $MgCl_2$		49.4 ± 1.0(2)	29.0 ± 1.0 (2)
	1.0M NaCl,0.5M KCl,50mM $MgCl_2$	62.8±.7(4)	46.8±0.5(7)	28.4 ± 1.1 (6)
	2.0M NaCl,1.0M KCl,100mM $MgCl_2$	65.0 (1)	50.3 (1)	35.6 (1)
1.5M	1.5M NaCl,0.75M KCl,75mM $MgCl_2$	62.7±1.6(2)	45.7±0.4(2)	30.2 ± 0.7 (2)

[1]These values are means of a number (in brackets) of separate determinations ± average deviation from mean. Most were determined with less than 5% error. $S_{20,w}$ values are calculated from S_{obs} and the measured density and viscosity of suspending buffer, assuming partial specific volumes for monosomes, 0.595; large subunit, 0.581; and small subunit, 0.713.

The S_{obs} values of the smaller subunit were more difficult to measure accurately because of the slower sedimentation and lower absorbance. It is unlikely that the apparent high value for the partial specific volume for the small subunit is real.

When the $S_{20,w}$ values for the monosomes and subunits were calculated for each salt concentration, it was found (Table II-2) that the values did not vary significantly with the ionic environment and the NaCl concentration of the growth media. E. coli ribosomes analyzed in dilute buffer had a sedimentation coefficient for the monosome of 68.5 S. Evaluation of the $S_{20,w}$ of E. coli ribosomes in buffer containing 0.5 M NaCl was not possible because the particles fell apart and aggregated into non-discrete sedimenting particles (Figure II-6).

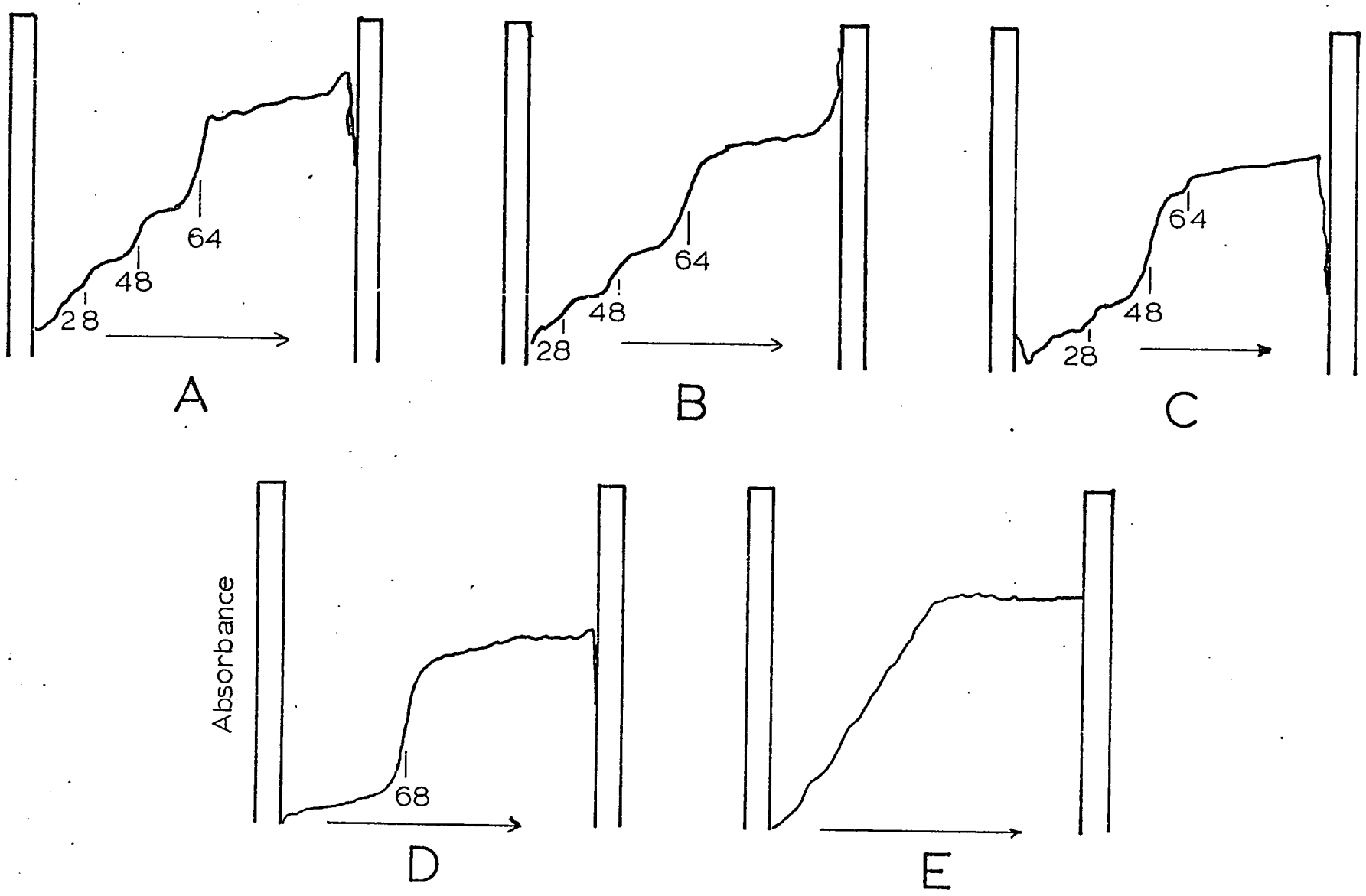
Using the data obtained from the sedimentation analysis, the frictional coefficient can be calculated. The frictional ratio (f/f_o) is a measure of the departure of the particle from the simple behavior of a perfect sphere and is an indirect expression of the asymmetry of the particle (Oncley, 1951). The frictional coefficient is evaluated in the following equation:

$$f/f_o = 1.19 \times 10^{-15} M^{2/3}(1-0.9882 \bar{v}_{20})/(S_{20,w} \bar{v}_{20}^{1/3}) \quad (1)$$

where: M, is the molecular weight of the particle; $\bar{v}_{20}$, is the partial specific volume of the particle at 20 C; $S_{20,w}$, is the standard sedimentation coefficient; f_o, is the frictional resistance of an unsolvated sphere; and f, is the frictional resistance of the particle being tested. The closer the ratio is

Figure II-6

Ultraviolet scans of V. costicola and E. coli ribosomes in the analytical ultracentrifuge at different salt concentrations. (A) V. costicola ribosomes in 3 mM Na-succinate, 10 mM $MgCl_2$, 10 mM Tris, pH 7.4, at 23 minutes after the start of the run. (B) V. costicola ribosomes in 0.5 M NaCl, 0.25 M KCl, 25 mM $MgCl_2$, 10 mM Tris, pH 7.4, 27 min after start of run. (C) V. costicola ribosomes in 2.0 M NaCl, 1.0 M KCl, 100 mM $MgCl_2$, 10 mM Tris, pH 7.4, after 31 min. (D) E. coli ribosomes in the same buffer as panel (A), but 15 min after start of run. (E) E. coli ribosomes in 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.4, after 27 minutes. Analytical ultracentrifuge was run at 36,000 rpm.


28
48
64
A
28
48
64
B
28
48
64
C
Absorbance
68
D
E

to 1.0, the more the experimental approaches a spherical shape.

The equation also requires that the molecular weight of the particle be known. One method of estimating the molecular weight (as done by Bayley and Kushner, 1964) of procaryotic ribosomes and their subunits was devised by Inouye and co-workers (1963). They found that for all procaryotic ribosomes tested, the molecular weight of the particles and the sedimentation coefficient were related by the following equation:

$$S_o = 0.69 \times 10^{-12} (1-\rho\bar{v}) M^{2/3} \qquad (2)$$

where: S_o, is the sedimentation coefficient in Svedberg units; ρ, is the density of the solution; $\bar{v}$, is the partial specific volume of the particle; and M, is the molecular weight. Using this formula, V. costicola ribosomes would have molecular weights of 0.75×10^6, 1.72×10^6, and 2.67×10^6 for the 28, 48, and 64 S particles respectively. If this formula was applied to the sedimentation properties of E. coli ribosomal particles, the resulting molecular weights for the 30, 50, and 70 S particles would be 0.91×10^6, 1.84×10^6, and 3.10×10^6, respectively. The formula correctly estimates the molecular weight of the small subunit of 0.90×10^6 (Hill et al, 1969), but grossly misjudges the molecular weight of the large subunit (1.55×10^6) and the monosome (2.65×10^6) as determined by molecular weight studies using sedimentation equilibrium (Hill et al, 1969; van Holde and Hill, 1974). For the purpose of using equation (1), it may be better to assume that the molecular weight of V. costicola ribosomes are approximately the same as E. coli ribosomes. This also avoids the problem of putting too much mathematical

emphasis on the sedimentation value itself.*

Using the molecular weights of E. coli ribosomal particles results in E. coli ribosomal frictional ratios of 1.77, 1.58, and 1.70 (Gabler et al, 1974) and V. costicola ribosomal particle ratios of 1.28, 1.67, and 1.71 for the small subunit, large subunit, and monosome, respectively. This frictional ratio is dependent upon the partial specific volumes of each particle. As stated previously, the 0.713 value for the 28 S V. costicola small subunit could be in error, resulting in the unusually low frictional ratio. Taking this into account, then, the shape properties of the V. costicola ribosomal particles resemble those of E. coli rather than those of H. cutirubrum (Table II-3).

All of these sucrose gradients and sedimentation analyses were based on the assumption that the measured cell-associated ions reflect the ionic environment of the ribosomes in situ. Later experiments with the protein synthesizing system from V. costicola (Chapter IV) indicated that maximum function in an in vitro situation occured at much lower salt concentrations and that the higher salts were very inhibitory to protein synthesis activity. An additional sedimentation experiment was performed in which the ribosomes were isolated in a manner identical to that used for achieving an extract for protein synthesis experiments (see Methods, Chap. IV). The sedimentation coefficients determined under these conditions were exactly the same as those found before ie. 28, 48, and 64 S. The only difference was that the monosome composed the major peak.

* This is all the more desirable since subsequent discussions have pointed out that the method used calculates the partial specific volume of the solvated particle, which may be quite different from the particle in water. The nature of the salt used in sedimentation studies can also affect the density. Bayley and Kushner (1964) found that H. cutirubrum ribosomal particles sedimented in high KCl concentrations had a mean density of 1.416 while those sedimented in high CsCl concentrations had a mean density of 1.551. High KCl and CsCl concentrations were not used in this work. The main value of the analytical ultracentrifugation studies was to establish the $S_{20,w}$ values of the monosome and subunits.

Table II-3

Physical properties of *E. coli*, *H. cutirubrum*, and *V. costicola* ribosomes.

	$S_{20,w}$	MW ($\times 10^6$)	$\bar{v}$	f/f_o
E. coli[1]				
small subunit	31.8 S	0.90	0.601	1.77
large subunit	50.2	1.55	0.592	1.58
monosome	70.5	2.65	0.606	1.70
V. costicola				
small subunit	28	0.75	0.713	1.28
large subunit	48	1.72	0.581	1.67
monosome	64	2.67	0.595	1.71
H. cutirubrum[2]				
small subunit	31	0.90	0.710	1.14
large subunit	52	1.90	0.710	1.14
monosome	70	3.00	0.710	1.15

[1] Data from van Holde and Hill (1974).

[2] Data from Bayley and Kushner (1964).

Discussion

Sucrose gradient analysis

One of the first characteristics observed is that the ribosomes of V. costicola show similar sucrose gradient profiles no matter what NaCl concentration the cells were grown or the salt concentration of the gradient. This is in contrast to the behavior of ribosomes from the extreme halophile, Halobacterium cutirubrum, which maintain 'normal' monosomes and subunits in very high KCl concentrations but alter drastically in lower salt concentrations (Bayley and Kushner, 1964). It is also in contrast to the behavior of Escherichia coli ribosomes whose gradient profiles and protein content are changed by salt concentrations of 0.15 M or higher (Spitnik-Elson and Atsmon, 1969; Atsmon et al, 1969; Phillips et al, 1969; and Dijoseph et al, 1971).

A good deal of material intermediate between the two subunits was observed in cells grown in low salt concentrations. Slight amounts of this material can be found in almost all gradients. The nature of this material is unknown, but from the data available for E. coli (Atsmon et al, 1969) this could reflect ribosomal particles deficient in proteins. The experiments analyzing the amount of protein in the sucrose gradient fractions support the conclusion that increased NaCl content of the gradient results in the release of protein from the ribosomes. The amount of protein released may not be enough to alter the sedimentation profile other than to show the slight

amounts of intermediate material. In those gradients where both RNA and protein were measured, the large and the small subunits had approximately the same RNA to protein ratios. These values are in close agreement with those found for E. coli (Spitnik-Elson and Atsmon, 1969) and H. cutirubrum (Bayley and Kushner, 1964). This loss of protein at the higher salt concentrations may indicate that the monosomes produced at the higher salt concentrations may not be true 70 S particles, but non-specific aggregates of protein-depleted subunits (Wydro et al, 1975).

One of the biggest problems in running sucrose gradients in this range of salt concentrations was finding an adequate marker for a reference point. E. coli ribosomes could be used in gradients containing 0.1 M salts or lower, but could not be used in the 0.5 M or higher salts. Using purified R_{17} phage of E. coli also presented problems. It was only after analytical centrifugation measurements had shown that V. costicola subunits retained the same $S_{20,w}$ over a wide range of salt concentrations that purified V. costicola large subunits could be used as a marker.

Analytical ultracentrifugation

Analytical ultracentrifugation confirmed the sucrose gradients in showing that the salt did not drastically alter ribosome structure. Ribosomes isolated from cells grown in different salt concentrations and analyzed in different buffers had standard sedimentation coefficients of 28, 48 S for the subunits and 64 S for the monosome. These values are somewhat lower

than those reported for E. coli (Hill et al, 1969) and H. cutirubrum (Bayley and Kushner, 1964). This difference could be explained in one of several ways. The first is that the ribosomes are smaller and therefore do not sediment as fast. The second is that the particles are the same size (molecular weight) as other ribosomes but have a more expanded conformation. This latter explanation is indicated by the higher frictional ratio of V. costicola ribosomes. Even if the molecular weight of the V. costicola particles is estimated by the method of Inouye et al (1963), the particles show a higher ratio than those of E. coli, indicating a more open structure.

A third possiblity is that all of the salt concentrations tested were too high, resulting in protein deficient particles, or too low, as in the case of the succinate buffer, also resulting in protein deficient particles. Later experiments with the in vitro protein synthesizing system revealed that maximum functioning of the ribosomes occured in 100 mM KCl or NH_4Cl and 20 mM $MgCl_2$. Analytical ultracentrifugation of purified ribosomes in this buffer resulted in sedimentation values exactly the same as before. If proteins are being removed by the higher salt concentrations, they do not alter the sedimentation behavior of the ribosomal particles. This is in agreement with studies performed by A. Matheson (personal communication), who found that the 30 S subunit of H. cutirubrum could lose a number of proteins without changing the sedimentation rate.

General conclusions

From the sucrose gradient data it appears that the ribosomes isolated from cells grown at different salt concentrations are similar. They seem to be affected by the salts in the same manner. If the salt concentrations are having an adverse effect on the ribosomes, then this effect is not changing the sedimentation coefficient outside of experimental error. Detailed analysis of several sucrose gradients revealed that the higher the salt concentration, the more protein was removed from the ribosomal particles. This would tend to indicate that the salt concentrations measured as intracellular are not indicative of the salt environment of the ribosomes *in situ*. One postulation could be that the medium ions are balanced by a high intracellular concentration of polyamines. This is supported by the sucrose gradient data showing the monosomes of *V. costicola* are more stable in the presence of spermine than in any of the other ion conditions tested.

Chapter III

Ribosomal Proteins of V. costicola

Introduction

The ribosome is a complex structure, whose proper functioning is influenced by the spatial arrangement of the ribosomal ribonucleic acid (rRNA) and the ribosomal proteins. Thus it seems that secondary, tertiary, and quartinary structure are important. For example, experiments with the extreme halophile, H. cutirubrum, have shown that magnesium is required for the secondary structure of the rRNA, and potassium is necessary for the proper binding of the ribosomal proteins (Bayley, 1966). E. coli ribosomes show similar requirements for these two ions, but at greatly reduced concentrations. The influence, then, of the intracellular ionic environment on the structure and functioning of the ribosome is of major importance.

Bayley (1966), investigating the amino acid composition of the halophilic ribosome, showed that lowering the potassium ion concentration removed some of the ribosomal proteins. He also showed that although most of the ribosomal proteins of H. cutirubrum are more acidic than those of E. coli, it was the most acidic ribosomal proteins that were sensitive to the low ionic buffers. Visentin et al (1972) developed a procedure in which they could reproducibly remove what they termed "split" proteins (those ribosomal proteins of H. cutirubrum that were removed by low salts) and the "core" proteins (those ribosomal proteins that remain after removal of the split proteins).

They found that for the ribosomes of H. cutirubrum, the less acidic proteins were harder to remove. They postulated that the split proteins represented the outermost proteins on the ribosome, and which are thus exposed to the high ionic environment in situ. Split ribosomal proteins are generally more acidic than the other ribosomal proteins in both procaryotes (Wittmann, 1974) and eucaryotes (Saleem and Atkinson, 1976).

The mechanism of halophilic adaptation to the high saline environment has been under investigation for some time (for reviews see: Kushner, 1968; Lanyi, 1974; and Brown, 1976). The increased acidity of H. cutirubrum ribosomal proteins was thought to be charge shielded by high ionic concentrations (Bayley, 1966). Lanyi (1974) proposed that the charge shielding effect of cations on the acidic proteins was complete at 0.2 M or less. Lanyi states that requirements for salt concentrations above this value are the result of other factors such as hydrophobic interactions. He showed that proteins from the extreme halophiles have a decreased hydophobicity along with increased protein acidity. These and other observations led Lanyi to the conclusion that hydrophobic interactions may play a more dominant role in the maintenance of halophilic protein structure. What, then, would be the composition of ribosomal proteins from an organism that can grow over a wide range of NaCl concentrations?

Even though there are changes in the bacterial ribosomes from organism to organism, there is evidence to suggest that certain polypeptide and polynucleotide regions of the ribosome

have been conserved during evolution. Experiments by Nomura et al (1968) have reconstituted biologically active 30 S ribosomal hybrids from the 16 S rRNA and the ribosomal proteins from different species of bacteria. On the assumption that this indicates conservation of protein structure, studying the peptide sequence of functionally similar ribosomal proteins from a variety of organisms, these areas of conservation could be pinpointed. This may elucidate those parts of the protein which are involved in the specific function of the protein since it is these areas that are most likely to be conserved.

This chapter describes an attempt to study the ribosomal proteins of V. costicola grown over a range of NaCl concentrations. Both the acidity and the proportion of hydrophobic amino acids of the ribosomal proteins was investigated and correlated with the halophilic nature of the organism. Two ribosomal proteins were purified and characterized. Furthermore, the partial amino acid sequence of one of the ribosomal proteins was determined and compared to the sequence data from equivalent proteins from other organisms.

Experimental Methods

Growth conditions

For the isolation of total ribosomal proteins, Vibrio costicola (NRC 37001) was grown in liquid culture medium containing: 0.5% Bacto-tryptone (DIFCO), 0.5% proteose peptone (DIF CO), NaCl, depending upon the desired final concentration, and distilled-deionized water. For most studies the cells were grown in a 25 liter fermenter (New Brunswick Sci. Co.) with 12 psi of bubbled air and vigorous stirring. A small amount of Anti-foam A (Dow Corning Ltd.) was added to prevent excessive foaming. When larger amounts of cells were needed for the isolation of individual ribosomal proteins, a 180 liter fermenter was used. Fermenters were inoculated with a 1% inoculum of a pre-culture grown to late logarithmic phase of growth. Cells grown at 30 C under these conditions had a generation time of 1.3 hours for 1.0 M NaCl; 2.5 hours for 0.5 M NaCl; and 6.2 hours for 3.0 M NaCl. Cells were harvested in mid- to late-logarithmic phase of growth with an optical density at 660 nm of between 0.8 (3.0 M NaCl) and 1.5 (0.5 and 1.0 M NaCl). This resulted in 50 to 75 g wet weight of cells per 25 liters. Cells were harvested by centrifugation at 16,300 xg for 10 min at 4 C in a Serval GSA rotor or by a Sharples continuous flow centrifuge. Cells were washed once in buffer containing: 0.6 M KCl, 40 mM $MgCl_2$, 10 mM Tris, pH 7.4, and NaCl corresponding to the growth medium.

Cell breakage and purification of ribosomes

Washed V. costicola cell pellets were suspended in an equal volume (1.0 ml per gram of wet cell weight) of 0.2 M NaCl, 0.2 M KCl, 40 mM $MgCl_2$, and 10 mM Tris, pH 7.4 (buffer 1) containing 1 µg/ml deoxyribonuclease (Worthington). The cell suspension was placed in an Eppenbach colloid mill with an equal volume of 12 micron glass beads and ground for 20 minutes at 8 C. Glass beads were allowed to settle out and the supernatant was centrifuged at 10,000 xg for 20 min at 4 C. The resulting supernatant was centrifuged twice at 39,000 xg for 20 min to remove whole cells and membrane fragments. Ribosomes were pelleted from the resulting supernatant by centrifugation at 250,000 xg for 3 hours in a Ti60 rotor (Beckman Inst. Co.). Loose membranous material was washed off the top of the ribosome pellet with a stream of buffer 1 from a Pasteur pipet. Ribosomal pellets were stored frozen at -70 C until needed.

Purification of total ribosomal proteins

Ribosomal pellets were suspended in buffer 1 using a teflon-glass homogenizer. Large aggregates were removed by centrifugation at 39,000 xg for 20 min at 4 C. The final ribosome concentration was between 200 and 250 A_{260} units per ml. Total ribosomal proteins were removed by one of two methods. In the first method, the ribosomal suspension was mixed with an equal volume of 7 M LiCl, 8 M urea, and 20 mM ethylenediamine tetraacetic acid and stirred at 4 C for 18 hours. The precipitated ribosomal RNA was removed by centrifugation at 100,000 xg

in a Ti50 rotor (Beckman) for 30 minutes at 4 C. The supernant solution containing the ribosomal proteins was mixed with an equal volume of 20% trichloroacetic acid and stirred at 4 C for 30 minutes. The precipitated ribosomal proteins were collected by centrifugation at 48,000 xg for 20 minutes at 4 C and enough of a mixture of 40mM TRIS-OH, (unbuffered), 8M urea, and 2mM beta-mercaptoethanol added to raise the pH to 7.0 (Howard et al, 1975). The proteins were then dialyzed against 3 changes of 8 liters of distilled-deionized water (12 hours each) and freeze dried. Proteins were stored in this form at -20 C.

The second method of total ribosomal protein extraction involved the acetic acid extraction of the purified ribosomes as described by Hardy et al (1969) and further modified by Howard et al (1975). The resulting ribosomal suspension was extensively dialyzed against water and dried as before. Removal of total ribosomal proteins by either method gave identical pH 8.7 gels.

Purification of acidic ribosomal proteins

Ribosomal pellets (prepared as before) were suspended in 60mM KCl, 10mM $MgCl_2$, and 20 mM TRIS (pH 7.8) to a concentrations of 105 A_{260} units per ml. After removal of large aggregates (39,000 xg for 20 minutes at 4 C), the solution was made 1.0M in NH_4Cl by the addition of three volumes of 4.0M NH_4Cl made up in the suspension buffer. The resulting ribo-nucleoprotein particles (ribosomal RNA and 'core' proteins) were pelleted by centrifugation at 48,000 xg for 20 minutes at 4 C. The supernatant containing the 'split' proteins was carefully decanted

off and the proteins precipitated by the addition of three volumes of cold (-20 C) acetone. After standing at -20 C overnight, as much of the acetone was decanted off as possible. The precipitated proteins were removed from the remaining acetone by centrifugation at 10,000 xg for 10 minutes at 4 C. The protein pellet was dissolved in 100 ml of 6M urea, 10mM TRIS (pH 6.0), and 6mM dithiotreitol (buffer 2). This solution was dialyzed against three 3 liter changes of buffer 2, each for 24 hours at 4 C. The solution was then adjusted to pH 8.0 and applied to the DEAE cellulose column.

DEAE cellulose separation of 'split proteins

Thirty grams of diethylaminoethyl cellulose (DEAE cellulose, SIGMA) was suspended in 750 ml of 0.5N HCl. This was stirred for 5 minutes and filtered using a glass fiber filter (Reeve-Angel). The DEAE cellulose was then washed with distilled deionized water until the effluent had a pH of 4.0. The DEAE cellulose was then suspended in 0.5N NaOH, stirred for 30 minutes, filtered, and washed with water until the effluent had a pH of 8.0. The resulting DEAE cellulose was suspended in 6M urea, 10mM TRIS (pH 8.0), and 6mM dithiothreitol (buffer 3) and poured into a 2.5 x 25cm column (Pharmacia). The DEAE cellulose was allowed to settle and then washed with 500ml of buffer 3 with a flow rate of 20ml/hr.

The 'split' proteins were applied to the column and washed with four volume of buffer 3. A two liter gradient 0-0.18M KCl (made up in buffer 3) was then pumped through the column with a flow rate of 20ml/hr and fractions collected every 30 minutes.

Every fraction was monitored for its absorption at 230nm and peak fractions analyzed for protein purity by gel electrophoresis in pH 8.7 urea gels. Conductance was measured in every tenth tube to monitor gradient linearity using a Radiometer conductivity meter. Pure protein fractions of individual proteins were pooled and dialyzed against for 6 liter changes of distilled deionized water (12 hours each). The protein was then freeze dried, dissolved in water using a trace amount of ammonium carbonate to raise the pH, dialyzed for 24 hours against 6 liters of water, and lyophilized. The purified proteins were stored in this form at -20 C.

Purification of urea

All urea used in the purification of the acidic ribosomal proteins was purified in the following manner. Six liters of 8M urea (Fisher) containing 6ml of methylamine and 300g of activated charcoal was stirred at 4 C overnight. The charcoal was removed by filtration through celite in a Buchner funnel. The filtrate was then filtered through a 5 micron and then a 0.45 micron Millipore filter. The resulting urea solution had an absorbance at 230nm of 0.24.

Gel electrophoresis

The ribosomal proteins were separated by their migration in 7.5% polyacrylamide gels at pH 4.5 and 8.7 (Leboy et al, 1964). Fifteen percent gels were made by using the same procedure but doubling the acrylamide concentration. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis for molecular weight determinations and molecular weight distribution of total ribosomal

proteins were done by the method of Maizel (1971).

Two dimensional electrophoresis was done by a modification of the Kaltschmidt and Wittmann (1970) system. The first dimension, pH 8.6, had an acrylamide concentration of 6% on the basic side and 10% on the acidic side to prevent loss of the more acidic halophilic ribosomal proteins. This dimension was electrophoresed for 16 hours at 3 ma/gel. The second dimension was an 18% acrylamide slab gel, pH 4.6. This dimension was electrophoresed for 40 hours at 80 ma/3 slabs.

Amino acid analysis and protein sequence

For amino acid analysis the proteins were hydrolyzed for 20 hours at 110 C in 6N HCl and the hydrolysates analyzed on a Durrum D-500 amino acid analyzer. Amide nitrogen of the total ribosomal proteins was measured by the method of Stegemann (1958).

Sequence analysis was performed by an automatic Edman degradation in a Beckman Model 890 C Sequenator. For sequence runs, the thiazolinone derivatives were hydrolyzed separately with 6N HCl and HI at 130 C for 22 hours and the amino acids formed were analyzed with the amino acid analyzer (Visentin et al, 1974).

Source of proteins used for comparison

All of the ribosomal proteins used for comparison were supplied by Dr. A.T. Matheson. The data for the amino acid compositions and sequences were supplied by Dr. M. Yaguchi.

Results

Total ribosomal proteins

Sucrose gradients described in the previous chapter indicated that proteins could be removed from the ribosome by high sodium chloride concentrations. Before analysis of the ribosomal proteins of V. costicola, the proper ionic conditions of isolation had to be established. This was done by growing the cells in 1.0 M NaCl and preparing the extracts and purifying the ribosomes in six different salt conditions. It has been shown that for E. coli, the specific monovalent cation affected the amounts of protein removed from the ribosome (Atsmon et al, 1969). Thus, to test the effect of different cations in V. costicola, sodium, potassium, and ammonium chlorides were used in low ionic concentrations (0.1 M) and in relatively high ionic concentrations (0.5 M)[1]. Electrophoretic separation of the resulting purified ribosomal proteins are shown in Figure III-1. All of the patterns appear very similar. The pH 8.7 urea polyacrylamide gel electrophoresis (PAGE) system was used because the population of ribosomal proteins most sensitive of salt extraction were the acidic ribosomal proteins (Visentin et al, 1972). Acidic proteins will move into the gel

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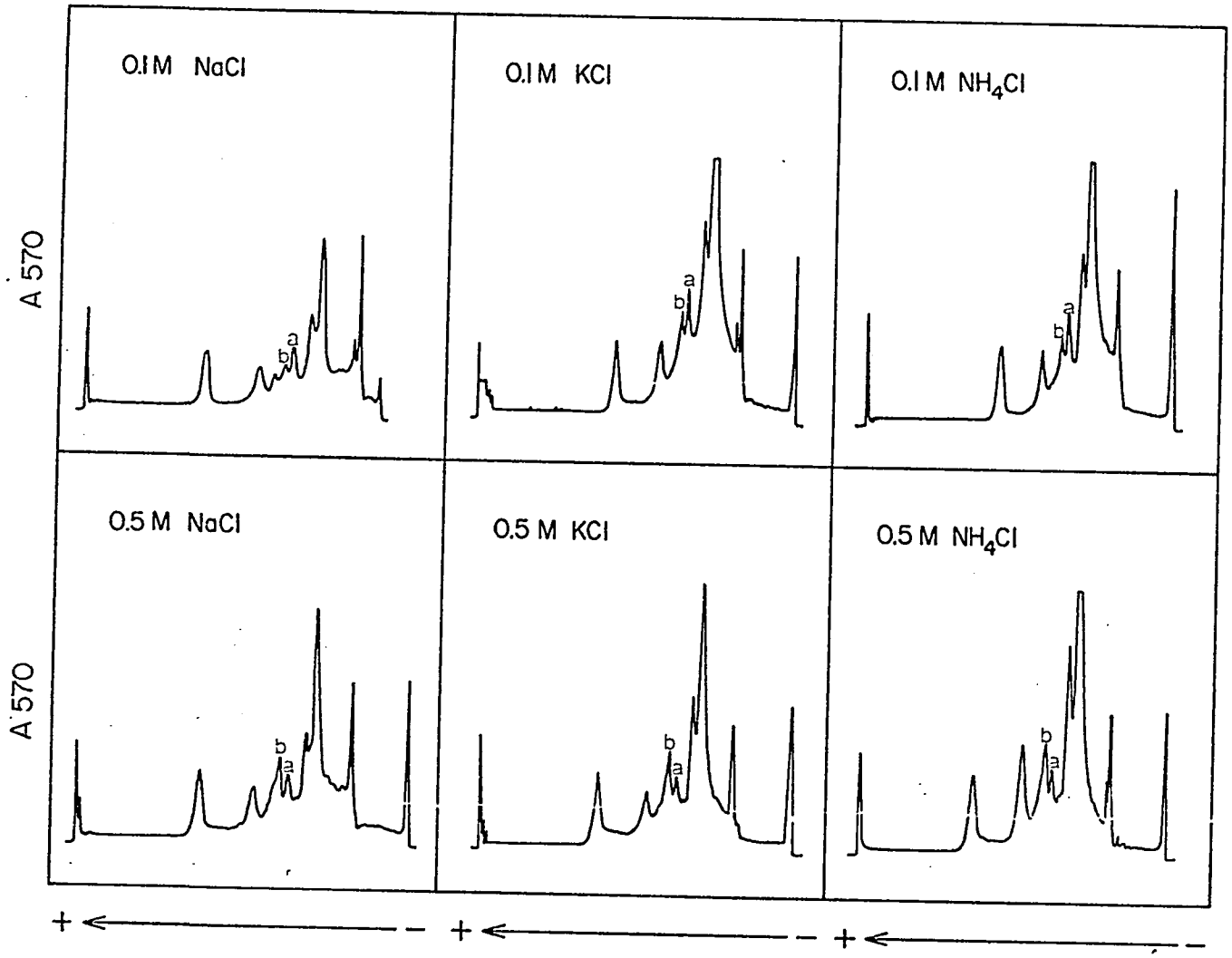
[1] When these experiments were done, it was already realized that protein synthesis took place at approximately 0.1 M. We were concerned with actively functioning ribosomes and hence did not test much higher salt concentrations.

Figure III-1

Effect of salt concentration in the isolation buffer on the distribution of total ribosomal proteins. V. costicola cells were grown in 1.0 M NaCl and the ribosomes purified in 20 mM $MgCl_2$, 10 mM Tris, pH 7.4, and NaCl, KCl, or NH_4Cl as indicated. Ribosomal proteins were extracted by the acetic acid method and run on pH 8.7 urea polyacrylamide gels. Gels were stained with Coomassie blue and scanned at 570 nm using a Gilford spectrophotometer equipped with a linear transport.

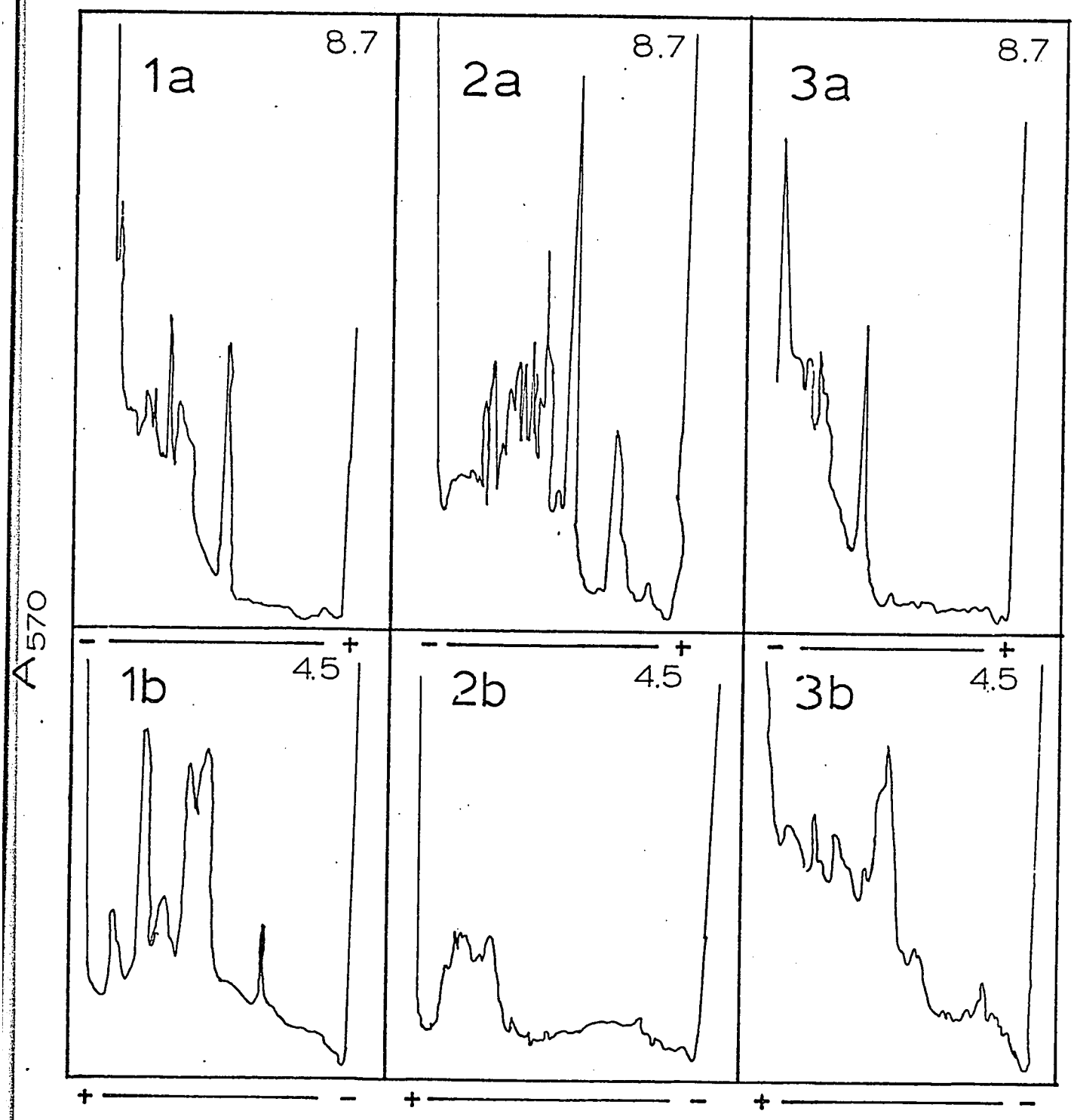

0.1 M NaCl
0.1 M KCl
0.1 M NH_4Cl
0.5 M NaCl
0.5 M KCl
0.5 M NH_4Cl
A 570
A 570
b
a
+
−

at pH 8.7. Only one minor difference in the gel patterns could be observed. Peak A, from the ribosomes isolated in the lower salt concentrations, was higher than peak B, but in the ribosomes isolated in the higher salt concentrations, peak B is higher than peak A. This indicates that either the lower salts facilitate the removal of the protein(s) associated with peak B or that the higher salts facilitate the removal of protein(s) associated with peak A. Data from the sucrose gradients performed in different salt concentrations (Chapter II) indicate that the higher salt concentrations are probably removing proteins from the ribosomes. The lower salt concentrations then, were considered to be more accurate in their representation of the ribosomal protein profile of the biologically active ribosomes (see Chapter IV) and further isolation of total proteins was done in low ionic strength buffers.

To test for the relative acidity of the V. costicola ribosomal proteins, the organism was grown in 1.0 M NaCl and the total ribosomal proteins purified. These purified ribosomal proteins were then separated on polyacrylamide gels of pH 8.7 and 4.5. Acidic proteins will migrate into the gel at pH 8.7 and basic proteins will migrate into the gel at pH 4.5. The electrophoretic patterns of total ribosomal proteins from V. costicola, E. coli, and H. cutirubrum are shown in Figure III-2. The patterns of the proteins in the pH 8.7 gels indicate that the V. costicola ribosomal proteins migrate further into the gel than the ribosomal proteins of E. coli, but less than the H. cutirubrum ribosomal proteins. In the pH 4.5 gels, V. costicola ribosomal

Figure III-2

The relative acidity of the total ribosomal proteins isolated from V. costicola. V. costicola cells were grown in 1.0 M NaCl and the ribosomes isolated in 0.2 M NaCl, 20 mM $MgCl_2$, and 10 mM Tris, pH 7.4. Ribosomal proteins were extracted by the LiCl-urea procedure and run on pH 8.7 gels (frame 1a) and pH 4.5 gels (1b). H. cutirubrum total ribosomal proteins (2a and 2b) and E. coli total ribosomal proteins (3a and 3b) were run for comparison. After electrophoresis, gels were stained with Coomassie blue, destained with 10% acetic acid, and scanned at 570 nm with a Gilford spectrophotometer equipped with a linear transport.


8.7
1a
8.7
2a
8.7
3a
A570
4.5
1b
4.5
2b
4.5
3b

proteins do not migrate as far as those of E. coli, but further than those of H. cutirubrum. These results indicate that the ribosomal proteins of V. costicola are more acidic than those of E. coli, but not as acidic as those of H. cutirubrum.

This method can only give qualitative evaluation of the acidic nature of the ribosomal proteins. The zwitterion characteristic of amino acids and thus proteins makes it possible for the same proteins to migrate into both the pH 8.7 and 4.5 gels. For a more quantitative determination of the acidic nature of the V. costicola ribosomal proteins, the amino acid composition was determined (Table III-1). The measure of protein acidity most commonly used is the base/acid (B/A) ratio (Visentin et al, 1972). This is calculated by dividing the mole percent of the total basic amino acids (histidine+lysine+arginine) by the mole percent of the total acidic amino acids (glutamate+aspartate). For a relatively neutral protein, the B/A would be equal to 1.0. Calculating the B/A ratio for V. costicola, E. coli, H.x. (Falkenberg et al, 1976), and H. cutirubrum results in values of 0.78, 0.99, 0.86, and 0.49 respectively. This method presents problems in that the digestion of the protein necessary for the amino acid analyzer, converts glutamine to glutamate and asparagine to aspartate. Glutamate and aspartate are acidic amino acids, but in the amide form of glutamine and asparagine they assume neutral properties. To calculate the amide concentration of V. costicola total ribosomal proteins, 1.0 mg of the proteins were analyzed for amide content by the method of Stegemann (1958). This gave a value of 5.9 moles of amide nitrogen

Table III-1

Amino acid composition of total ribosomal proteins.

(values expressed as moles percent)

	V. costicola	E. coli[1]	H.x[1]	H. cutirubrum[1]
Asp	9.7	8.3	8.7	12.7
Thr	4.8	5.2	5.4	5.9
Ser	4.0	4.4	4.9	5.3
Glu	11.4	10.1	11.9	13.9
Pro	3.7	3.7	3.6	4.0
Gly	8.8	8.2	9.5	9.8
Ala	11.4	11.0	10.6	11.2
Cys	-	0.5	-	0.7
Val	8.8	9.6	8.3	8.6
Met	2.5	2.4	2.5	1.3
Iso	5.5	5.5	4.6	3.4
Leu	7.9	7.4	7.6	6.4
Try	2.0	1.8	2.1	1.8
Phe	3.2	3.0	2.6	1.9
His	2.1	1.9	1.8	1.9
Lys	7.7	9.0	8.3	4.4
Arg	6.6	7.3	7.6	6.8
Amide N	5.9	7.1	6.2	4.7
B/A	0.78	0.99	0.86	0.49
B/A corrected for amide	1.08	1.58	1.23	0.69
$H\phi_{avg}$[2]	1.044	1.057	0.999	0.850

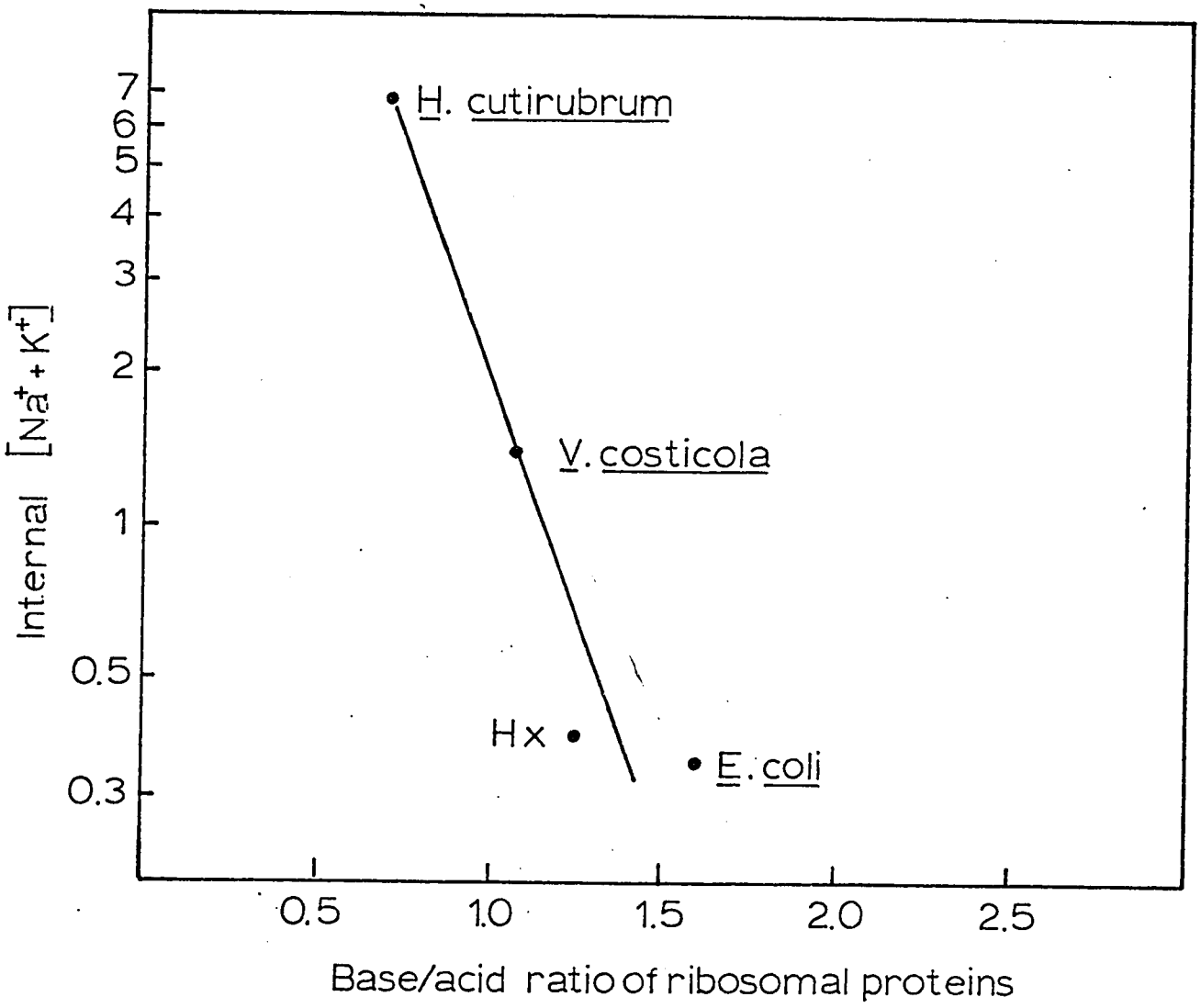
[1] Data from Falkenberg et al (1976)

[2] Values expressed as kilocalories.

per mole of total V. costicola ribosomal protein. The average molecular weight of the V. costicola ribosomal proteins was assumed to be 17,320, the average molecular weight of E. coli ribosomal proteins (Wittmann, 1974). This molecular weight assumption was based on the observations of Sun et al (1972) that all of the procaryotic ribosomes studied have the same molecular weight distribution of ribosomal proteins and on the SDS-gel electrophoresis of total V. costicola ribosomal proteins which show a similar distribution to those of E. coli (Figure III-5). Correcting for the amide concentration, the B/A ratios for the total ribosomal proteins from V. costicola, E. coli, H.x., and H. cutirubrum are 1.08, 1.58, 1.23, and 0.69, respectively. Quantitatively, then, V. costicola ribosomal proteins have an acidic character exactly between that of E. coli and H. cutirubrum. To correlate this with the halophilic nature of the organism, the internal ion concentration was used as an index. V. costicola grown at its optimum of 1.0 M NaCl has an internal $Na^+ + K^+$ concentration of 1.41 (Shindler et al, 1977). E. coli grown at 0.09 M NaCl has an internal $Na^+ + K^+$ concentration of 0.35 M (Schultz et al, 1962). The intracellular $Na^+ + K^+$ of H.x. and H. cutirubrum grown at their optima (1.0 and 4.0 M NaCl) are 0.39 and 6.12 M, respectively (Falkenberg et al, 1976). If the B/A ratio of the ribosomal proteins is plotted against the logarithm of the internal $Na^+ + K^+$ concentration, a straight line results through the halophiles (Figure III-3) indicating a relationship between the internal salt concentration of the organism and the acidity of the ribosomal proteins.

Figure III-3

Correlation between the internal cation concentration and the Base/Acid ratio of the total ribosomal proteins. The internal $Na^+ + K^+$ concentrations (from literature values) for V. costicola grown in 1.0 M NaCl (Shindler et al, 1977), E. coli grown in 0.09 M NaCl (Schultz et al, 1967), H. cutirubrum and H.x. grown at their respective optimums of 4.0 and 1.0 M NaCl (Matheson et al, 1976) were plotted on semi-log paper against the Base/Acid ratio of the total ribosomal proteins isolated from these organisms (Table III-1).

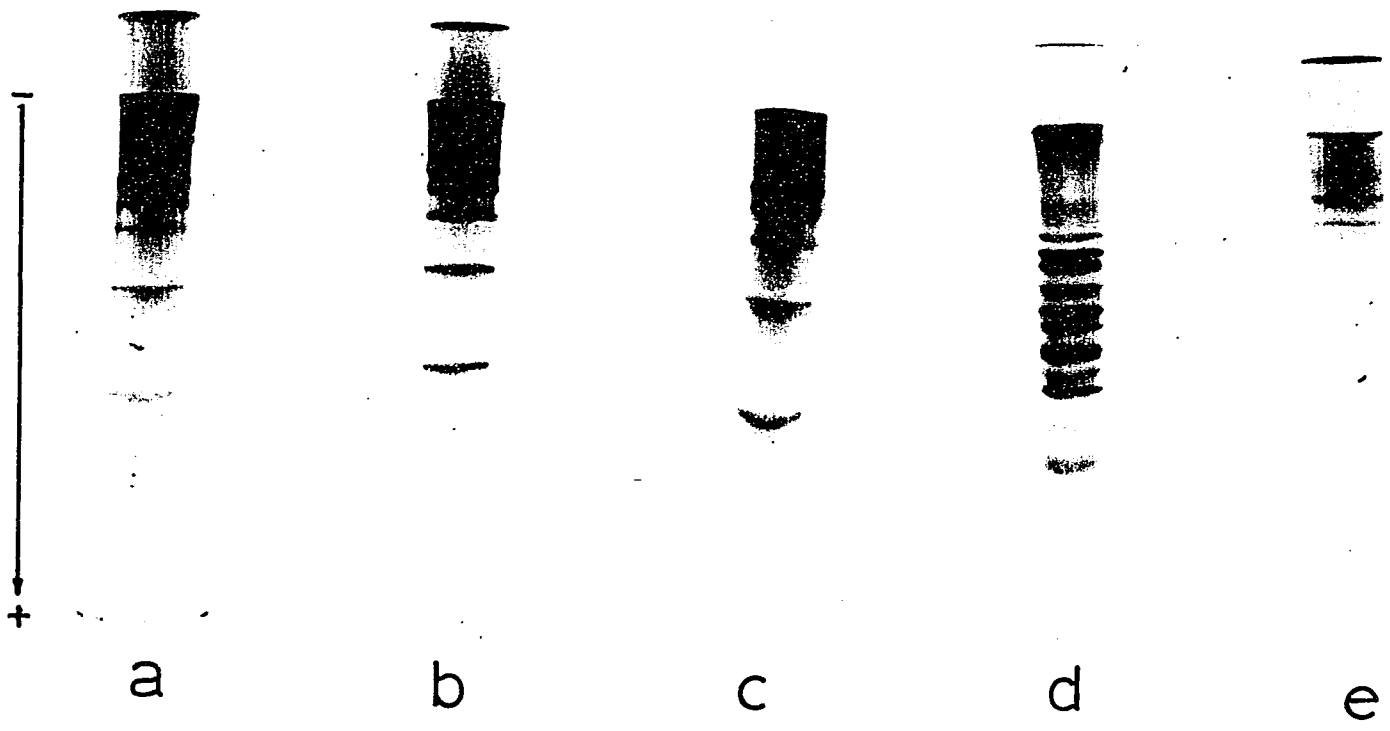

H. cutirubrum
V. costicola
Hx
E. coli
Internal [Na+ + K+]
7
6
5
4
3
2
1
0.5
0.3
0.5
1.0
1.5
2.0
2.5
Base/acid ratio of ribosomal proteins

Another structural parameter that can be measured from the amino acid composition is the average hydrophobicity, $H\phi_{avg}$ (Bigelow, 1967). The average hydrophobicity is based on the free-energy of transfer of the amino acid side chains from an organic environment to an aqueous environment. This value is a measure of the non-polarity of the protein molecules. The values are expressed in kilocalories and the higher the value, the more non-polar the molecule. Calculating the $H\phi_{avg}$ for the total ribosomal proteins results in values of: 1.044, 1.057, 0.999, and 0.850 kcal for V. costicola, E. coli, H.x., and H. cutirubrum, respectively.

The internal $Na^+ + K^+$ concentration of V. costicola increases from 1.41 M, when the cells are grown in 1.0 M NaCl, to 2.15 M when the cells are grown in 3.0 M NaCl (Shindler et al, 1977). Since there seems to be a correlation between the internal $Na^+ + K^+$ concentration and the acidity of the ribosomal proteins, it seemed interesting to test whether the ribosomal proteins of V. costicola became more acidic if the organism was grown in higher salt concentrations. V. costicola was grown in 0.5, 1.0, and 3.0 M NaCl and the ribosomal proteins isolated. Falkenberg et al (1976) found that there could be differences in the ribosomal protein populations due to different ionic conditions of isolation. To avoid this, identical conditions of isolation (0.2 M KCl, 20 mM $MgCl_2$, 10 mM Tris, pH 7.6) were used. Figure III-4 is the electrophoretic patterns (pH 8.7) of the total ribosomal proteins from V. costicola grown in different NaCl concentrations. Gel electrophoresis in pH 8.7 gels was used because it was the shift

Figure III-4

The effect of medium NaCl concentration on the acidity of the total ribosomal proteins from V. costicola. Cells were grown in (a) 0.5 M, (b) 1.0 M, and (c) 3.0 M NaCl and the ribosomes isolated in 0.2 M KCl, 20 mM $MgCl_2$, and 10 mM Tris, pH 7.4. Extraction of the ribosomal proteins was by the acetic acid method. Proteins were run on 7½% pH 8.7 urea polyacrylamide gels and stained with Coomassie blue. H. cutirubrum total ribosomal proteins (d) and E. coli total ribosomal proteins (e) are shown for comparison.


−
+
a
b
c
d
e

in acidity of the ribosomal proteins that was of interest, and the acidic proteins migrate into the gel at this pH. Figure III-5 is the molecular weight distribution of the same ribosomal proteins but run in an SDS-gel system. All protein bands in one salt concentration of V. costicola were discernible in the other salt concentrations by examination of the gels by eye, although they are resolved poorly by the photograph. From the patterns in the pH 8.7 and the SDS-gel systems there appears to be no change in the ribosomal proteins when V. costicola is grown in different salt concentrations.

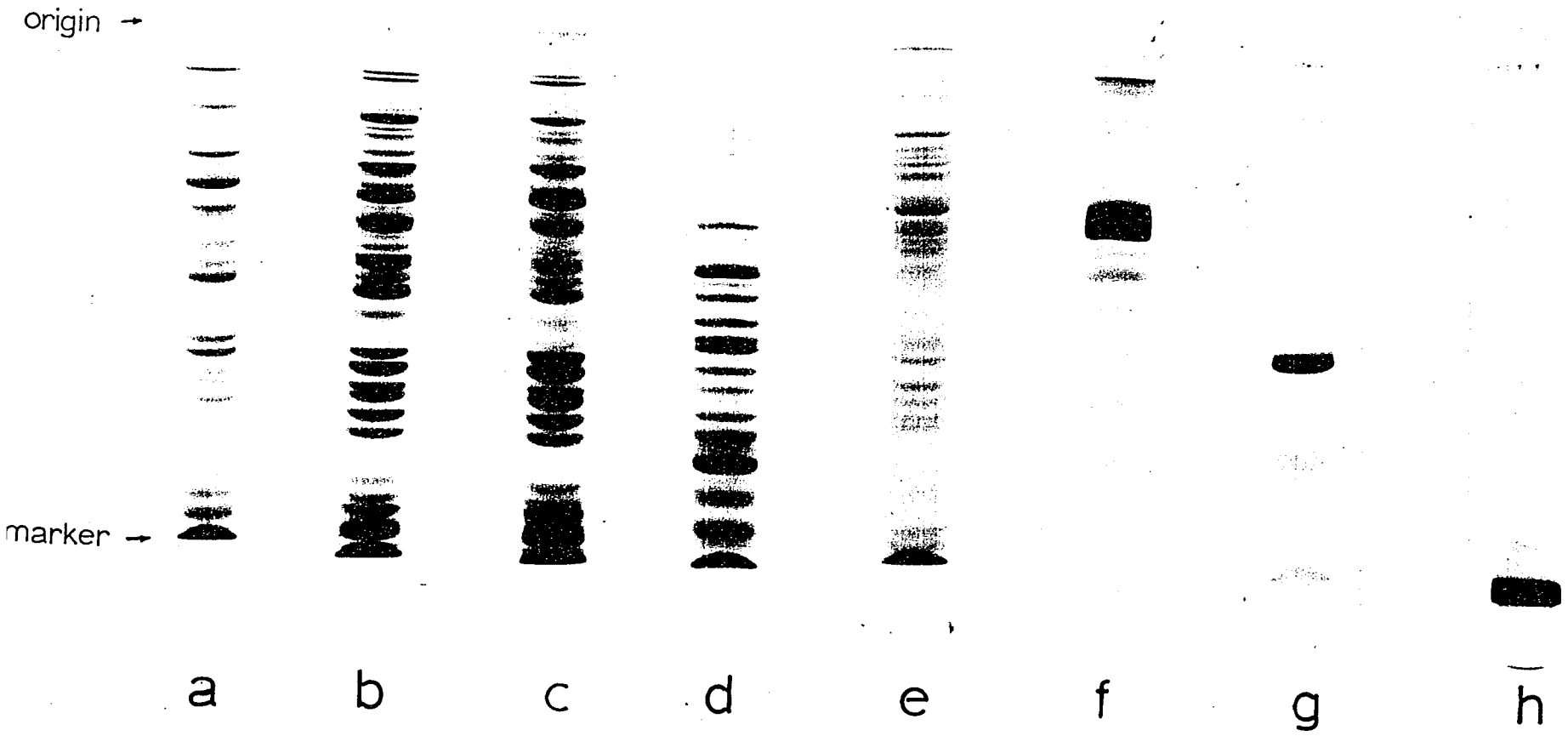
Purification of ribosomal proteins

The correlation between the acidity of the ribosomal proteins and the halophilic nature of the organism raised some questions as to the evolutionary relationship between these organisms. One of the ways that evolutionary relationships have been determined is by the comparison of sequences of functionally similar proteins. Such has been done for cytochrome C, hemoglobin, and fibrinopeptides (Dickerson, 1971). Since some sequence data was available on equivalent ribosomal proteins from E. coli, H.x., and H. cutirubrum, similar data on an equivalent protein from V. costicola might better define the relationship of V. costicola to these other organisms.

The electrophoretic patterns in the pH 8.7 PAGE (Figure III-4) show one protein band that migrates further than any other bands. In E. coli, this protein is the most acidic ribosomal protein (L7/12) and is characterized by its high alanine content (Terhorst et al, 1972). A similar protein has been isolated from

Figure III-5

Molecular weight distribution of total ribosomal proteins of V. costicola grown in different salt concentrations. V. costicola was grown in different salt concentrations and the ribosomes and ribosomal proteins isolated as described in Figure III-4. The total ribosomal proteins were run on SDS-polyacrylamide gels and stained with Coomassie blue: (a) total ribosomal proteins from V. costicola grown in 0.5 M NaCl, (b) total ribosomal proteins from V. costicola grown in 1.0 M NaCl, (c) total ribosomal proteins from V. costicola grown in 3.0 M NaCl, (d) total ribosomal proteins from H. cutirubrum, (e) total ribosomal proteins from E. coli, (f) bovine serum albumin, MW 65,000, (g) deoxyribonuclease, MW 31,000, (h) ribonuclease A, MW 13,700.


origin
marker
a
b
c
d
e
f
g
h

a thermophile, Bacillus stearothermophilis (Visentin et al, 1974) and the extreme halophile, H. cutirubrum (Oda et al, 1974). Another characteristic of this protein in E. coli is that it can be washed from the ribosome, along with 8 other proteins, with 1.0 M NH_4Cl and 50% ethanol (Terhorst et al, 1972). Purified ribosomes from V. costicola grown in 1.0 M NaCl were washed with 1.0 M NH_4Cl and 50% ethanol. The ribosomal proteins thus removed were separated on a DEAE cellulose column (see Methods). The elution profile is shown in Figure III-6. From the DEAE cellulose column, two proteins were eluted in pure form. Figure III-7 shows the electrophoretic patterns of the purified ribosomal proteins following each step of purification.

The first ribosomal protein eluted from the DEAE cellulose column had an apparent molecular weight of 43,500 as judged by SDS-gel electrophoresis (Figure III-8). This is a relatively high molecular weight for a ribosomal protein, so first indications were that it might be analogous to the S1 protein of E. coli which has a molecular weight of 65,000 (Wittmann, 1974). Further indication that this V. costicola protein was analogous to S1 was that S1 is also removed during a NH_4Cl-ethanol wash of E. coli ribosomes and that it cross-reacted with antibody formed against S1 in Ouchterlony double diffusion tests (A. Matheson, personal communication). All of the E. coli ribosomal proteins have been shown to be immunologically unique, that is, they do not cross-react with each other (Stöffler and Wittmann, 1971).

The results of amino acid analysis of this protein along

Figure III-6

Elution profile of ribosomal proteins on a DEAE cellulose column. *V. costicola* "split" ribosomal proteins washed from the ribosomes with a NH_4Cl-ethanol wash (see Figure III-7) were separated on a DEAE cellulose column with a 2 liter 0-0.18 M KCl gradient. Protein was monitored at 230 nm and the gradient was monitored by conductivity. The positions of the two ribosomal proteins eluted in pure form are indicated. The peaks labeled A and B could be two forms of the most acidic ribosomal protein as will be discussed in the text.

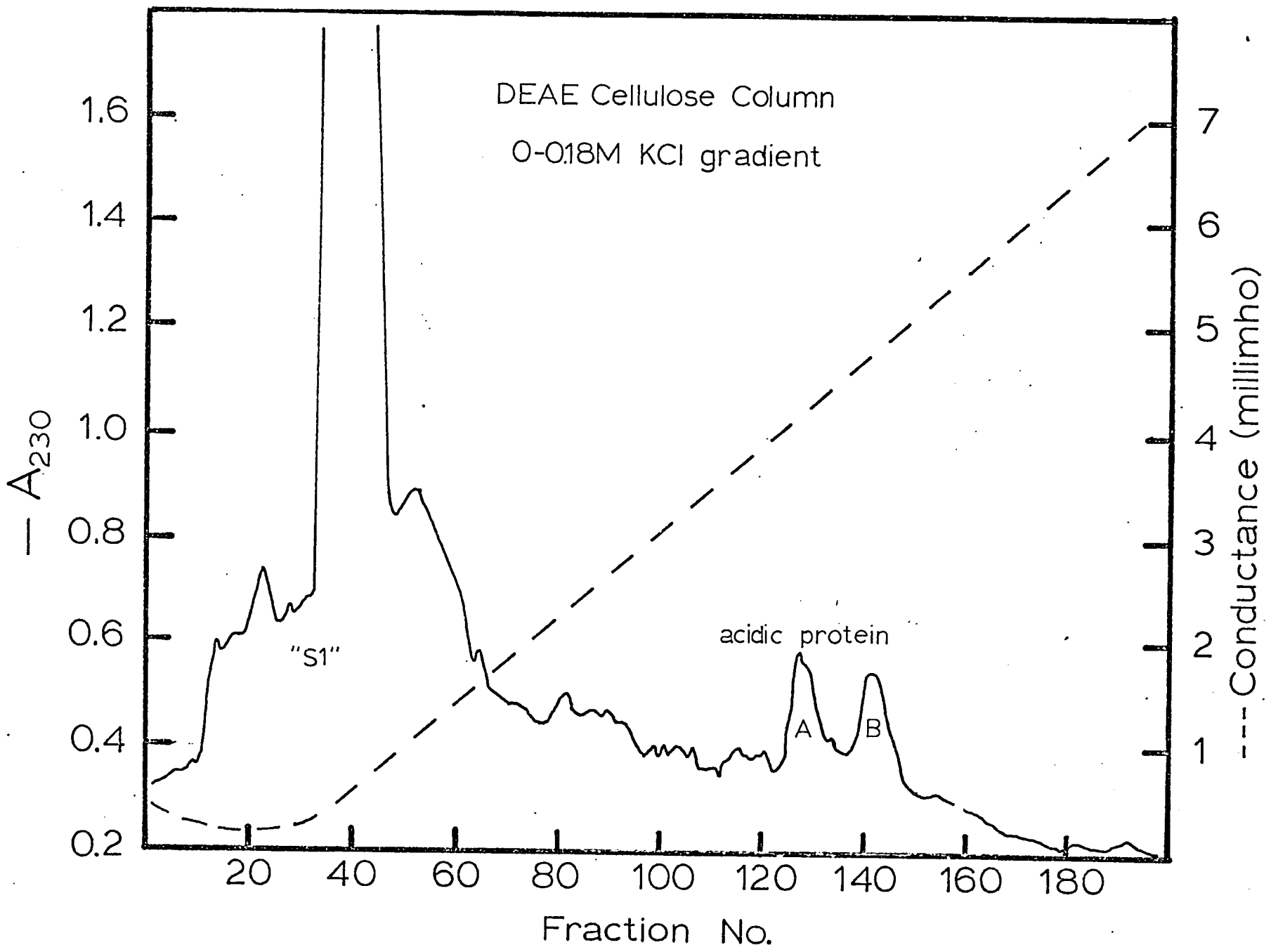

DEAE Cellulose Column
0-0.18M KCl gradient
"S1"
acidic protein
A
B
A_{230}
Conductance (millimho)
Fraction No.
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
7
6
5
4
3
2
1
20
40
60
80
100
120
140
160
180

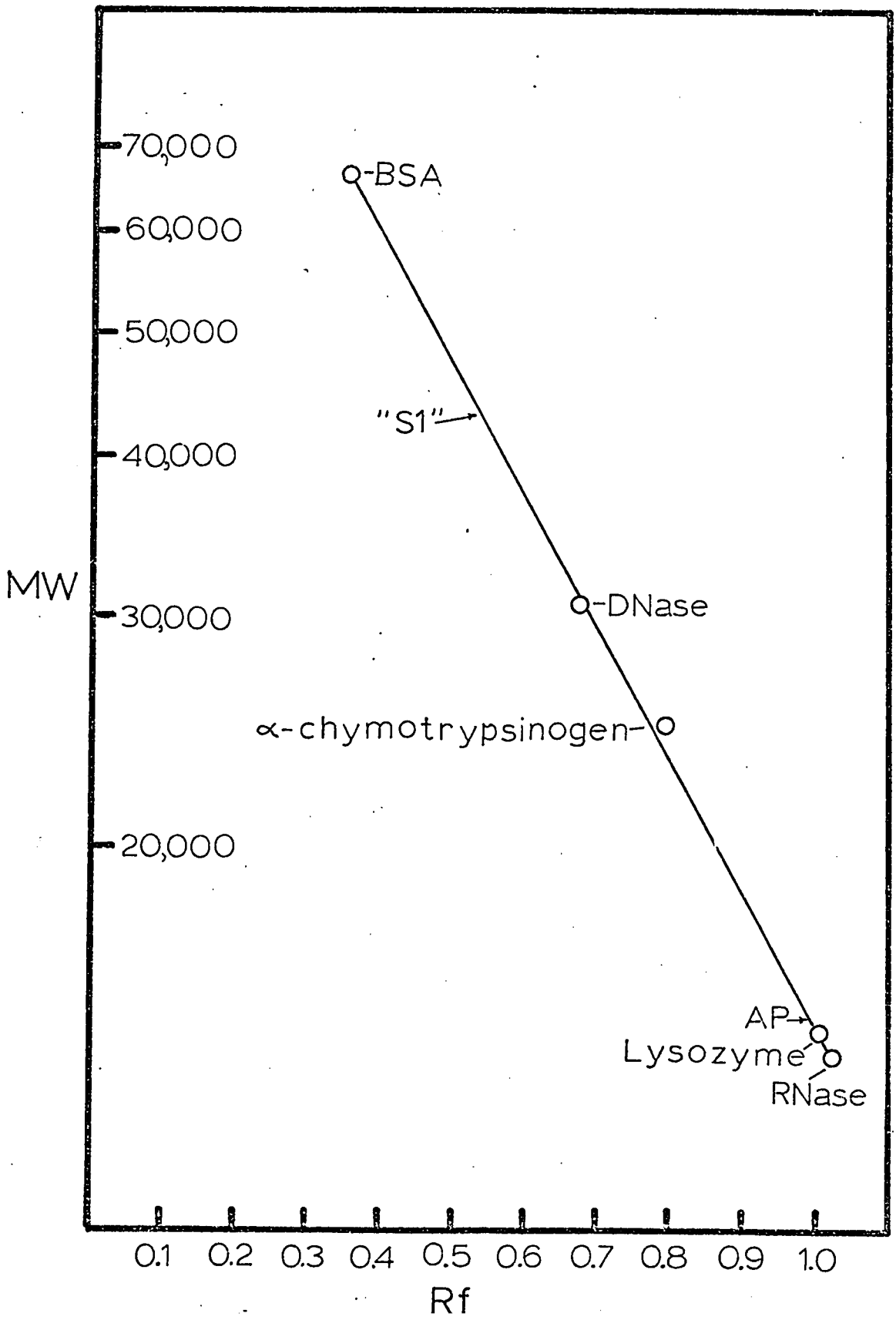
Figure III-7

Gel electrophoresis showing the purification of the ribosomal proteins. Samples were electrophoresed in pH 8.7 urea polyacrylamide gels, stained with Coomassie blue, and destained with 10% acetic acid. (a) total ribosomal proteins, (b) "split" ribosomal proteins (removed by NH_4Cl-ethanol wash), (c) the high molecular weight ribosomal protein analogous to *E. coli* S1, (d) the most acidic ribosomal protein (protein B) analogous to *E. coli* L7/12.

a
b
c
d
-
+

Figure III-8

Determination of molecular weights of purified ribosomal proteins by SDS-gel electrophoresis. Purified ribosomal proteins of V. costicola were run on SDS gels along with molecular weight standards. BSA, bovine serum albumin MW 65,000; "S1", V. costicola ribosomal protein equivalent to E. coli S1; DNase, deoxyribonuclease MW 31,000; alpha-chymotrypsinogen MW 26,000; AP, V. costicola acidic ribosomal protein (protein B); lysozyme MW 14,400; and RNase, ribonuclease A MW 13,700.


70,000
60,000
50,000
40,000
MW
30,000
20,000
BSA
"S1"
DNase
α-chymotrypsinogen
AP
Lysozyme
RNase
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Rf

with E. coli S1 and an equivalent protein from H.x. are shown in Table III-2. No analogous protein had been identified in H. cutirubrum and B. stearothermophilis has been shown to lack any protein similar to S1 (Isono and Isono, 1976). The B/A ratios for these analogous proteins were 0.59, 0.54, and 0.50 for E. coli, H.x., and V. costicola, respectively. Although the B/A ratio does not vary greatly, V. costicola 'S1' remains the more acidic. The average hydrophobicity calculated from the amino acid compositions are 1.049, 0.975, and 0.963 kcal for V. costicola, E. coli, and H.x., respectively.

Using the method of Schachman (1957) for determining the partial specific volume of a protein by its amino acid composition results in a $\bar{v}$ = 0.734 for the V. costicola protein which is very close to the 0.737 value determined experimentally for the E. coli S1 (Craven et al, 1969). Since comparative data was not available for the extreme halophiles no further characterization of this protein was done.

The most acidic protein eluted from the DEAE cellulose column gave two peaks (A and B) (Figure III-6). The fractions from this area of the column were analyzed for purity on 15% pH 8.7 urea PAGE. The first peak (A) migrated as a faster moving band than the second peak (B). The first peak (A) always contained some of the second peak. At tube 134 (Figure III-6), the protein bands from peak A and B were in equal amounts and as the fractions increased, the amount of peak A rapidly decreased until tube 138 showed only the protein associated with peak B. For ease of explanation, the protein from peak A will be referred to

Table III-2

Amino acid composition of "S1" equivalent ribosomal proteins.

(values expressed as mole percent)

	V. costicola	*E. coli*[1]	H.x.[2]
Asp	9.9	11.6	9.7
Thr	6.4	4.3	6.3
Ser	3.5	4.4	4.6
Glu	12.9	14.1	12.6
Pro	4.6	1.9	3.0
Gly	7.8	8.9	10.9
Ala	13.2	9.3	11.4
Cys	-	0	-
Val	6.2	11.3	8.9
Met	3.3	1.0	4.3
Isc	5.1	5.3	5.5
Leu	8.8	8.2	7.3
Tyr	3.0	1.1	1.4
Phe	3.8	3.1	1.9
His	2.2	1.5	0.7
Lys	4.8	8.2	6.7
Arg	4.5	5.5	4.7
B/A	0.50	0.59	0.54
$H\phi_{avg}$[3]	1.049	0.975	0.963

[1] Data from Craven *et al* (1969).

[2] Data from Falkenberg *et al*, unpublished.

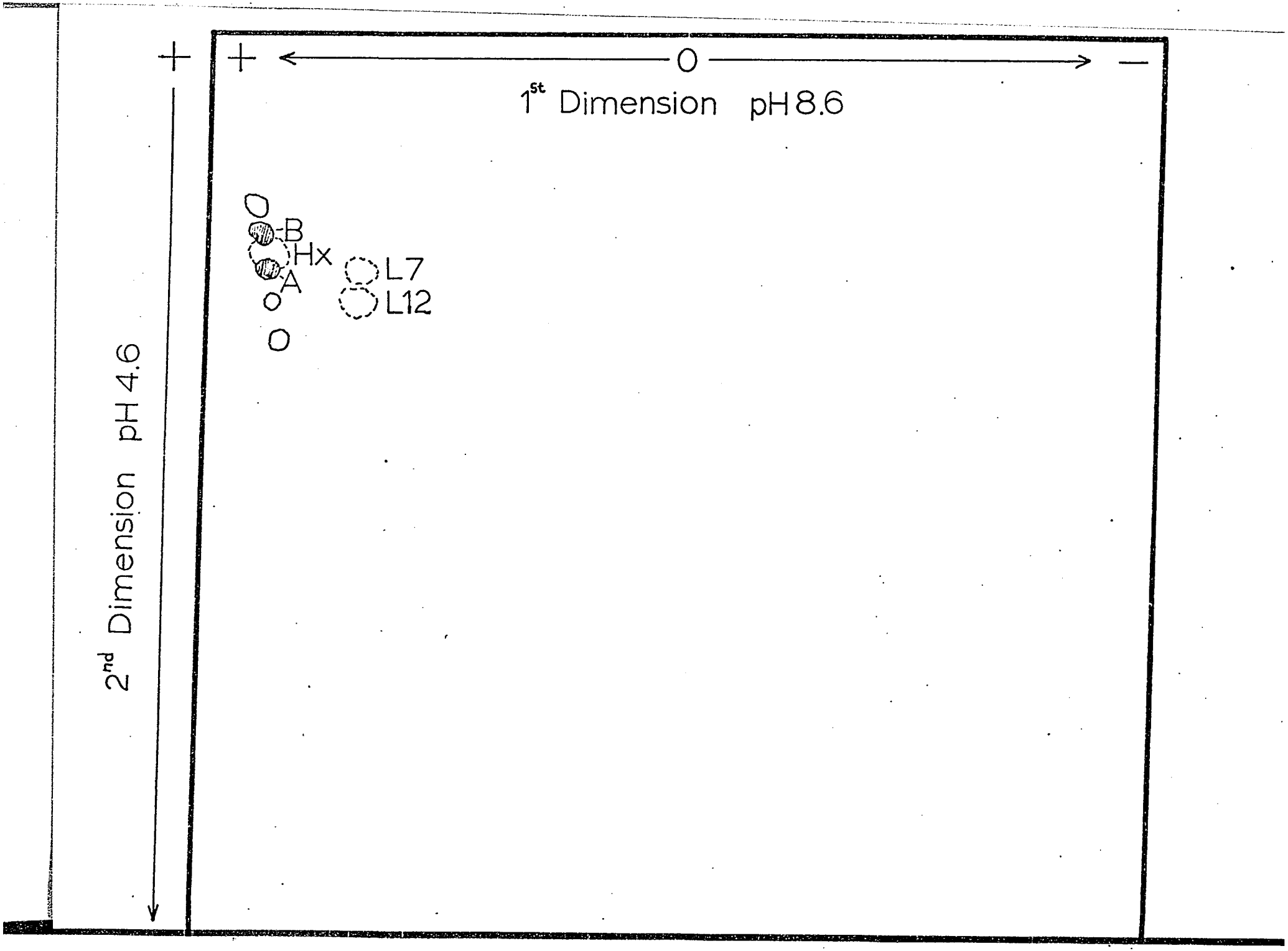
[3] Data expressed as kilocalories.

as protein A, and the protein from peak B as protein B.

It was first thought that V. costicola had two forms of the acidic protein as is the case with E. coli. In E. coli, the difference between the two acidic proteins is that one (L7) has an acetylated N-terminal serine, while the other (L12) has a free N-terminal serine (Terhorst et al, 1972). Other than the acetylated serine, the amino acid sequences of the two proteins are identical (Terhorst et al, 1973). Proteins A and B were run on SDS gels to determine if there was a detectable difference in molecular weight. Both proteins A and B migrated to a distance corresponding to a molecular weight of 14,500 * 1,000. The amino acid compositions of the two proteins were also identical. As a further check on the purity of proteins A and B, they were run on the two dimensional electrophoresis system of Kaltschmidt and Wittmann (1970) as modified in the Methods section. Protein A separated into two major spots and two minor spots, and protein B into one major spot and two minor spots. One of the minor spots of protein B corresponded to one of the major spots of protein A and one of the major spots of protein A corresponded to one of the minor spots of B. A schematic representation of the two-dimensional electrophoresis patterns is shown in Figure III-9 along with the relative positions of L7, L12, and the H.x. acidic protein. The minor spots of protein B together represent less than 10% of the total material. If these two proteins are similar to the L7/12 of E. coli, then similarities should be evident by their sequence. Small amounts of proteins A and B were placed in the automatic sequenator and the process

Figure III-9

Two dimensional electrophoresis of V. costicola acidic ribosomal protein. The two peaks (A and B) of the acidic ribosomal protein from V. costicola eluted from the DEAE cellulose column were electrophoresed in the two dimensional system of Kaltschmidt and Wittmann (1970) as modified in the Methods section. The spot labeled A is one of the major spots associated with the first acidic peak from the DEAE column (peak A). The spot labeled B is the major spot from the second peak (B) eluted from the DEAE column. The relative positions of E. coli L7 and L12 and the acidic ribosomal protein of H.x. are indicated. The two lower unlabeled spots were the minor contaminants of A, while the upper unlabeled spot was a minor contaminant of B.


+
+
0
−
1st Dimension pH 8.6
2nd Dimension pH 4.6
B
Hx
A
L7
L12

run to sequence the first four residues. Both proteins could be sequenced thus indicating that neither had a blocked N-terminus. Such a blockage would prevent sequencing by the Edmann degradation used in the automatic sequenator. These results could also mean that protein A contained enough of protein B to show sequencing. It is therefore still possible that V. costicola has two forms of the acidic protein as does E. coli. Another possible explanation is that somehow the isolation conditions changed the secondary structure of some of the acidic protein resulting in different behavioral characteristics. Since protein B seemed to be the purer protein, it was used in all of the following studies.

The acidic protein isolated from V. costicola had a molecular weight of 14,500 as determined by SDS-PAGE (Figure III-8) and 12,900 from the amino acid composition. These are close to the value reported for E. coli L7/12 (13,400) (Wittmann, 1974). The amino acid composition of the V. costicola acidic protein (Table III-3) revealed a high (18%) alanine content, which is also a characteristic of E. coli L7/12 (23%) (Terhorst et al, 1973). The V. costicola acidic protein also contains some monomethyllysine which is yet another characteristic of L7/12. Thus, since the most acidic ribosomal protein of V. costicola has a high alanine content, a relatively low molecular weight, the ability to be removed by NH_4Cl-ethanol from the ribosome, and some methyllysine, this protein and the E. coli L7/12 are probably analogous.

The calculation of partial specific vomume gives the V. costicola acidic protein a $\bar{v} = 0.741$, indistinguishable from the 0.740 value for L7/12 (Wong and Paradies, 1974). Calculating

Table III-3

Amino acid composition of "L7/12" equivalent ribosomal proteins.

(values expressed as mole percent)

	V. costicola	E. coli[1]	H.x.[2]	H. cutirubrum[3]
Asp	6.2	6.2	6.0	19.5
Thr	3.4	2.6	4.8	2.0
Ser	4.1	5.3	4.9	3.7
Glu	20.1	15.0	19.4	18.4
Pro	3.0	1.7	1.4	2.8
Gly	9.4	7.6	10.3	8.1
Ala	17.8	23.4	17.5	24.2
Cys	-	-	-	-
Val	9.6	14.0	10.2	6.1
Met	2.5	2.5	3.3	0.9
Iso	4.1	5.0	2.2	2.7
Leu	8.0	7.0	7.5	7.2
Tyr	0.6	0	0	1.8
Phe	1.7	1.6	1.6	0.8
His	0.6	0	0	0
Lys	7.0	10.5	9.4	1.0
Arg	1.9	0.9	1.0	0.9
B/A	0.36	0.54	0.41	0.05
$H\phi_{avg}$[4]	0.917	1.024	0.841	0.726

[1] Data from Terhorst et al (1972).

[2] Data from Falkenberg et al, unpublished.

[3] Data from Strom et al (1975).

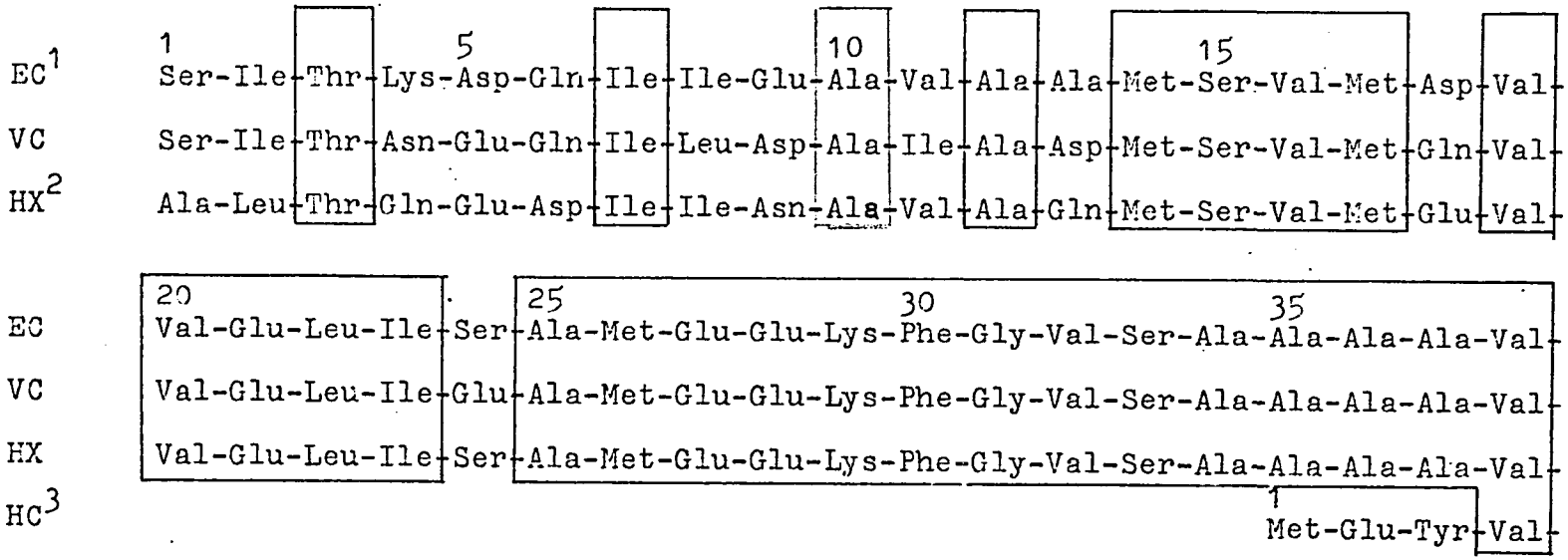
[4] Values expressed in kilocalories.

the B/A ratio for the proteins in Table III-3 results in values of: 0.54, 0.41, 0.36, and 0.05 for E. coli, H.x., V. costicola, and H. cutirubrum, respectively. In this case, the V. costicola acidic protein closely resembles that of E. coli rather than being halfway between E. coli and H. cutirubrum. It is still more acidic than the L7/12. There is one relationship that the V. costicola acidic protein has in common with H. cutirubrum. In the amino acid composition; H. cutirubrum acidic protein has two tyrosine residues which are absent in the E. coli L7/12. The V. costicola acidic protein contains one residue of tyrosine. V. costicola also shows one residue of histidine which does not occur in any other L7/12 equivalent protein. The average hydrophobicity calculated from the amino acid composition of these proteins were 0.917, 1.024, 0.841, and 0.726 kcal for V. costicola, E. coli, H.x., and H. cutirubrum, respectively.

Since sequence data was already available on several equivalent acidic proteins, as much of the V. costicola acidic protein was sequenced as possible using the automatic sequenator. Thirty-eight residues from the N-terminal end of the protein could accurately be determined. The sequence of the V. costicola protein is shown in Figure III-10 along with the sequences of analogous proteins from E. coli, H.x., and H. cutirubrum. The protein sequences are arranged so that homologous regions are aligned. From the sequences of these proteins, an indication of the percent homology can be obtained. This is calculated by dividing the number of homologous residues by the total number of residues sequenced (38 in this case) and multiplying the result by 100.

Figure III-10

Amino acid sequence of acidic ribosomal protein (equivalent to E. coli L12) from V. costicola, E. coli, H.x., and H. cutirubrum. The amino acid composition of the first 38 residues of the V. costicola acidic ribosomal protein was determined by an Edmann degradation in an automatic sequenator. For comparison, the sequence data from the other halophiles and E. coli are included. VC, Vibrio costicola; EC, Escherichia coli; H.x., unclassified moderate halophile; and HC, Halobacterium cutirubrum. Proteins are positioned so that regions of homology are aligned.



[1]From Terhorst et al (1973).

[2]From Falkenberg et al, unpublished.

[3]From Oda et al (1974).

A summary of these calculations is presented in Table III-4.

When compared to L7/12, the sequence of the V. costicola acidic protein has some interesting features. The L7/12 has a high (50-60%) alpha helix content, while the other ribosomal proteins of E. coli have an average of 25% alpha helix (Möller et al, 1970). If the sequenced segment of the V. costicola acidic protein is analyzed by the method of Chou and Fasman (1974) for predicting alpha helix, then the protein segment has exactly the same helical content as L7/12 (Figure III-11). This means that the amino acid changes that have occured between V. costicola acidic protein and the L7/12 have similar 'helical potential' resulting in conservation of secondary structure.

The N-terminal region (residues 1-55) of the L7/12 protein molecule is characterized by its high negative charge and hydrophobic residues (Terhorst et al, 1973; Wittmann, 1974). The amino acid composition of this end of the acidic protein of V. costicola has decreased the hydrophobicity by one residue and increased the negative charge by two residues. Both of these characteristics may be related to the mechanism in which halophilic proteins have adapted to their saline environment (Lanyi, 1974).

"Genetic distances" between organisms can be calculated from sequence data. This method was devised by Fitch (1966) in which he used the codon assignments for each amino acid and calculated the minimum number of mutations that could take place to change one amino acid to another. In position 4 of the E. coli and V. costicola sequence, to change the lysine (AAA or AAG) to asparagine (AAU or AAC) or vice versa, would require only one mutation, while the amino acid change that occurs in position 18, aspartate (GAU

Table III-4

Percent homology and genetic distances between acidic ribosomal proteins.

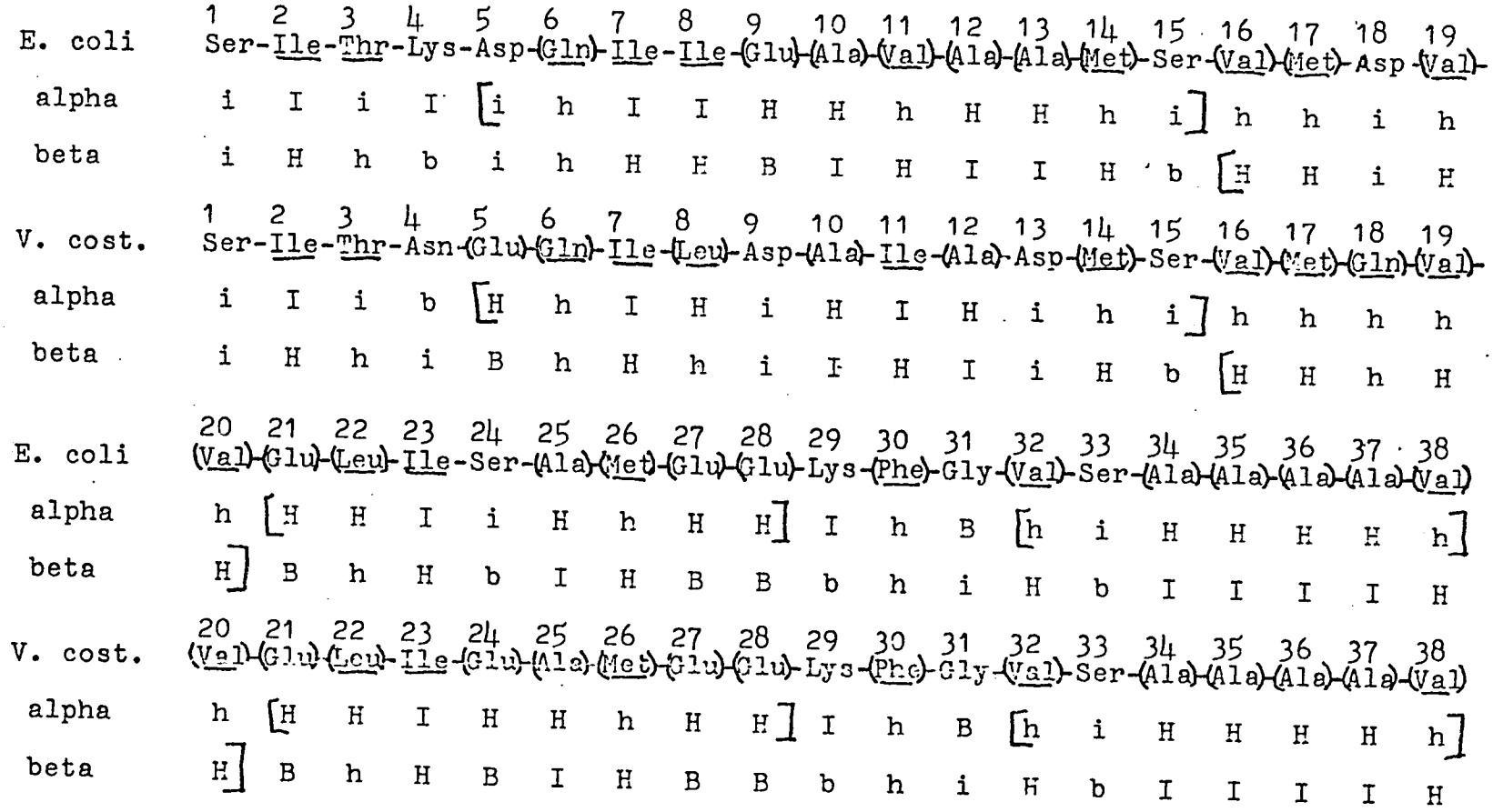
Compared proteins	amino acid[1] changes	% Homology	Minimum # mutations[2]
E. coli-*V. costicola*	8	79	11
E. coli - H.x.	10	74	12
V. costicola - H.x.	12	68	15

[1] Out of the first 38 residues.

[2] Calculated by the method of Fitch (1966).

Figure III-11

Predictive analysis of helical and sheet regions in the acidic ribosomal proteins of V. costicola and E. coli based on the amino acid sequence. Assignments in the first row under the residue refer to the α-helical potenial and in the second row to the beta sheet potential. Assignments are: H, strong former; h, former; I, weak former; i, indifferent; b, breaker; B, strong breaker. Helical and beta residues are also enclosed and underlined, respectively. This assignment and the rules for prediction are based on the method of Chou and Fasman (1975). Predicted α-helix and β-sheet regions are inclosed in brackets in their respective rows.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
E. coli Ser-Ile-Thr-Lys-Asp-Gln-Ile-Ile-Glu-Ala-Val-Ala-Ala-Met-Ser-Val-Met-Asp-Val-
alpha i I i I [i h I I H H h H H h i] h h i h
beta i H h b i h H H B I H I I H b [H H i H
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
V. cost. Ser-Ile-Thr-Asn-Glu-Gln-Ile-Leu-Asp-Ala-Ile-Ala-Asp-Met-Ser-Val-Met-Gln-Val-
alpha i I i b [H h I H i H I H i h i] h h h h
beta i H h i B h H h i I H I i H b [H H h H
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
E. coli (Val)-Glu-Leu-Ile-Ser-Ala-Met-Glu-Glu-Lys-Phe-Gly-Val-Ser-Ala-Ala-Ala-Ala-Val
alpha h [H H I i H h H H] I h B [h i H H H H h]
beta H] B h H b I H B B b h i H b I I I I H
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
V. cost. (Val)-Glu-Leu-Ile-Glu-Ala-Met-Glu-Glu-Lys-Phe-Gly-Val-Ser-Ala-Ala-Ala-Ala-Val
alpha h [H H I H H h H H] I h B [h i H H H H h]
beta H] B h H B I H B B b h i H b I I I I H

or GAC) to glutamine (CAA or CAG) would require at least two mutations. The genetic distance, then, is the total minimum number of mutations required to convert one protein into another. These totals, based on the first 38 residues of the sequence, are shown in Table III-4. Of interest is that the two moderate halophiles are more closely related to *E. coli* than they are to each other. *H. cutirubrum* was not included in this comparison since it is "missing" the first 34 residues (Oda *et al*, 1974).

Discussion

When trying to draw general conclusions from the data presented on the moderately halophilic bacteria, one must discuss possible mechanisms of halophilic adaptation to the salt environment. Several investigators who have worked with halophilic enzymes propose that the increased number of acidic residues found in the proteins act as a charge shield (Baxter, 1959; Brown, 1964). The cations, supposedly, act to cancel the charges on these acidic amino acids allowing proper folding to occur. However, Lanyi (1974) contends that the charge shielding effect is complete once the cation concentration reaches 0.2 M. Thus, charge shielding no longer occurs and the effects of the salts on hydrophobic interactions become important.

Hydrophobic or apolar groups in proteins tend to avoid interaction with the aqueous environment and sequester themselves in the interior of the protein molecule (Fisher, 1964). This tendency to avoid solvation in an aqueous medium is due to the decreased entropy associated with the interaction of these apolar groups with water. The water molecules will order themselves around the apolar groups resulting in a decreased entropy. Different salts have different effects on the solvation of these apolar groups. "Salting-out" salts such as chlorides inhibit solvation by increasing the structure of water and making the apolar group-water interaction less favorable. The hydrophobic interactions of a protein, then, would be stabilized by increased concentrations of the appropriate salts which increase the water structure. Hochachka and Somero (1973) indicate that the contribution

of hydrophobic interactions in stabilizing the protein tertiary structure is sizable. Probably more than half of the stabilizing force is due to hydrophobic interactions. A protein with fewer hydrophobic residues, then, would need higher "salting-out" salt concentrations in order to increase the stability of the protein structure. Lanyi (1974) showed that the proteins of the extreme halophiles, along with having a higher protein acidity, have a lower protein hydrophobicity, The halophilic proteins probably not only need high salt concentrations for charge cancelation, but also for increasing the stability of the fewer hydrophobic interactions contained within the protein molecule.

Calculations of the B/A ratio and the average hydrophobicity from the amino acid compositions of the total ribosomal proteins, the S1 analogous proteins, and the L7/12 analogous proteins shows some interesting trends. The proteins of H. cutirubrum are more acidic and less hydrophobic. The two moderate halophiles, however, show different trends. V. costicola proteins, although much more acidic the E. coli ribosomal proteins, are only slightly less hydrophobic. In the S1 analogous protein, V. costicola is more hydrophobic than E. coli. In the other moderate halophile, the trends are reversed. The ribosomal proteins from H.x. are only slightly more acidic but are less hydrophobic than those of E. coli (Table III-5). These opposite trends in the two moderate halophiles could be explained if their mechanisms of adaptation to life in saline envrionments were different. V. costicola internal ion concentrations have been shown to change with the NaCl of the growth medium, ie the actual ion concentrations of the internal

Table III-5

Acidic and hydrophobic character of total and purified ribosomal proteins.

Organism	Ribosomal protein	Base/Acid[1]	$H\Phi_{avg}$[2]
V. costicola	total[3]	1.08[4]	1.044
E. coli	total	1.58	1.057
H.x.	total	1.23	0.999
H. cutirubrum	total	0.69	0.850
V. costicola	"S1"[5]	0.50	1.049
E. coli	S1	0.59	0.975
H.x.	"S1"	0.54	0.963
V. costicola	"L7/12"[6]	0.36	0.917
E. coli	L7/12	0.54	1.024
H.x.	"L7/12"	0.41	0.841
H. cutirubrum	"L7/12"	0.05	0.726

[1] Base/Acid ratio is the mole percent of basic residues divided by the mole percent of acidic residues.

[2] Average hydrophobicity c lculated by the method of Bigelow (1967) expressed as kilocalories.

[3] Total ribosomal protein values are calculated from Table III-1.

[4] Values of the total ribosomal proteins have been corrected for amide content.

[5] Values calculated from the data in Table III-2.

[6] Values calculated from the data in Table III-3.

environment approaches that of the medium (Shindler et al, 1977). This means that the cellular processes could be exposed to high concentrations of cations. The ribosomal proteins being much more acidic than those of E. coli might be able to cope with the ions by a charge shielding mechanism. The internal ion concentration of the other moderate halophile, H.x., does not contain such a high cation concentration even if the cells are grown in 4.25 M NaCl (Matheson et al, 1976). Its mechanism of adaptation to the high saline environment might be to have a high internal non-ionic solute such as glycerol which is found in some halophilic algae (Ben-Amotz, 1974). The high non-ionic solute would not involve a charge shielding, but may increase the structuring of water so that the reduced number of hydrophobic interactions would be more stable. This explanation, although purely speculative might help account for the genetic distance relationship of the two moderate halophiles, both of which are closer to E. coli than to each other.

CHAPTER IV

In Vitro Protein Synthesis

Introduction

Protein synthesis is a complex, coordinated series of events involving a multitude of interactions. Studies of several bacterial protein-synthesizing systems have revealed that some of these interactions show a dependence on the presence of cations (Lubin, 1963; Rauser and Bayley, 1966; Bayley and Griffith, 1968; Landau et al, 1977). For example, E. coli requires between 60-160 mM K^+ or NH_4^+ and between 7-15 mM Mg^{2+}, depending on the messenger RNA used, for in vitro protein synthesis (Modelell, 1971). On the other hand, the extreme halophile, H. cutirubrum, requires 3.8 M K^+ and 40 mM Mg^{2+} for in vitro protein. Amino acid incorporation is also stimulated by the addition of 1.0 M NaCl and 0.4 M NH_4Cl (Bayley and Griffith, 1968). Since these two organisms can be regarded as opposite extremes in the cation requirement range, it would be interesting to determine the ionic requirements for the protein synthesizing system of a moderately halophilic bacterium. Are the cation requirements of such a bacterium truly moderate? Do they fall into the intermediate range between the cation requirements of the E. coli and H. cutirubrum protein synthesizing systems?

V. costicola was chosen for these studies primarily because the bacterium has been classified as a moderate halophile with respect to its NaCl requirements for growth. Furthermore, measurements of the intracellular ion concentrations indicate that this organism is able to maintain an internal

milieu that has approximately the same ionic strength as the external environment (Shindler *et al*, 1977). It is possible that, like the extreme halophiles, the salts required for *in vitro* protein synthesis are approximately the same as those measured as intracellular. Also, data is available concerning the effects of salts on the physical characteristics of *V. costicola* ribosomes (Chapter II). Some of the characteristics of the ribosomal proteins of this organism have also been investigated (Chapter III).

Thus, in this chapter, the development and characterization of the *in vitro* protein synthesizing system of *V. costicola* will be discussed as well as the effects of salts on the stability and activity of the system.

EXPERIMENTAL METHODS

Growth of cells

Vibrio costicola (NRC 37001) was grown in liquid culture medium containing: 0.5% proteose peptone, 0.5% tryptone, 1.0 or 3.0 M NaCl, and distilled-deionized water. Five hundred ml of media were dispensed in 2800 liter Fernbach flasks and incubated at 30 C on a reciprocating shaker. Cells were harvested in mid-logarithmic phase of growth at an optical density of approximately 0.8 at 660 nm. Before harvesting, the temperature of the culture was rapidly lowered below 8 C. This was accomplished by immersing the culture flask into a dry ice-ethanol bath. The culture was then harvested at 10,000 xg for 10 min at 4 C. Cells were then washed once in buffer containing; 0.6 M KCl, 40 mM $MgCl_2$, 10 mM Tris (pH 7.4), and NaCl corresponding to the growth medium. The resulting cell pellets were stored frozen at -70 C.

Isolation of the S-30 extract

Frozen cell pellets were thawed at 4 C. The cells were then mixed with two times their wet weight of levigated alumina (Norton Abrasives) and ground in a pre-chilled mortar for approximately 10 min. The paste was washed from the mortar with a volume of buffer (100 mM KCl, 20 mM $MgCl_2$, and 10 mM tris, pH 7.6) corresponding to two times the wet weight of the cell pellet. The alumina and remaining whole cells were removed by centrifugation at 10,000 xg for 10 min at 4 C. The resulting supernatant was incubated on ice for 10 min with DNase (final concentration 3 µg/ml; Worthington) then centrifuged at 30,000 xg

for 35 min at 4 C. The upper 4/5 of the supernatant was carefully removed with a Pasteur pipet and dialyzed for 1-2 hours at 4 C against at least 500 volumes of buffer containing: 100 mM KCl, NH_4Cl, or NaCl, 20 mM $MgCl_2$, and 10 mM tris, pH 7.6. Dialysis was performed in Spectrapore #1 (Fisher Sci. Co.) dialysis tubing (molecular weight cutoff 6,000-8,000), 14.6 mm diameter. After dialysis, the extract was centrifuged at 30,000 xg for 20 min. The upper 4/5 of the resulting supernatant (S-30) was used in the in vitro protein synthesizing system. This S-30 fraction was stored in the form of 0.5 ml aliquots at -70 C.

Assay system

The assay system employed was modeled after the one used for E. coli by Modelell (1971). The stock solutions were made as follows: 1) NRG (10X) contained: 150 mM phosphoenolpyruvate, 20 mM adenosine triphosphate (Na^+ salt), 10 mM guanosine triphosphate (Na^+ salt), and 300 µg/ml, pyruvate kinase (Miles Lab.). The pH of the solution was adjusted to 7.0 with 1 N NaOH and stored as 0.5 ml aliquotes. 2) concentrated salts contained: 1.0 M NH_4Cl, 120 mM $MgCl_2$, and 460 mM tris, pH 7.6. 3) reduced glutathione, 0.15 M, pH adjusted to 7.0 with 1 N NaOH. 4) poly-uridylic acid (Miles Lab.), 6 mg/ml, stored as 0.5 ml aliquots; and 5) ^{14}C-phenylalanine (485-522 mCi/mmole, Amersham-Searle), 50 µCi/ml. All stock solutions were stored frozen at -20 C until used.

When assays were to be performed, the stock solutions were thawed and stored on ice. A 'pre-mix' solution was made which

contained the following:

330 µl NRG (10X)
165 µl reduced glutathione
165 µl ^{14}C-phenylalanine
495 µl poly-U
165 µl distilled-deionized water

Each assay system contained the following: 120 µl of the "premix", 30 µl of the concentrated salts, 60 µl of distilled-deionized water, and 90 µl of the dialyzed S-30 extract. The final concentrations of the system components are shown in Table IV-1. When different salts and salt concentrations were tested, the adjustments were made in the stock 'concentrated salts' mixture so that the final concentrations, including those of salts added by the extract, were as desired. Any additions to the assay system, such as antibiotics, were as aqueous solutions and the amount of water added to the reaction mixture was adjusted to keep the final volume constant.

The complete assay system was incubated at 30 C in a circulating water bath, and at 0,2,4,6, and 8 min time intervals 50 µl aliquots were removed and added to 3 ml of cold 5% (w/v) trichloroacetic acid. The samples were heated in a 90 C water bath for 20 min. The resulting precipitates were washed onto 0.45 micron membrane filters (Millipore, Gelman, or Amicon) or GF/A Whatmann glass fiber filters with three volumes of cold 5% trichloroacetic acid. The filters were dried under a heat lamp and counted in a Beckman Model 233 liquid scintillation counter with 5 ml of Hydromix (Yorktown Research) scintillation

Table IV-1

Components of the *in vitro* protein synthesizing system directed by polyuridylic acid.

Component	Concentration
Tris-HCl (pH 7.6)	50 mM
NH_4Cl	130 mM
$MgCl_2$	18 mM
Reduced glutathione	7.5 mM
ATP	2.0 mM
GTP	1.0 mM
Phosphoenol pyruvate	15 mM
Pyruvate kinase	30 µg/ml
^{14}C-phenylalanine	9.6 uM
Polyuridylic acid	0.9 mg/ml
Dialyzed S-30 extract	0.3 vol. (150-200 A_{260}/ml)

fluor. The counting efficiency of ^{14}C by this method using the ^{3}H-^{14}C preset window was 87.5%. The 0 time value was subtracted from the other time intervals and the picomoles of phenylalanine calculated from the resulting figures. A plot of picomoles phenylalanine versus time was made and the rate of incorporation determined from the most linear portion of the graph. The rate was then corrected for the concentration of the extract by dividing the rate by the amount of A_{260} material that would be in the 50 µl aliquot.

Endogenous assay system

When the in vitro protein synthesis system was tested using endogenous messenger (cellular mRNA attached to the ribosomes or free in the cytoplasm), the assay system was the same as the poly-U directed system with the following exceptions: the poly-U was omitted and water used in its place; the radioactive phenylalanine was replaced with either uniformly labeled ^{14}C-amino acid mixture (10 mCi/mmole, Amersham-Searle) or ^{14}C-Chlorella hydrolysate (54 Ci/mAtom).

Determination of poly-phenylalanine

The amounts of poly-phenylalanine produced by the in vitro protein synthesizing system was determined either by the paper chromatography method of Gavrilova and Smolyaninov (1971) or by the column chromatography method of Pestka et al (1969).

Preparation of E. coli extracts

E. coli S-30 extracts were prepared by the method described by Modelell (1971). The strain used was E. coli MRE 600

(a gift from Dr. Kogut) which is lacking RNase I.

RESULTS

Development of the in vitro protein synthesizing system

Initial attempts to measure *in vitro* protein synthesis of *V. costicola* extracts in salt concentrations resembling those estimated as intracellular (Shindler *et al*, 1977) were unsuccessful. Varying the relative concentrations of sodium and potassium, but keeping the total ionic concentration constant, still resulted in no detectable incorporation. However, measurable incorporation of radioactive amino acids did occur in salt and assay component concentrations used for the *in vitro* protein synthesizing system of *E. coli* (Modelell, 1971). This time dependent incorporation is shown in Figure IV-1.

The length of linear incorporation raised some concern. Consequently, attempts were made to increase the length of incorporation. Increasing the concentrations of ATP, GTP, and phenylalanine increased the amount of phenylalanine incorporated in the poly-U directed system, but did not increase the period of linearity. Increasing the pyruvate kinase, phosphoenol pyruvate, and glutathione concentrations did not affect the poly-U directed system. However, increased concentrations of phosphoenol pyruvate and pyruvate kinase did increase the linearity of the endogenous messenger system (W. Madira, personal communication). Increasing the concentration of poly-U in the poly-U directed system increased the period of linear incorporation as is shown in Figure IV-2.

The higher concentrations of ATP, GTP, pyruvate kinase,

Figure IV-1

Time dependent incorporation of phenylalanine by poly-U directed S-30 extracts of V. costicola. Extracts of V. costicola were prepared as described in the Methods section and assayed in the in vitro protein synthesizing system for E. coli as described by Modelell (1971). Final concentrations of salts were 90 mM KCl and 15 mM $MgCl_2$. Samples were taken at one minute intervals and analyzed as explained in the Methods.

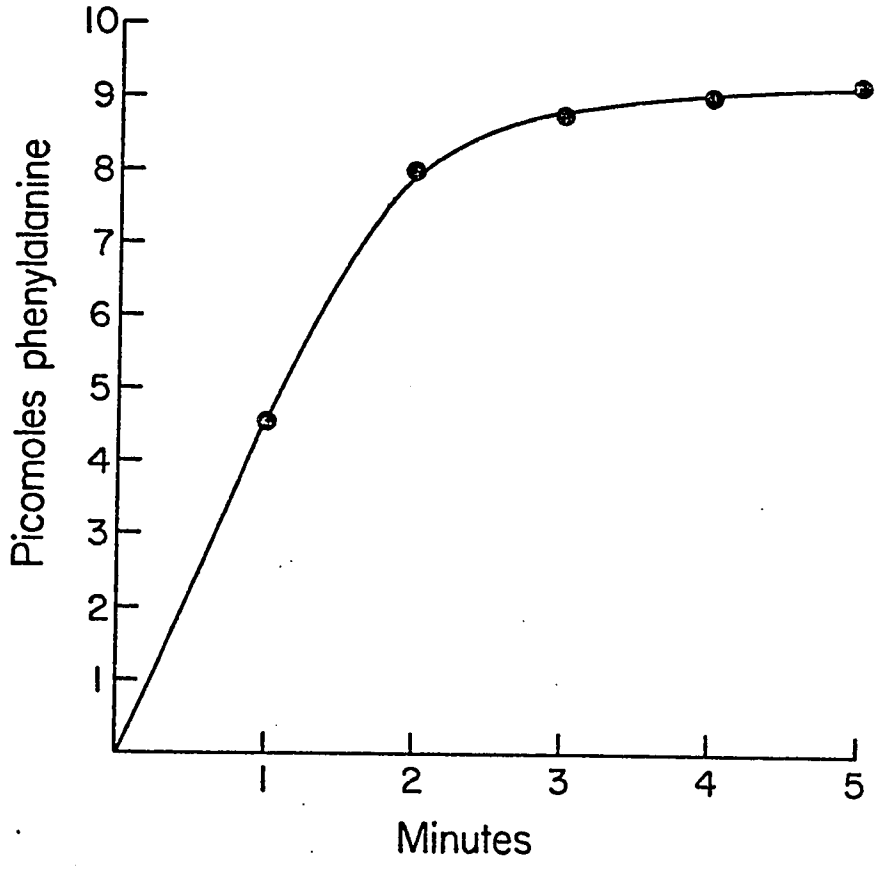
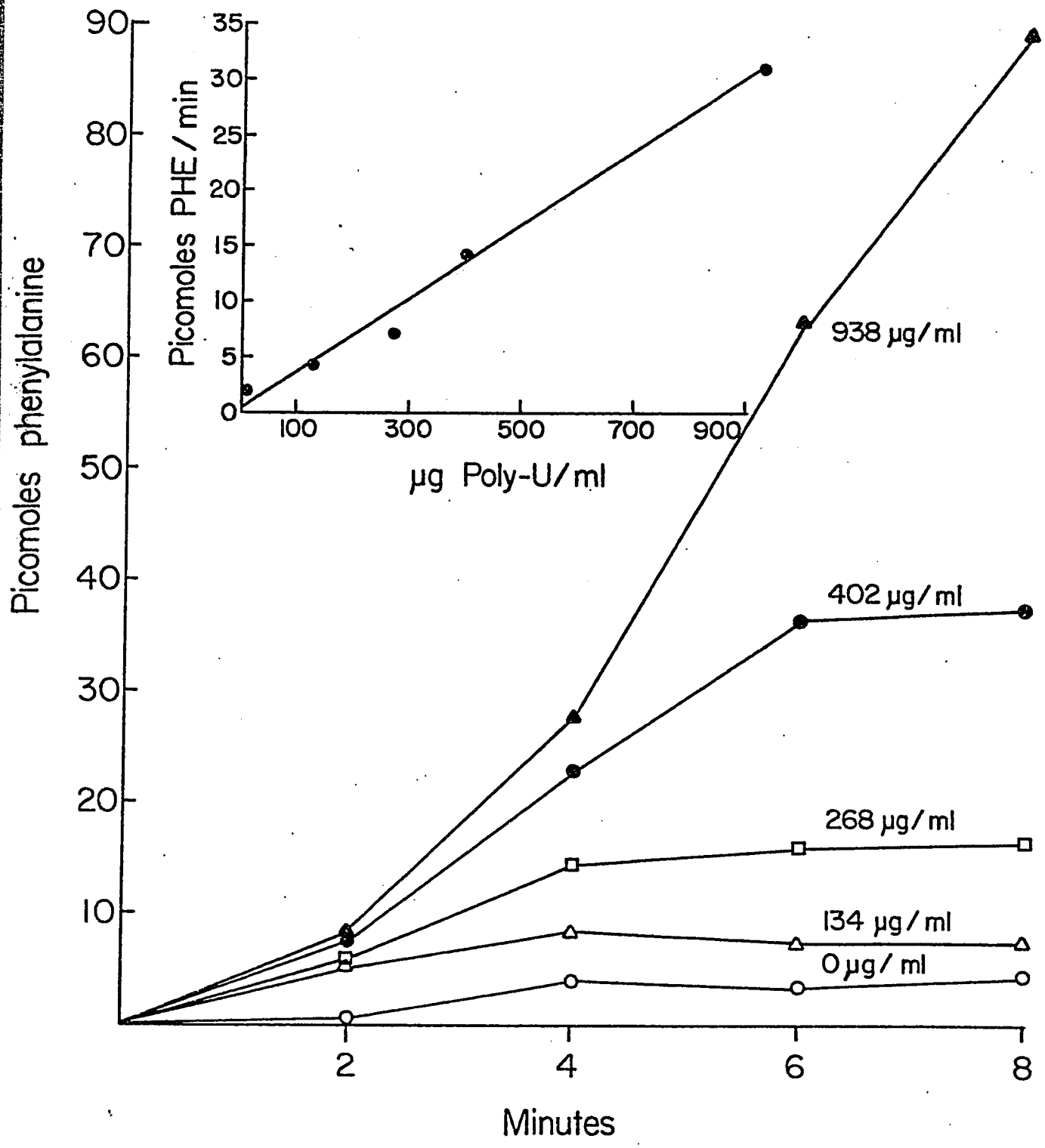

Picomoles phenylalanine
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
Minutes

Figure IV-2

The effect of polyuridylic acid concentration on the *in vitro* protein synthesis system of *V. costicola*. The amount of phenylalanine incorporated versus time was measured in various concentrations of poly-U. The rate of phenylalanine incorporation was then plotted against poly-U concentration (inset). The assay salts and conditions are the same as described in the Methods section.


Picomoles phenylalanine
90
80
70
60
50
40
30
20
10
2
4
6
8
Minutes
938 µg/ml
402 µg/ml
268 µg/ml
134 µg/ml
0 µg/ml
Picomoles PHE/min
35
30
25
20
15
10
5
0
100
300
500
700
900
µg Poly-U/ml

phosphoenol pyruvate, phenylalanine, and poly-U (shown in Table IV-1) were used in all subsequent assays. Higher salt concentrations, although lowering the activity, did not increase the length of linear incorporation. A comparison of the E. coli and V. costicola poly-U directed systems is shown in Figure IV-3 using the system component values shown in Table IV-1.

Since the length of incorporation was short, it had to be established whether the incorporation of radioactive phenylalanine into trichloroacetic acid insoluble material actually represented de novo polypeptide synthesis or whether it was a result from non-specific adsorption. The latter could conceivably result from radioactive phenylalanine forming complexes with protein components of the V. costicola extract. These complexes could remain after hot trichloroacetic acid extraction.

An indirect method of showing that the poly-U directed phenylalanine incorporation is indeed de novo polypeptide synthesis is the demonstration that the system is energy, poly-U, and extract dependent (Table IV-2). The incorporation should also be sensitive to inhibitors (Table IV-3). Aurin tricarboxylic acid showed an inhibition response in the V. costicola system similar to that reported for E. coli (Apirion et al, 1971; Figure IV-4).

A more direct means of evaluating the poly-U directed incorporation of phenylalanine is the measurement of the amount of oligophenylalanine produced. Two methods were used to determine quantities of polyphenylalanines. The method of Gavriiova and Smolyaninov (1971) involves descending chromatogra-

Figure IV-3

Comparison of the *in vitro* protein synthesis between extracts of *E. coli* and *V. costicola*. An *E. coli* S-30 extract was prepared by the method of Modelell (1971). It was assayed along with a *V. costicola* S-30 extract in the *in vitro* protein synthesizing system described in the Methods section. The amount of phenylalanine incorporated was corrected for the absorbance at 260 nm of each extract so that they could directly be compared.

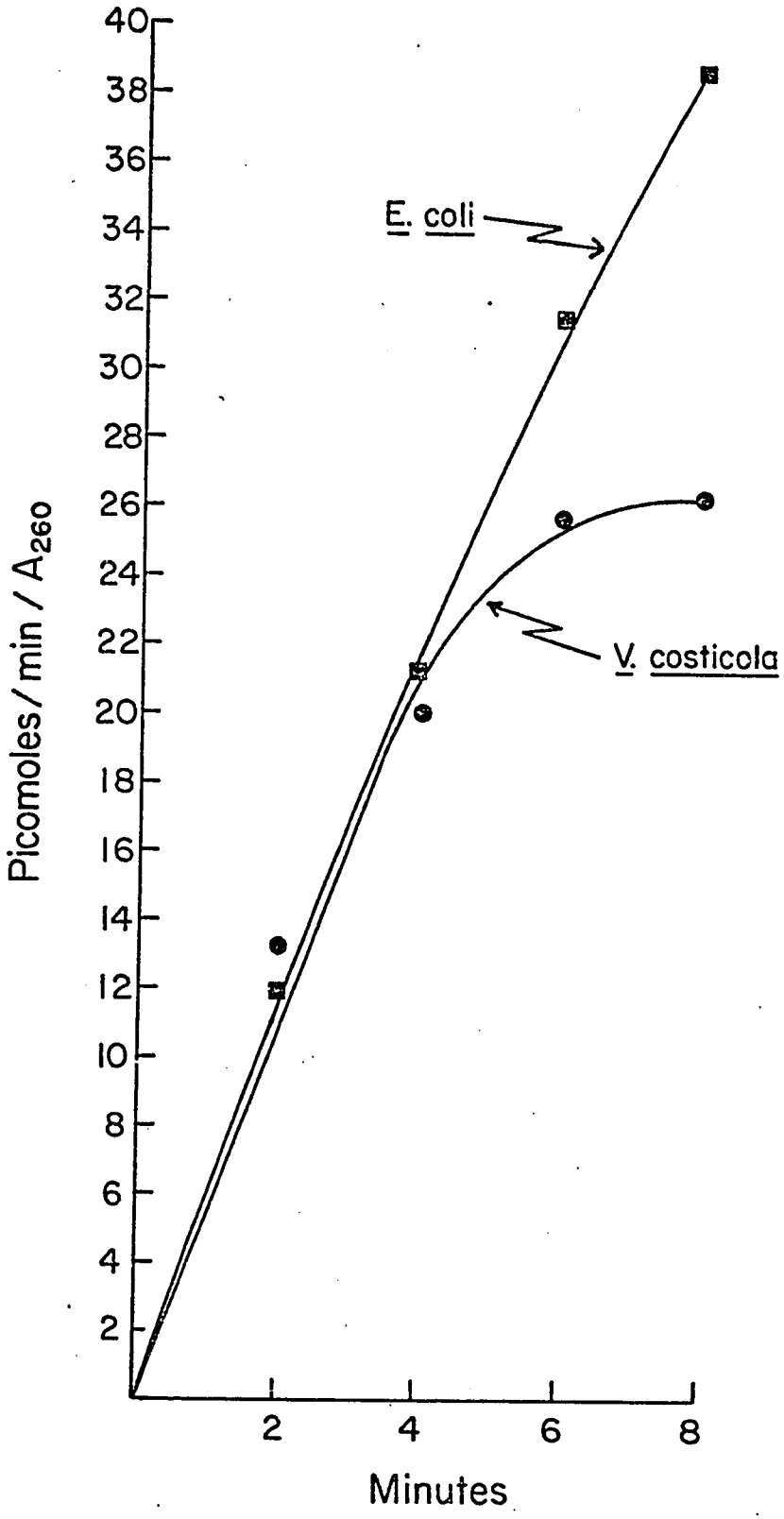

40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
Picomoles / min / A_{260}
E. coli
V. costicola
2
4
6
8
Minutes

Table IV-2

Effects of system components on the incorporation of ^{14}C-phenylalanine into trichloroacetic acid insoluble material.

	picomoles phe/min/A_{260}	percent activity
Complete[1]	0.81	100
Complete -ATP	0.03	4
Complete -GTP	0.17	21
Complete -poly-U	0.10	12
Complete -S-30 extract	0	0

[1] See Table IV-1.

Table IV-3

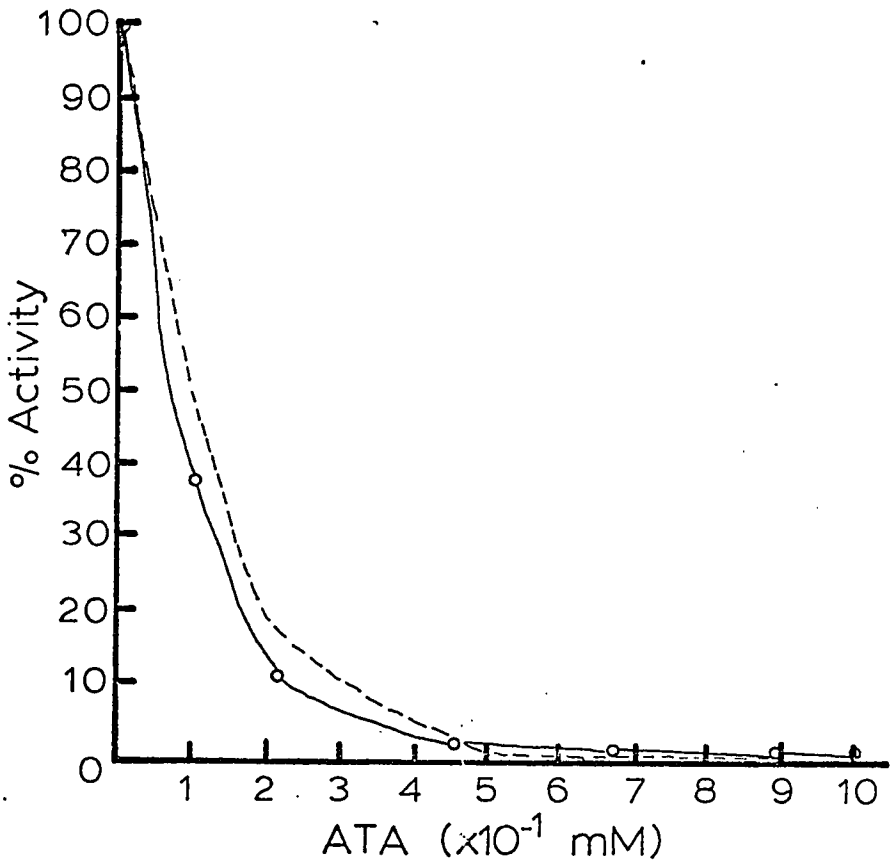
The effect of inhibitors on the phenylalanine incorporation of *V. costicola* extracts.

Additions	Percent inhibition[1]
none	0
Ribonuclease A (40 µg/ml)	100
Chloramphenicol (15 µg/ml)	100
Puromycin (25 µg/ml)	70
Aurin tricarboxylic acid (105 µg/ml)	62
Aurin tricarboxylic acid (445 µg/ml)	98

[1] Assays were performed as described in the Methods section. Percent inhibition was calculated from the rate of linear incorporation of ^{14}C-phenylalanine as compared to the rate of a control without the inhibitor present. 0% inhibition (100% activity) was 52.3 picomoles phenylalanine incorporated per min.

Figure IV-4

Inhibition of the V. costicola in vitro protein synthesizing system by aurin tricarboxylic acid. The assay system was the same as that described in the Methods section except that an aqueous solution of aurin tricarboxylic acid (ATA) was added and the amount of water adjusted to maintain a constant volume. The inhibition of the V. costicola system (solid line) and the E. coli system (dashed line, from Apirion et al, 1971) are shown as a function of ATA concentration.


100
90
80
70
60
50
40
30
20
10
0
% Activity
1
2
3
4
5
6
7
8
9
10
ATA (x10⁻¹ mM)

phy. Reference compounds of phe, phe_2, phe_3, and phe_4 were run on paper strips with incubated reaction mixtures and the reference compounds localized by their reaction with ninhydrin. The reference spots were then cut from the paper and analyzed for radioactivity. The method of Pestka et al (1969) involves the separation of oligophenylalanines by elution with different concentrations of formamide from a benzoated diethylaminoethyl cellulose column. The results of both methods are shown in Table IV-4.

Characterization of the in vitro protein synthesizing system

The concentration of Tris in the final assay system was 50 mM, a concentration which has been shown to inhibit some enzyme systems (Good et al, 1966). Due to the high magnesium concentration (20 mM), phosphate buffer could not be used. Therefore, other organic buffers and Tris concentrations were tried. N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and Morpholinopropane sulfonic acid (MOPS) buffers were used. Tris at a concentration of 50 mM had the highest incorporation rate (Table IV-5). Lower concentrations of Tris were probably not as effective in maintaining the pH. Higher tris concentrations and the other organic buffers were slightly inhibitory.

Optimum protein synthesis occured at approximately pH 7.6 (Figure IV-5). This is slightly lower than the 7.8 optimum reported for E. coli (Modelell, 1971) and the 7.6-8.0 optimum for H. cutirubrum (Bayley, 1976). The curve shows an almost biphasic

Table IV-4

Oligophenylalanines produced by the poly-U directed *in vitro* protein synthesizing system of *V. costicola*.

	% Total ^{14}C-phenylalanine		% Incorporated ^{14}C-phenylalanine	
	paper[1]	column[2]	paper	column
phe	93.4	93.0	-	-
phe-phe	3.6	4.1	55.4	58.0
phe-phe-phe	1.2	1.8	18.5	25.0
>phe-phe-phe	1.7	1.1	26.2	17.0

[1] Measured by the paper chromatography method of Gavrilova and Smolyaninov (1971).

[2] Measured by the BD-cellulose column chromatography method of Pestka *et al* (1969).

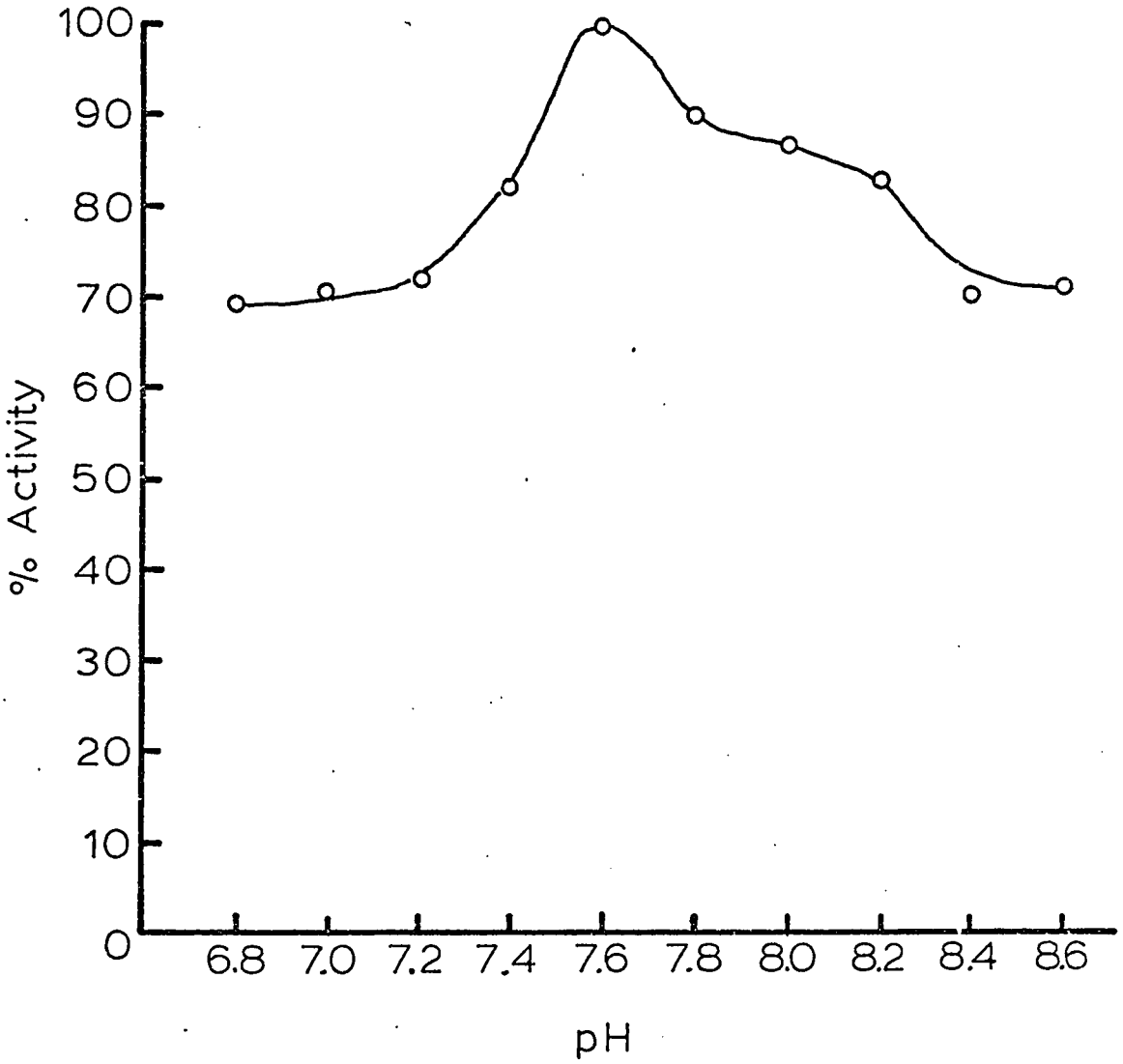
Table IV-5

The effect of different buffers on *in vitro* protein synthesis.

Buffer	^{14}C-phenylalanine incorporated per minute	% Maximum activity
10 mM Tris	4.5	26
50 mM Tris	17.1	100
100 mM Tris	13.8	81
50 mM HEPES	13.5	79
50 mM MOPS	11.4	67

Figure IV-5

The effect of pH on the <u>in vitro</u> protein synthesizing system. The assay conditions are the same as those described in the Methods section. The pH of the Tris buffer was adjusted to the values indicated. The activity is expressed as percent of the maximum activity, 18.7 picomoles phenylalanine incorporated/ min/ A_{260} of the S-30 extract.


100
90
80
70
60
50
40
30
20
10
0
% Activity
6.8
7.0
7.2
7.4
7.6
7.8
8.0
8.2
8.4
8.6
pH

character suggesting that different components of the protein synthesizing system have different responses to pH.

Figure IV-6 shows the effects of incubation temperatures on the rate of protein synthesis. The amount of phenylalanine incorporated after eight minutes of incubation was approximately the same at all temperatures. The temperature of incubation appears to affect only the rate of attainment of the total amount of phenylalanine incorporated. Since 30 C is the optimum temperature for growth of this bacterium, all subsequent assays were performed at this temperature.

Effects of salts on the rate of protein synthesis

The salt responses of the poly-U directed in vitro protein synthesis system are shown in Figure IV-7. In all cases the maximum activity occured at approximately 130-150 mM salt. NH_4Cl showed the highest activity followed by KCl and then NaCl. The optimum $MgCl_2$ concentration was 18 mM (Figure IV-7 inset). The salt requirements of the E. coli in vitro protein synthesizing system directed by poly-U have been reported to be 60-100 mM KCl or NH_4Cl and approximately 15 mM Mg^{2+} (Modelell, 1971). The system from H. cutirubrum has an optimum of 3.8 M KCl and 40 mM Mg^{2+} (Bayley and Griffiths, 1968a).

The optimum salt concentrations for E. coli in vitro protein synthesis directed by natural messenger have been found to be somewhat lower, 60 mM KCl or NH_4Cl and 7-11 mM Mg^{2+} (Tissiere et al, 1960), than those for synthetic messenger. To check whether there is a similar discrepency in the V. costicola

Figure IV-6

The effect of incubation temperature on the *in vitro* protein synthesis of *V. costicola*. The assay system is the same as described in the Methods section. The assay mixture was incubated at the temperatures indicated. Sampling and treatment of samples for radioactive measurements was not changed. The rate of linear incorporation of phenylalanine was plotted against temperature (inset).

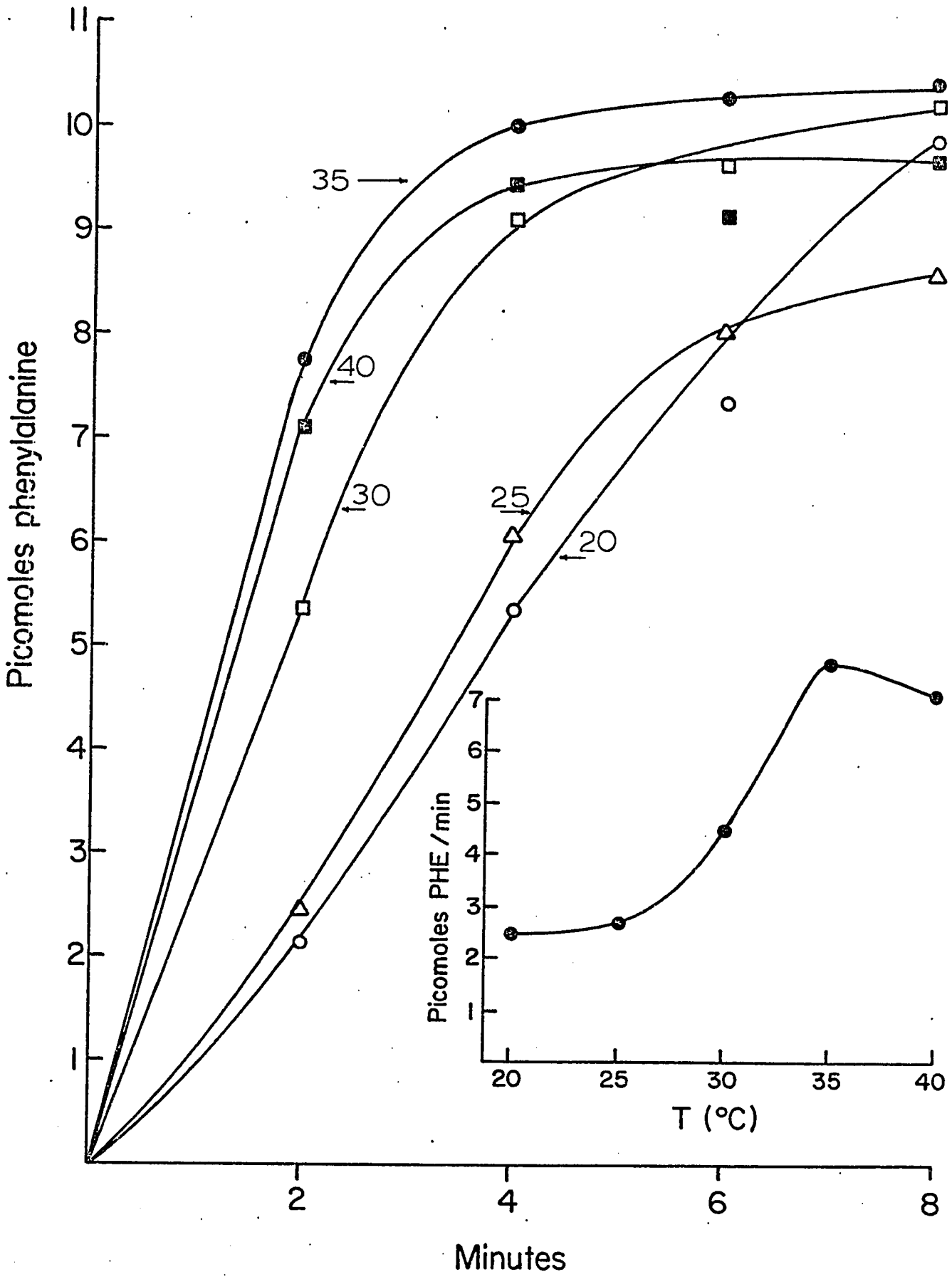
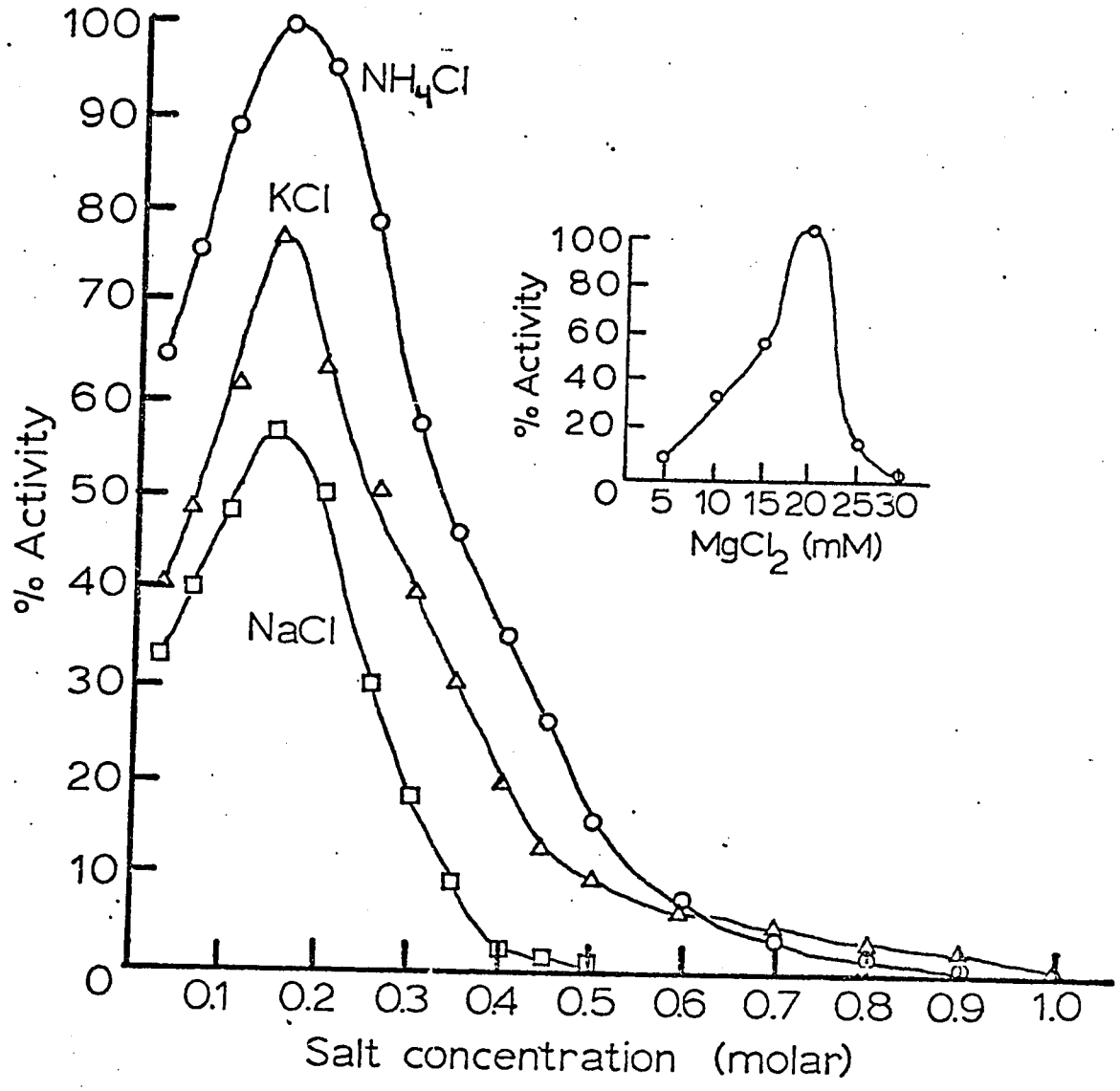

Picomoles phenylalanine
Minutes
35
40
30
25
20
Picomoles PHE / min
T (°C)

Figure IV-7

The effect of NaCl, KCl, NH_4Cl, and $MgCl_2$ concentrations on the poly-U dependent ^{14}C-phenylalanine incorporation. S-30 extracts were dialyzed for 1 hour against 1-2 liters of 100 mM of the respective monovalent cation plus 20 mM $MgCl_2$ and 10 mM Tris, pH 7.6. The magnesium optimum was determined using 100 mM NH_4Cl as the monovalent cation. 100% activity represents 22.2 picomoles of phenylalanine incorporated/min/A_{260} of the S-30 extract.


100
90
80
70
60
50
40
30
20
10
0
% Activity
NH4Cl
KCl
NaCl
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Salt concentration (molar)
100
80
60
40
20
0
% Activity
5
10
15
20
25
30
MgCl2 (mM)

protein synthesizing system, the salt response of the in vitro system was tested using endogenous messenger (Figure IV-8). The salt response was almost identical to that of the poly-U directed system. The only difference was that the endogenous system was inhibited by all NaCl concentrations tested.

In the abovementioned experiments, the response for a particular salt was measured with S-30 extracts dialyzed against that same salt. It is therefore possible that removing the other salts could result in adverse effects on the protein synthesizing system. This could manifest itself in the alteration of the relative activities in each of the salts. To check for this possibility, S-30 extracts were dialyzed against one salt and tested for activity in each of the other salts (Table IV-6). The relative activities (expressed as percent of the optimum) of the protein synthesis system were shown to have similar trends no matter what salt was used for dialysis.

It seemed possible that the inhibition by high salt concentrations in the V. costicola protein-synthesizing system could be explained if the increased monovalent cations were displacing the divalent cation, magnesium. If this were the case, raising the magnesium concentration might overcome the inhibition. The results obtained, however, did not verify this hypothesis (Table IV-7). At 450 mM NaCl, the Mg^{2+} optimum was still approximately 18 mM.

Stability of the in vitro protein synthesis system in different salts

Since the V. costicola extracts had to be dialyzed before

Figure IV-8

Effects of NaCl, KCl, NH_4Cl, and $MgCl_2$ on the amino acid incorporation dependent on "endogenous" messenger. V. costicola, grown in 1.0 M NaCl, cells were lysed osmotically by suspending the cell pellets in buffer containing: 200 mM KCl; 10 mM Tris, pH 7.6; and 5 mg/ml lysozyme. After 2-3 minutes incubation on ice, the ionic concentration and composition was restored to 100 mM KCl; 10 mM Tris, pH 7.6; and 18 mM $MgCl_2$. S-30 extracts were prepared and dialyzed as in Methods section. The values are expressed as percent of maximum activity, maximum activity being 300 cpm/min/A_{260}. Data supplied by M. Kogut and W. Madira, Dept. Biochemistry, King's College, Univ. of London).

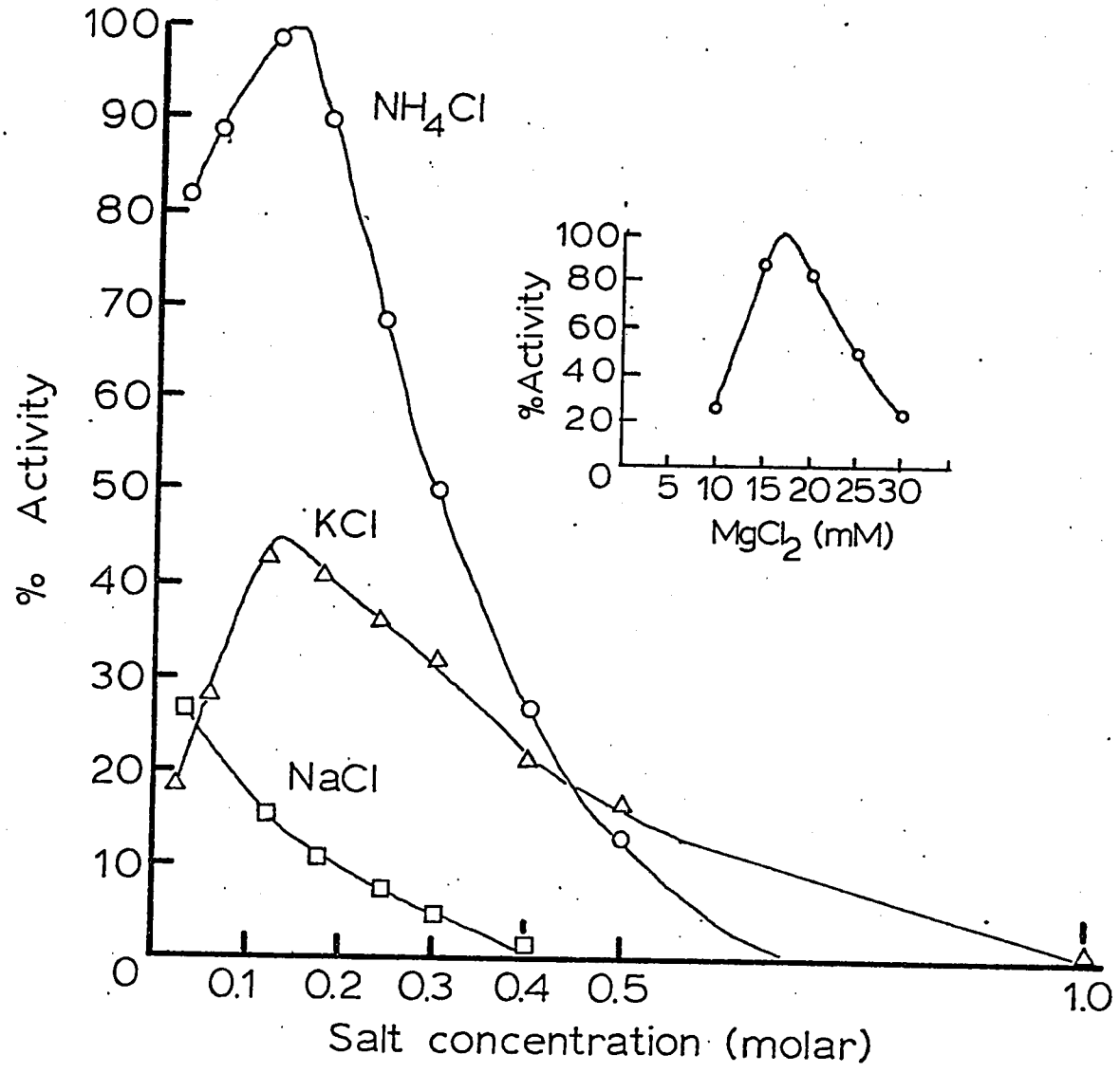

100
90
80
70
60
50
40
30
20
10
0
% Activity
NH4Cl
KCl
NaCl
0.1
0.2
0.3
0.4
0.5
1.0
Salt concentration (molar)
100
80
60
40
20
0
%Activity
5 10 15 20 25 30
MgCl2 (mM)

Table IV-6

The effect of dialysis salt on the relative salt optimum

Dialysis salt[1]	Assay salt[2] NH_4Cl	KCl	NaCl
NH_4Cl	100	75.8	54.6
KCl	100	96.8	79.8
NaCl	100	62.4	39.8

[1] S-30 extracts were dialyzed against 100 mM of the indicated salt plus 20 mM $MgCl_2$ and 10 mM Tris, pH 7.6.

[2] Dialyzed extracts were assayed with 100 mM of the salt indicated. When the assay salt was the same as the dialysis salt, then corrections were made to keep the assay concentration at 100 mM. The values are expressed as the percent maximum activity of that particular extract. For the NH_4Cl dialyzed extract the activity was 14.6 picomoles phenylalanine incorporated per min per A_{260}. The values for the KCl and NaCl extracts were 28.2 and 37.2 picomoles/min/A_{260} respectively.

Table IV-7

Effect of Mg^{2+} on high salt inhibition of protein synthesis

Assay salts[1]	picomoles phenylalanine/minute
150 mM NaCl 18 mM $MgCl_2$	107.0
450 mM NaCl	
7.5 mM $MgCl_2$	1.4
18.0 mM $MgCl_2$	18.0
36.0 mM $MgCl_2$	7.2
52.0 mM $MgCl_2$	0.3

[1] S-30 extracts dialyzed against 100 mM NaCl for 1 hour and assayed in the system outlined in the Methods section except for the final concentration of salts.

the salt response could be measured, it was thought possible that the relative activities could reflect the stability of the extracts in different salts. The stability of the protein synthesizing system was tested with various salts at different concentrations and temperatures (Table IV-8). All salt concentrations which inhibited protein synthesis could, however, stabilize the system. Furthermore, the system was usually more stable at lower temperatures. Since dialysis was performed at 4 C, the maximum loss of activity would be approximately 2% during the 1-2 hour dialysis period and thus would not affect the salt response studies.

Effect of salt on the accuracy of translation

The accuracy of translation was checked by substituting ^{14}C-leucine for the ^{14}C-phenylalanine in the poly-U directed assay system. Leucine was used because its codon assignments differ from phenylalanine usually by one nucleotide (Nirenberg et al, 1966). The system was run with and without poly-U at 100, 300, and 500 mM KCl (Table IV-9). In every case, poly-U stimulated the incorporation of phenylalanine. However, as the KCl concentration increased, the amount of stimulation decreased. Leucine incorporation, on the other hand, was not stimulated to any great extent at 100 mM. As the concentration of KCl increased, the stimulation of leucine incorporation went up. At 500 mM KCl, the rate of leucine incorporation increased 2.6 times in the presence of poly-U. In general, the rates of leucine incorporation are higher than those of phenylalanine. Possibly this was

Table IV-8

The effects of Na^+, K^+, and NH_4^+ chlorides on the stability of the poly-U dependent incorporation of ^{14}C-phenylalanine

Salt concentration	$t_{1/2}$, 30 C	$t_{1/2}$, 20 C	$t_{1/2}$, 3 C
0.1 M NH_4Cl	22 min	160 min	510 min
1.0 M NH_4Cl	63 min	-	-
0.1 M KCl	40 min	57 min	405 min
1.0 M KCl	20 min	-	-
0.1 M NaCl	30 min	45 min	-
1.0 M NaCl	48 min	58 min	1095 min

[1] 400 µl of the S-30 extract, dialyzed against 100 mM of the appropriate salt was incubated with solid salt to raise the concentration to the desired level. 90 µl aliquots of this mixture were removed at various time intervals and assayed as described in the Methods except that for the 1.0 M salt concentrations, no monovalent ions were added to the reaction mixture. This resulted in a final concentration of 0.3 M salt. The $t_{1/2}$ is the time required for the extract to lose half of the original activity. The - symbol indicates that the extract lost no activity over the time period tested (usually 8 hours).

Table IV-9

The effect of salt concentration on the fidelity of translation

KCl concentration	Phenylalanine[1] incorporation	Leucine[1] incorporation
100 mM + poly-U	10.1	27.4
- poly-U	0.55	22.7
300 mM + poly-U	3.1	16.4
- poly-U	0.84	10.3
500 mM + poly-U	0.89	10.7
- poly-U	0.84	4.1

[1] Values expressed as picomoles (phenylalanine or leucine) incorporated per minute per A_{260} of extract.

because the specific activity of the ^{14}C-leucine (10 mCi/mmole) was lower than that of ^{14}C-phenylalanine (522 mCi/mmole). This required approximately 50 times more leucine to be present in the assay mixture for the attainment of the same concentration of radioactive carbon as is present in the phenylalanine assay. However, in 0.15 M salts, increasing the phenylalanine concentration three fold did not increase the rate of incorporation.

DISCUSSION

After a long period of trial and error, an *in vitro* protein synthesizing system for *V. costicola* was developed. This amino acid incorporating system resembles other bacterial systems in being energy dependent (Bayley and Griffith, 1968; Lamborg and Zamicnik, 1960). In *V. costicola* the actual levels of ATP and GTP for maximum activity are higher than in *E. coli*. However, with these higher concentrations, the rate of incorporation is able to approach that of the *E. coli* system.

The system is also dependent on the presence of poly-U for the incorporation of phenylalanine into oligophenylalanines. In initial experiments, the *V. costicola* poly-U directed incorporation was linear for 2-3 minutes. This time interval of linear incorporation raised some concern since literature values for *E. coli* show a range of linear incorporation from 10 min (Coleman, 1967) to 20-40 min (Modelell, 1971). However, Matthaei and Nirenberg (1961) report that linear incorporation can vary with the specific batch of commercially prepared poly-U which may contain as much as 0.4% RNase. Since increasing the concentration of the poly-U several fold resulted in an increased length of linear incorporation in the *V. costicola* system, the presence of an active RNase in the poly-U preparation or in the S-30 extract seemed possible. Experiments performed with *E. coli* MRE 600 (a mutant lacking RNase I) extracts showed linear incorporation for the eight minute time period tested. It is therefore felt that the *Vibrio costicola* extract contained

endogenous RNase activity. Even though the incorporation period was short, analysis of the endproducts revealed that oligophenylalanines were being synthesizied.

The V. costicola system was sensitive to inhibition by chloramphenicol, unlike the in vitro system of H. cutirubrum (Bayley and Griffith, 1968). Furthermore, the V. costicola system was sensitive to puromycin, aurin tricarboxylic acid, and ribonuclease.

Probably the most striking finding in the V. costicola protein synthesizing system was the response to salts. The salt optimum was considerably less than the total ionic concentration measured by determining ions/ml cell water and quite close to the salt optima reported for several non-halophilic bacteria. Lubin (1963) found that for the poly-U directed system in E. coli, NH_4Cl was 2-3 times more efficient in stimulating incorporation than KCl and the optimum was around 60 mM. A more detailed study by Conway (1964) agreed that NH_4Cl was more efficient than KCl, but the optimum concentration was 160 mM. If this latter value is correct, then it is coincident with the value obtained for V. costicola. The optimum KCl concentration of the in vitro protein synthesizing system from Bacillus amyloliquefaciens using endogenous messenger was 85 mM (Coleman, 1969).

The role of cations in protein synthesis is not clearly understood. They seem to be indispensible, for when E. coli ribosomes are depleted of K^+ and NH_4^+, they lose the ability to perform a number of ribosomal reactions and to bind certain antibiotics. Activity can be restored by replacing the depleted monovalent cations. Either NH_4^+, K^+, Rb^+, or Cs^+ ions can pres-

erve or restore activity, while Na^+ and Li^+ can not (Spitnik-Elson and Elson, 1976). It has also been noted by Lubin (1963) and Conway (1964) that there was a specific monovalent cation requirement for the initiation of protein synthesis in E. coli. Furthermore, Spyrides (1964) found that binding of tRNA to the ribosomes of E. coli was dependent upon the presence of NH_4^+ ions. Rauser and Bayley (1968) report similar findings with H. cutirubrum. Although NaCl is inhibitory to the E. coli system, it increases the rate of incorporation and fidelity in H. cutirubrum (Bayley and Griffiths, 1968a). Low concentrations of NaCl shows stimulation of incorporation in a poly-U directed system in V. costicola, but were inhibitory to the endogenous system.

The magnesium optimum for the V. costicola system directed by either natural or synthetic messenger was 18 mM. This is comparable with the 15-18 mM requirement of the E. coli poly-U directed system (Tissiere et al, 1960). This all leads to the conclusion that in both monovalent and divalent cation requirements, the V. costicola in vitro protein synthesis system is almost identical to those of the non-halophile E. coli.

The optimum salt concentration for in vitro protein synthesis of V. costicola is well below that measured as intracellular. Indeed, in salt concentrations resembling those reported as intracellular (Shindler et al, 1977), protein synthesis is completely inhibited. This would suggest that perhaps these ions are not free in the cytoplasm of intact cells. The method by which the intracellular ion concentrations were measured does

not distinguish between those ions free in the cytoplasm and those bound to cellular structures. It is thus quite possible that the protein synthesis machinery *in situ* is situated in a region of low ionic concentrations. Studies by Shindler (1976) on permeabilized cells of *V. costicola* also indicate that some of the ions are bound to the cell envelope. This implies that there must be regions of the cell which have a higher concentration of these ions. Masui and Wada (1973) report that substantial amounts of Na^+ and K^+ are bound to the cell envelopes of a marine pseudomonad. In *Bacillus amyloliquefaciens*, 80% of the cellular Na^+ ions are bound to the cell envelope, while most of the K^+ ions were considered to be intracellular (Coleman, 1974). It therefore seems plausible that the cytoplasm of this organism does not contain the high salt concentrations that are implied by the values measured by Shindler *et al* (1977).

Chapter V

General Discussion and Speculation

The mechanism of adaptation of the extreme halophiles has been extensively studied over the past few years. It is currently thought that this mechanism is not to exclude salt but rather to alter the cellular structures in such a way so that they are able to function under the extreme environmental conditions (Brown, 1976). This is reflected in the measured intracellular ion concentrations and the requirement of the cell envelope, enzymes, and ribosomes of the extreme halophiles for high salt concentrations. The biochemical basis for this high salt requirement is still under investigation. At present, the proteins of the extreme halophiles have been shown to be much more acidic and much less hydrophobic than those of non-halophiles (Lanyi, 1974). Whether these are the only biochemical adaptations, and the relative importance of each are still unknown.

The moderate halophiles are not as unusual as the extreme halophiles which are found only in very high salt concentrations. The moderate halophiles include a large number of marine bacteria which have demonstrated the ability to grow over a wide range of salt concentrations (Forsyth *et al*, 1971). The mechanism of adaptation of the moderate halophiles is poorly understood. Studies in D.J. Kushner's laboratory have concentrated on defining the mechanism of adaptation of one moderate halophile, *Vibrio costicola*, which is considered an interesting representative moderate

halophiles. From the studies of Shindler (1976) and this thesis, I propose the following mechanism of adaptation.

The moderate halophile, V. costicola, does not follow the pattern of the extreme halophiles in maintaining an intracellular ion concentration equal to that of the environment. Although the intracellular ion concentrations of this organism were high (Shindler, 1976; Shindler et al, 1977), there is no evidence that these are the ion concentrations free in the cytoplasm, hence the term "cell-associated" ions has been introduced (Shindler et al, 1977). From the studies of Masui and Wada (1973), Shindler (1976), Coleman (1974) and Marquis and Carstensen (1973), it is proposed that the majority of ions measured as cell-associated in V. costicola, are bound to the cell envelope. This is also supported by a survey of moderately halophilic enzyme studies in which Shindler (1976) found that the enzymes supposedly associated with the cell membrane of moderate halophiles (cytochrome oxidase, lactate dehydrogenase, and alkaline phosphatase) required much higher salt concentrations for activity than soluble enzymes (isocitrate dehydrogenase, pyruvate kinase, and threonine deaminase).

Low concentrations of free cytoplasmic ions are also implicated by the low salt optimum of the protein synthesizing system of V. costicola. Not only is the activity of the protein synthesis system reduced when the salt concentrations are raised above the optimum, but the accuracy of translation of the messenger is also reduced (Chapter IV). Thus, protein synthesis may provide a physiological index of the ion concentrations free to interact

with the soluble cell components.

Most of the enzymes studied from moderate halophiles exhibit the ability to withstand high salt concentrations. This follows from the studies of the stabilities of the moderately halophilic enzymes over a wide range of salt concentrations (Shindler, 1976). The ribosomes (Chapter II) and the protein synthesizing system (Chapter IV) are also stable over a wide range of salt concentrations. This could be interpreted as a mechanism of survival. When cells are placed in new ionic conditions (ie. by placing cells grown in one salt concentration into media containing a different salt concentration) a bacterial cell undergoes a period of adjustment (Brown, 1976). During this period, the bacterial cell comes to grip with the new ionic conditions and makes the appropriate adjustments (ie. increasing the polyol content as in the halophilic algae, Ben-Amots, 1975). Ginzberg et al (1970) have shown that even transferring cells of an extreme halophile from one culture flask to another with the same ionic composition results in a tremendous influx of ions that are gradually extruded to maintain the high gradient in logarithmic-phase cells. It is during this period of adjustment that stability of the cellular components to high salt concentrations would be advantageous. Cellular components already present in the cell could still function, although at greatly reduced activity. In intertidal pools and estuaries, the ionic conditions are not stable but constantly changing so that those cells capable of such a salt tolerance would survive, while those whose components cannot function at high salt concentrations would not.

If the intracellular salt concentrations of a moderate halophile, such as V. costicola, are low, then what is maintaining the osmotic balance inside the cell? Ben-Amotz (1975) has shown that some halophilic algae maintain a cellular osmotic pressure by containing large amounts of polyols such as glycerol. As yet, no such mechanism has been demonstrated in bacteria. Measures (1975) puts forth the idea that in bacteria, increases in the amino acid pools are responsible for osmoregulation. Tempest et al (1970) showed that increases in the salt content of the growth medium of several bacteria, results in an extensive and rapid increase in the glutamate intracellular pool. Such a mechanism could exist in the moderate halophiles. Clearly, more work in this area is needed in order to more clearly define the moderately halophilic mechanism of adaptation.

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Appendix

The determination of the intracellular cation concentrations of Vibrio costicola grown in complex media were done as part of this thesis research. The procedures and results were thoroughly discussed in D.B. Shindler's Ph.D. thesis (1976), so they are not repeated here. D.B. Shindler was concerned, however, with the intracellular ion concentrations of V. costicola grown in minimal media. The range of salt concentrations over which this organism can grow is dependent upon the complexity of the growth medium. Since the ribosomes were to be studied from V. costicola grown over the widest possible salt range, this necessitated the use of a complex medium. It was possible that the intracellular ion concentrations were different when the cells were grown in a complex medium. Therefore, the intracellular ion concentrations of V. costicola grown in various NaCl concentrations in a minimal media plus casamino acids or in a complex medium were determined. The following is a reprint of an article published combining the intracellular ion data.

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Cell-Bound Cations of the Moderately Halophilic Bacterium *Vibrio costicola*

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Over most of the range of salt concentrations in which the moderately halophilic bacterium *Vibrio costicola* could grow, the sum of the cell-associated Na^+ + K^+ ions was at least as high as in the external medium. This is in contrast to other moderate halophiles, which have substantially lower internal than external salt concentrations for most of their growth range. The relative amounts of Na^+ and K^+ in *V. costicola* varied with environmental conditions. The K^+/Na^+ ratio fell during anaerobic incubation or when cells were poisoned. As Na^+ ions left the cells, K^+ ions entered. However, movement of these ions was not tightly coupled, since K^+ content of cells could increase without a corresponding decrease in Na^+ content. The Mg^{2+} contents of cells varied little with environmental conditions.

Most microorganisms that can grow in concentrated solutes probably do not do so by maintaining a dilute internal milieu. It has been pointed out (3) that such a mechanism, which implies impermeability to water, is very unlikely. Furthermore, in a number of microorganisms that can grow in high solute concentrations, the internal solute concentration is at least as high (or the internal water activity, a_w, is at least as low) as the external concentration (6; reviewed by Brown [4]). However, the internal solute composition of such cells (as of all cells) is always different from the outside. It is well known that the extremely halophilic bacteria growing in 4 M NaCl contain lower amounts of Na^+ ions and high amounts of K^+ ions (up to 5 M or higher; 7, 11, 12, 14). Salt-tolerant or halophilic yeasts and algae may contain low concentrations of salt but high concentrations of nonionic solutes (1, 4). Nonhalophilic bacteria may use amino acids for osmotic regulation (18). The internal solute concentrations of moderately halophilic bacteria, which grow over a wide range of salt concentrations, have been less studied, but evidence is beginning to accumulate that the salt contents of these cells may be less than that of the external medium. Masui and Wada (16) found that in a moderately halophilic pseudomonad the internal concentrations of Na^+, K^+, and Cl^- were about 1.0, 0.8, and 0.8 M, respectively, after growth in media containing 1.0, 2.0, or 3.0 M NaCl and 5.5 mM KCl. Matheson et al. (17) found that a facultatively halophilic bacterium with a wide salt tolerance could maintain an internal ionic (Na^+ + K^+) concentration substantially lower than that of the external medium.

We have been studying physiological properties of the moderate halophile *Vibrio costicola*, including the behavior of its ribosomes (28), its protein-synthesizing systems, and some of its regulatory enzymes (work in preparation). Very little is known about the internal composition of this organism, except for Christian and Waltho's report in 1962 that stationary-phase cells grown in 1 M NaCl had a total of about 0.9 M Na^+ + K^+ ions (7). We present here a study of the internal ionic contents of *V. costicola* as influenced by the composition of the growth medium and by the physiological state of the cells.

(This paper is taken in part from a thesis submitted by D. B. S. to the University of Ottawa in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacteria. *V. costicola* NRC 37001 came from the National Research Council of Canada culture collection.

Media and culture conditions. Cells were maintained on agar slants containing: 0.5% proteose peptone (Difco), 0.5% tryptone (Difco), and 1 M NaCl. Before growing large culture volumes in synthetic media, cells were precultured twice in small volumes of these media. One percent inocula were used. For the experiments reported, cells were grown at 30°C with vigorous aeration (i.e., 500 ml of culture medium in a 2,800-ml Fernbach flask

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shaken in a reciprocating shaker at 150 to 200 strokes/min) to the late-exponential growth phase or as indicated. The synthetic medium used was either the fourfold-buffered medium (SGP) of Forsyth and Kushner (9) or one that contained an equimolar amount of disodium phosphate substituted for the dipotassium phosphate (SGS). In addition to the specific molar concentration of NaCl added, the SGS medium contained 0.14 M Na^+, 0.008 M K^+, and 0.41 mM Mg^{2+}, and the SGP medium contained 0.15 M K^+ and 0.41 mM Mg^{2+}. Other media used are specified in Results.

Suspension preparation. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 20°C, washed in an isotonic buffer [NaCl as in the medium plus 0.2 M NaCl, 0.008 M KCl, 0.41 mM $MgCl_2$, and 0.05 M tris(hydroxymethyl)aminomethane, pH 7.5] and then resuspended, using a syringe fitted with a 14-gauge cannula, to a concentration of 20 to 40 mg/ml (wet cell weight). This procedure did not cause cell damage as measured by the release of material absorbing at 260 nm.

These suspensions with either 25 mM glucose, 25 mM ethanol, or "poisons" (10 mM each iodoacetate and sodium cyanide) were agitated in a New Brunswick G-76 gyratory water bath shaker at 250 rpm for 30 min at 30°C. In some experiments, nitrogen or oxygen gas was passed through the head space of the incubating vessels. At appropiate time intervals, sets of three 10-ml samples were removed for the determination of ionic contents, intercellular space (ICS), and concentration of cells (milligrams per milliliter, wet weight and dry weight).

Determination of the ionic content. Each sample was pelleted in a 50-ml polycarbonate centrifuge tube by centrifugation at 25,000 × *g* for 10 min at 20°C. The supernatant was discarded, and the inside walls of the tube were carefully rinsed with distilled deionized water and wiped dry with tissue paper. The cell pellet was resuspended in 24 volumes of deionized water per g (wet weight). To this suspension was added an equal volume of 10% (wt/vol) trichloroacetic acid. The suspension was mixed thoroughly, heated at 95°C for 15 min, and then centrifuged at 25,000 × *g* for 10 min at 4°C. The supernatant was decanted into plastic or polycarbonate containers for dilution and analysis. A Perkin-Elmer 403 atomic absorption spectrophotometer was used for the emission analysis of Na^+, K^+ and Mg^{2+}. To obtain an ionic composition similar to that of the samples, standards for sodium and potassium were made up from a stock solution containing equal amounts of both ions. Magnesium standards contained 10-fold-excess Na^+ and K^+ ions.

Choice of a solute for determining ICS. ICS was measured as previously described (22) with some minor changes. [*carboxyl*-^{14}C]inulin (New England Nuclear), [^{14}C]sucrose (Amersham/Searle), and [8-^{14}C]adenosine 5'-triphosphate (ATP; Amersham/Searle) were tested as nonpenetrating radioactive solutes. Portions (0.2 ml) of the two supernatants S_1 and S_2 (22) were counted on a Beckman LS-230 liquid scintillation counter in 10 ml of Aquasol (New England Nuclear) plus 1 ml of water. Unlabeled solutes, phosphate and blue dextran (Pharmacia, Ltd.), were also tested. These were measured colorimetrically (blue dextran by absorbance at 620 nm; phosphate by the Fiske-SubbaRow method [15]).

Since *V. costicola* lyses at low ionic strength, it was not possible to measure cell-associated ions in cells washed onto filters. Estimates of these ions in packed cell pellets depend on accurate determinations of the extracellular volume. Different solutes have been used to measure this volume. Large molecules such as albumin, dextrans, and inulin (7, 10, 11, 14, 20) probably cannot penetrate further than the outside wall surface of most cells (21, 22). Extremely halophilic organisms are an interesting exception in that they appear permeable to large molecules such as inulin (10). Small molecules such as sucrose (5) or phosphate (19, 20) probably penetrate through the outer cell layers to the cell membrane (19). With *V. costicola* grown in 1 M NaCl, blue dextran and inulin gave ICSs of 0.27 to 0.28 ml/g of wet pellet, representing 40% penetration. ATP and phosphate gave larger ICSs (0.43 to 0.45 ml/g) and apparently deeper penetration (62 to 66%). This pattern of penetration is similar to that of a marine pseudomonad (5, 24) and of *Staphylococcus aureus* (20). We think that the first two solutes penetrated to the wall and the other solutes to the cytoplasmic membrane. Sucrose could not be used to measure ICSs because *V. costicola* rapidly metabolized it.

Later work showed that ATP substantially penetrated cells grown in 0.6 or 1.6 M NaCl. We thought it likely that phosphate could be taken up actively under certain conditions, and hence we distrusted these ions for routine determinations of ICS. Separate experiments showed that inulin was not retained by cells washed onto filters after several hours of exposure. This compound was used for most ICS measurements. Inulin ICS values were consistent for cells harvested in one phase of growth, though they were about 10% higher in mid-logarithmic than in late-logarithmic cells. Time course studies at intervals up to 60 min showed that inulin ICS did not vary with time for any one batch of cells. ICS values were not affected by aerating the cells with or without glucose or poisons.

Pellet wet- and dry-weight determinations. Samples (10 ml) for pellet weights were centrifuged at 25,000 × *g* for 10 min in Corex no. 8441 15-ml centrifuge tubes. After supernatants were removed, the inside tube walls were rinsed with deionized water and wiped with tissue. A jet of gently flowing dry air was directed inside the tubes until the walls were dry (1 to 3 min). The wet weights of the pellets were determined. After 24 to 36 h at 105°C, the tubes attained constant weight and the dry weights of the pellets were determined.

Molar cell-associated ion concentrations. These were calculated by the formula:

$$C = \frac{A - (\mathrm{ICS} \times B)}{[(1 - D) - (W \times \mathrm{ICS})] \times F}$$

where A is pellet ion concentration in milligrams per gram of wet pellet weight; B is ion (milligrams per milliliter) in ICS solution; C is intracellular ion concentration in moles per kilogram of cell water; D is

grams (dry weight) per gram (wet weight) of pellet; F is molecular weight of the ion; ICS is intercellular space in milliliters per gram (wet weight); and W is water content of the intercellular solutions (grams per milliliter) estimated from tables (27). The formula resembles those used by others (7, 11, 14, 21, 22, 26). It differs only in taking the water content of intercellular solutions into account, which makes a very small difference in the calculation.

RESULTS

Effects of metabolic state on cell-associated ions. Thompson and MacLeod (24) found that the amounts of cell-associated ions of a marine bacterium were greatly affected by the metabolic state of the cell suspensions. Washed and pelleted cells could have an internal distribution of ions quite different from that of active cells in culture. To see whether this was also true for *V. costicola*, its ionic contents were determined after incubations of thick suspensions under different conditions, including aeration, aeration with energy sources (glucose or ethanol), aeration with metabolic poisons, and gassing with oxygen or nitrogen.

Cells grown in 1 M NaCl SGS or SGP medium, washed and resuspended in isotonic buffer, typically contained 1 M Na^+ and 0.4 M K^+. When the thick suspension was aerated for 30 min by vigorous shaking, the potassium concentration increased while the sodium concentration did not change significantly (Fig. 1). Agitation under N_2 caused a small drop in potassium levels and a larger increase in sodium levels. The total sodium and potassium levels as well as the K^+/Na^+ ratios were higher in aerated cells than in cells incubated under conditions that inhibited aerobic metabolism. There were probably no major changes in ion distribution during the 10 min of centrifugation required to pellet the cells, since the changes due to the nitrogen treatment took approximately 15 min to begin (Fig. 1).

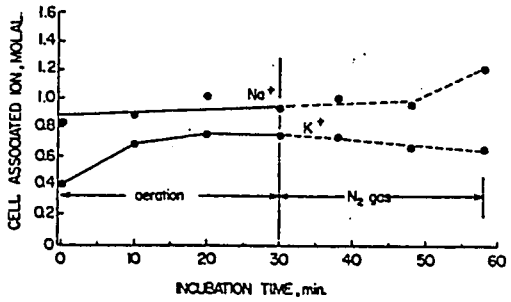

FIG. 1. *Effects of aeration and subsequent incubation under N_2 gas on V. costicola cell-associated Na^+ and K^+. Cells grown to late-exponential phase in SGP medium containing 1 M NaCl were washed and suspended in the isotonic buffer.*

Effect of the medium NaCl concentration on cellular cations. The effects of the concentration of NaCl in synthetic media (SGS and SGP) on intracellular cations are shown in Table 1. In NaCl concentrations up to 1.6 M, the sum of internal Na^+ and K^+ ions was higher than that of the external ions. Only in 2 M NaCl, the highest concentration in which cells could grow well in the synthetic media, was the total $Na^+ + K^+$ concentration slightly lower than the external NaCl concentration.

The internal ions increased with external NaCl concentrations, though not proportionally. The K^+/Na^+ ratio decreased with increasing NaCl concentration in the growth medium. As might be expected (see Fig. 1), posioned cells had the lowest K^+/Na^+ ratio, less than one-half that of aerated cells. Actively metabolizing *V. costicola* cells, on the other hand, maintained 0.5 to 0.6 molal cellular K^+ in addition to an amount of Na^+ that resulted in the cellular ion concentration being equal to or greater than the medium Na^+ content.

Cellular Mg^{2+} concentrations were not greatly affected by the treatments or medium NaCl concentrations (Table 1). Poisoned cells had slightly higher Mg^{2+} contents than aerated cells. Increasing medium NaCl caused a small decrease in cellular Mg^{2+}.

To observe the effects of yet higher external NaCl concentrations, it was necessary to grow *V. costicola* in complex media. Cells grown in such media, however, appear to be fragile. During the preparation of six out of ten thick suspensions (from different cultures), the cells lysed. High inulin ICS values of 0.6 to 0.8 ml/g of wet cells, approaching 100% penetration of the total fluid space, and low pellet dry weight-to-wet weight ratios (D values) indicated that these cells had lost an effective permeability barrier. In the remaining experiments, ICS and D values for the pellets were closer to those of cells grown in defined salts-glucose media. The cause of lysis in the other experiments was not investigated further. With those cells that did not lyse after being grown in complex media, the total $Na^+ + K^+$ concentration also increased with the medium salt concentration (Table 2). At higher salt concentrations, the $Na^+ + K^+$ concentration was less than the NaCl concentration of the medium. A considerable amount of K^+ was contained in cells grown at medium NaCl concentrations of 1.0 to 1.6 M; cells had less K^+ at lower and higher medium salt concentrations.

TABLE 1. *Effect of medium NaCl concentration on cell-associated cations*

Medium	NaCl (M)	Treatment	Cell-associated cations (molal)				
			Na^+	K^+	Mg^{2+}	K^+/Na^+	$Na^+ + K^+$
SGP	0.6	None[a]	0.77	0.52		0.68	1.29
	1.0		0.95	0.66		0.69	1.61
	1.6		1.71	0.52		0.30	2.23
SGS	0.6	Aerated for 30 min[b]	0.65	0.72	0.053	1.10	1.37
	1.0		0.89	0.82	0.048	0.92	1.71
	1.6		1.39	0.57	0.037	0.41	1.96
	2.0		1.29	0.55	0.038	0.41	1.84
SGS	0.6		0.51	0.52	0.051	1.04	1.03
	1.0		0.58	0.66	0.056	1.13	1.24
	1.6		1.09	0.59	0.038	0.54	1.68
	2.0		0.90	0.57	0.042	0.63	1.47
SGS	0.6	Poisoned[b]	0.94	0.40	0.062	0.43	1.34
	1.0		0.97	0.33	0.056	0.34	1.30
	1.6		1.36	0.24	0.040	0.17	1.60
	2.0		1.52	0.28	0.046	0.18	1.80

[a] Cells grown to mid-exponential phase and washed with SGP medium without glucose, containing the appropriate NaCl concentration, and then pelleted directly for ionic analyses.

[b] Cells grown to mid-exponential phase and washed in isotonic buffer containing the same Na^+, K^+, and Mg^{2+} concentrations as the growth medium and resuspended in this buffer; 25 mM glucose or 10 mM NaCN and iodoacetate were used. All values are the mean of duplicate determinations that agreed closely (usually within 5%).

TABLE 2. *Cell-associated ions of V. costicola grown in complex media*[a]

Medium, and ion concentrations	Cell-associated cations (molal)					Cell $Na^+ + K^+$/medium $Na^+ + K^+$	D[b]	ICS (mg/g of wet pellet)
	Na^+	K^+	Mg^{2+}	K^+/Na^+	$Na^+ + K^+$			
SGS-CAA[c]								
0.5 M NaCl (0.8 M Na^+, 0.008 M K^+)	0.65	0.39	0.046	0.61	1.04	1.29	0.41	0.24
1.0 M NaCl (1.3 M Na^+, 0.008 M K^+)	0.39	1.02	0.021	2.6	1.41	1.08	0.31	0.50
1.5 M NaCl (1.8 M Na^+, 0.008 M K^+)	0.518	1.01	0.098	1.9	1.52	0.84	0.35	0.40
PPT[d]								
3.0 M NaCl (3.1 M Na^+, 0.009 M K^+)	1.78	0.37	0.028	0.21	2.15	0.69	0.33	0.26

[a] Results given here are from those experiments in which the cells did not show evidence of lysis, i.e., $D \leq 0.24$, ICS ≤ 0.50. Cells washed and aerated in thick suspension in fresh growth.

[b] Pellet dry weight/wet weight.

[c] 0.5% (wt/vol) Casamino Acids with the SGS formulation.

[d] 1% (wt/vol) proteose peptone + 1% (wt/vol) tryptone.

DISCUSSION

Estimating cellular ions by using small molecules such as ATP or inorganic phosphate to determine ICS involves the assumption that all the intracellular ions are contained in the space bound by the permeability barrier, i.e., the membrane. If, however, a significant portion of cellular ions is associated with cell wall material external to the membrane, these ions will be included in the assay if ICS is measured by larger molecules, such as inulin.

Although different experimental techniques were used, our estimates of ICS values are comparable to those found for *Staphylococcus aureus* (19), *Escherichia coli* (21), *Vibrio alginolyticus* (26), and *Halobacterium* strains (albumin and dextran spaces, since inulin seems to penetrate these cells; 10, 14).

Actively metabolizing *V. costicola* could keep a 75- to 100-fold concentration gradient of K^+ with respect to the medium. The NaCl concentration of the medium did not alter the cellular K^+ concentration but had a large effect on the cellular Na^+ concentration. At external concentrations above 1.0 M, the concentration

of Na^+ was greater than that of K^+. Substantial amounts of cellular Na^+ are usually not found in actively metabolizing nonhalophilic bacteria such as *E. coli* (21) or *Streptococcus faecalis* (13), but have been noted in several halophilic bacteria.

In the few moderately halophilic bacteria previously studied, the internal salt concentration was usually less than that outside the cells (16, 17). *V. costicola* differs from these in maintaining internal $Na^+ + K^+$ concentrations at least as high as the external concentrations over most of the range of salt concentration in which it grows.

The cellular K^+ concentration was markedly reduced when cells were poisoned, indicating that metabolic energy was essential for cells to concentrate these ions. The decrease in cellular K^+ was more than compensated for by increases in Na^+ in poisoned cells. Although in general there was an exchange of cellular Na^+ for K^+, the Na^+ and K^+ translocation mechanisms were not directly linked to each other, because cellular K^+ could increase without a corresponding decrease in cellular Na^+. Studies on ion transport in *S. faecalis* (13), in *E. coli* (21), and in a marine pseudomonad (24) indicate that bacterial transports of Na^+ and K^+ are not tightly coupled.

The intracellular Mg^{2+} contents were unaffected by metabolic changes of *V. costicola*. Under conditions where the cell K^+ increased in chemostat cultures, the Mg^{2+} of *Aerobacter aerogenes* varied little (23). In *V. alginolyticus*, Mg^{2+} was not affected by washing with solutions containing various NaCl concentrations (26). In *V. costicola*, the maintenance of the 100-fold gradient of cellular Mg^{2+} with respect to the medium may be explained by tight binding to cell structures such as nucleic acids, ribosomes, and cell envelopes.

Though *V. costicola* usually has at least as high a total salt concentration inside as outside, some preliminary experiments suggest that these salts are not free in the cytoplasm. When cells were treated with cetyltrimethyl ammonium bromide, the cell-bound Na^+ and K^+ ions did not come to equilibrium with the external ions: the K^+ remained high and the Na^+ increased severalfold. A higher binding of Na^+ ions was also observed in those cells that lysed after growth in complex media. Masui and Wada (16) showed that the isolated cell envelope of the moderately halophilic pseudomonad no. 101 contained a large amount of Na^+ and somewhat less K^+. The distribution of ions in the nonhalophile *Bacillus amyloliquefaciens* was studied by Coleman (8). Most (80%) of the cellular Na^+ and all of the Ca^{2+} and Cl^- were bound to the envelope, whereas most of the Mg^{2+} and K^+ were considered to be intracellular, but probably associated with the phosphate groups of deoxyribonucleic acid and ribosomes. Very recently, Beveridge and Murray (2) showed that the cell walls of *B. subtilis* can bind considerable amounts of Na^+ and K^+ ions, as well as Mg^{2+} ions and a number of other cations. These results argue against the concept that all or even most of the cellular Na^+ and K^+ ions of different bacterial species are free in the cytoplasm. This conclusion is also supported by recent studies of the salt requirements and tolerances of *V. costicola* enzymes. The activities of a number of enzymes, as well as in vitro protein synthesis, are strongly inhibited by levels of salt well below those measured here as total intracellular concentrations (D. B. Shindler, Ph.D. thesis, University of Ottawa, Ottawa, Canada, 1976; R. M. Wydro, W. Madira, M. Kogut, T. Hiramatsu, and D. J. Kushner, manuscript in preparation). It seems possible that most of the cellular ions in these bacteria are somehow separated from salt-sensitive structures.

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