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UNIVERSITY OF OTTAWA

To my wife Andrée

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ABSTRACT

In vitro protein synthesis in *Vibrio costicola* (polyuridilic acid [poly(U)]-directed incorporation of phenylalanine) was studied. The extent of protein synthesis is limited by the number of ribosomes present. Density gradient centrifugation experiments suggest that after run-off of the ribosomes from the artificial messenger, the 50S subunit is unable to attach to the 30S-messenger complex.

As shown previously (Kamekura and Kushner, 1984), Cl^- ions inhibited protein synthesis; indeed, the highest rate of synthesis took place in the lowest attainable Cl^- concentration (37 mM). The inhibitory effects were partly reversed by glutamate and betaine, both of which are concentrated within cells of *V. costicola*. The strongest reversal was seen when both glutamate and betaine were present. Cl^- ions can prevent binding of ribosomes to poly(U) and displace ribosomes already bound to this artificial messenger. The effects of Cl^- ions on binding were also reversed by glutamate and betaine. Cl^- ions do not affect accuracy of translation; they were shown previously (Kamekura and Kushner, 1984) not to affect phenylalanyl-tRNA synthetase. It was also found that washing ribosomes with inhibitory NaCl concentrations did not interfere with their ability to carry out protein synthesis later in optimal (low) salt concentrations. On the contrary, these ribosomes were more active than before being washed. It is

concluded that the main site of action of Cl^- in this system is on the binding of ribosomes to the messenger RNA.

Because the poly(U) system is directed by an artificial mRNA, it differs in many ways from the natural process. Therefore, I studied the protein synthesizing machinery of V. costicola with in vitro protein synthesis systems representing more closely the normal physiological conditions under which this essential process normally occurs; that is, using cell-free protein synthesis systems directed by natural mRNAs. One system, the endogenous system, was directed by a mixture of unidentified messengers present in V. costicola, the mRNAs endogenous to these cells. This system was initially described by Wydro et al. (1977) and I have now studied it in more detail. In another system, I have shown that the viral mRNA, that of the coliphage R17, directs the incorporation of [^{14}C]valine with cellular extracts of V. costicola. Both systems were shown to carry out de novo synthesis.

The ionic requirements of both systems were similar; both were most active at NH_4^+ (as ammonium glutamate) and Mg^{++} concentrations of 250-300 mM and 8 mM, respectively. NH_4^+ supplied in its chloride form did not support high levels of activity in both systems. As was the case with the poly(U) system, Cl^- also inhibited protein synthesis in these systems. It was also found that added sodium or potassium glutamate inhibited the R17 RNA system but stimulated the endogenous system. These differences are probably not due to a change in

the secondary structure of the viral RNA (because of increasing salt concentrations), since these salts also inhibited in vitro protein synthesis directed by formaldehyde-treated R17 RNA. From these studies, it is believed that Cl^- is more deleterious to the initiation than to the elongation of protein synthesis, and that glutamate stimulates elongation. Betaine stimulated the activity of both systems, but did not show the same protective effects against Cl^- as those observed in the poly(U) system.

In an attempt to study more closely the effects of Cl^- on the initiation of protein synthesis, I have successfully isolated a crude preparation of initiation factors of V. costicola. These factors were released from the ribosomes at a NH_4Cl concentration of at least 3.0 M, a concentration much higher than that required for the isolation of the initiation factors of E. coli (1.0 M). Cl^- was more inhibitory to the retention of [^{14}C]fmet-tRNA on nitrocellulose filters, than to the retention of [^{3}H]R17 RNA; the retention of these substances was presumably due to the action of IF_2 and IF_3, respectively.

The enzymes choline dehydrogenase and betaine aldehyde dehydrogenase, which are involved in the synthesis of betaine from choline, were identified in V. costicola. Choline dehydrogenase is an O_2-dependent membrane bound enzyme and it oxidizes not only choline but also betaine aldehyde (at 30% the rate of choline oxidation). Betaine aldehyde dehydrogenase is a soluble NAD-dependent enzyme. Both enzymes are probably

inducibly formed in the presence of choline and seem to be osmoregulated. Choline dehydrogenase is inhibited by high Cl^- concentrations but functions well at high Na^+ or K^+. On the other hand, betaine aldehyde dehydrogenase is stimulated by NH_4Cl (0-1.0 M) and KCl (0-2.0 M) and is inhibited only by 30% by as much as 2.0 M NaCl.

V. costicola accumulates betaine in response to increasing external NaCl; the intracellular betaine concentration in cells grown in media containing 1.0 and 3.0 M NaCl is 0.25 and 1.2 M, respectively. This accumulation of betaine is also affected by the external concentration of choline. At the lowest choline concentration (0.002%), there is no difference in betaine concentration between cells grown in media containing 0.5, 1.0 and 2.0 M NaCl, while cells grown in the presence of 3.0 M NaCl have only slightly more (0.4 M). These results suggest that betaine may not be the only compatible solute in V. costicola and/or that it may be synthesized through an alternative pathway, and that these cells may also employ other means to maintain turgor pressure.

RÉSUMÉ

J'ai étudié la synthèse protéique in vitro [l'incorporation de la phénylalanine dirigée par le poly(U)] chez la bactérie Vibrio costicola. Dans ce système, le nombre de ribosomes détermine la durée de la synthèse protéique. D'après des études de centrifugation en gradient de densité, il semble que la sous-unité 50S n'est plus capable de s'associer au complexe 30S-ARNm lorsque les ribosomes se detachent de la matrice de poly(U).

Comme Kamekura et Kushner (1984) l'ont déjà démontré, le Cl^- inhibe la synthèse des protéines; en effet, le taux de synthèse le plus rapide est obtenu lorsque la concentration de Cl^- est à son minimum (37 mM). Les effets inhibiteurs des ions de Cl^- peuvent être partiellement supprimés par le glutamate et la bétaïne. Ces deux substances sont normalement présentes à de fortes concentrations à l'intérieur de V. costicola. Cette protection est maximale lorsque le glutamate et la bétaïne sont présents simultanément. Le Cl^- empêche la fixation des ribosomes à la matrice polu(U) et, de plus, cause la dissociation des ribosomes qui sont déjà fixés au messager artificiel. L'effet du Cl^- sur la fixation des ribosomes a aussi été renversé par le glutamate et la bétaïne. Les ions de Cl^- n'ont aucun effet sur l'ambiguïté du code, et Kamekura et Kushner (1984) ont déjà démontré que ces ions n'affectaient pas la phénylalanyl-ARNt synthétase. De plus, j'ai découvert que laver les ribosomes avec une solution de NaCl de concentration

inhibitrice n'a aucun effet négatif sur leur capacité de participer à la synthèse des protéines dans une solution à faible concentration de sel. Au contraire, ces ribosomes étaient plus actifs qu'avant qu'ils ne soient lavés. Il a été conclu que, dans ce système, le Cl^- inhibe la synthèse peptidique au niveau de la fixation des ribosomes à l'ARNm.

La synthèse protéique in vitro dirigée par le poly(U), qui est un messager artificiel, diffère considérablement du processus naturel. J'ai donc étudié la synthèse des protéines chez V. costicola à l'aide de systèmes in vitro qui reflètent plus précisément les conditions physiologiques dans lesquelles ce processus a normalement lieu, c.-à-d. en utilisant des systèmes de traduction dirigés par des ARNm naturels. Un de ces systèmes, le système endogène, était dirigée par un mélange de ARNm inconnu, les ARNm endogènes à V. costicola. Ce système a été initialement décrit par Wydro et al. (1977), et j'en ai maintenant fait une étude plus approfondie. Dans un autre système, j'ai démontré que de l'ARNm viral, celui du coliphage R17, peut diriger l'incorporation de la valine par des extraits cellulaires de V. costicola. Il a été démontré que la polymérisation dans ces deux systèmes est effectivement le résultat de la synthèse protéique de novo.

Dans les deux systèmes, les conditions ioniques requises sont semblables: 0.25-0.3 M de NH_4^+ et 8 mM de Mg^{++}. Lorsque le NH^{4+} était fourni sous forme de chlorure, la synthèse des protéines était pratiquement inexistente. Comme pour le système

dirigé par le poly(U), le Cl^- a inhibé la synthèse dans ces deux systèmes. De plus, le sodium glutamate et le potassium glutamate ont aussi inhibés la synthèse des protéines dans le système dirigé par l'ARN de R17, tandis quils ont stimulés la synthèse protéique dans le système endogène. Ces différences ne sont probablement pas attribuables à un changement de la structure secondaire de l'ARN viral (à cause de l'augmentation de la teneur de sel), puisque ces sels ont aussi inhibés la synthèse protéique dirigée par lÄRN de R17, qui avait été traité au préalable avec de la formaldéhyde. D'après ces études, on croit que le Cl^- endommage davantage l'initiation des chaînes peptidiques que le cycle d'élongation de la synthèse protéique et que le glutamate stimule l'élongation des chaînes peptidiques. La bétaïne a stimulé la synthèse des protéines dans les deux systèmes, mais elle n'a pas démontré le même pouvoir protecteur envers les ions de Cl^- que celui observé dans le système dirigé par le poly(U).

Afin d'étudier plus à fond l'effet des ions Cl^- sur l'initiation des chaînes peptidiques, j'ai réussi à isoler une préparation brute des facteurs d'initiation de V. costicola. Ces facteurs ont été extraits des ribosomes à l'aide de solutions de NH_4Cl d'une concentration d'au moins 3.0 M, une valeur nettement supérieure à celle nécessaire pour extraire ces mêmes facteurs des ribosomes de Escherichia coli (1.0 M). Le Cl^- était plus inhibiteur envers la rétention , sur des filtres de nitrocellulose, de fmet-ARNt qu'envers la rétention de l'ARN

de R17; de toute évidence, ces substances étaient retenues par le filtre grâce aux actions de IF_2 et IF_3, respectivement.

Chez V. costicola, les enzymes choline déshydrogénase et bétaïne aldéhyde déshydrogénase, qui sont impliquées dans la synthèse de la bétaïne à partir de la choline, ont été identifiés. La choline déshydrogénase est un enzyme membranaire qui requiet de l'oxygène pour fonctionner. Cet enzyme, en plus d'oxider la choline peut aussi oxider la bétaïne aldéhyde (à 30% du taux d'oxidation de la choline). La bétaïne aldéhyde déshydrogénase est un enzyme soluble qui requiert la présence de NAD pour son bon fonctionnement. Ces deux enzymes sont probablement synthétisés par induction en présence de choline, et il semble qu'ils soient aussi sous un contrôle d'osmorégulation. La choline déshydrogénase est inhibée par de fortes concentrations de Na^+ et de K^+. Par ailleurs, la bétaïne aldéhyde déshydrogénase est stimulé par le NH_4Cl (0-1.0 M) et le KCl (0-2.0 M) et n'est que légèrement inhibée (30%) par une concentration de NaCl aussi forte que 2.0 M.

V. costicola accumule de la bétaïne en réponse à une augmentation de la concentration externe de NaCl: la concentration intracellulaire de bétaïne dans des cellules cultivées en présence de 1.0 et de 3.0 M NaCl est de 0.25 et 1.2 M, respectivement. Cette accumulation de bétaïne est aussi affectée par la concentration externe de choline. A la plus faible concentration externe de choline (0.002%), la concentration intracellulaire de bétaïne est la même, que les

cellules aient été cultivées dans un milieu contenant 0.5, 1.0 ou 2.0 M NaCl. Cependant, les cellules cultivées en présence de 3.0 M NaCl contiennent un niveau un peu plus élevé de bétaïne. Ces résultats semblent indiquer que la bétaïne n'est probablement pas le seul soluté compatible chez V. costicola, ou encore qu'elle est synthétisée par une voie métabolique différente. Il se peut aussi que ces cellules utilisent d'autres moyens pour maintenir leur pression osmotique interne à un niveau plus élevé que la pression osmotique du milieu externe.

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INTRODUCTION

Halotolerant and halophilic microorganisms

The term "halophilic" describes organisms that require salt for growth. In the microbial world, halophilism can be expressed to varying degrees and consequently halophilic microorganisms have been grouped according to the salt concentration at which they are best able to grow (Table 1).

The slight halophiles, as the name suggests, grow best in the presence of low salt concentrations (0.2-0.5 M NaCl). Many marine microorganisms belong to this group. Seawater also contains numerous moderate halophiles (Forsyth et al., 1971; Ventosa et al., 1984) such as Vibrio parahaemolyticus. These grow best at salt concentrations between 0.5 and 2.5 M NaCl. Moderate halophiles can also be isolated from salty soil, salty mud, and salty food (Kushner, 1978; Larsen, 1986; Rodriguez-Valera, 1986). In fact, several of these microorganisms are responsible for the spoilage of certain food. Moderate halophiles are adapted to greater variations in the salt concentration of the external medium than the other halophiles (Kushner, 1978; Kamekura, 1986; Larsen, 1986). This is a reflection of the wide range of salt concentrations to which they are exposed in their natural environments (Larsen, 1986).

Microorganisms growing best at salt concentrations between 2.5 and 5.2 M NaCl (saturated) are the extreme halophiles

Table 1. Salt response of different microorganisms (Kushner, 1985).

Category	Reaction	Examples
Nonhalophile	Grows best in media containing less than 0.2 M salt	- most normal eubacterial and most freshwater microorganisms
Slight halophile	Grows best in media containing 0.2-0.5 M salt	- many marine microorganisms
Moderate halophile	Grows best in media containing 0.5-2.5 M salt	- Vibrio costicola, - Paracoccus halodenitrificans, - Pseudomonas species.
Borderline extreme halophile	Grows best in media containing 1.5-4.0 M salt	- Ectothiorhodospira halophila, - Actinopolyspora halophila, - Halobacterium volcanii, - H. mediterranei
Extreme halophile	Grows best in media containing 2.5-5.2 M (saturated) salt	- Halobacterium salinarium, - Halococcus morrhuae
Halotolerant	Nonhalophile that can tolerate salt. If the growth range extends above 2.5 M salt, it may be considered extremely halotolerant.	- Staphylococcus epidermidis, - solute-tolerant yeasts, fungi, and algae. - Halomonas elongata

(Table 1). They have been of much interest in recent years not only because of their ability to grow at such high salt concentrations, but also because they are members of the archaebacteria (Woese, 1981). Members of the genus Halobacterium have an additional feature that attracted a lot of attention, the purple membrane and its protein bacteriorhodopsin (Stoeckenius, 1979). The discovery of bacteriorhodopsin has revealed new ways of transducing the energy of light through biological membranes. Other microorganisms have been classified as borderline extreme halophiles since their minimum salt requirement is higher than the moderate halophiles while the highest salt concentration at which they can grow is not saturation, a characteristic of extreme halophiles.

The extreme halophiles have been mainly isolated from salterns, the ponds used to prepare solar salt from seawater, and from salt lakes, such as the Great Salt Lake and the Wadi Natrun Lakes (Kushner, 1985; Grant and Ross, 1986). It should be noted that the term "salty environments" does not necessarily imply that Na^+ is the major cation. For example, the Dead Sea contains more Mg^+ than Na^+ (Kushner, 1985). In addition, different environments may have very different ionic conditions. The Wadi Natrun Lakes have HCO_3^-/CO_3^{2-} high enough to maintain pH values greater than 11 (Kushner, 1985).

To this list one can add the nonhalophiles which include most normal eubacteria and freshwater microorganisms, and the

halotolerant microorganisms which do not require NaCl for growth but can tolerate high concentrations of it.

The moderate halophile V. costicola

The moderate halophiles have attracted a lot of attention mainly because of their ability to grow, in contrast to the extreme halophiles, over a wide range of NaCl concentrations (Kushner, 1978; Kamekura, 1986; Larsen, 1986). This characteristic makes them good candidates for studies of haloadaptation, and Vibrio costicola has been, by far, the most studied.

V. costicola is a gram negative, facultative anaerobic, curved rod. This organism was originally isolated from pork ribs curing in salt brine (Smith, 1938). It can grow in media containing from 0.4 to 3.5 M NaCl with its optimum growth occurring at a concentration of 1 M NaCl (Flannery et al., 1952; Forsyth and Kushner, 1970; Kamekura et al. 1985). This ability to grow at higher NaCl concentrations is not due to a selection of more resistant cells, but rather is a result of individual adaptation (Forsyth and Kushner, 1970).

Intracellular ionic composition of halophilic eubacteria

To understand the effects of changing environmental salt conditions on the growth and on the cellular components of V. costicola, it is important to at least have a basic knowledge of the intracellular ionic conditions of these cells (Table 2). As

Table 2. Effect of medium NaCl concentration on cell-associated ions of V. costicola (Shindler et al, 1977).

Ion concentration (M)					
In medium[a]			Cell-associated		
Na^+	K^+	$Na^+ + K^+$	Na^+	K^+	$Na^+ + K^+$
0.6	.008	0.6	0.51	0.52	1.03
1.0	.008	1.0	0.58	0.66	1.24
1.6	.008	1.6	1.09	0.59	1.68
2.0	.008	2.0	0.90	0.57	1.47
3.0	.008	3.0	1.78	0.37	2.15

[a] From 0.6 to 2.0 M NaCl, the cells were grown in the chemically defined medium SGS (Forsyth and Kushner, 1970) and, at 3.0 M NaCl, they were grown in the complex medium PPT (Shindler et al., 1977).

in most cells, actively metabolizing V. costicola accumulates K^+ ions. This accumulation is energy-dependent since poisoning the cells significantly decreases the amount of intracellular K^+ (Shindler et al., 1977). Changing external NaCl concentrations have no effect on internal K^+ concentrations. The intracellular Na^+ concentration is always lower than its external concentration, but increasing Na^+ ions in the medium results in increased levels of internal Na^+ ions (Table 2).

In V. costicola, the sum of the cell-associated K^+ and Na^+ ions is, in general, at least equal to the external NaCl concentration; that is, it increases with increasing external NaCl concentration (Table 2). Although this is also observed in other moderate halophiles (Oren, 1986a, 1986b; Rengpipat et al., 1988), this increase is not a characteristic of all halophiles. In the moderate halophile Pseudomonas halosaccharolytica, the sum of Na^+ and K^+ remains constant over a wide range of NaCl concentrations (Masui and Wada, 1973), while in an unidentified moderate halophile (Matheson et al., 1976) and in the extremely halotolerant Halomonas elongata (Vreeland et al., 1983), it is much lower than the outside NaCl.

The anionic composition of halophilic and nonhalophilic microorganisms is not well defined. In most eubacteria studied, the cell-associated Cl^- is usually less than 10% that outside the cell and lower than the sum of the cell-associated Na^+ and K^+ (Kushner, 1988). This is also true for the moderate halophiles Paracoccus halodenitrificans (Christian and Waltho,

1962), P. halosaccharolytica (Masui and Wada, 1973) and Vibrio alginolyticus (Unemoto and Hayashi, 1979). As for V. costicola, cells grown in media containing 1 and 3 M NaCl contain 0.2 M (Kamekura and Kushner, 1984) and 1.5 M (Kushner and Kamekura, 1988) Cl^- ions, respectively. However, it appears that strictly anaerobic moderate halophiles do not follow this rule; it was shown that the internal and external concentrations of Cl^- ions were equal in Haloanaerobium praevalens and Halobacteroides halobius (Oren, 1986b) and in Halobacteroides acetoethylicus (Rengpipat et al., 1988). Therefore, these anaerobes behave like the extreme halophilic archaebacteria in which the Cl^- ion concentration is the same as the external one (Kushner, 1978, 1985).

In V. costicola and other moderate halophiles that exclude Cl^- ions, what is the counterion to the high cell-associated Na^+ and K^+ ions? In V. costicola, it is not glutamic or aspartic acid since their concentrations are much lower than the intracellular Na^+ and K^+ (Kamekura and Kushner, 1984). The enzymes of the tricarboxylic acid cycle of V. costicola do not function in the presence of 0.5 M or higher sodium or potassium nitrates or bicarbonates (Kushner, 1988); it therefore seems unlikely that these are the balancing anions. In view of all these results, it may well be that the actual concentration of "free" intracellular cations is lower than their measured cell-associated concentrations. As suggested by Kushner (1988), the cell-associated cations could be bound to the envelope so that

the internal environment is really low in salts. Many studies on gram positive bacteria have shown that bacterial envelopes, which are negatively charged structures, can in fact bind monovalent cations in surprisingly large quantities (Beveridge and Murray, 1980).

Enzymes of halophilic eubacteria

The uncertainty about the ionic composition (nature and state of the different ions) of V. costicola and moderate halophiles in general is reflected in the paradoxical results obtained with numerous intracellular enzymes. While most enzymes of extreme halophiles require high salt concentrations for proper functioning (Kushner, 1985), as would be expected from their high internal ionic composition (up to 5 M KCl and 1 M NaCl), different intracellular enzymes of moderate halophiles may respond differently to NaCl. For example, threonine deaminase of V. costicola works best in the absence of salts (Kushner, 1986), while aspartate transcarbamylase from the same bacterium shows maximal activity at 1.5 M NaCl or 1.0 M KCl (Ahonkhai et al., 1989).

As a group, the intracellular enzymes of moderate halophiles studied so far seem to function well in, or to require, salt concentrations lower than what is normally found in the cells (Kushner and Kamekura, 1988). However, these studies were normally done with chloride salts since it had been tacitly assumed that Cl^- was the major counterion. We now know

that Cl^- ions are excluded from these cells and, therefore, the inhibitions may have been due to the Cl^- ion itself rather than to the salt (Kushner and Kamekura, 1988). Therefore, interpretation of these results must be made with great care and, in future experiments, salts other than chloride salts should also be studied.

In contrast with the intracellular enzymes, many, but not all, of the membrane-associated enzymes of halophilic eubacteria studied so far behave as if they are most active in ionic conditions approximating those of the external medium (Kushner and Kamekura, 1988). These requirements may simply be the result of their orientation in the membrane. If they are exposed to the external environment, they must be able to work in such an environment. If they face the interior of the cell they may behave more like intracellular enzymes. For example, the purified 5'-nucleotidase of V. costicola, which has been shown to be in contact with the external medium, requires 2.0 to 3.0 M NaCl for optimal activity (Bengis-Garber and Kushner, 1981). However, the cytochrome c reductase of P. halodenitrificans is inhibited by as little as 0.05 M NaCl (Miller and Hochstein, 1972), and its nitrate reductase functions optimally in the absence of any salt (Rosso et al., 1973). Although there is no such information, these last two enzymes are probably not exposed to the external environment.

Not surprisingly, extracellular enzymes of moderate halophiles, such as amylases, nucleases and B-lactamases,

function under the same salt conditions as the ones found in the medium (Onishi and Hidaka, 1978; Kamekura and Onishi, 1978; Onishi and Sonoda, 1979; Onishi et al., 1984; Kamekura, 1986; Kushner and Kamekura, 1988); otherwise, they would serve no purpose.

Envelopes of halophilic eubacteria

The ability of moderate halophiles to grow over a wide range of salt concentrations is believed to be due to their ability to modify cellular constituents, i.e. there are phenotypic adaptations to cope with the external conditions. It is not, as it was once postulated, due to the synthesis of different subsets of proteins or structures, that would each work over a certain range of salt concentrations. This is especially well illustrated in the changes observed in the cellular envelopes of halophiles in response to changing external salt concentrations.

In the moderate halophiles P. halosaccharolytica (Ohno et al., 1979), V. alginolyticus (Ohno et al., 1976), V. costicola (Hanna et al., 1984) and a Paracoccus sp. (Hiramatsu et al., 1980), there is a net increase in the proportion of negatively-charged phospholipids with increasing external NaCl. In V. costicola, this change is the result of an alteration in the biosynthetic rates of the particular classes of phospholipids, rather than a degradation of the already existing phospholipids (Russell et al., 1985). Protein synthesis is not required for

these events to take place (Kogut and Russell, 1984; Adams et al., 1987). Furthermore, the stimulus to which V. costicola responds is, at least in part, a change in osmotic pressure since changing the osmotic pressure of the medium with sucrose triggered comparable changes in phospholipid composition and synthesis as those observed with NaCl (Russell et al., 1986; Adams et al., 1987).

It is believed that these negatively-charged phospholipids are required to neutralize the increased concentration of counterions at the membrane surface (Kushner, 1978; Hiramatsu et al., 1980; Russell et al., 1986; Kates, 1986). They may also be involved in the regulation of the membrane permeability to cations (Kanemasa et al., 1972; Ohno et al., 1976; Thirkell and Summerfield, 1977;) since negatively-charged phospholipids have been shown to increase selectivity in the membrane permeability of cations (Hopfer et al., 1970; Papahadjopoulos, 1971; Haest et al., 1972).

The fatty acid composition of the cell envelope of moderate halophiles can also be affected by changing environmental salt conditions. In P. halosaccharolytica (Ohno et al., 1979) and Flavobacterium halmephilum (Monteoliva-Sanchez and Ramos-Cormenzana, 1986), cyclopropane fatty acids increase with increasing external salt concentration which results in an increased membrane fluidity. This change in membrane fluidity is also observed in the gram positive halotolerants Staphylococcus epidermidis (Komaratat and Kates, 1975) and a

Planococcus sp. (Miller, 1985) via an increase in the proportion of major branched fatty acids chains. In V. costicola, there is a decrease of total mono unsaturated fatty acids in cells grown at concentrations other than the optimal 1 M NaCl (Hanna et al., 1984), thus decreasing membrane fluidity (Melchior, 1982).

Finally, different external NaCl concentrations can also cause a change in envelope proteins. In P. halosaccharolytica, the excess of acidic amino acids of the outer membrane proteins, as well as the ratio of polar/non polar amino acids, is greater at its optimum salt concentration (2.0 M) for growth (Hiramatsu et al., 1980). In V. costicola, there is an outer membrane protein of yet unknown function that accumulates with increasing NaCl concentrations so that, at 3.0 M, it is the major outer membrane protein (Kushner and Kamekura, 1988). In the nonhalophile Anabaena sp. strain L-31 and in the halotolerant Anabaena torulosa, an increase in the external salinity causes an alteration in their protein profiles of membrane and cytoplasmic fractions (Apte and Bhagwat, 1989). The synthesis of different proteins is either enhanced or repressed and the synthesis of a specific set of proteins is induced de novo. The role of the proteins preferentially synthesized during salt-stress has not yet been identified.

In Escherichia coli, the relative amounts of its two major outer membrane proteins, OmpF and OmpC, are osmoregulated (Kawaji et al., 1979; van Alphen and Lugtenberg, 1977; Jovanovich et al, 1988). As the osmolarity of the medium

increases, the amount of OmpF decreases and the amount of OmpC increases; the total amount of both porins remaining constant. This control is at the transcriptional level and involves the gene products of the ompR and envZ genes (Hall and Silhavy, 1981a, 1981b; Matsuyama et al., 1986).

It should not be surprising that the composition of the cell envelope changes in response to changing external salt conditions since the envelope is in direct contact with the external medium and, therefore, the first structure to experience new environmental conditions. It more than likely plays an important role in haloadaptation. A good example is the fact that active transport of the amino acid analogue, -aminoisobutyric acid (AIB) changes depending at which NaCl concentrations the cells are grown (Kushner et al., 1983). Cells grown in the presence of 1.0 M NaCl can transport AIB at higher salt concentrations (3.0 M and 4.0 M) than cells grown in the presence of 0.5 M NaCl. In addition, incubating the latter in 1.0 M NaCl, under conditions that prevent growth and protein synthesis, enables them to transport AIB at the higher salt concentrations. Kushner et al. (1983) postulated that this development of salt-resistant active transport may be linked to the changes that occur in the membrane composition and/or to changes in internal solutes, such as the accumulation of a compatible solute (see below).

Ribosomes and *in vitro* protein synthesis of halophilic eubacteria

The properties of *V. costicola* ribosomes have also been studied extensively (Wydro *et al.*, 1975). The monosomes have a sedimentation coefficient of 64S and they, in turn, are composed of two subunits of 48S and 28S. The ribosomal proteins of *V. costicola* are more acidic than those of *E. coli* but less acidic than those of the archaebacterial extreme halophile *Halobacterium cutirubrum* (Wydro, 1977). The same is true for the ribosomal proteins of the unidentified moderate halophile NRC 41227 (Falkenberg *et al.*, 1976). It has been postulated that the increased proportion of acidic proteins from the nonhalophilic *E. coli* to the extreme halophile is required by the cells to adapt to the increasingly higher internal K^+ environment (Falkenberg *et al.*, 1976).

Comparisons of the equivalent of the L7/L12 ribosomal protein of *E. coli* in *V. costicola* and the moderate halophile NRC 41227 with similar proteins of *E. coli* and *H. cutirubrum* have revealed analogies between the proteins of the two moderate halophiles and *E. coli* but not with the protein of *H. cutirubrum*. These included similarities in the secondary structures of the proteins (Willick *et al.*, 1978) and in the amino acid sequence of the first 38 residues of the N-terminal end (Falkenberg *et al.*, 1979). Homologies between halophilic and nonhalophilic ribosomal proteins in eubacteria is now well established (Matheson, 1985), and as Kushner and Kamekura (1988)

pointed out: "In this respect, the evolutionary position of moderate halophiles seems more important than their salty environments."

Ribosomes of V. costicola also differ from those of E. coli and archaebacterial halophiles in their ability to maintain a constant sedimentation pattern over a wide range of NaCl concentrations. Analytical ultracentrifuge studies in sucrose gradients have shown that the sedimentation pattern of V. costicola ribosomes were relatively the same in gradients of different ionic composition: from one containing 10 mM $MgCl_2$ to one with 2.0 M NaCl, 1.0 M KCl and 100 mM $MgCl_2$ (Wydro et al., 1975). These results were the same for ribosomes isolated from cells grown at NaCl concentrations anywhere between 0.5-3.5 M. In comparison, the ribosomes of the extreme halophile H. cutirubrum require very high ionic conditions (3.0 M KCl and 0.1 M Mg^{++}) for stability; at lower concentrations they are drastically altered (Bailey and Kushner, 1964; Kushner, 1985). At the other end of the spectrum, the sedimentation pattern of E. coli ribosomes changes at salt concentrations above 0.5 M; the ribosomes aggregate at a concentration of 1.0 M (Wydro et al., 1975).

The fact that ribosomes of V. costicola are able to retain their integrity over a wide range of salt concentrations indicates their ability to tolerate major changes in the intracellular ionic conditions that would result from shifting external salt concentrations. Therefore, one would suspect that

protein synthesis would also be taking place over such a wide range of salt concentrations. However, as Wydro et al. (1977) discovered, the requirements for in vitro protein synthesis were much more selective.

With cellular extracts of halophilic archaebacteria, optimal activity for in vitro protein synthesis takes place under ionic conditions approaching those found inside the cell (Bayley and Griffiths, 1968; Saruyama and Nierhaus, 1985; Sanz et al., 1988); a similar situation was therefore expected with V. costicola. However, poly(U)-directed incorporation of phenylalanine by cellular extracts of V. costicola, that had been grown in the presence of 1.0 M NaCl and therefore had a cell-associated Na^+ and K^+ around 1.0 M, showed optimal activity at salts concentrations of 0.1-0.2 M and virtually no activity at 0.6 M (Wydro et al., 1977). Therefore, it seemed that the salt concentration present in the cell would prevent protein synthesis from taking place.

Kamekura and Kushner (1984) later showed that in vitro protein synthesis was not only inhibited by 0.6 M NaCl but also by 0.6 M NaF, NaBr and NaI. However, they also showed that 0.6 M solutions of sodium glutamate, acetate, aspartate, lactate, formate and sulfate supported relatively high levels of activity. Actually, sodium glutamate stimulated protein synthesis up to 0.6 M. Consequently, Kamekura and Kushner (1984) concluded that the nature of the anion had a profound effect on protein synthesis at higher salt concentrations and

that the inhibitory action of NaCl was solely due to the action of Cl^-. In fact, as opposed to Wydro et al. (1977) who had assumed that the major counterion to the high cell-associated Na^+ and K^+ was Cl^-, as is the case for the extreme halophiles, and which had led them to the hypothesis that protein synthesis occurred in a secluded area of the cell, Kamekura and Kushner (1984) showed that V. costicola largely excludes Cl^- ions, confirming the earlier results of Christian and Waltho (1962).

The stimulatory effect exerted by sodium glutamate on poly(U)-directed in vitro protein synthesis by V. costicola does not occur with E. coli. In fact, sodium glutamate is inhibitory to protein synthesis by cellular extracts of E. coli. Through hybridization studies with ribosomes and soluble fractions (enzyme-containing fractions) from E. coli and V. costicola, it has been found that the glutamate-sensitive factor of V. costicola is present in the soluble fraction and not in the ribosomal fraction (Kamekura and Kushner, 1984).

Even though V. costicola excludes Cl^-, it still appears that this ion is present in sufficient amount, especially when grown at higher salt concentrations, to have an inhibitory effect on the cellular protein machinery: 0.2 M and 1.5 M Cl^- in cells grown in media containing 1.0 and 3.0 M NaCl, respectively. The Cl^- sensitive site of poly(U)-directed in vitro protein synthesis has not yet been identified although it is known that it is not the enzyme phenylalanyl-tRNA synthetase

(Kamekura and Kushner, 1984). In fact, the enzyme is almost as active in 1 M NaCl as in its absence.

Kamekura and Kushner (1984) have also shown that the inhibitory activity of Cl^- ions on *in vitro* protein synthesis can be partly counteracted by betaine, a compatible solute in *V. costicola*. Glutamate was also shown to counteract the inhibitory action of Cl^- in *V. costicola* (Kamekura and Kushner, 1984). The mechanism by which this protection is accomplished is still unknown.

Osmoregulation and compatible solutes

Life in high salt concentrations presents a particular problem. In nonhalophilic bacteria, the internal osmotic pressure is normally higher than the external one which leads to a positive turgor pressure within the cell, i.e. H_2O wants to enter the cell to equilibrate the osmotic pressure. This pressure is resisted by the rigid cell wall which prevents the cell from bursting. However, if a cell is present in a medium of greater osmotic pressure than its own, the water will rush out of the cell causing plasmolysis.

Plasmolysis can be prevented if the cell can raise its own osmotic pressure to a level higher than that of the external medium. Bacteria can achieve this by accumulating, through uptake or synthesis, an osmotically active solute. This accumulation restores the positive turgor pressure within the cell and prevents plasmolysis. This concept is the basis of

osmoregulation: "the maintenance of turgor pressure and/or cell volume within limits necessary for growth and multiplication of an organism" (Brown et al., 1986).

Halophilic environments have a much higher solute concentration than nonhalophilic environments and, therefore, a much greater osmotic pressure. Consequently, halophilic microorganisms must constantly ensure that their internal osmotic pressure is higher than the outside one. The most striking example is the strategy employed by the extremely halophilic archaebacteria. They maintain a high internal solute concentration by accumulating KCl. H. cutirubrum can accumulate as much as 5.32 M KCl when grown in a media containing 3.3 M NaCl (Matheson et al., 1976) while Halobacterium salinarium accumulates 4.6 M KCl in the presence of 4.0 M NaCl (Christian and Waltho, 1962). The gradient of K^+ ions between the inside and the outside of these cells can be as high as 1000:1 (Ginzburg et al., 1971). The demand for this ion is so great that it can actually become growth-limiting if its concentration in the medium is too low (Gochnauer and Kushner, 1969). As stated previously, K^+ in extreme halophiles is required at high concentrations for the stability and activity of enzymes, ribosomes and membranes, as well as for protein synthesis. This shows that the high levels of K^+ required to maintain turgor pressure are not inhibitory to their different metabolic functions. For this reason, KCl is called a compatible solute; that is, a compound that through its accumulation will maintain

turgor pressure, as well as not interfere with the proper functioning of the cellular metabolic processes even when it is present at high concentrations (Brown, 1976; Kushner, 1978).

There is a great variety of compatible solutes. Polyols (and sugars) and proline are the major ones encountered in eucaryotic algae (Wegmann, 1986). The extremely halotolerant algae Dunaliella, for example, accumulates large amounts of glycerol in response to increasing external salt concentrations (Ben-Amotz and Avron, 1973; Borowitzka and Brown, 1974; Goyal et al., 1988). Glycerol is also the main compatible solute in osmotolerant yeasts such as Saccharomyces rouxii, Saccharomyces cerevisiae and Debaryomyces hansenii (Brown, 1978; Adler and Gustafsson, 1980; Edgley and Brown, 1983; Lars et al., 1988; Blomberg and Adler, 1989).

Extensive surveys of the compatible solutes that accumulate in cyanobacteria (Mackay et al., 1984; Reed et al., 1984a, 1984b, 1986; Reed and Stewart, 1985) have revealed that the least halotolerant isolates, mainly from freshwater, accumulate the disaccharides sucrose and trehalose; that the intermediate halotolerant, such as marine isolates accumulate glucosylglycerol; and that the most hypersaline strains accumulate glycine betaine (betaine) or glutamate betaine. Therefore, the nature of the compatible solute has a determining role in the osmotolerance of cyanobacteria.

In the other eubacteria, the main compatible solutes are

K^+, amino acids and betaine. Among the amino acids, proline and glutamate are the most common ones although glutamine, and γ-aminobutyric acid fulfill this role in some bacteria (Table 3). Betaine has been found to accumulate in every moderate halophile so far examined, as well as in some nonhalophiles, under osmotic stress (Imhoff and Rodriguez-Valera, 1984; Imhoff, 1986; Le Rudulier and Bernard, 1986). In addition to the large amount of betaine found in the halophilic Ectothiorhodospira halochloris (Galinski and Truper, 1982) and in a moderately halophilic Micrococcus sp. (Imhoff, 1986), large quantities of a new amino acid, ectoine, were also found in them (Imhoff, 1986). As in cyanobacteria, it appears that there is a correlation between the nature of the compatible solutes and the halotolerance (or halophilism) of bacteria; the best compatible solute being betaine, followed by proline, glutamate and K^+ ions (Imhoff, 1986; Meury, 1988). Thus, bacteria accumulating betaine are much better adapted to life in high salt concentrations than the ones accumulating proline or glutamate.

Bacteria that accumulate ionic compatible solutes, such as K^+ or glutamate, must simultaneously accumulate a counterion to maintain ionic balance. Therefore, in some instances, glutamate may be accumulated more as a counterion to increasing levels of K^+ ions than as the actual primary compatible solute. Other solutes, such as betaine or proline, have the advantage of being neutral molecules at physiological pH's.

Table 3. Accumulation of amino acids in response to osmotic shock in different bacteria (Reproduced from Hamaide, 1984).

Organism	Amino acid(s) accumulated	Reference
Nonhalophile		
Escherichia coli	glutamate, proline, GABA[1]	Measures (1975)
Serratia marcescens	glutamate, proline, GABA	Measures (1975)
Erwinia carotovara	glutamate	Tempest et al. (1970)
Pseudomonas aeruginosa	glutamate	Measures (1975)
Peudomonas fluorescens	glutamate	Tempest et al. (1970)
Klebsiella aerogenes	glutamate, proline glutamate glutamate, glutamine	Measures (1975) Tempest et al. (1970) Brown & Stanley (1972)
Salmonella oranienburg	proline glutamate, glutamine	Christian (1955) Measures (1975)
Salmonella typhimurium	proline glutamate, glutamine	Csonka (1980) Csonka (1981)
Sarcina lutea	proline	Measures (1975)
Clostridium sporogenes	glutamate, proline, GABA	Measures (1975)
Streptococcus faecalis	GABA, proline	Measures (1975)
Micrococcus lysodeikticus	proline	Measures (1975)
Lactobacillus plantarum	glutamate, proline	Measures (1975)

Organism	Amino acid(s) accumulated	Reference
Bacillus megaterium	glutamate, proline, GABA	Tempest *et al.* (1970)
	proline	Measures (1975)
B. subtilis	proline	Measures (1975)
B. subtilis var. Niger	glutamate, proline GABA	Tempest *et al.* (1970)
	proline	Brown & Stanley (1972)
B. polymixa	glutamate, proline, GABA	Tempest *et al.* (1970)
B. cereus	proline	Measures (1975)
Marine bacteria		
Vibrio alginolyticus	glutamate, proline	Unemoto & Hayashi (1979)
V. parahaemolyticus	glutamate	Measures (1975)
Beneckea harveyi	glutamate	Makemson & Hastings (1979)
Halotolerant bacteria		
Staphylococcus aureus	proline	Measures (1975)
	proline	Koujima *et al.* (1978)
	glutamine, proline	Anderson & Witter (1982)
Rhizobium WR 1001	glutamate	Hua *et al.* (1982)
Rhizobium sp UMKL 20	glutamate	Yap & Lim (1983)
Rhizobium japonicum 191	glutamate	Yelton *et al.* (1983)
Halomonas elongata	glutamate, glutamine	Vreeland *et al.* (1983)

1 GABA, γ-aminobutyric acid

Although the problem of life at high salt concentrations appears to be, at first sight, a problem mainly restricted to the halophilic microorganisms, many nonhalophilic and halotolerant microoganisms must occasionally face these conditions in nature. Therefore, a lot of information on compatible solutes and osmoregulation has been obtained from studies on these latter microorganisms.

It is well established that the presence of betaine or proline, in minimal media of normally inhibitory salt concentrations, stimulates growth of a number of enterobacteria (Le Rudulier and Bouillard, 1983). It extends the growth rate of these bacteria towards higher salt concentrations. Klebsiella pneumoniae cannot grow well in the presence of 0.8 M NaCl but, with as little as 0.5 mM betaine in the medium, the inhibitory action of NaCl is reversed (Le Rudulier and Valentine, 1982; Le Rudulier and Bouillard, 1983). The same is observed with E. coli and Salmonella typhimurium: 1 mM betaine stimulates their growth at 0.8 M NaCl, a normally inhibitory concentration (Le Rudulier and Bouillard, 1983; Perroud and Le Rudulier, 1985). Compatible solutes can also increase growth rates of bacteria under osmotic stress. Betaine (1 mM) caused a 7-fold and a 4-fold increase in growth rates of K. pneumoniae and S. typhimurium, respectively, in the presence of 0.8 M NaCl (Le Rudulier and Bouillard, 1983). Smaller generation times in the presence of 1 mM betaine were also observed with numerous strains of Rhizobium meliloti at a NaCl concentration of 0.65 M

(Le Rudulier and Bernard, 1986). Proline may also exhibit a similar protective role on bacteria but not as efficiently as betaine (Le Rudulier and Bouillard, 1983; Strom et al., 1986).

It should be noted that a bacterium may accumulate more than one kind of compatible solute and that different degrees of osmotolerance are conferred upon the microorganism depending on the solute accumulated (Dinnbier et al., 1988; Giaever et al., 1988). In E. coli, accumulation of betaine provides the highest degree of osmotic tolerance. Proline is the next most effective compatible solute. Under osmotic stress, and in the absence of exogenously supplied betaine or proline, E. coli accumulates trehalose and glutamate. This confers low level of osmotic tolerance (Strom et al., 1986; Larsen et al., 1987; Dinnbier et al., 1988). Unlike betaine and proline, trehalose and glutamate have no detectable osmoprotective effects when supplied exogenously (Strom et al., 1986; Rod et al., 1988). In addition, E. coli mutants, defective in trehalose synthesis, have an impaired osmotic tolerance in minimal media without betaine and other osmoprotectants (Larsen et al., 1987). These results indicate that trehalose and glutamate synthesis are endogenous mechanisms, that is, they are synthesized de novo as opposed to being transported in. This is surprising since transport is less energy-demanding than biosynthesis (Imhoff, 1986).

All living cells accumulate K^+ ions to achieve internal concentrations much higher than the outside concentration. For

example, the intracellular K^+ concentration of V. costicola is about 75 times higher than the external K^+ concentration (Shindler et al., 1977). Thus, K^+ ions may contribute, at least partly, to the maintenance of a positive turgor pressure within the cell. In some cases, the internal K^+ concentration can actually be shown to be affected by changing external salt concentrations. In Klebsiella aerogenes, K^+ increases from 125 mM, in the absence of osmotic stress, to 625 mM when the cells are grown in a medium containing 1 M NaCl (Measures, 1975). In E. coli, intracellular K^+ increases by ~.11 M for each 0.2 osM increase in medium osmolarity (Epstein, 1986).

In V. costicola, the K^+ ion concentration (0.6 M) remains constant regardless of the amount of NaCl present in the medium (0.5-3.0 M) (Shindler et al., 1977). The reason may be that, as in other bacteria, high concentrations of K^+ are deleterious to normal cellular enzymes and functions (Sutherland et al., 1986; Larsen et al., 1987). In the cyanobacterium Aphanothece halophytica, K^+ can reach concentrations of 0.75 M to osmotically balance increasing environmental salinity (Miller et al., 1976). However, many of its enzymes are severely inhibited by K^+ concentrations higher than 0.4 M (Pavlicek and Yopp, 1982). Most of the intracellular enzymes of V. costicola studied so far do not function well in vitro at KCl or NaCl concentrations higher than 0.5 M, but this may be the result of Cl^- inhibition, rather than K^+ or Na^+ inhibition, since most of these enzymes are not as strongly inhibited by sodium glutamate

(Kushner and Baddeley, unpublished results; Kushner and Ahonkhai, unpublished results).

Betaine, on the other hand, seems to have little effect on the activity of numerous cellular enzymes (Imhoff and Rodriguez-Valera, 1984). In fact, its presence can have a protective role against ionic inactivation of cellular enzymes. In vitro activities of the enzymes 6-phosphate dehydrogenase of A. halophytica (Pavlicek and Yopp, 1982) and glutamine synthetase of the halotolerant Synechocystis sp. DUN52 (Warr et al., 1984) are restored in the presence of betaine at K^+ concentrations that would normally be inhibitory. Pollard and Wyn Jones (1979) have also shown that betaine restores enzyme activity of a number of plant enzymes at high salt concentrations. Glycerol, the compatible solute of the green algae Dunaliella tertiolecta, partially protects the enzyme glucose-6-phosphate dehydrogenase and the NADP-specific glycerol dehydrogenase from inhibition by high salt concentrations (Borowitzka and Brown, 1974). Therefore, betaine and glycerol, and probably other compatible solutes, not only prevent dehydration of the cell during osmotic stresses, but may also serve to protect intracellular enzymes from inhibition by accumulated K^+.

Other protective roles of betaine have also been identified. It can relieve the inhibition of respiration caused by high NaCl concentrations in the moderate halophile Ba1 (Rafaeli-Eshkol and Avi-Dor, 1968); relieve the effects of high NaCl concentrations on active transport in V. costicola (Kushner

et al., 1983); relieve the inhibition of nitrogen fixation by high salt concentrations in K. pneumoniae (Bouillard and Le Rudulier, 1983); and reverse the effects of osmotic stress on DNA replication and cellular division of E. coli (Meury, 1988).

Direct measurements of intracellular betaine concentrations in cells grown at different salt concentrations support the idea that betaine is of general importance for osmotic adaptation of most eubacteria. In a survey of moderate halophiles, including V. costicola, Imhoff and Rodriguez-Valera (1984) determined that, as a group, moderate halophiles contain ca. 200 mM betaine at 3% salts, ca. 650 to 850 mM at 10% salts and ca. or greater than 1000 mM at 20% salts. They also observed a 13-fold, 7-fold and 2-fold increase in betaine concentration in the non-halophiles E. coli, B. subtilis and S. epidermidis, respectively, between cells grown in media with no salt and cells grown in media containing 0.514 M NaCl. In K. pneumoniae, betaine concentration increases from 13 mM to 615 mM when the external NaCl concentration is raised from 0 to 0.8 M NaCl (Le Rudulier and Bouillard, 1983). Lactobacillus acidophilus grown in the absence of osmotic stress contains 4 mM betaine while cells grown in a medium containing 1 M KCl have 430 mM (Hutkins et al., 1987). In A. halophytica betaine levels increase by 70-75% when the NaCl in the medium is increased from 1.0 to 2.25 M (Yopp et al., 1983).

Accumulation of intracellular betaine can be accomplished by either transporting exogenous betaine or by synthesizing it

from a precursor present in the medium, such as choline. Betaine transport systems have only been identified in few microorgamisms (Stalmach et al., 1983; Cairney et al, 1985a, 1985b; May et al., 1986; Nobile and Deshusses, 1986; Moore et al., 1987; Gloux and Le Rudulier, 1989) and, of those, the transport systems of E. coli and S. typhimurium have been the most studied. In E. coli and S. typhimurium, there are two distinct transport systems, the proP locus, which encodes a low affinity betaine uptake system, and the proU locus, which encodes a high affinity transport system (Cairney et al., 1985a; 1985b; May et al., 1986). Both of these systems also have low affinity for the other important osmoprotectant, proline (Anderson et al., 1980; Stalmach et al., 1983; Grothe et al., 1986).

Initially, a 32 kilodalton (kd) betaine-binding periplasmic protein was identified as a gene product of the proU locus in both E. coli and S. typhimurium (May et al., 1986; Barron et al., 1987; Higgins et al, 1987b). More recently, it was shown that this locus is, in fact, composed of three different genes, proV, proW and proX, organized in a single operon (Faatz et al., 1988; Dattananda and Gowrishankar, 1989; Gowrishankar, 1989). Their respective gene products are proteins of molecular weight of 44, 35 and 33 kd (Dattananda and Gowrishankar, 1989), the last of which is a previously identified periplasmic binding protein. From the nucleotide sequence of proW, Gowrishankar (1989) established that its gene product, the 35-kd protein,

contained numerous hydrophobic stretches capable of spanning the membrane, and could therefore be a membrane protein. He also found nucleotide sequence homologies between proV and the nucleotidyl triphosphate-binding domains of corresponding component proteins of other porters. Therefore, he postulated that the proU locus encodes for a betaine-multicomponent-protein-dependent transport system in which the betaine-periplasmic-binding protein (ProX) would present the substrate, bound in the periplasm, to the membrane protein (ProW) for transport across the cytoplasmic membrane, and that ProV, probably a peripheral membrane protein found on the cytoplasmic surface of the membrane, would be involved in the coupling between high energy phosphate bond hydrolysis and the work done by the porter.

ProU expression is osmotically regulated at the transcriptional level: it is fully repressed at low osmolarities, but its expression is induced over 100-fold under osmotic stress (Cairney et al., 1985b; Dunlap and Csonka, 1985; Barron et al., 1986). The intracellular signal for proU induction is the concentration of K^+ (Sutherland et al., 1986; Higgins et al., 1987a; Ohwada and Sagisaka, 1988). The sequence of events that leads to this induction is best understood in E. coli. At external osmolarities lower than 600-700 mosM, K^+ accumulation is enough to maintain turgor pressure in this bacterium (Meury, 1988). K^+ uptake is mediated by two independent transport systems: the Trk system, a low affinity

system, and the Kdp system, a high affinity system (Rhoads et al., 1976). The Kdp system is specifically induced when accumulation of K^+ by the Trk system is not sufficient to maintain turgor pressure (Laimins et al., 1981). The induction signal of the Kdp system is a lowering of cell turgor (Laimins et al., 1981; Sutherland et al., 1986), that is, upon experiencing an increase in external salinity, the water movement out of the cell lowers the turgor pressure and triggers the expression of Kdp.

When the external osmolarity exceeds 700 mosM, K^+ ions are no longer able to maintain turgor and E. coli cells have to adapt differently. They then resort to the accumulation of a second compatible solute, preferably betaine, to restore turgor pressure. ProU expression is therefore induced by the accumulated intracellular K^+ (Sutherland et al., 1986; Higgins et al, 1987a). This mechanism allows the cell to accumulate betaine only in cases where K^+ cannot act alone. Cairney et al. (1985b) have shown that proU expression of S. typhimurium does not occur until the external NaCl concentration reaches ca. 0.2 M. It should be noted that even though K^+ may play a secondary role in restoring turgor pressure under high osmotic stress, its importance lies in its role immediately following the osmotic upshock and its subsequent activation of the proU system. Therefore, by accumulating K^+, the cell is able to counteract plasmolysis rapidly and prepare itself for long term osmoadaptation (Yancey et al., 1982).

This serial induction of proU presents several advantages. First, K^+, as opposed to betaine, is almost always available to cells and, therefore, represents the most appropriate primary osmoprotectant to restore turgor pressure at low osmotic shock. Second, the accumulation of betaine at higher external osmolarities reduces the need to accumulate potentially deleterious K^+; high K^+ concentrations can be inhibitory to cellular enzymes and normal cellular processes. Third, accumulation of betaine may actually protect enzymes from ionic inactivation by the accumulated K^+.

The mechanism by which K^+ induces proU expression is not well understood. K^+ could interact with a regulatory protein and induce specific conformational changes that would alter its interaction at the proU promoter/operator region. However, no such protein has been identified. A second possible mechanism may involve a K^+-induced change in the DNA structure or topology that could influence the interaction between RNA polymerase and the proU promoter. In fact, Higgins et al. (1988) have presented data that suggests that intracellular K^+ can affect DNA supercoiling of E. coli and proU expression. They suggested that the rapid accumulation of K^+, as the cell's primary response to osmotic upshock, alters DNA supercoiling and induces proU expression by enhancing the productive interaction of RNA polymerase at the proU promoter. Consistent with this model, Higgins et al. (1988) have also found that betaine restores chromosomal supercoiling, and since it is known that betaine

accumulation is correspondingly accompanied by a decrease in intracellular K^+ ions (Sutherland et al., 1986; Ohwada and Sagisaka, 1988), this could be the mechanism by which proU expression is reduced.

In addition to the regulatory mechanism discussed above, there is also some evidence that transport of betaine via the proU system is regulated at the level of transport activity. Even when fully induced, function of the transport system is only detected at high osmotic stress which suggests that the proteins only adopt an active conformation at high salt concentrations (Cairney et al., 1985b).

In the event that betaine is not present in the external media, some bacteria can synthesize it. E. coli (Landfald and Strom, 1986), P. aeruginosa (Nagasawa et al., 1975, 1976), Arthrobacter globiformis (Ikuta et al., 1977) and R. melitoti (Tombras Smith et al., 1988) are capable of doing so, provided that choline is present in the external media (Figure 1). No biosynthetic pathways that would suggest that these bacteria can synthesize betaine de novo have been identified. The synthesis of betaine from choline involves two enzymes, choline dehydrogenase (oxidase) and betaine aldehyde dehydrogenase (Figure 1). In all the microorganisms mentioned above, these enzymes are inducibly synthesized in the presence of choline.

In E. coli, choline dehydrogenase and betaine aldehyde dehydrogenase are also osmoregulated: they are only expressed under osmotic stress in the presence of choline (Landfald and

Strom, 1986). This control is believed to be at the transcriptional level, since chloramphenicol prevents the appearance of these enzymes in osmotically stressed E. coli (Landfald and Strom, 1986).

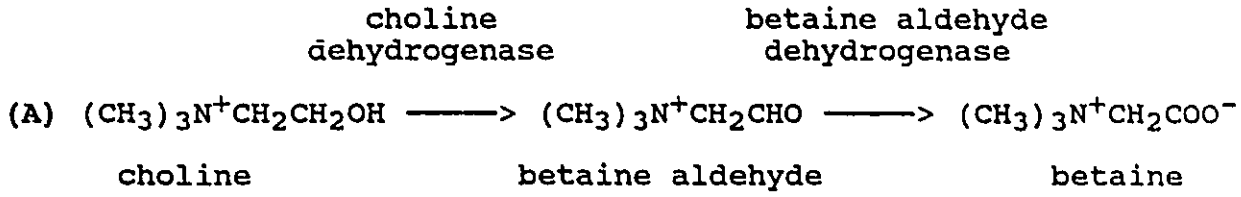
Other enzymes have also been found to be osmotically regulated. Under osmotic stress and in the absence of exogenous betaine, proline and choline, E. coli accumulates trehalose. The enzyme trehalose-phosphate synthase utilizes UDP-glucose and glucose-6-phosphate to form trehalose. This enzyme is osmotically regulated at both the gene and the protein level. K^+ may have an important role in this regulation since it stimulates enzyme activity by at least 5-fold (Giaever et al., 1988).

In the cyanobacterium Aphanothece halophytica, betaine is synthesized through a series of transmethylation using S-adenosylmethionine (AdoMet) as methyl donor, glycine as methyl acceptor, and an AdoMet-dependent methyl transferase enzyme (Figure 1) (Sibley and Yopp, 1987). In this bacterium, K^+ appears to regulate betaine synthesis: following an osmotic upshock (1.0 to 2.5 M NaCl), there is a large increase in intracellular K^+ (0.3 to 0.6 M) within the first 12 hours (Miller et al, 1976), followed by betaine accumulation (0.8 to 1.3 M) (Yopp et al, 1983). Thus, betaine synthesis occurs under conditions of elevated K^+. Regulation by K^+ appears to be through a second enzyme, S-adenosylhomocysteine hydrolase (AdoHcy hydrolase), which catalyses the reversible hydrolysis of

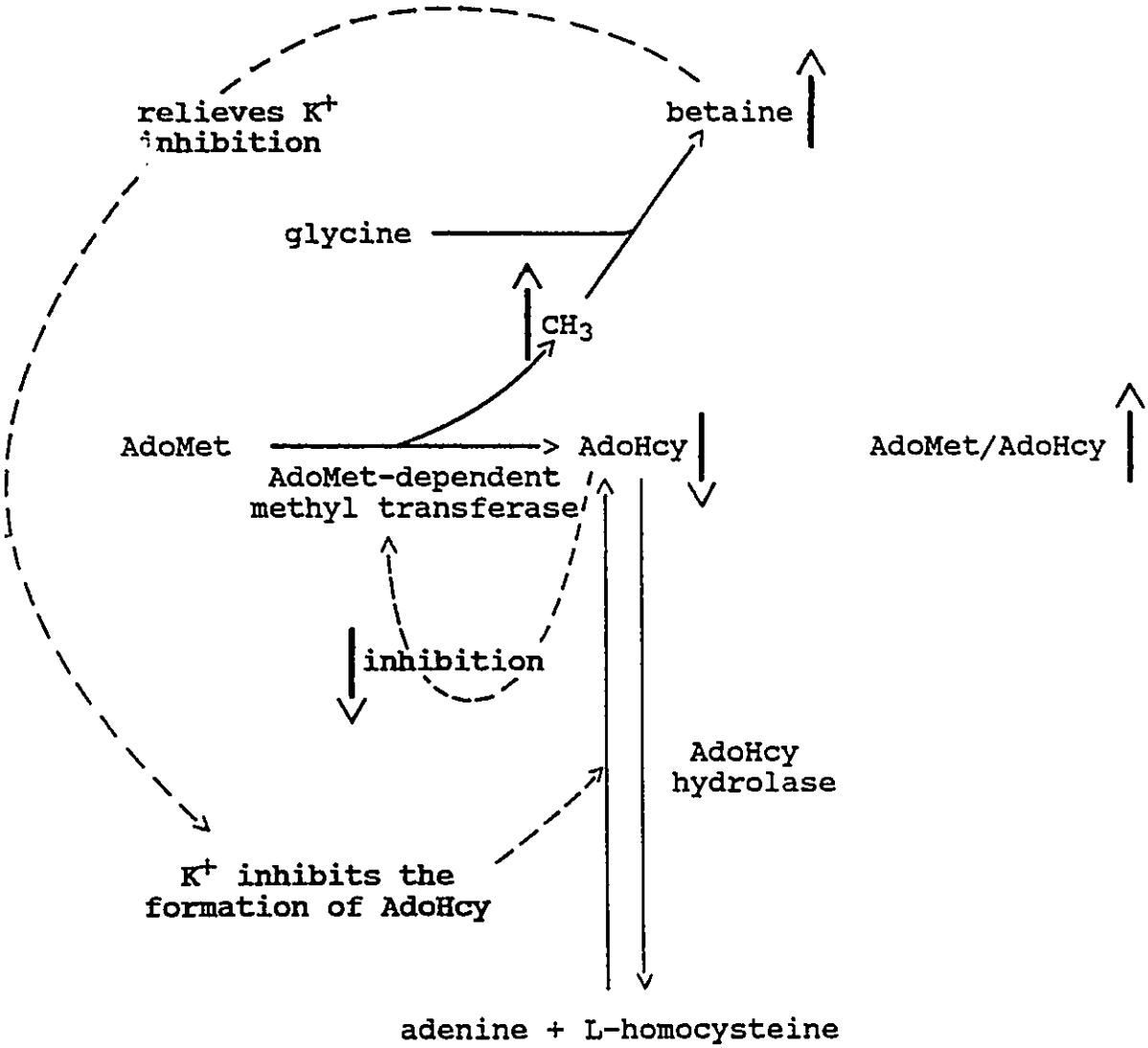
Figure 1. (A) Choline-betaine oxidation pathway in Escherichia coli (Landfald and Strom, 1986).

(B) Regulation of betaine synthesis in the cyanobacterium, Aphanothece halophytica. The thick arrows indicate an increase or a decrease of the different components under conditions of elevated K^+ (Sibley and Yopp, 1987).

(A)


choline dehydrogenase
betaine aldehyde dehydrogenase
$(CH_3)_3N^+CH_2CH_2OH$
$(CH_3)_3N^+CH_2CHO$
$(CH_3)_3N^+CH_2COO^-$
choline
betaine aldehyde
betaine

(B)


relieves K^+ inhibition
betaine
glycine
CH_3
AdoMet
AdoHcy
AdoMet-dependent methyl transferase
AdoMet/AdoHcy
inhibition
AdoHcy hydrolase
K^+ inhibits the formation of AdoHcy
adenine + L-homocysteine

S-adenosylhomocysteine (AdoHcy), the product of the AdoMet mediated transmethylation, to adenine and L-homocysteine. AdoHcy is a strong competitive inhibitor of many AdoMet-dependent methyl transferases (Glick et al, 1975; Pugh et al, 1977; Schneider and Vance, 1979) and therefore, the ratio of AdoMet/AdoHcy is of critical importance in the control of transmethylation. Sibley and Yopp (1987) have shown that K^+ inhibits the synthesis of AdoHcy (from adenosine and homocysteine), but has no effect on its hydrolysis. This unidirectional inhibitory effect of K^+ can be reverted by high concentrations of betaine. Therefore, by selectively inhibiting AdoHcy synthesis, K^+ would change the AdoMet/AdoHcy ratio in the direction favoring methylation and consequently betaine synthesis. Betaine synthesis would then continue until high levels of betaine were reached which would then reverse the inhibition of K^+ on the hydrolase enzyme, allowing it to restore levels of AdoHcy and thus inhibiting transmethylation, that is, betaine synthesis.

Goals of thesis

Protein synthesis is a central and essential process in any cell. As I have presented in this introduction, paradoxical results have been obtained with the poly(U)-directed *in vitro* protein synthesis system of the moderate halophile *V. costicola*; that is, protein synthesis was inhibited by Cl^- concentrations lower than what had been measured in these cells (Wydro *et al*, 1977; Kamekura and Kushner, 1984). Therefore, it appears that Cl^- is present in sufficient amount in these cells to have an inhibitory effect on the cellular protein machinery. Consequently, one goal of this thesis was to study in more detail the inhibitory effects of Cl^- on *in vitro* protein synthesis of *V. costicola* and the possible mechanisms available to this bacterium to protect its cellular protein machinery against Cl^-.

In this introduction, I have also emphasized the importance of betaine in bacteria living under osmotic stress. Not only is betaine the most efficient compatible solute involved in osmoregulation, but it can also protect several metabolic processes against the inhibitory effects of high salt concentrations. Therefore, another goal of this thesis was to study the pathway and control of betaine synthesis in *V. costicola*. These studies should reveal valuable information on the role of betaine in osmoregulation in these cells.

MATERIALS AND METHODS

POLY(U)-DIRECTED IN VITRO PROTEIN SYNTHESIS

Bacterial cultures

Vibrio costicola NCR 37001 was grown in 1.0 M NaCl PPT medium [1% proteose peptone #3 (Difco Laboratories), 1% tryptone (Difco) and 1.0 M NaCl]. Five hundred ml of 1.0 M NaCl PPT, in 2.8-liter Fernbach flasks, were inoculated with 1.0 ml of an overnight culture of V. costicola, grown in 1.0 M NaCl PPT. The flasks were incubated at 30°C with shaking until the cells reached late exponential phase (7-8 hours). The cells were then harvested by centrifugation (8,000 x g_{max} for 10 min) and washed twice with an isotonic washing buffer [1.2 M NaCl, 8 mM KCl, 0.41 mM $MgCl_2$, 0.05 M Tris (pH 7.5 adjusted with HCl)].

Preparation of cellular extracts and ribosomes

The washed cell pellet (5-10 g) was ground for 20 min with twice its weight of alumina. The resulting cellular paste was extracted for 5 min with one volume of low-salt extraction buffer [124 mM NH_4Cl, 20 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6), 3 mM spermidine-trihydrochloride and 6 mM β-mercaptoethanol] per g of pellet and 3 µg/ml of RNAse-free DNAse I. Large cellular debris and alumina were removed by centrifugation (8,000 x g_{max} for 10 min) and the supernatant further centrifuged at 30,000 x g_{max}

for 30 min. The resulting supernatant (S-30) was dialyzed for 2 h at 4°C against 4 liters of extraction buffer, clarified by centrifugation (30,000 x g_{max} for 30 min) and stored in small aliquots at -70°C.

In some cases the S-30 extract was further fractionated, by ultracentrifugation at 150,000 x g_{max} (Beckman, type 42.1 rotor) for 3 h at 4°C, into an S-150 fraction and a ribosomal fraction. The upper two-thirds of the supernatant (S-150) was removed carefully, dialyzed for 2 hours against 4 liters of extraction buffer, clarified by centrifugation (30,000 x g_{max} for 30 min) and stored in small aliquots at -70°C. The ribosomal pellet was washed twice with extraction buffer and then stored at -70°C.

In vitro protein synthesis assay

The poly(U)-directed incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid (TCA)-insoluble material was measured as described by Kamekura and Kushner (1984) except for the following changes to the reaction mixture. Because of the inhibitory action of Cl^-, NH_4Cl, $MgCl_2$ and Tris-HCl, previously used in the preparation of the reaction mixture, were substituted by ammonium glutamate, $MgSO_4$ and Tris-sulfate; 0.6 M glutamate and sulfate anions having been shown to be less inhibitory to protein synthesis than Cl^- ions (Kamekura and Kushner, 1984). Therefore, the reaction mixture (0.4 ml) contained the following: 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 124 mM ammonium glutamate, 18 mM Mg^{++} ($MgSO_4$), 7.5 mM

reduced glutathione, 82 mM Tris-sulfate (pH 7.6), 1.2 mg/ml of polyuridilic acid [poly(U)], 1.96 μM [^{14}C]phenylalanine (512 mCi/mmole; Dupont, NEN Research Products), various concentrations of solutes and 0.2 volume (80 μl) of S-30 or 70 μl of S-150 and 10 μl (4 A_{260} units) of ribosomes. These resulted in a reaction mixture containing only 37 mM Cl^- after adding 0.2 volume of extract.

The reaction mixture was incubated at 30°C. At appropriate time intervals 50 μl samples were removed and spotted on a Whatmann 3MM filter paper (12.5 cm diameter, divided into 2.2-cm squares) placed above a hot plate to accelerate evaporation of water. After drying, the radioactive proteins were precipitated by placing the filter paper into hot TCA (10%) for 20 min. The filter paper was then washed twice with 100 ml of 5% TCA on a Buchner funnel. The paper was cut into squares and each square placed into a scintillation vial to be dried (80°C for 2 h). Finally, 5 ml of scintillation cocktail (Universol Cocktail, ICN Biomedicals Canada, Ltd.) were added to each vial and the radioactivity counted (2000CA LSC, Packard Instruments Canada Ltd.)

Fidelity of translation

Fidelity of translation of the <u>in vitro</u> protein synthesizing system at different NaCl concentrations was studied by measuring the incorporation of [^{14}C]phenylalanine (256 mCi/mmole; NEN Research Products), [^{14}C]leucine (276 mCi/mmole;

NEN research Products) or [^{14}C]valine (225 mCi/mmole; ICN Biomedicals Canada, Ltd.) in the presence and absence of poly(U).

Binding of poly(U) to ribosomes

A reaction mixture (0.4 ml) containing 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 124 mM ammonium glutamate, 18 mM Mg^{++} ($MgSO_4$), 7.5 mM reduced glutathione, 82 mM Tris-sulfate (pH 7.6), 1.96 µM [^{12}C]phenylalanine, ribosomes (20 A_{260} units) and various concentrations of solutes (as indicated in the legend to each figure) was incubated for 2 min at 30°C. Poly[5-^{3}H]uridilic acid (2-4 Ci/mmole of UMP; NEN Research Products) was added to a final concentration of 4 µCi/ml, and the reaction mixture was incubated for another 2 min. Then 300 µl of the reaction mixture was loaded on top of a linear sucrose gradient containing the same solute(s) as the reaction mixture: 124 mM ammonium glutamate, 18 mM Mg^{++}, 82 mM Tris-sulfate (pH 7.6) and various concentrations of solutes.

The presence and nature of a solute in a sucrose gradient changes the density and viscosity of the gradient. Therefore, the conditions of centrifugation were different for each gradient. In the binding experiments described in this thesis, when different concentrations and combinations of NaCl, sodium glutamate and betaine were used, the range of the sucrose gradient and the temperature of centrifugation were adjusted to obtain similar ribosomal profiles under each condition. This

information is given in the legend to each figure. The centrifugation itself was always done at 25,000 rpm (110,000 x g_{max}) for 16 hours in a SW-41 Beckman rotor.

After centrifugation, the gradients were fractionated (0.13 ml/fraction) and each fraction diluted with 2 ml of H_2O; 1 ml was added to 4 ml of Universol Cocktail and the radioactivity counted, while the remainder was used to read the A_{260} (Spectronic 1001, Milton Roy Company).

For locating the 70S, 50S and 30S ribosomal particles, parallel experiments were carried out in which purified ribosomes of V. costicola were centrifuged in appropriate gradients in the presence of 18 mM or 1 mM Mg^{++}; with 1 mM Mg^{++} the 70S ribosomes dissociated into 50S and 30S subunits (Wydro et al., 1975) [Note that I am using 70S, 50S and 30S to indicate the usual forms of ribosomes, though the actual "S" values determined for V. costicola were slightly different: 64S, 48S and 28S (Wydro et al., 1975).]

IN VITRO PROTEIN SYNTHESIS DIRECTED BY NATURAL mRNAs

Preparation of cellular extracts, ribosomes and initiation factors

The cellular extracts, S-30 and S-150, and the ribosomes were prepared as described for the poly(U) system with the exception that a different low-salt extraction buffer was used. It contained the following: 124 mM ammonium glutamate, 8 mM magnesium acetate, 10 mM Tris-acetate (pH 7.6), 3 mM spermidine-trihydrochloride and 6 mM β-mercaptoethanol.

Crude initiation factors were prepared following the method described for E. coli (Steitz, 1979), with some modifications to adapt it to V. costicola. Ribosomes were homogenized in extraction buffer (15 ml/10 g wet weight of cells), in which the ammonium glutamate had been replaced with 3.0-3.5 M NH_4Cl (high-salt extraction buffer), and left at 4°C overnight. These high-salt washed ribosomes were spun down (150,000 x g_{max} for 3 h at 4°C), washed once with low-salt extraction buffer and stored at -70°C. To the supernatant, solid $(NH_4)_2SO_4$ (0.52 g/ml) was added and the solution stirred in the cold for 2 hours. The precipitated proteins were centrifuged (15,000 x g_{max} for 30 min), dissolved in extraction buffer (0.5 ml/10 g original wet weight of cells), dialyzed overnight against several liters of the same buffer, clarified by centrifugation and then stored at -70°C.

Growth of phage R17 and isolation of its RNA

E. coli CSH39 (accession number HER 1290) and the bacteriophage R17 (accession number HER 1290) were obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses, Laval University, Quebec, P.Q., Canada. E. coli was grown with maximal aeration at 37°C in 2-liter Erlenmeyer flasks containing 250 ml of 4YT medium [32 g tryptone, 20 g yeast extract and 5 g NaCl per litre (Osborn et al., 1970)] up to an optical density of 0.4 (550 nm). The bacterial culture was then made 5 mM in $CaCl_2$ (Osborn et al. 1970), infected with R17 at a multiplicity of 0.15 and incubated for an additional 4 hours. With this incubation time, titers of 7×10^{11} PFU/ml were routinely obtained. The titers were determined as described by Osborn et al. (1970). For labeling the R17 RNA, 400 µCi of [6-^{3}H]uridine (29 Ci/mmole; NEN Research Products) per liter of media was added at the time of infection (Albrecht et al., 1969).

All of the following operations were carried out at 4°C. At the end of the incubation, EDTA (50 mM) and lysozyme (100 µg/ml) were added to promote further lysis (Webster et al., 1967). The viscosity of the solution was then lowered by adding $MgSO_4$ and DNase to final concentrations of 200 mM and 2 µg/ml, respectively (Webster et al., 1967). The bacteriophage was then collected according to Steitz (1979). It was precipitated overnight by adding 330 g $(NH_4)_2SO_4$ per liter of medium and recovered by centrifugation (25,000 x g_{max} for 30 min). The pellet was well resuspended in 100 ml standard sodium

citrate buffer [SSC; 0.15 M NaCl, 0.015 M sodium citrate (pH 7.2)] per liter of lysate and the solution clarified by centrifugation (25,000 x g_{max} for 10 min). The phage (20 ml/tube) was then pelleted at 100,000 x g_{max} (Beckman, Type 42.1 rotor) for 4 hours and each pellet resuspended overnight in 2 ml SSC.

The phage was further purified by two successive sedimentations through CsCl step gradients (Yamamoto et al., 1970). After each CsCl centrifugation, the visible phage band was removed, diluted to 20 ml with SSC, pelleted at 100,000 x g_{max} for 4 hours and resuspended in SSC (2-4 ml). The RNA was then extracted according to Steitz (1979): solubilization of the bacteriophage with 1% sodium dodecyl sulfate (SDS), followed by phenol (SSC-saturated) extraction and ethanol precipitation.

Treatment of R17 RNA with formaldehyde

Partial denaturation of R17 RNA was done according to Lodish (1970). The reaction mixture (500 µl) contained 9 µM Na_2HPO_4, 1 µM NaH_2PO_4, 0.2 M NaCl, 1.0 M formaldehyde and 500 µg of R17 RNA. In control reactions, formaldehyde was omitted. The reaction mixtures were incubated at 37°C for 15 min and then chilled on ice. The RNA was precipitated with 1 ml of ethanol at -20°C for 20 min and collected by centrifugation. It was dissolved in 500 µl of 0.2 M Tris-HCl (pH 7.0) and reprecipitated with ethanol. The RNA was then washed once with 80% ethanol, then with 95% ethanol and finally dried in vacuo.

Formaldehyde-treated R17 RNA was used the same day in *in vitro* protein synthesis assays.

In vitro protein synthesis assays

For the R17 RNA-directed *in vitro* protein-synthesizing system, the complete reaction mixture (50 µl) contained 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 225 mM ammonium glutamate (including the ammonium glutamate contributed by the cellular extracts and ribosomal preparations), 8 mM Mg^{++} (as magnesium acetate), 7.5 mM reduced glutathione, 82 mM Tris-acetate (pH 7.6), 1.25 µM $tRNA_f^{met}$ (from *E. coli* MRE 600; Boehringher Mannheim), 0.03 mM [^{14}C]valine (225 mCi/mmole; ICN Biomedicals Canada, Ltd.), 0.05 mM each of the other 19 amino acids, 100 µg R17 RNA, 0.2 volume of S-30 (ca. 0.8 mg protein) or 10 µl of S-150 (ca. 0.5 mg protein) and 2 A_{260} units of ribosomes or 10 µl of S-150, 4 A_{260} units of NH_4Cl-washed ribosomes and 39 µg of crude initiation factors.

The complete reaction mixture (50 µl) for the *in vitro* translation system directed by endogenous mRNAs of *V. costicola* contained 15 mM phosphoenolpyruvate, 2 mM ATP, 1 mM GTP, 300 mM ammonium glutamate (including the ammonium glutamate contributed by the S-30), 8 mM Mg^{++} (as magnesium acetate), 7.5 mM reduced glutathione, 82 mM Tris-acetate (pH 7.6), [U-^{14}C]amino acids (1.74 mCi/mg; ICN Biomedicals Canada, Ltd.) at a concentration of 0.05 mM each (adjusted with unlabeled amino acids), and 0.4 volume of S-30 (ca. 1.5 mg protein).

In both systems, protein synthesis was followed by measuring the amount of radiolabeled amino acid(s) incorporated into hot TCA-insoluble material. Reaction mixtures were incubated at 30°C for the times indicated. Reactions were stopped by adding 500 µl of 5% TCA, the mixtures heated at 90°C for 20 minutes, cooled in ice and filtered (Millipore filters, Type HA, 45 µm). The filters were washed twice with 5 ml of 5% TCA, dried, and mixed with 4 ml of Universol Cocktail. The radioactivity trapped on the filters was then counted.

Assay of formylmethionyl-tRNA synthetase

The enzyme reaction was performed in a mixture of the same composition as that for R17 RNA-directed protein synthesis except that R17 RNA was not included and that [^{14}C]valine and the other 19 amino acids were replaced by 0.07 mM of [^{14}C]methionine (285 mCi/mmole; Amersham). S-150 was used as the source of enzyme. Incubation was at 30°C for 20 min. Synthetase activity was measured by determining the amount of radioactivity rendered insoluble by 500 µl of cold 10% TCA and retained on Millipore filters (Type HA, 45 µm) (Ellis and Conway, 1984).

Binding of [^{14}C]fmet-tRNA and [^{3}H]R17 RNA by initiation factors

Binding of [^{14}C]fmet-tRNA (Van der Hofstad et al., 1979) and [^{3}H]R17 RNA (Johnson and Szekely, 1979) was measured by determining the amount of radioactivity retained on

nitrocellulose filters (Schleicher and Schuell, BA 85). The reaction mixture (50 µl) contained the following: 225 mM ammonium glutamate, 8 mM Mg^{++} (as magnesium acetate), 82 mM Tris-acetate (pH 7.6), 7.5 mM reduced glutathione, 40 µg of crude initiation factors and 11 pmole [^{14}C]fmet-tRNA (550 DPM/pmole) or 100 µg [^{3}H]R17 RNA (900 DPM/µg). The reaction mixture was incubated at 30°C for 15 min, cooled on ice, filtered and washed twice with 3 ml of ice-cold buffer [225 mM ammonium glutamate, 8 mM magnesium acetate, 82 mM Tris-acetate (pH 7.6) and 6 mM β-mercaptoethanol]. The filter was then dried and the radioactivity counted.

Synthesis of [^{14}C]fmet-tRNA

The charging and formylation reactions were done according to Voorma et al. (1971) with modifications of the reaction mixture to adapt it to V. costicola. The reaction mixture (1 ml) contained the following: 225 mM ammonium glutamate, 8mM magnesium acetate, 82 mM Tris-acetate (pH 7.6), 6 mM β-mercaptoethanol, 1.5 mM ATP, 10 mM phosphoenolpyruvate, 5 A_{260} units $tRNA_f^{met}$ (from E. coli MRE 600; Boehringher Mannheim), 10 µg folinic acid, 3.5 mM [^{14}C]methionine (225 mCi/mmole; ICN Biomedicals Canada, Ltd.) and 250 µl of S-150 of V. costicola. Incubation was at 30°C for 15 min. The initiator tRNA, fmet-tRNA, was then isolated and purified by phenol extraction and ethanol precipitation as described by Voorma et al. (1971)

Gel electrophoresis and autoradiography

After incubation, the reaction mixture of the R17 RNA-directed protein synthesis system was mixed with 0.125 volume of sample buffer [250 mM Tris-HCl (pH 6.8), 5% SDS, 5% 2-mercaptoethanol, 50% glycerol and 0.1% bromophenol blue] and 0.2 A_{260} units of R17, as carrier, and heated for 3 min at 90°C (Ellis and Conway, 1984). Fifty µl samples were used for electrophoresis. SDS-PAGE profiles of A and coat proteins were obtained simultaneously by denaturing purified R17 (0.2 A_{260} units) with 0.125 volume of sample buffer.

Gel slabs for SDS-PAGE, with linear gradients of polyacrylamide (10-15%) and glycerol (0-13%) (Ellis and Conway, 1984), were prepared according to Laemmli (1970) and run at 25 mA. Proteins were fixed and stained with Coomassie blue R250 (0.1%) in water:methanol:glacial acetic acid (5:5:2). The gels were destained in a solution of 12.5% isopropanol and 10% acetic acid and then dried *in vacuo* on Whatmann 3MM paper. The dried gels were autoradiographed at -80°C with Kodak X-Ray film (X-OMAT AR) and with an intensifying screen.

OXIDATIVE PATHWAY OF CHOLINE TO BETAINE

Bacterial cultures

V. costicola was grown at 30°C with vigorous shaking in a chemically defined minimal medium (CDMM) (Kamekura et al., 1985).

CDMM contained (per 100 ml): 2.0 g sodium glutamate monohydrate, 0.01 g KH_2PO_4, 0.02 g KCl, 2.0 g $MgSO_4$-$7H_2O$, 5 µg biotin, 40 µg thiamine hydrochloride, and choline chloride and NaCl as needed for different molarities, final pH 7.3. The medium was autoclaved and cooled and a 25% glucose solution, autoclaved separately, was added to give a final glucose concentration of 1.0%.

Uptake of choline by V. costicola

Cells were grown in 100 ml of CDMM to which 5 µCi of radiolabeled choline chloride (7.2 mCi/mmole; NEN Research Products) had been added. Choline chloride labeled in different positions was used in separate experiments: $[(^{14}CH_3)_3\text{-}N\text{-}CH_2\text{-}CH_2OH]Cl$ and $[(CH_3)_3\text{-}N\text{-}^{14}CH_2\text{-}CH_2OH]Cl$. Choline uptake was followed by its disappearance from the media. Samples (5 ml) were removed at different time intervals during the incubation, the cells quickly spun down (8,000 x g_{max} for 10 min), and the radioactivity of the supernatant (100 µl) determined.

The cell pellet was used to identify the labeled material accumulated in these cells. The pellet was extracted with 100 ul of 0.7 M perchloric acid containing 1 mM each of cold betaine and betaine aldehyde. Then, 125 µl of 1.0 M $NaHSO_3$ (pH adjusted to 7.5 with NaOH) was added and the solution centrifuged at 10,000 x g_{max} for 15 min. The deproteinized sample was tested for the presence of radioactive betaine aldehyde (glycine betaine aldehyde), betaine (glycine betaine) or choline by ion-exchange chromatography, as described below for the choline dehydrogenase assay.

Disruption and centrifugal fractionation of cells

Cells grown in CDMM were harvested at late exponential phase and washed twice with washing buffer [8 mM KCl, 0.41 mM $MgCl_2$, 0.05 M Tris-HCl (pH 7.5) and NaCl to the same molarity as the growth medium]. Late exponential phase was reached after 24, 24, 36 and 120 hours of incubation for cells grown in 0.5, 1.0, 2.0 and 3.0 M NaCl CDMM, respectively. Freshly washed-cells (20 g) were suspended into 60 ml of disruption buffer [50 mM Tris-HCl (pH 7.6), 10% glycerol, 0.25 mg/ml of lysozyme and 10 mM EDTA] and stirred at room temperature for 20 min (Bengis-Garber and Kushner, 1981). After the lysozyme treatment, $MgCl_2$, DNAse and RNAse were added to a final concentration of 10 mM, 30 µg/ml and 30 µg/ml, respectively (Landfald and Strom, 1986). The cell suspension was then placed in a cell disruption bomb (Parr Instrument Co., Moline, III) and

kept under argon at a pressure of 1,500 psi for 15 min at 4°C (Bengis-Garber and Kushner, 1981). The pressure was released and the procedure repeated two more times.

The disrupted suspension was centrifuged at 12,000 x g_{max} for 15 min to remove whole cells and large fragments. The supernatant was then centrifuged at 150,000 x g_{max} for 90 min. The high speed supernatant was collected (S-150) and the pellet (membrane fraction) washed once by resuspending it in buffer [50 mM Tris-HCl (pH 7.6) and 10% glycerol] and centrifuging it at 150,000 x g_{max} for 60 min (Bengis-Garber and Kushner, 1981). The final pellet was resuspended in the same buffer at a concentration of ca. 50 mg of protein/ml.

Solubilized membranes were obtained according to Landfald and Strom (1986) by dissolving the final membrane pellet in buffer [50 mM Tris-HCl (pH 7.6) and 10% glycerol (Bengis-Garber and Kushner, 1981)] that also contained 0.3% Triton X-100. The solution was stirred for 20 min at 4°C and the undissolved material removed by centrifugation (40,000 x g_{max} for 20 min).

Choline dehydrogenase assay

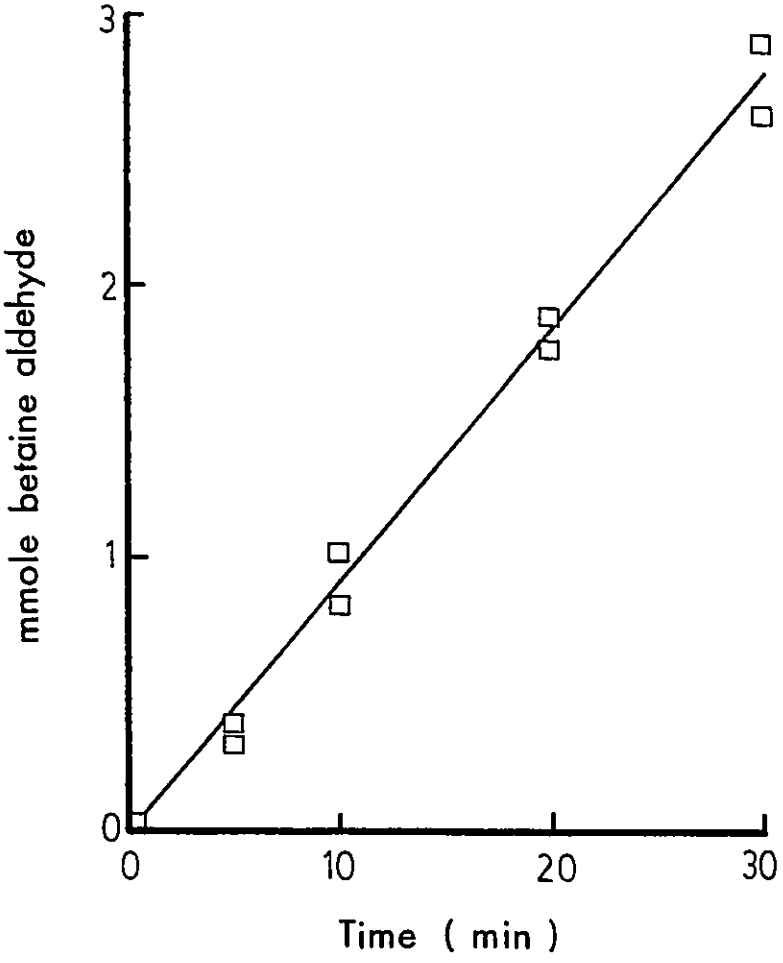
This assay was developped by Landfald and Strom (1986). The reaction mixture (1 ml) contained 100 mM Tris-acetate (pH 7.6), 10 mM [^{14}C]choline (20 nCi/µmole; NEN Research Products) and 20-:.5 mg of cell protein. Incubation was at 30°C for 30 min with maximal agitation. In the presence of the enzyme, synthesis of betaine aldehyde was linear over the entire

incubation period (Figure 2). When enzyme solubilized with Triton X-100 was tested, 0.5 mM of phenazine methosulfate was included in the reaction mixture as an artificial electron acceptor. The reaction was stopped by the addition of 400 µl of 0.7 M perchloric acid containing 1 mM each of unlabeled glycine and glycine betaine. Then, 500 µl of 1.0 M $NaHSO_3$ were added and the deproteinized sample centrifuged (10,000 x g_{max} for 15 min). Sodium bisulfite reacted with betaine aldehyde formed during the reaction mixture. The resulting addition product was separated from choline and betaine by ion-exchange chromatography on Dowex 50X4-200 (Sigma) in the H^+ form. Five hundred µl of the deproteinized sample were routinely applied to the column (0.5 by 4 cm).

The bisulfite-betaine aldehyde complex is not retained on the column while betaine is eluted with 2.0 ml of 2.0 M NH_3 and choline with 2.0 ml of 0.5 N NaOH. Fractions of 1.0 ml were collected in scintillation vials, to which 4 ml of Universol Cocktail were added and the radioactivity counted. Enzyme activity was expressed in nanomoles of total betaine aldehyde and betaine formed per minute at 30°C.

To perform the reaction under anaerobic conditions, the membrane preparation and the remaining components of the reaction mixture were flushed with N_2 in separate tubes. The appropriate amount of membrane preparation was then added to the reaction mixture and the incubation carried out under N_2.

Figure 2. Time course experiment showing the oxidation of choline to betaine aldehyde by the enzyme choline dehydrogenase of V. costicola.


3
2
1
0
mmole betaine aldehyde
0
10
20
30
Time (min)

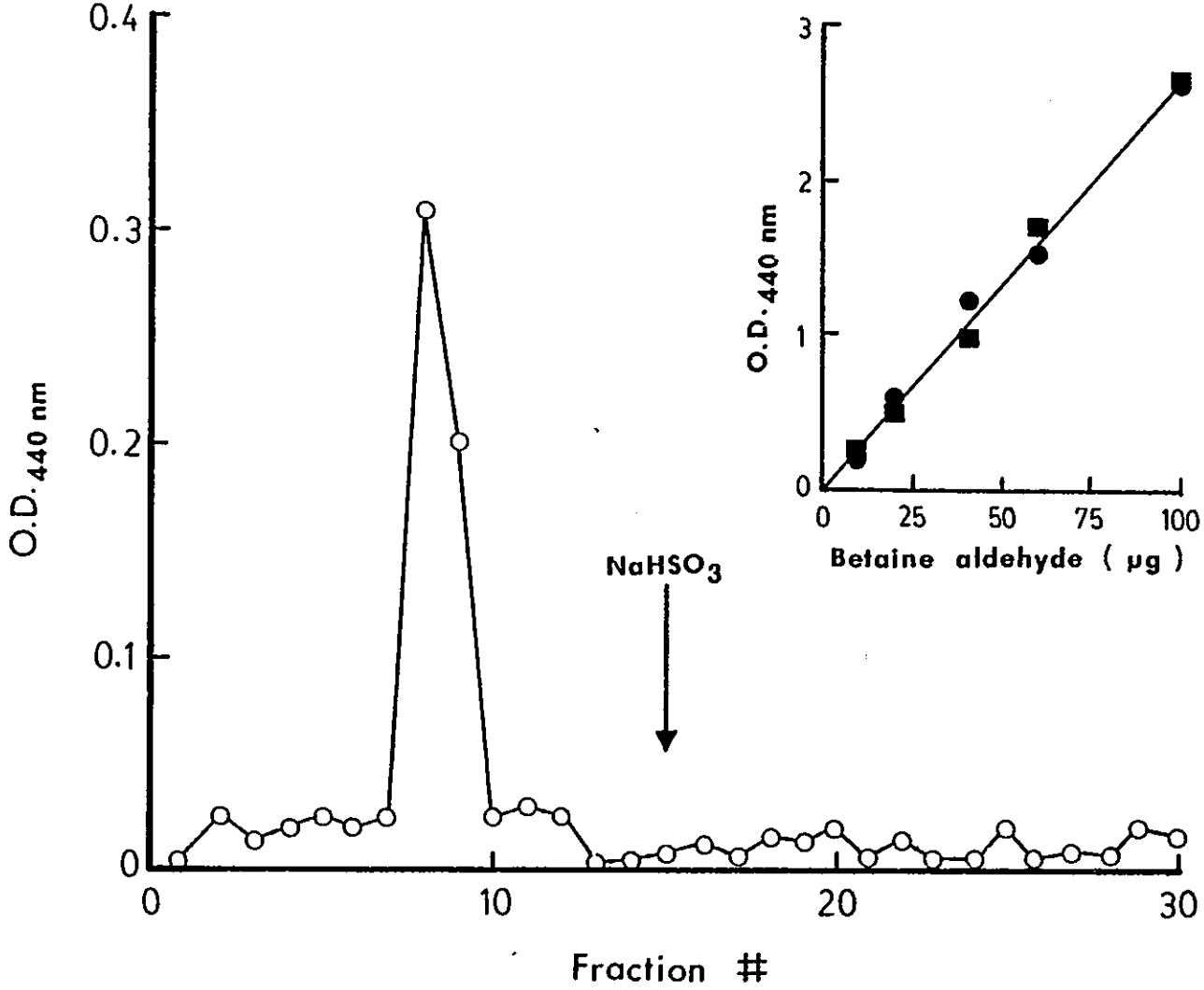
Landfald and Strom (1986) have reported that the complex formed between $NaHSO_3$ and betaine aldehyde should be retained on the column and then eluted with 2.0 ml of 0.2 M $NaHSO_3$. However, in my hands, the addition product did not bind to the resin. It is interesting to note that the amount of 1.0 M $NaHSO_3$ (500 µl) recommended by Landfald and Strom (1986) to neutralize the deproteinized sample gives a final concentration of $NaHSO_3$ of 0.26 M, a concentration higher than that apparently required to elute the addition product from the column.

To ensure that betaine aldehyde was, in fact, not retained on the column, a control experiment was done with a solution (100 µl) containing 100 mM Tris-acetate (pH 7.6), 20 µg unlabeled betaine aldehyde and 0.26 M $NaHSO_3$. This solution was applied to the column and then eluted with 2.0 ml of 0.2 M $NaHSO_3$. Fractions of 0.5 ml were collected and the amount of betaine aldehyde present in each fraction was determined by the colorimetric method described by Ikuta et al. (1977). To a 0.5 ml sample, 0.5 ml of 12% TCA and 1.0 ml of a 1% 2,4-dinitrophenyl-hydrazine (in 2 N HCl) solution were added. This solution was heated in a boiling water bath for exactly 5 min and then cooled (ca. 20°C) in tap water; 3 ml of 1.2 N NaOH were added and the solution incubated at room temperature for exactly 10 min, after which the A_{440} was read.

Standard curves of betaine aldehyde alone and of betaine aldehyde in the presence of 0.26 M $NaHSO_3$ were identical (Figure 3, inset), showing that the addition of bisulfite did

Figure 3. Elution diagram of the addition product betaine aldehyde-sodium bisulfite on Dowex 50X4-200. The amount of betaine aldehyde in each fraction was determined according to the spectrophotometric method of Ikuta et al (1977).
Fraction #1: application of the sample.
Fraction #15: elution with 0.2 M $NaHSO_3$.

Inset: standard curves of betaine aldehyde alone (●) and betaine aldehyde in the presence of 0.26 M $NaHSO_3$ (■).


0.4
0.3
0.2
0.1
0
O.D.440 nm
NaHSO3
0
10
20
30
Fraction #
3
2
1
0
O.D.440 nm
0
25
50
75
100
Betaine aldehyde (µg)

not interfere with the colorimetric determination of betaine aldehyde. Figure 3 shows a peak shortly after the application of the sample and before the elution with $NaHSO_3$. There is no peak after the addition of the bisulfite solution to the column. Determination of the amount of betaine aldehyde collected in fractions #8 and #9 gave a value of 20 µg. Therefore, betaine aldehyde was not retained on the column under those conditions.

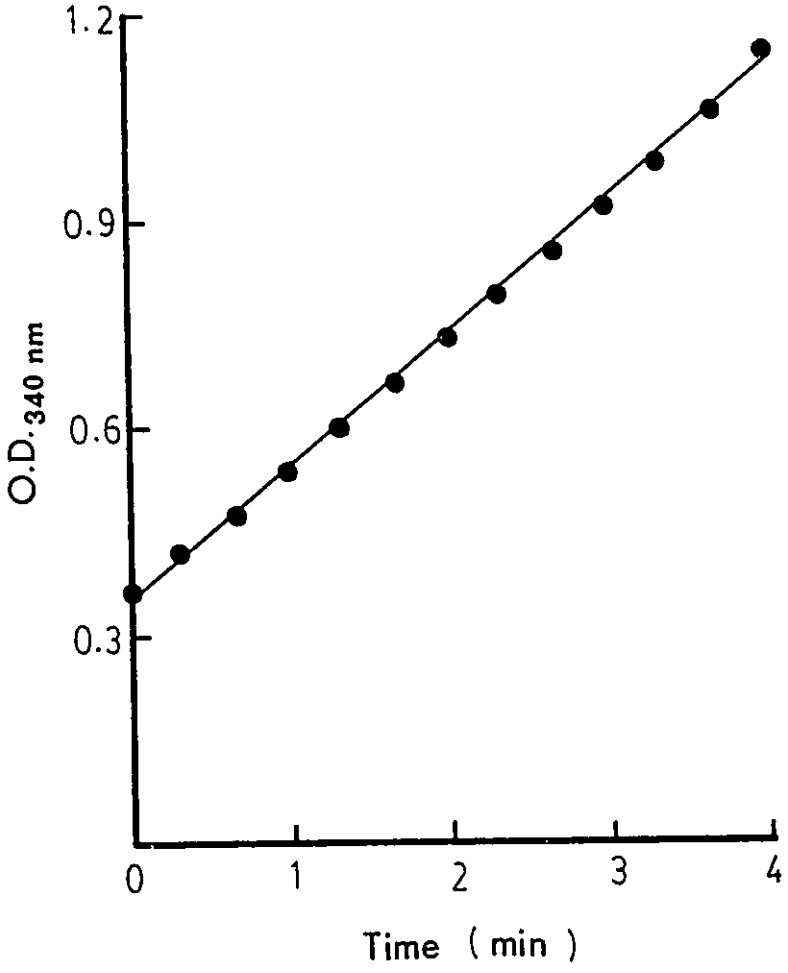
Betaine aldehyde dehydrogenase assay

The activity of betaine aldehyde dehydrogenase, an NAD-dependent enzyme, was assayed by the spectrophotometric determination of NADH at 340 nm. The reaction mixture (1 ml) contained 100 mM Tris-acetate, 5 mM betaine aldehyde, 5 mM NAD and 0.3-0.7 mg of cell protein (S-150). Incubation was at room temperature and the change in absorbancy at 340 nm was followed for 4 min. In the presence of the enzyme, reduction of NAD was linear over the entire incubation period (Figure 4). Enzyme activity was expressed in nanomoles of betaine formed per minute at 25°C.

Protein content determination

Prior to the Lowry assay (Lowry et al., 1951), samples (200 µl) were treated with 2 ml of cold acetone-methanol (5:2) to remove the Tris buffer (Bengis-Garber and Gronet-Elhanan, 1979). The precipitated proteins were solubilized in 1% SDS as a

Figure 4. Time course experiment showing the reduction of NAD by the enzyme betaine aldehyde dehydrogenase of V. costicola.


1.2
0.9
0.6
0.3
O.D.340 nm
0
1
2
3
4
Time (min)

substitute for NaOH. Bovine serum albumin (BSA) dissolved in 1% SDS was used as the standard.

Betaine determination

Cells grown in CDMM were harvested at late exponential phase, washed twice with washing buffer [8 mM KCl, 0.2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 50 mM Tris-HCl (pH 7.2), and NaCl at the same molarity as the growth medium] and then resuspended in the same buffer to an O.D. (650 nm) of 2.4-2.9. An aliquot of the cell suspension (1-10 ml) was centrifuged (8,000 x g_{max} for 10 min) and the cell pellet extracted with 1.0 ml of 10% $HClO_4$. The solution was neutralized with 10% KOH. This treatment also precipitated perchlorate ions ($KClO_4$). The precipitate was removed by centrifugation (10,000 x g_{max} for 15 min) and the amount of betaine in the supernatant was determined according to Wall et al. (1960).

The volume of different aliquots (20-100 µl) of the supernatant was brought up to 500 µl with 1.87 N H_2SO_4. Two hundred µl of KI-I_2 solution (20.0 g KI + 15.7 g I_2 in 100 ml H_2O) were added and the samples incubated on ice for 90 min. During this incubation, the samples were shaken frequently. After the incubation, 2 ml of H_2O and 7.0 ml of 1,2-dichloroethane (both were kept on ice) were added and gently mixed with a stream of air for 90 sec. The top layer was removed and the A_{365} of the bottom layer was read. This reading was always taken exactly 2 min after the addition of

1,2-dichloroethane. Betaine (0-50 μg) dissolved in H_2SO_4 was used as the standard.

RESULTS

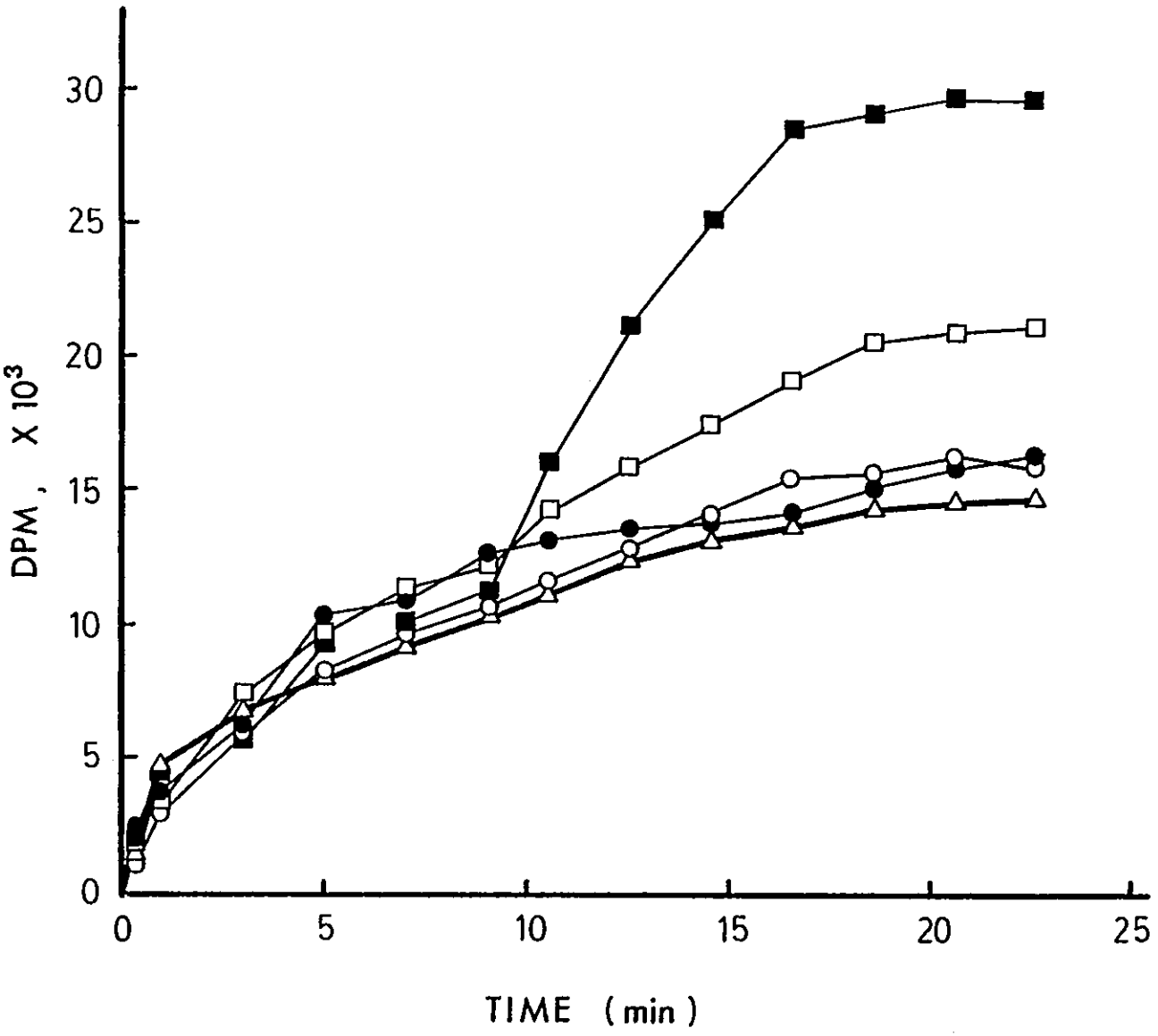
All the results presented in this thesis are mean values obtained from at least two separate experiments. In experiments where the results are expressed as percent (%) relative activity, the replicates never differed by more than 10% (in terms of relative activity), unless otherwise stated in the legend to each figure. In experiments where the results are expressed as DPM, the standard deviation of the data points that were obtained with more than two replicates was always less than 10% the mean value. The results of the ultracentrifugation studies, with sucrose gradients, are not mean values but rather "typical" results of experiments that were replicated.

POLY(U)-DIRECTED IN VITRO PROTEIN SYNTHESIS

Importance of different parts of the protein-synthesizing system

Time course experiments showed that even when active incorporation of [^{14}C]phenylalanine took place, the rate of incorporation fell quickly (Figure 5). Adding more poly(U) or more [^{14}C]phenylalanine at 9 min did not restore the rate of incorporation; adding more energy source (ATP, GTP and phosphoenolpyruvate) caused some stimulation, but the largest effect was obtained by adding fresh ribosomes (Figure 5). This suggests that ribosomes are not recycled in this system.

Figure 5. Importance of different parts of the poly(U) protein synthesizing system. Time course experiments showing the effects of adding more poly(U), ○ ; more [^{14}C]phenylalanine, ● ; more energy source (ATP, GTP and phosphoenolpyruvate), □ ; or more ribosomes, ■ ; at 9 min of incubation. Control (no addition) △ . The amounts of all constituents added were the same as those originally present.


30
25
20
15
10
5
0
DPM , X 10³
0
5
10
15
20
25
TIME (min)

As expected, no incorporation of phenylalanine occurred if the S-150 fraction or the ribosomes were omitted (not shown).

Effects of Cl^- ions on protein synthesis

In these experiments, a new incubation mixture was prepared that contained no Cl^- ions (see Materials and Methods, p. 39). Adding the extract itself brought the Cl^- concentration to 37 mM. The activities of extracts measured in this system were substantially higher (about fivefold) than those measured in the system previously used by Kamekura and Kushner (1984) which contained 279 mM Cl^-. I found that <u>all</u> concentrations of NaCl added to the new incubation system inhibited protein synthesis (see legend to Figure 6).

The possibility that the inhibition exerted by NaCl was due to the dilution, during the extraction procedure, of an essential intracellular substance, that would be necessary to carry out protein synthesis in the presence of high salt concentrations, was investigated. Cellular extracts prepared with half the amount of extraction buffer and then diluted to varying degrees responded similarly to the presence of NaCl during protein synthesis (Figure 6). The inhibition of protein synthesis was due to the Cl^- rather than to the Na^+, since adding different concentrations of sodium glutamate usually stimulated protein synthesis (Figure 7, inset).

Figure 6. Effects of NaCl on cell-free poly(U)-directed protein synthesis by extracts of V. costicola of different concentrations. Symbols: 2X concentrated extract, ○ ; normal S-30, □ ; 0.66X S-30, ● ; 0.5X S-30, ■ . Incubation time: 15 min.

Results are expressed as percentage of controls (without NaCl). 100% activity represents the incorporation of 23, 19, 17 and 13 pmole of [^{14}C]phenylalanine into hot TCA-insoluble material per 10 µl of 2X, 1X, 0.66X and 0.5X S-30, respectively

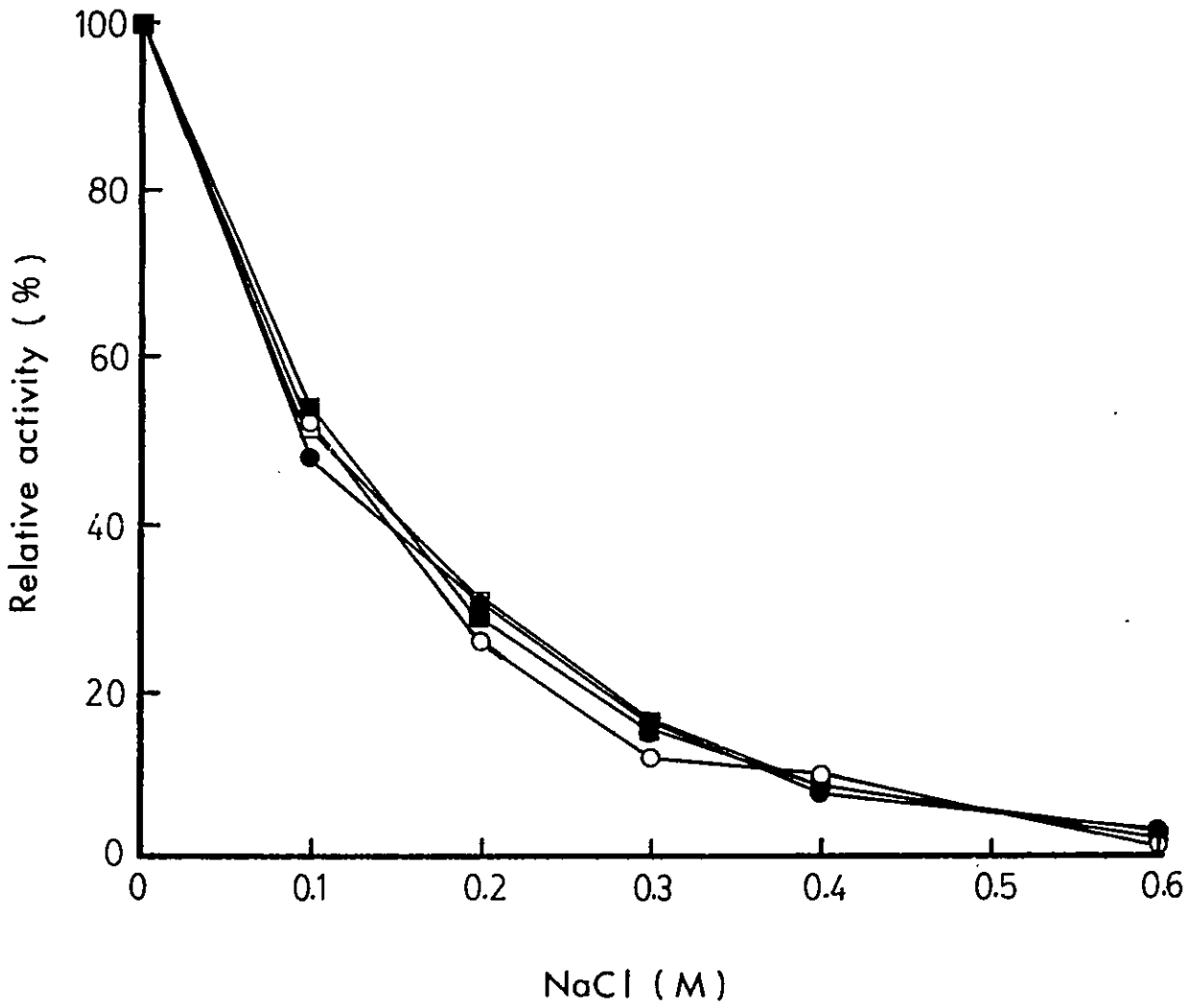
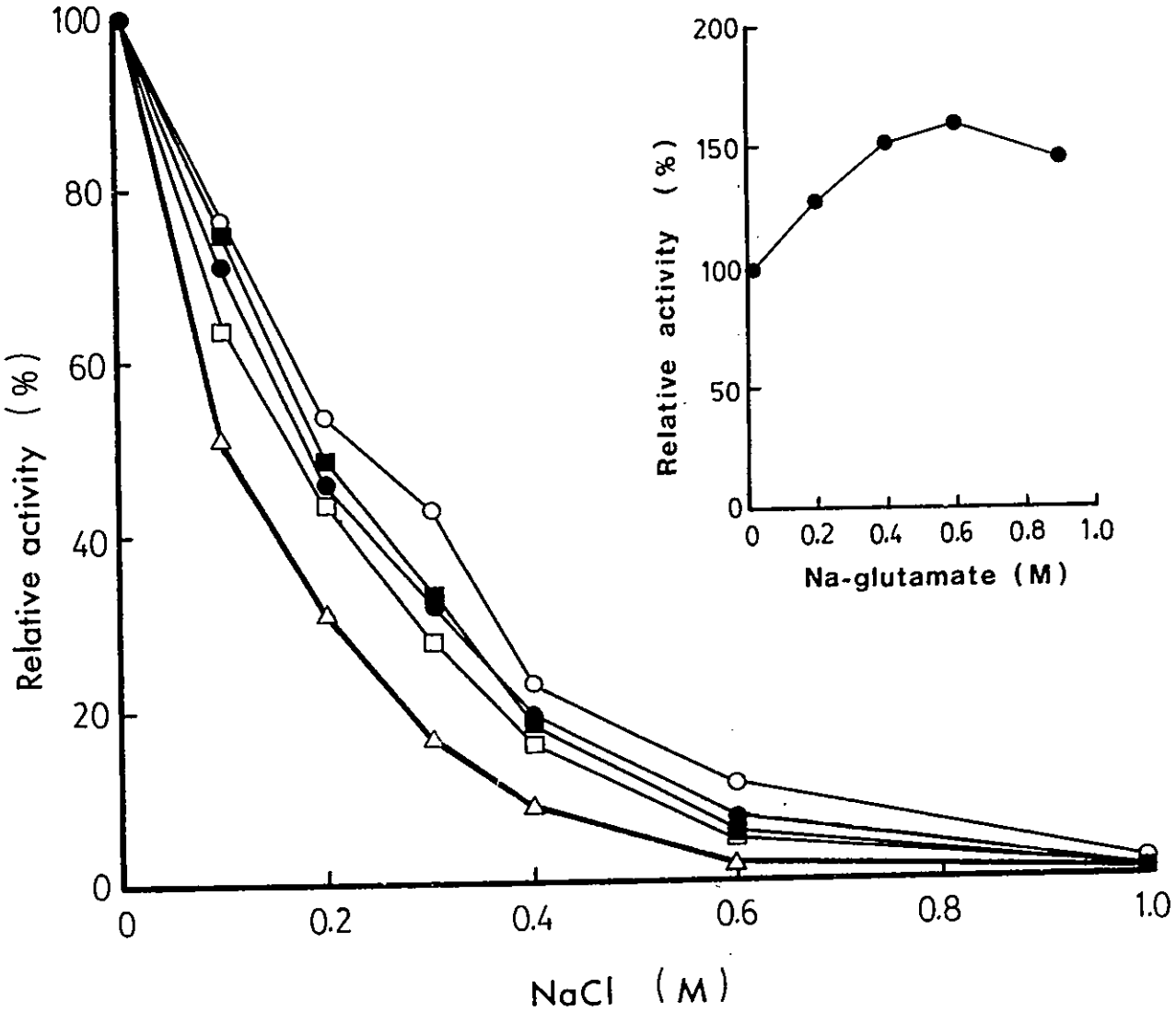

100
80
60
40
20
0
Relative activity (%)
0
0.1
0.2
0.3
0.4
0.5
0.6
NaCl (M)

Figure 7. Effects of sodium glutamate concentrations on poly(U)-directed protein synthesis by S-30 of V. costicola at increasing NaCl concentrations. The concentrations of added sodium glutamate were: △ , 0; □ , 0.2 M; ● , 0.4 M; ○ , 0.6 M; and ■ , 0.9 M. Incubation time: 15 min. Results are expressed as percentage of controls (without NaCl).

Inserted figure shows the effect of sodium glutamate concentrations on in vitro protein synthesis in the absence of NaCl. Incubation time: 15min. 100% activity represents incorporation of 20 pmole of [^{14}C]phenylalanine into hot TCA-insoluble material per 10 µl of S-30.


Relative activity (%)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)
Relative activity (%)
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1.0
Na-glutamate (M)

Fidelity of translation

Table 4 shows the effects of different NaCl concentrations on the fidelity of translation of the protein synthesizing system used here. In addition to that of phenylalanine, incorporation of valine and leucine were studied whose codons (GUU and UUG respectively) differ in only one nucleotide from that of phenylalanine (UUU). In the absence of poly(U) there was virtually no endogenous incorporation of any of the amino acids. In the presence of poly(U), only phenylalanine was significantly incorporated. Increasing concentrations of NaCl reduced the extent of phenylalanine incorporation. Since the presence of poly(U) did not stimulate the incorporation of leucine or valine with increasing NaCl concentrations [ratio of amino acid incorporated in the presence and absence of poly(U) did not increase], the fidelity of this translation system is not affected by increasing Cl^- ions.

Effects of glutamate and betaine on inhibition by Cl^- ions

Since cells grown in 1.0 M NaCl contain about 100 mM glutamate (Kamekura and Kushner, 1984), the concentration of glutamate (124 mM) used here may be considered an endogenous value. All solute concentrations shown in the experiments described below are added to solutions containing this level of glutamate.

I observed that all added concentrations of glutamate stimulated <u>in vitro</u> protein synthesis and counteracted the

Table 4. Poly(U)-directed incorporation of [^{14}C]phenylalanine, [^{14}C]leucine, and [^{14}C]valine by 10 µl of S-30 extracts of *V. costicola* at increasing Cl^- concentrations.

Concentration of Cl^- (mM)	Poly(U)	Amount of amino acid incorporated (pmole)		
		Phe	leu	val
0	+	47.3	0.5	0.0
	-	0.6	0.2	0.2
100	+	26.3	0.2	0.0
	-	0.4	0.0	0.2
300	+	10.8	0.0	0.0
	-	0.2	0.1	0.0
500	+	1.5	0.0	0.0
	-	0.3	0.0	0.0
1,000	+	0.0	0.0	0.0
	-	0.0	0.0	0.2

inhibitory effects of NaCl. The effects were generally higher in higher glutamate concentrations (Figure 7).

It was shown earlier that betaine can partly overcome the inhibitory action of Cl^- ions, even in the absence of glutamate (Kamekura and Kushner, 1984). In the presence of 124 mM glutamate, the effect increased with increasing betaine concentrations (Figure 8) up to the highest betaine concentration studied (1.2 M). The physiological concentration of betaine is about 0.5 M in these cells (M. Klein and D.J. Kushner, unpublished data), a value similar to that reported by Imhoff and Rodriguez-Valera (1984). This concentration causes a near-maximal stimulation of activity and has a definite sparing action on Cl^- inhibition. Adding more glutamate, in the presence of 0.5 M betaine, further decreased the inhibition by Cl^- (Figure 9).

Binding of poly(U) to ribosomes and ribosomal subunits

In an attempt to locate the site of action of Cl^- ions, the effects of NaCl and other substances on the binding of radiolabeled poly(U), an artificial mRNA, to the ribosomes of V. costicola was studied.

Figure 10 is an example of the results routinely obtained in experiments designed to locate the different ribosomal particles in a sucrose gradient. At the higher Mg^{++} concentration (18mM) there was one major peak and two minor ones. At the lower Mg^{++} concentration, the larger peak

Figure 8. Effects of betaine concentrations on poly(U)-directed protein synthesis by S-30 of V. costicola at increasing NaCl concentrations. The concentrations of added betaine were: △ , 0; □ , 0.3 M; ● , 0.6 M; ■ , 0.9 M; and ○ , 1.2 M. Incubation time: 15 min. Results are expressed as percentage of controls (without NaCl).

Inserted figure shows the effect of betaine concentrations on in vitro protein synthesis in the absence of NaCl. Incubation time: 15 min. 100% activity represents incorporation of 20 pmole of [^{14}C]phenylalanine into hot TCA-insoluble material per 10 µl of S-30.

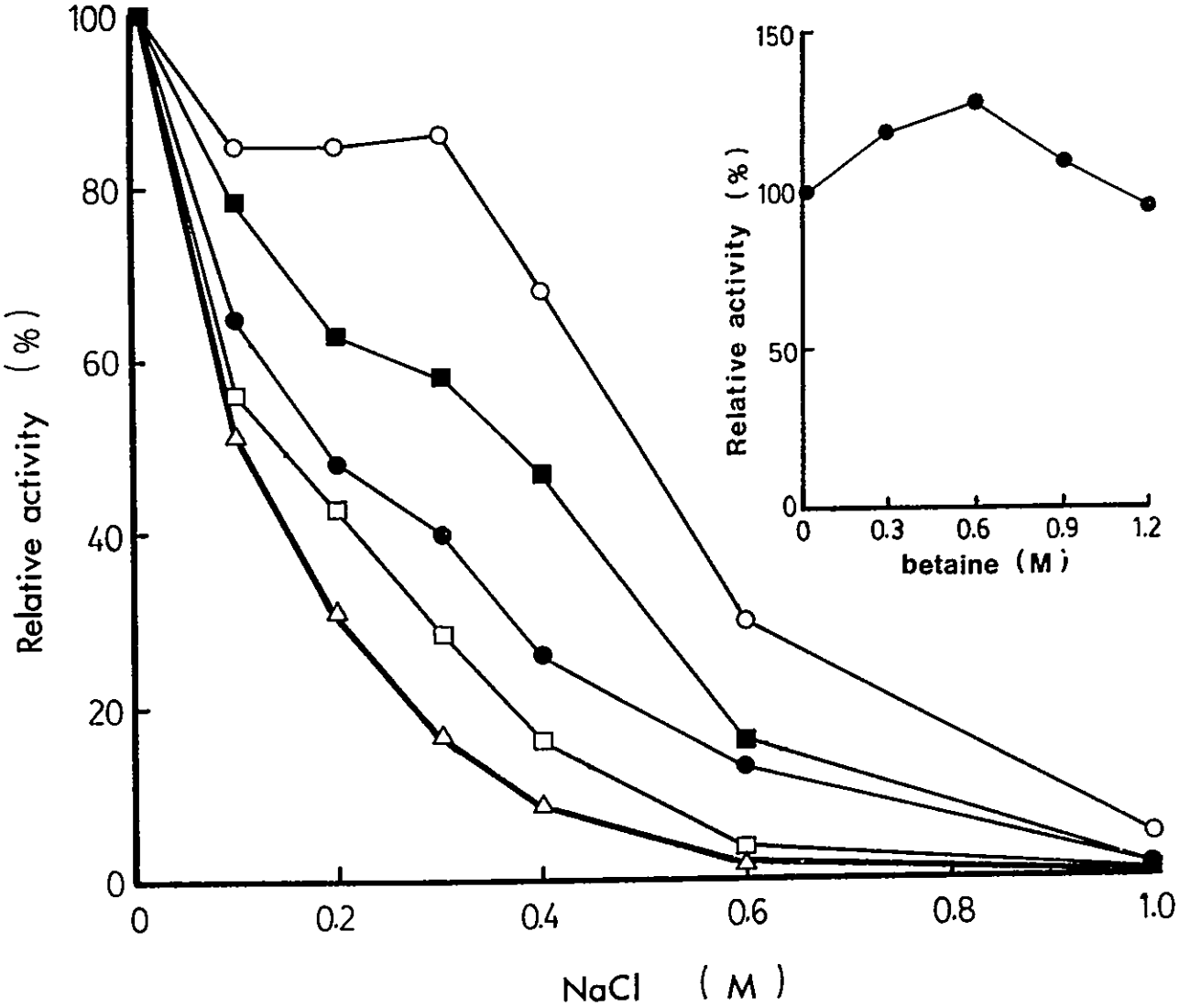

Relative activity (%)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)
Relative activity (%)
150
100
50
0
0
0.3
0.6
0.9
1.2
betaine (M)

Figure 9. Effects of betaine and/or sodium glutamate concentrations on poly(U)-directed protein synthesis by S-30 of V. costicola at increasing NaCl concentrations. Symbols: △ , no added salts; □ , 0.2 M sodium glutamate; ● , 0.5 M betaine; ■ , 0.2 M sodium glutamate and 0.5 M betaine. Incubation time: 15 min.

Results are expressed as percentage of controls (without NaCl). 100% activity is the incorporation of [^{14}C]phenylalanine by 10 µl of S-30 extract: 20 pmole in the absence of added solute; 25 pmole in the presence of 0.2 M sodium glutamate; 24 pmole in the presence of 0.5 M betaine; and 29 pmole in the presence of 0.2 M sodium glutamate and 0.5 M betaine.

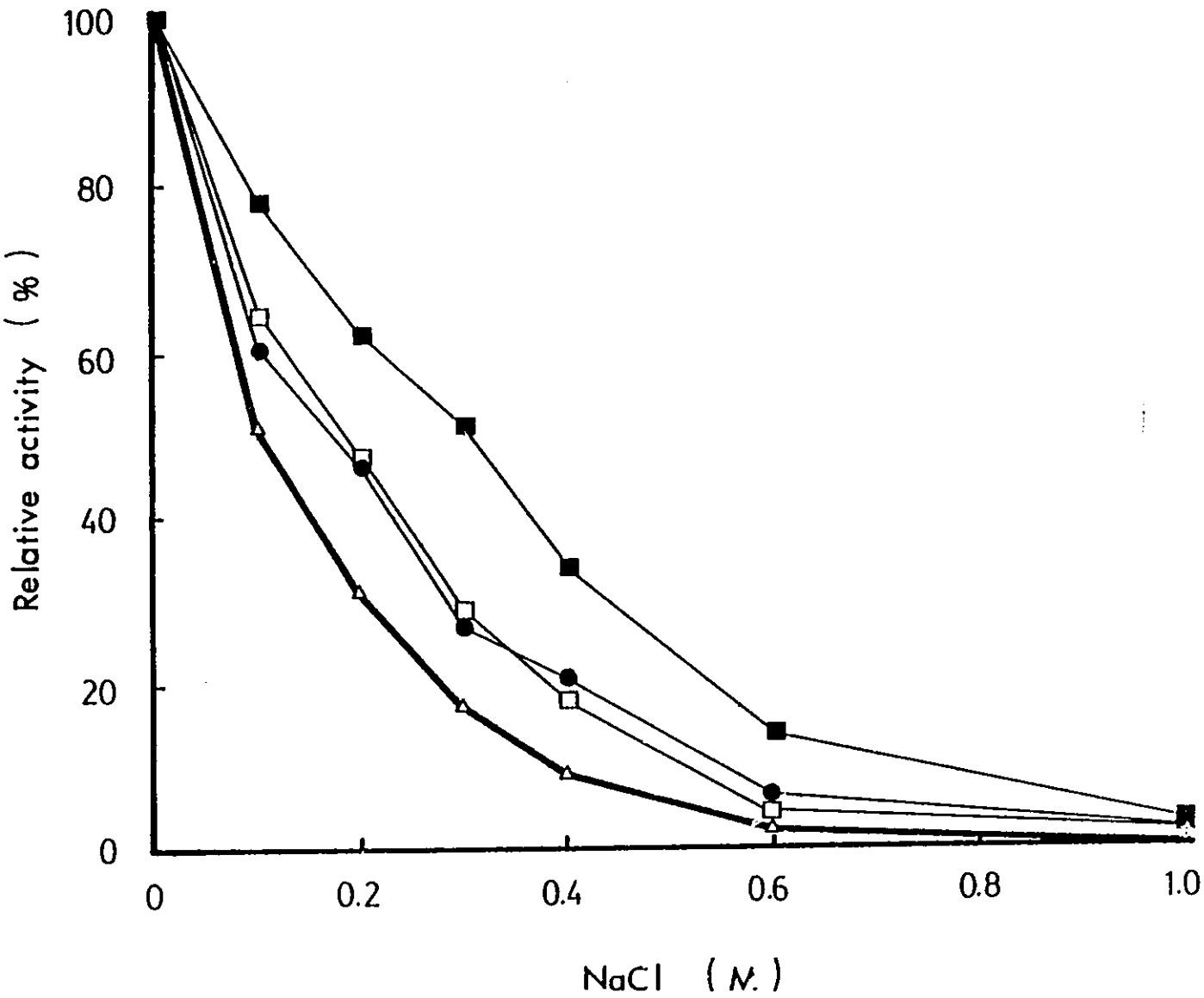
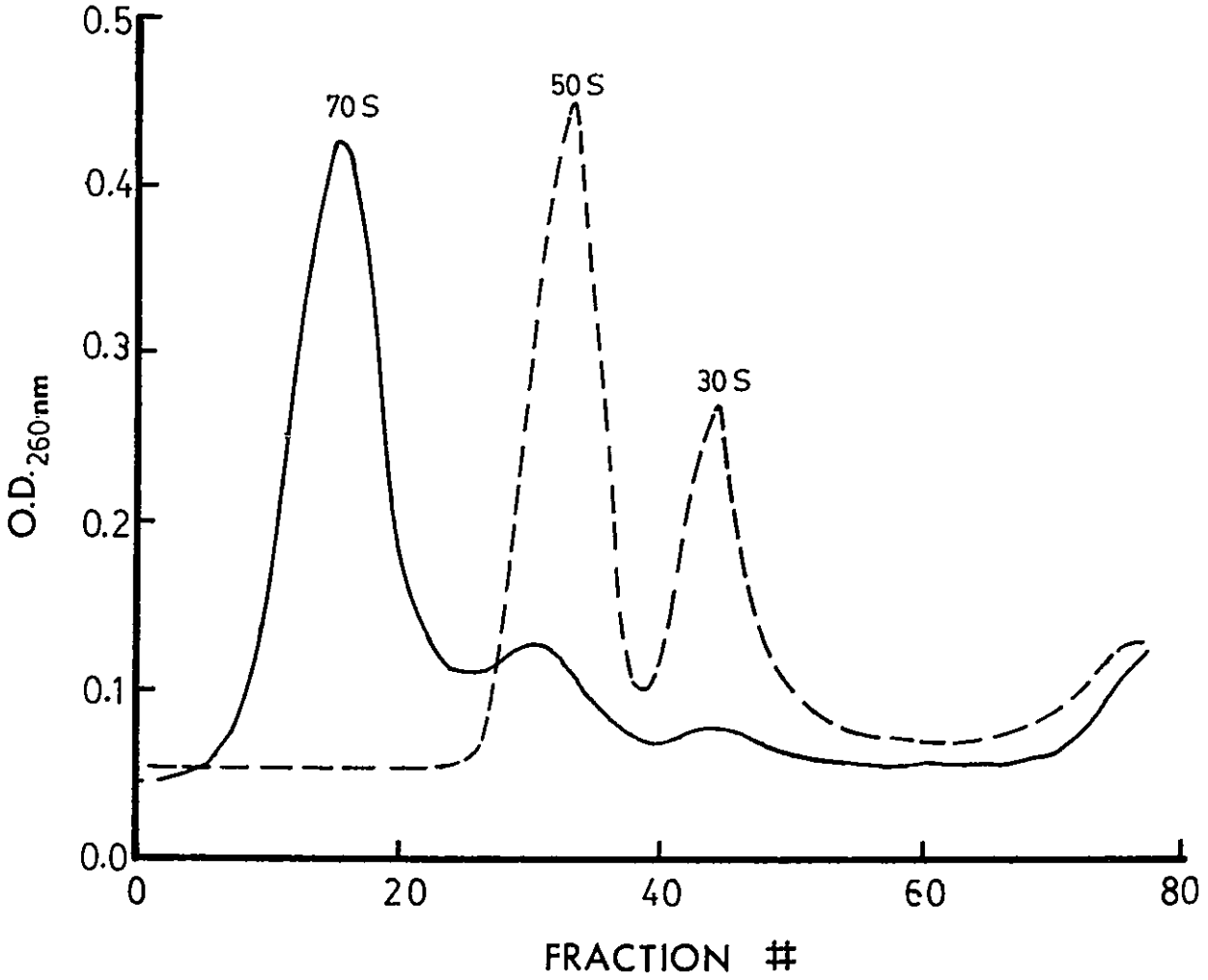

100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
NaCl (M.)

Figure 10. Sucrose density gradients (15-30%, centrifuged at 4°C) of V. costicola ribosomes at Mg^{++} concentrations of 18 (——) and 1 mM (----).


0.5
0.4
0.3
0.2
0.1
0.0
O.D.260nm
70 S
50 S
30 S
0
20
40
60
80
FRACTION #

disappeared, while the two minor peaks increased in size. These results showed the characteristic dissociation of 70S ribosomes into 50S and 30S subunits at low Mg^{++}, thus revealing the position of each ribosomal particle in the gradient.

Density gradient centrifugation showed that [^{3}H]poly(U) cosedimented with ribosomes. When the S-150 fraction was omitted from the reaction mixture, poly(U) cosedimented with, and was presumably bound to, the 70S and the 30S subunits (Figure 11B). However, with the additional presence of the S-150 fraction, poly(U) cosedimented only with the 30S subunits (Figure 11A). This suggests that factors in the S-150 fraction, probably elongation factors, caused the ribosomes to run off the artificial messenger. It also suggests that after runoff, although the 30S subunit was still able to attach to the messenger, the 50S subunit could not attach to the 30S-messenger complex. In further studies of poly(U) binding to ribosomes, the S-150 fraction was omitted.

I found that 0.6 M NaCl, which completely inhibits <u>in vitro</u> protein synthesis, also completely inhibits binding of ribosomes and 30S subunits to poly(U) (Figure 12A and 12B).

In the presence of 0.6 M sodium glutamate, a concentration which best protects <u>in vitro</u> protein synthesis, significant binding was still detected in the presence of 0.6 M NaCl (Figure 13B), although not to the same extent as in its absence (Figure 13A). The amount of binding detected in the presence of 0.6 M

Figure 11. Sucrose density gradients (15-30%, centrifuged at 4°C) showing binding of [^{3}H]poly(U) to V. costicola ribosomes (A) with S-150 present in the reaction mixture and (B) without S-150. O.D., optical density.

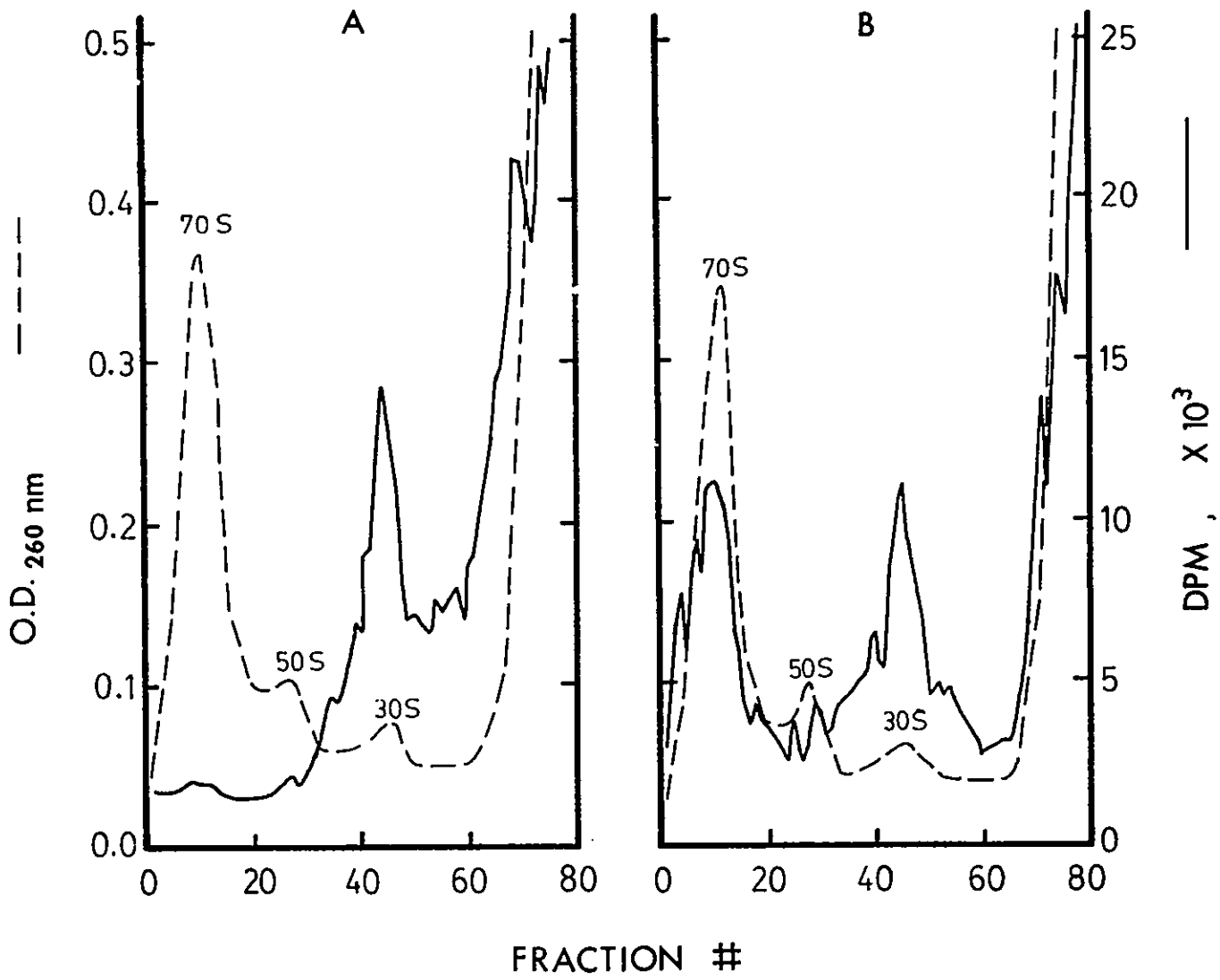

A
B
70 S
50 S
30S
70S
50S
30S
0.5
0.4
0.3
0.2
0.1
0.0
25
20
15
10
5
0
0
20
40
60
80
O.D. 260 nm
DPM , X 10³
FRACTION #

Figure 12. Sucrose density gradients showing binding of [^{3}H]poly(U) to V. costicola ribosomes in the presence of (A) no added solute in a 15-30% sucrose gradient (centrifuged at 4^{o}C) and (B) 0.6 M NaCl in a 5-20% sucrose gradient (centrifuged at 4^{o}C).

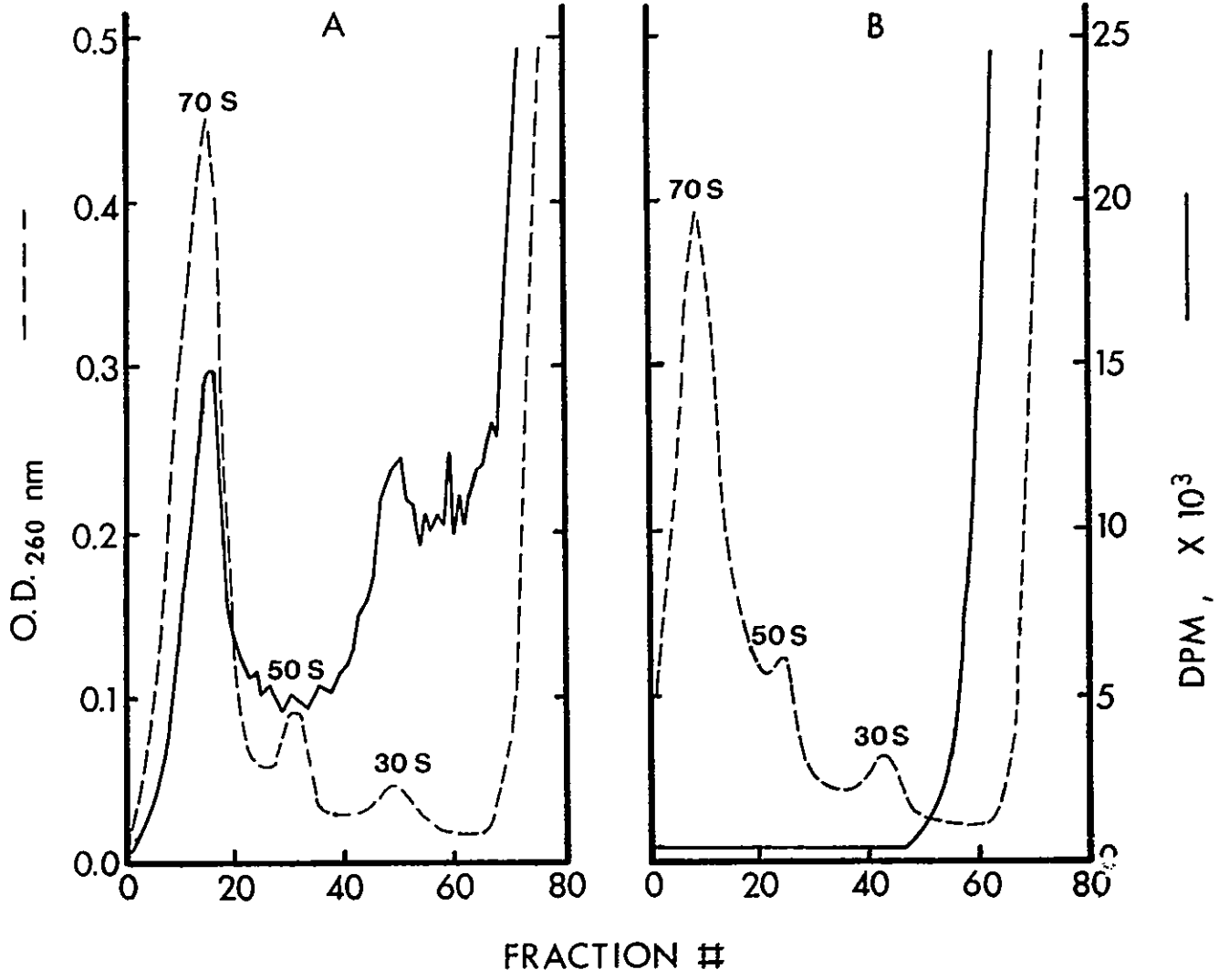
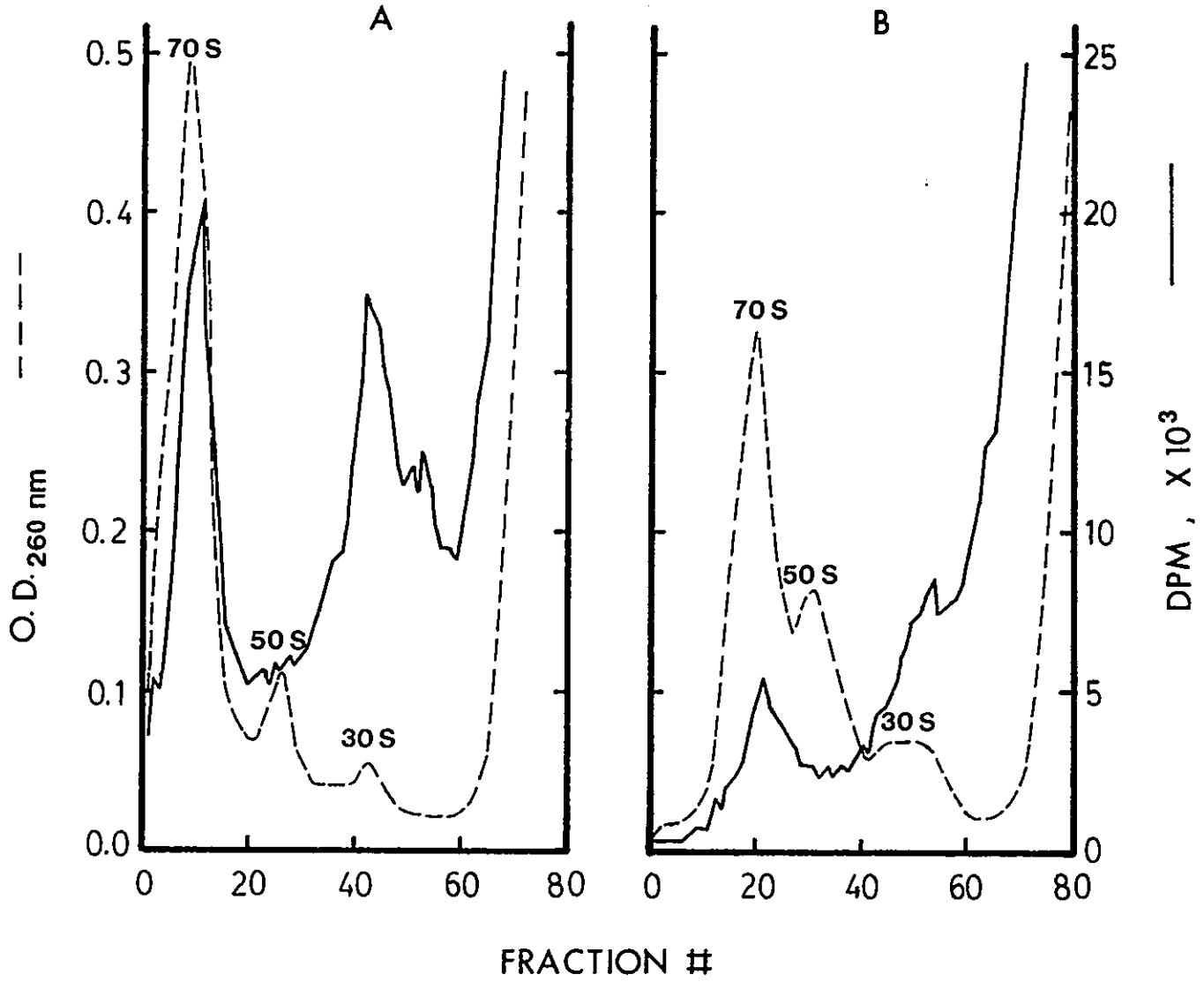

A
B
70 S
50 S
30 S
70 S
50 S
30 S
0.5
0.4
0.3
0.2
0.1
0.0
25
20
15
10
5
0
0
20
40
60
80
O.D.260 nm
DPM, X 10³
FRACTION #

Figure 13. Sucrose density gradients showing binding of [^{3}H]poly(U) to V. costicola ribosomes in the presence of (A) 0.6 M sodium glutamate and (B) 0.6 M sodium glutamate and 0.6 M NaCl. (A) and (B) were centrifuged in 5-20% sucrose gradients at 12.5^{o}C.


A
B
70 S
50 S
30 S
70 S
50 S
30 S
0.5
0.4
0.3
0.2
0.1
0.0
25
20
15
10
5
0
0
20
40
60
80
0
20
40
60
80
O. D. 260 nm
DPM , X 10³
FRACTION #

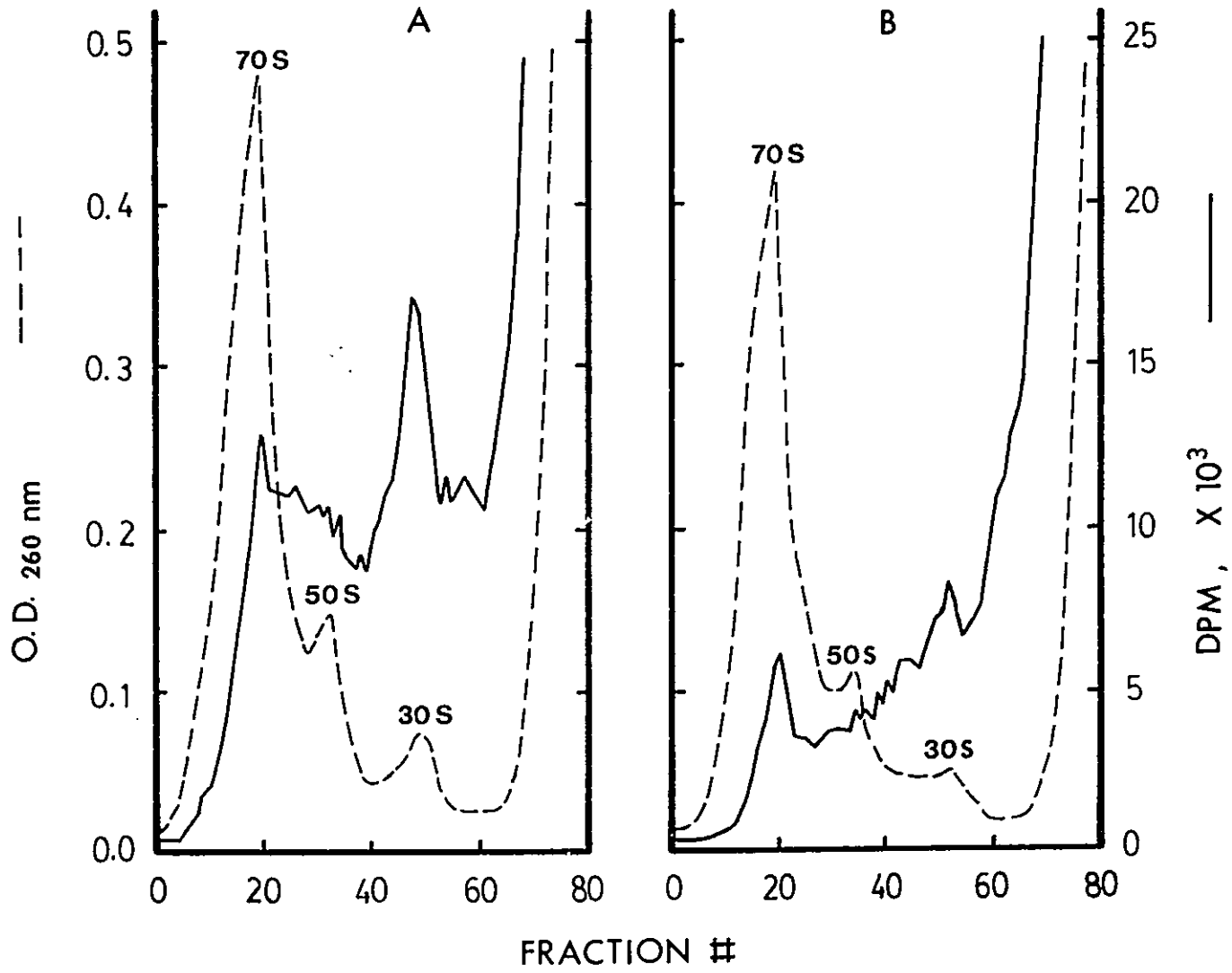
sodium glutamate alone, confirmed that it is Cl^-, and not Na^+, that is inhibitory to the binding between ribosomes and poly(U).

Similar results were obtained for betaine. In the presence of 1.2 M betaine, a concentration which affords maximal protection, poly(U) cosedimented with both 70S and 30S ribosomal particles (Figure 14A). With the addition of 0.6 M NaCl, a smaller but significant amount of poly(U) was found associated with each ribosomal particles (Figure 14B).

I then studied the ability of Cl^- to cause the dissociation of already bound poly(U) from ribosomes. A reaction mixture containing 0.6 M sodium glutamate was first incubated at 30°C for 2 min. Following this treatment, the binding pattern was the same as shown previously (Figure 13A), but after making the reaction mixture 0.6 M NaCl and incubating it for an additional 2 min, the amount of poly(U) bound to the ribosomal particles decreased to what had been observed previously when both salts were present at the beginning of the reaction (Figure 13B). Therefore, not only does Cl^- inhibit the binding of poly(U) to the ribosomes, but it also causes the release of already bound poly(U).

Although the sedimentation behavior of the ribosomes does not change at high Cl^- ion concentrations (Wydro et al., 1975), some ribosomal proteins may still be lost (Wydro, 1977). However, this loss did not damage the protein-synthesizing ability of the ribosomes. Ribosomes resuspended in 0.6 M NaCl and then washed in low salt extraction buffer to remove the

Figure 14. Sucrose density gradients showing binding of [^{3}H]poly(U) to V. costicola ribosomes in the presence of (A) 1.2 M betaine and (B) 1.2 M betaine and 0.6 M NaCl. (A) and (B) were centrifuged in 5-20% sucrose gradients at 7.5^{o}C.


A
B
70S
50S
30S
70S
50S
30S
0.5
0.4
0.3
0.2
0.1
0.0
25
20
15
10
5
0
0
20
40
60
80
0
20
40
60
80
O.D. 260 nm
DPM, X 10³
FRACTION #

residual NaCl could carry out more active protein synthesis in the standard (low salt) reaction mixture (Figure 15). The increased activity varied from 60 to 100% in different experiments. The mechanism of this increase has not yet been explored further, but the results do show that Cl^- ions cause no permanent damage to the ribosomes. In addition, in vitro protein synthesis with NaCl-washed ribosomes was as strongly inhibited as with ribosomes not exposed to 0.6 M NaCl (Figure 16). Thus, washing the ribosomes with 0.6 M NaCl did not protect, even slightly, protein synthesis against Cl^- inhibition, even though it increased their ability to carry out protein synthesis.

Figure 15. Incorporation of [^{14}C]phenylalanine into hot TCA-insoluble material by V. costicola ribosomes washed and not washed in 0.6 M NaCl. Symbols: ● , with NaCl; ○ , without NaCl.

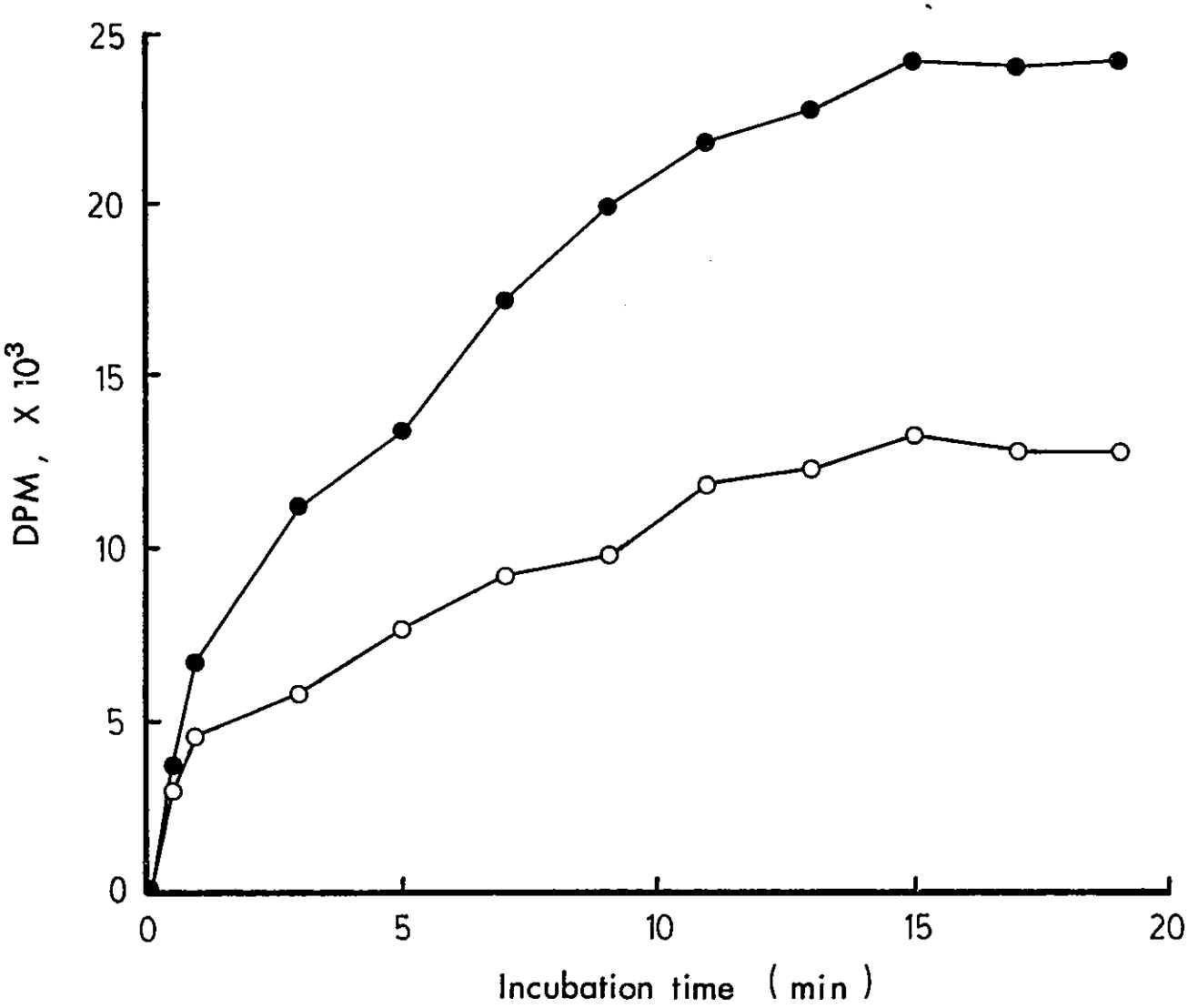
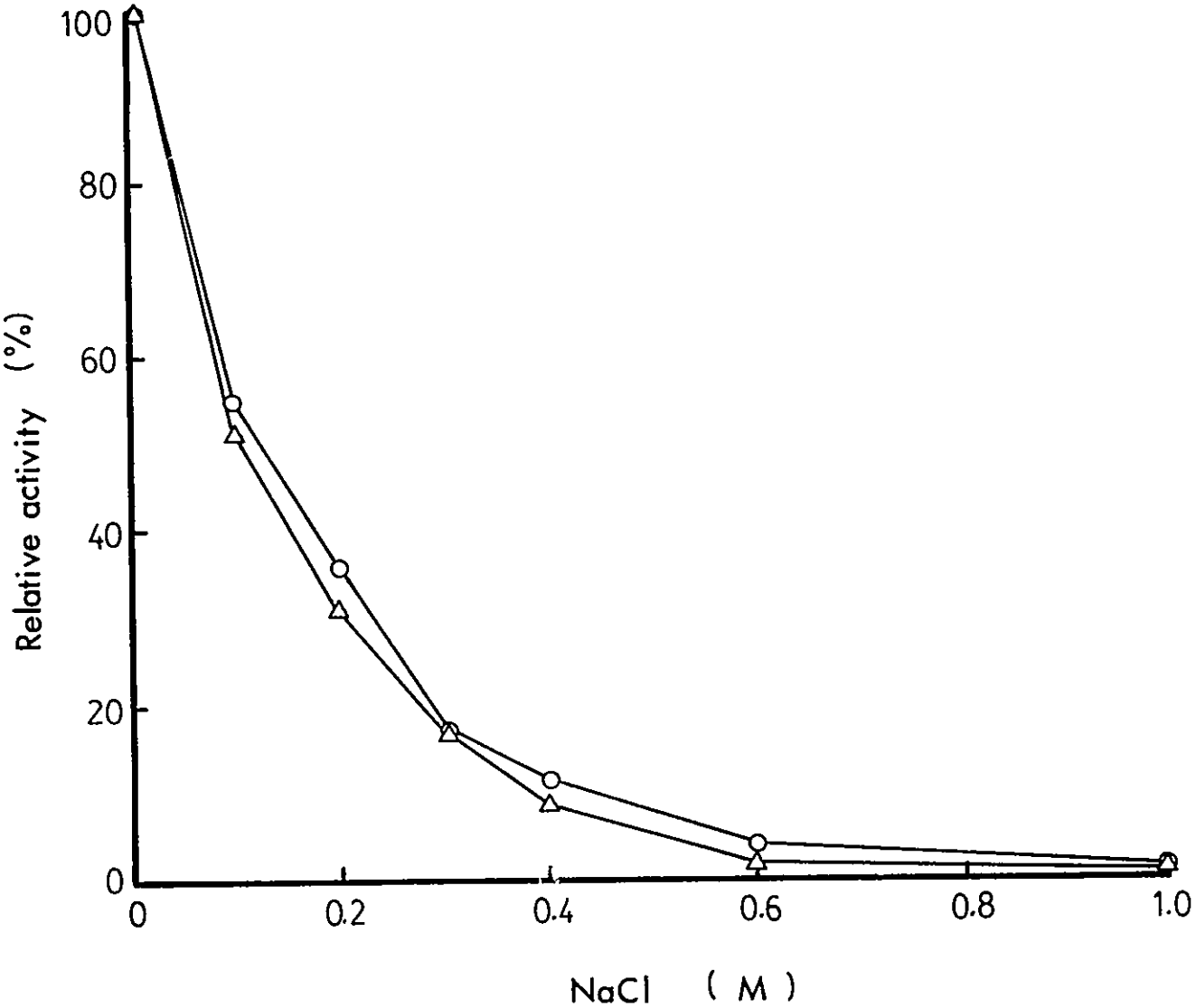

25
20
15
10
5
0
DPM, X 10³
0
5
10
15
20
Incubation time (min)

Figure 16. Effect of NaCl concentrations on poly(U)-directed protein synthesis by V. costicola ribosomes washed and not washed in 0.6 M NaCl. Symbols: △ , with NaCl; ○ , without NaCl. Incubation time: 15 min.

Results are expressed as percentage of controls (without NaCl). 100% activity represents incorporation of 23 and 12 pmole of [^{14}C]phenylalanine into hot TCA-insoluble material by 0.6 M NaCl-washed and normal ribosomes, respectively.


100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)

IN VITRO PROTEIN SYNTHESIS DIRECTED BY NATURAL mRNAs

Characterization of cell-free translation systems directed by natural mRNAs.

The RNA of the R17 phage of E. coli was able to act as a messenger for in vitro protein synthesis by extracts of V. costicola. The incorporation of [^{14}C]valine, one of the most predominant amino acid found in the coat protein of this phage (Min Jou et al., 1972), was absolutely dependent on the presence of this RNA (Figure 17). Valine incorporation was also absolutely dependent on ribosomes and on substances found in the S-150 supernatant (Figure 17), as well as on a source of energy (Figure 18). Final incorporation increased with increasing concentrations of ribosomes, mRNA and S-150 supernatant (Figure 17). Though incorporation was not absolutely dependent on added $tRNA_f^{met}$, adding this substance increased incorporation (Figure 17).

In an in vitro system containing ribosomes and S-150, incorporation of [^{14}C]valine was linear for the first 30 min (Figure 18). When S-30 fractions were used, which contained both ribosomes and S-150 fraction, valine incorporation reached at least as high a plateau as the former system within 10 min of incubation (not shown). Consequently, in experiments to determine total valine incorporation, the reaction mixture was

Figure 17. The effects of concentration of different parts of the R17 RNA-directed protein-synthesizing system of V. costicola. Symbols: ● , ribosomes; ○ , $tRNA_f^{met}$; □ , R17 RNA; ■ , S-150. Incubation time: 30 min.

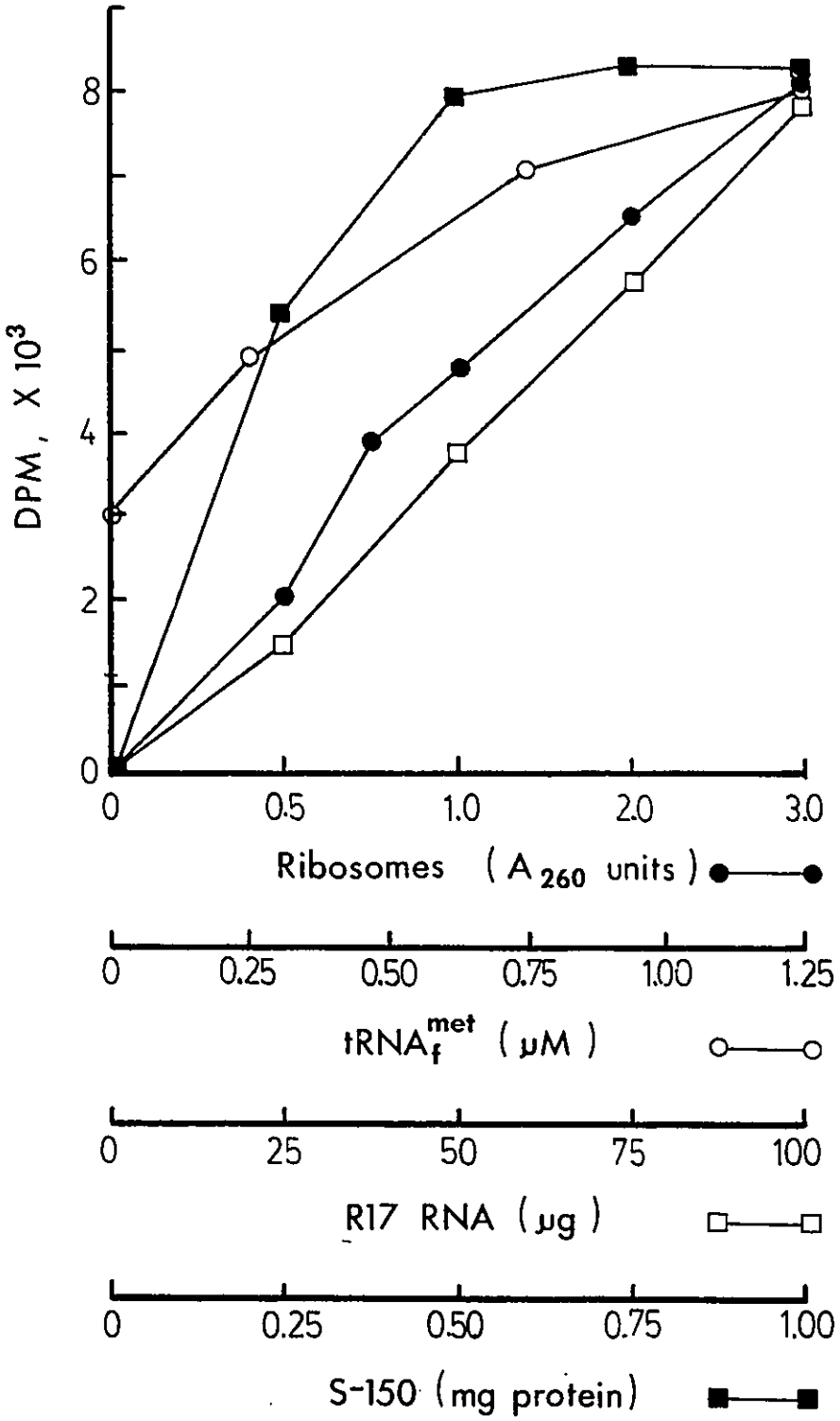
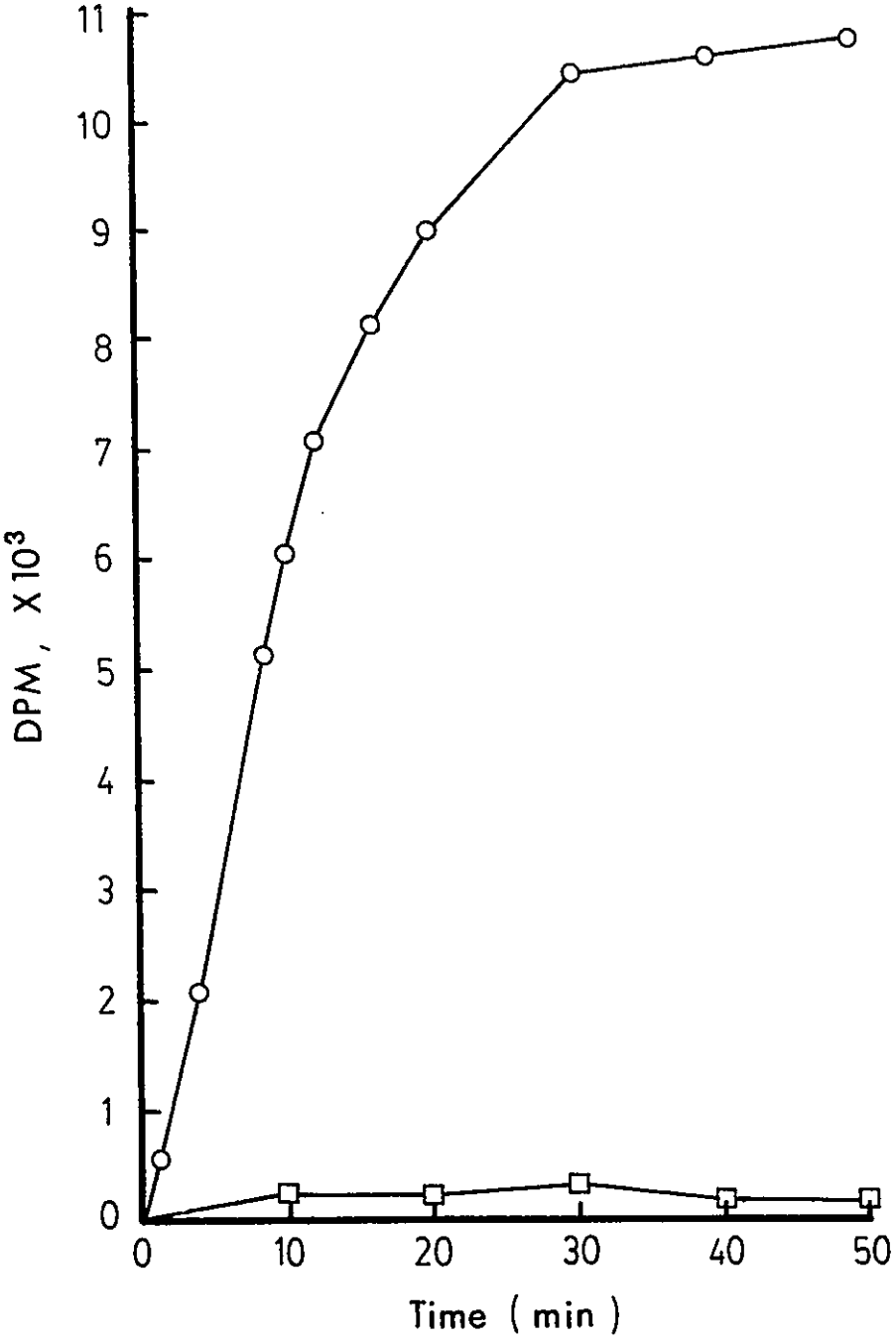

DPM, X 10³
8
6
4
2
0
0
0.5
1.0
2.0
3.0
Ribosomes (A 260 units)
0
0.25
0.50
0.75
1.00
1.25
tRNA f met (µM)
0
25
50
75
100
R17 RNA (µg)
0
0.25
0.50
0.75
1.00
S-150 (mg protein)

Figure 18. Time course experiment of the R17 RNA-directed protein-synthesizing system in the presence and absence of an energy source (ATP, GTP and phosphoenolpyruvate) by S-150 and ribosomes of V. costicola. Symbols: ○ , with energy; □ , without energy.


11
10
9
8
7
6
5
4
3
2
1
0
DPM, X 10³
0
10
20
30
40
50
Time (min)

incubated for 10 min in the presence of S-30 fractions and for 30 min in the presence of S-150 fractions plus ribosomes.

SDS-PAGE electrophoresis showed that the newly synthesized proteins made in the presence of R17 RNA comigrated with purified R17 coat protein (Figure 19).

In vitro protein synthesis directed by endogenous mRNAs of V. costicola was also dependent on time and on the amount of cell extract (Figure 20). Incorporation reached a plateau within 10 min of incubation. This system did not respond to increasing amounts of $tRNA_f^{met}$ (Figure 20).

Effects of protein synthesis inhibitors

The concentrations of the different protein synthesis inhibitors tested in these systems were determined from previous published studies. Chloramphenicol, at a concentration of 10^{-4} M, completely inhibits f2 RNA-directed protein synthesis by cellular extracts of E. coli (Kucan and Lipmann, 1964). Neomycin (3×10^{-4} M) inhibits peptidyl-puromycin synthesis with polyribosomes from E. coli (Pestka, 1972).

Kasugamycin (4×10^{-4} M) and aurin tricarboxylic acid (ATA) (4×10^{-5} M) selectively inhibit initiation of f2 RNA-directed protein synthesis by cellular extracts of E. coli (Grollman and Stewart, 1968; Okuyama et al., 1971).

R17 RNA-directed incorporation of [^{14}C]valine was strongly inhibited by every inhibitor tested (Table 5). Chloramphenicol and neomycin were also very effective in stopping endogenous

Figure 19. Autoradiogram of translated products of the R17 RNA-directed protein-synthesizing system by S-30 of V. costicola. The time of incubation is indicated above each lane. First lane on the right: SDS-PAGE profile of purified A and coat proteins of R17 electrophoresed alongside the radioactive translated products.

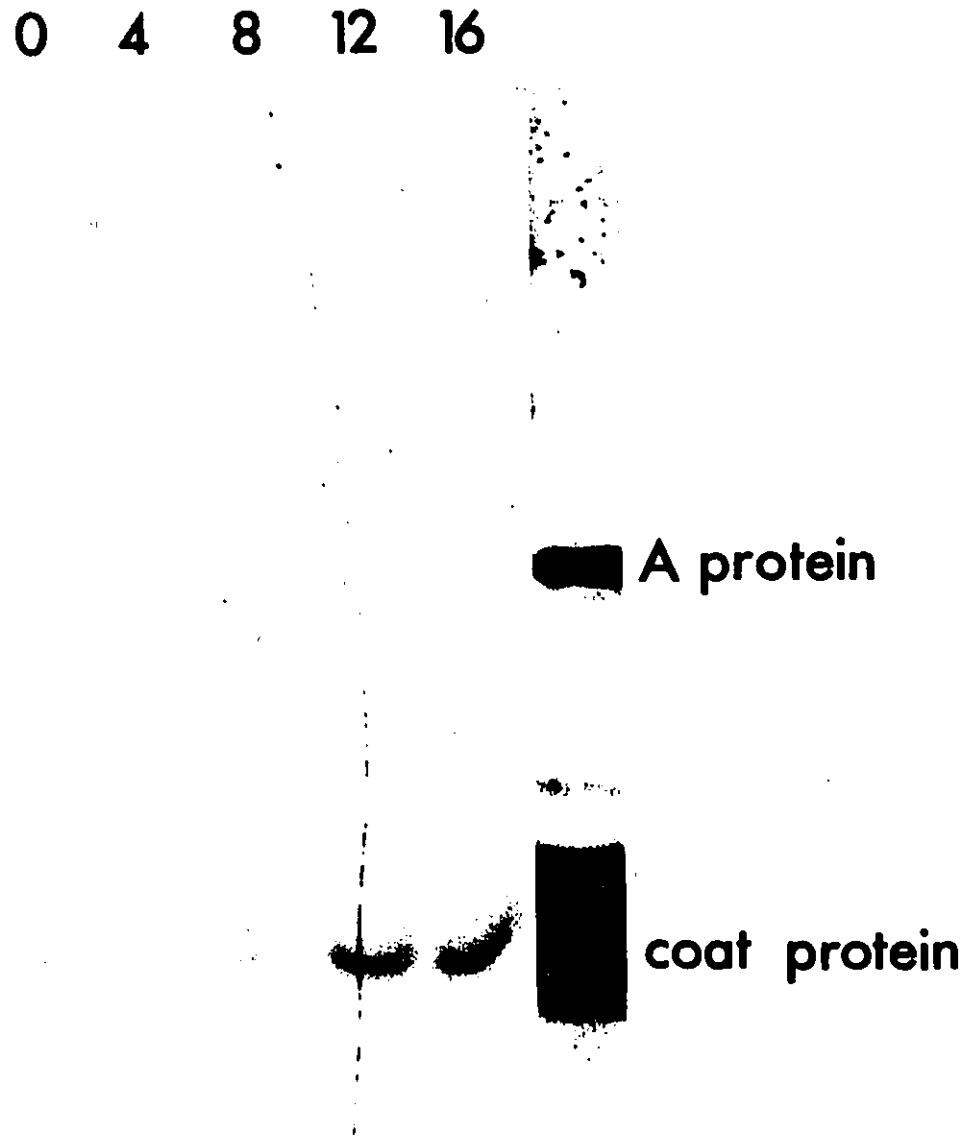

0 4 8 12 16
A protein
coat protein

Figure 20. Time course experiment of endogenous translation by S-30 of V. costicola and the effects of increasing concentrations of S-30 and $tRNA_f^{met}$. Symbols: ● , time course; ■ , S-30; ○ , $tRNA_f^{met}$. For the effects of S-30 and $tRNA_f^{met}$, the incubation time was 10 min.

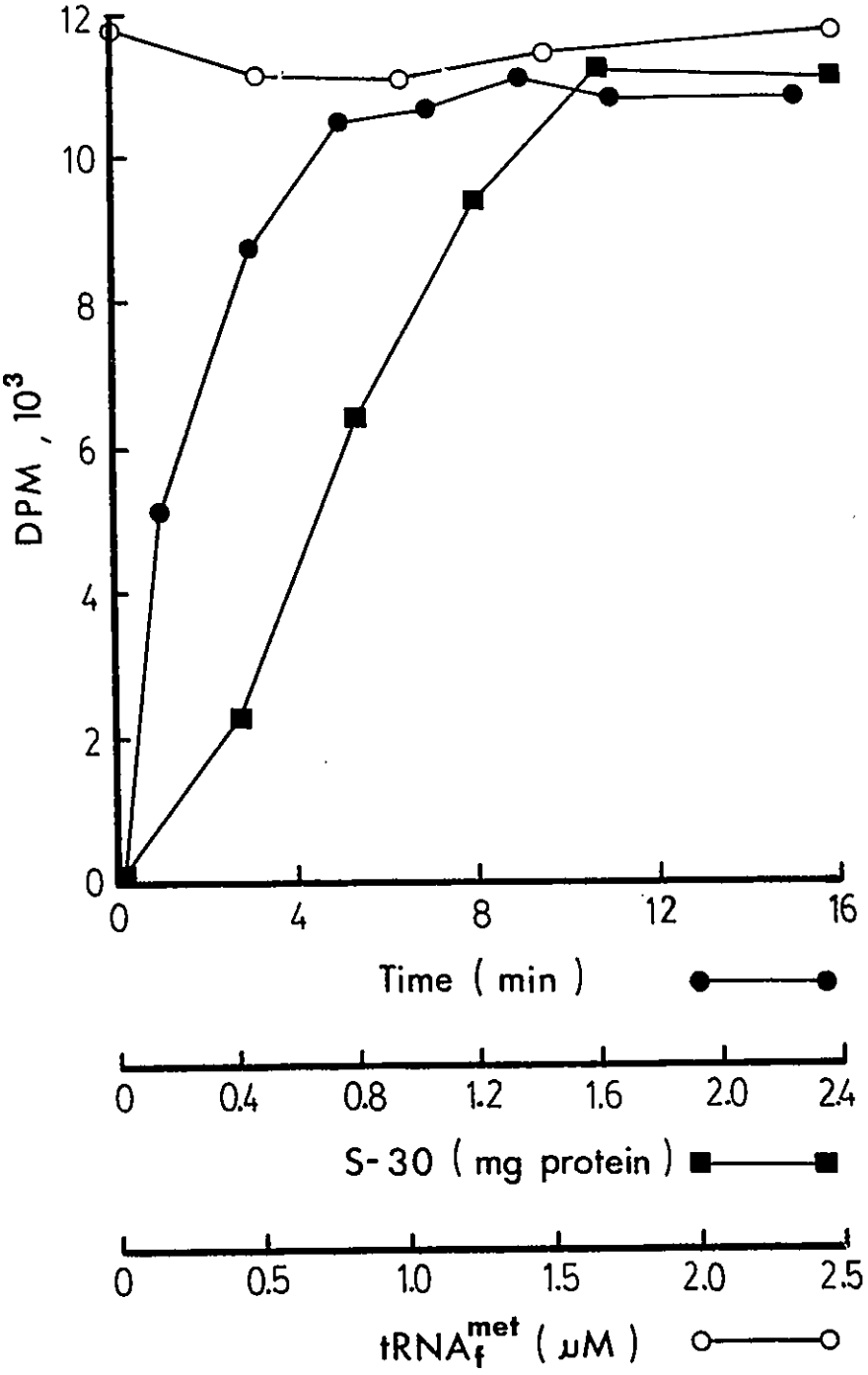

12
10
8
6
4
2
0
DPM , 10^3
0
4
8
12
16
Time (min)
0
0.4
0.8
1.2
1.6
2.0
2.4
S-30 (mg protein)
0
0.5
1.0
1.5
2.0
2.5
$tRNA_f^{met}$ (μM)

Table 5. Effects of protein synthesis inhibitors on the incorporation of radiolabeled amino acids in the R17 RNA system and in the endogenous system.

Inhibitor	concentration	% inhibition (S.D.[1]) endogenous translation	% inhibition (S.D.[1]) R17 RNA-directed translation
chloramphenicol	4×10^{-4} M	92 (1)	93 (1)
neomycin	4×10^{-4} M	81 (6)	98 (1)
kasugamycin	4×10^{-4} M	32 (4)	80 (5)
ATA[2]	4×10^{-5} M	5	80
RNAse[2]	40 µg/ml	100	100

[1] S.D., standard deviation obtained from 3 separate experiments.

[2] Results of ATA and RNAse are the means of 2 separate experiments.

translation. However, kasugamycin and ATA did not inhibit endogenous translation as effectively as R17 RNA-directed incorporation of [^{14}C]valine (Table 5). Pre-incubation (5 min) of the R17 RNA reaction mixture, without S-30, in the presence of RNAse completely inhibited protein synthesis. The same was true for the endogenous system after pre-incubating the S-30 fraction with RNAse for 5 min (Table 5).

Ionic requirements of the translation systems

The R17 RNA-directed protein-synthesizing system required Mg^{++} and NH_4^+ ions (ammonium glutamate) at concentrations of 8 mM and 225 mM (Figure 21), respectively, for optimal activity. Potassium glutamate could partially replace ammonium glutamate (Figure 21). However, sodium glutamate, NaCl, KCl and NH_4Cl did not support any levels of protein synthesis (not shown).

The translation of endogenous mRNAs also worked best at a Mg^{++} ion concentration of 8 mM, but required slightly more ammonium glutamate (300 mM) than the R17 system for optimal activity (Figure 22). Ammonium glutamate could be replaced quite efficiently by potassium glutamate, partially by NH_4Cl and KCl (at lower optimal concentrations) and not at all by sodium glutamate and NaCl (Figure 22).

Effects of added solutes on protein synthesis

Above the optimum concentration of ammonium glutamate, all added salts were inhibitory to the R17 RNA-directed translation

Figure 21. Ionic requirements of the R17 RNA-directed protein synthesizing system of V. costicola (S-30). Symbols: ammonium glutamate, ▲ ; potassium glutamate, ■ . Inserted figure shows the effects of Mg^{++} on in vitro protein synthesis. Incubation time: 10 min.

Results are expressed as percentage of maximum activity. 100% activity represents incorporation of 50 pmole of [^{14}C]valine into hot TCA-insoluble material per 10 µl of S-30.

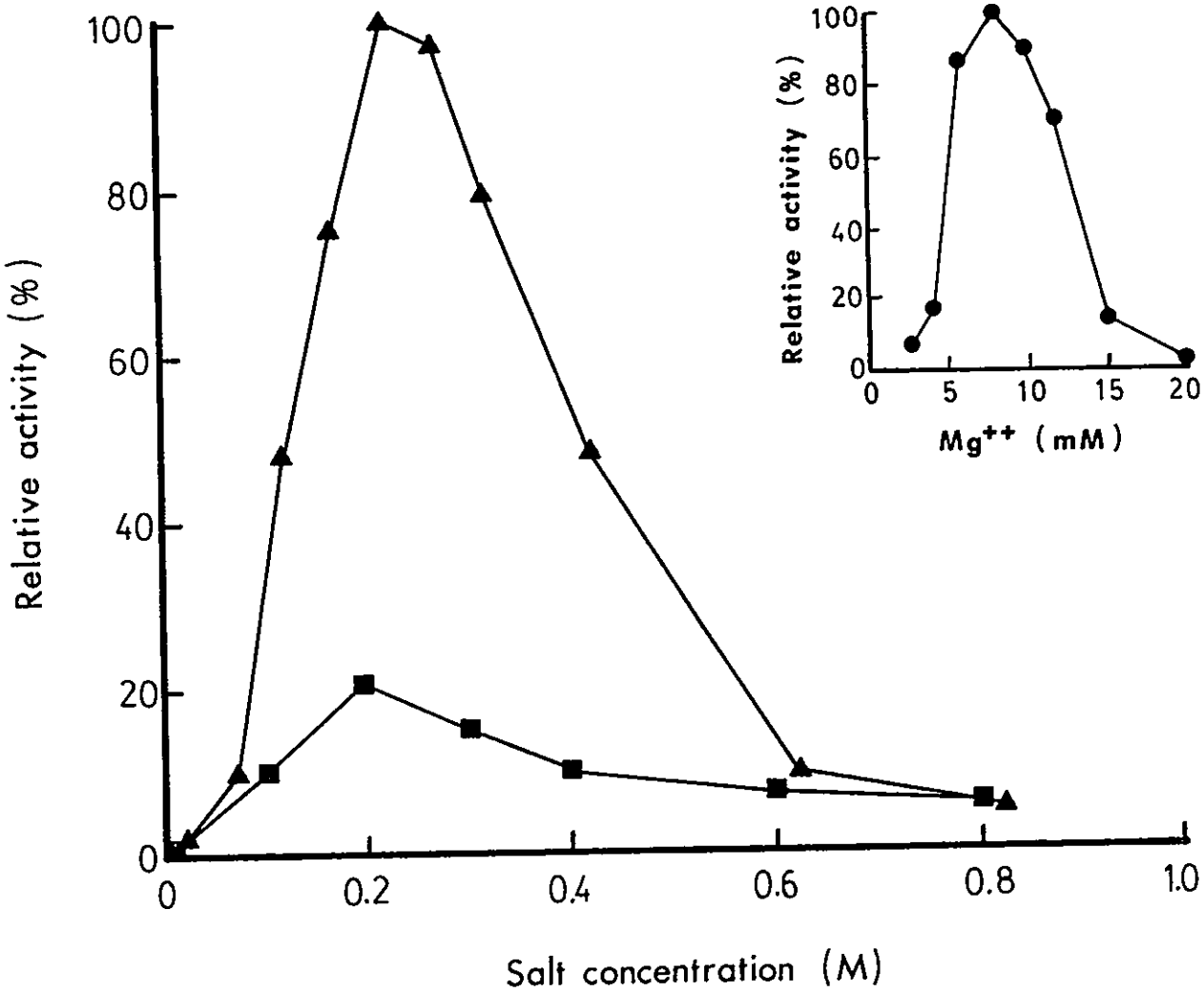
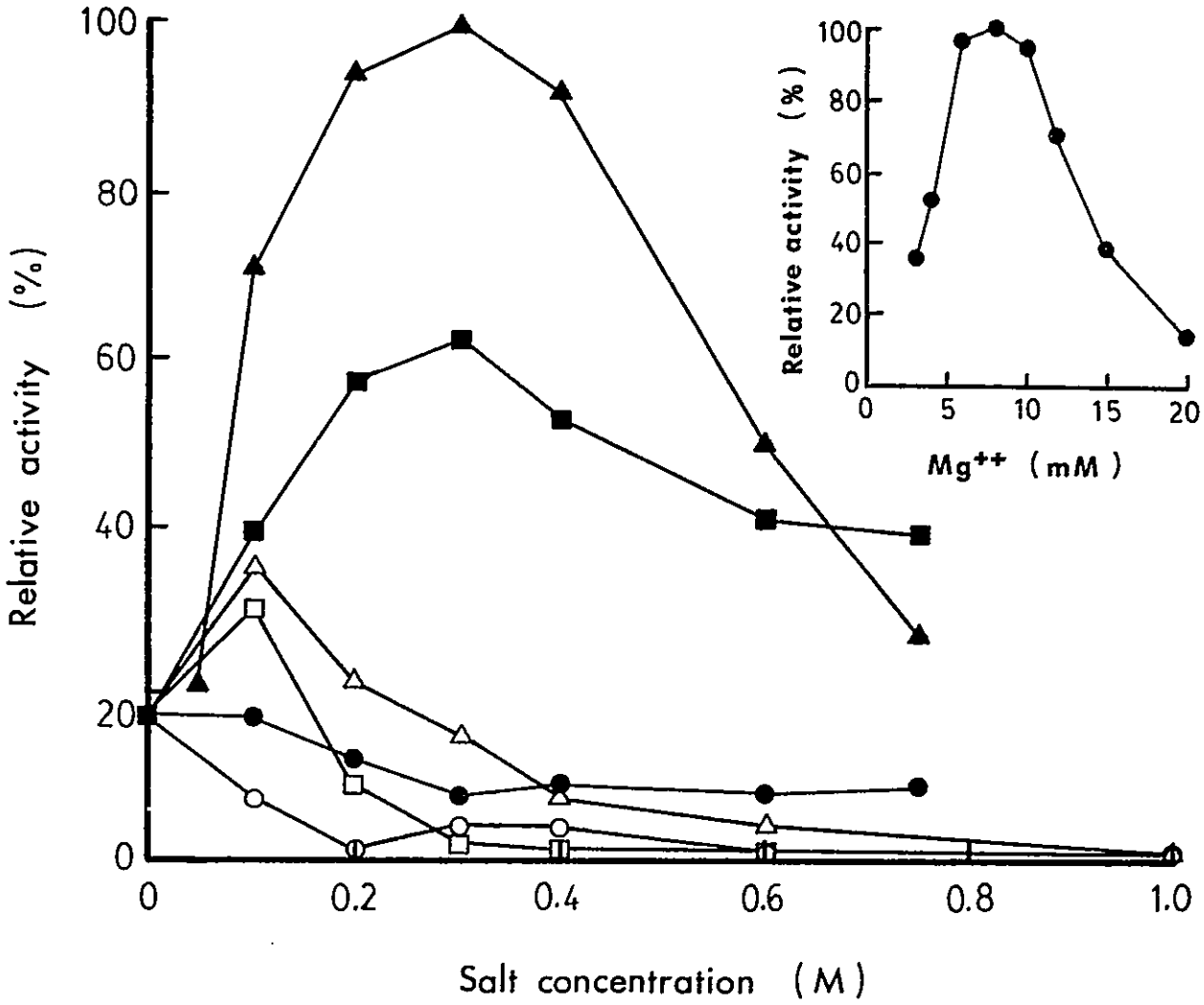

Relative activity (%)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)
Relative activity (%)
100
80
60
40
20
0
0
5
10
15
20
Mg++ (mM)

Figure 22. Ionic requirements of the *in vitro* endogenous translation system of *V. costicola* (S-30). Symbols: ammonium glutamate, ▲ ; potassium glutamate, ■ ; sodium glutamate, ● ; NH_4Cl, △ ; KCl, □ ; NaCl, ○ . Inserted Figure shows the effects of Mg^{++} on *in vitro* protein synthesis. Incubation time: 10 min.

Results are expressed as percentage of maximum activity, 100% being 13,500 DPM per 20 µl of S-30.


Relative activity (%)
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)
Relative activity (%)
100
80
60
40
20
0
0
5
10
15
20
Mg++ (mM)

system (Figure 23). The inhibition was strongest with any of the chloride salts [inhibitions by KCl and NH_4Cl (not shown) were similar to the inhibition observed with NaCl], followed by sodium glutamate, ammonium glutamate and potassium glutamate.

Added NaCl, KCl, NH_4Cl and ammonium glutamate were also inhibitory to protein synthesis directed by endogenous mRNAs (Figure 24). The inhibition exerted by KCl and NH_4Cl (not shown) was the same as that by NaCl. However, sodium glutamate and potassium glutamate were both stimulatory at concentrations lower than 0.4 M and most effective at 0.2 M (Figure 24).

These results confirmed the toxic nature of the Cl^- ions on in vitro protein synthesis by V. costicola, effects previously observed with poly(U) as artificial mRNA.

I considered the possibility that inhibition of the R17 system was due to changes in the secondary structure of the RNA with increasing salt concentrations. These changes could have resulted in a shielding of the normally available ribosome binding sites. To test this hypothesis, R17 RNA treated with formaldehyde was used. This treatment partially denatures and unfolds the RNA. Figure 25 (inset) shows that the R17 RNA was indeed partially unfolded. There was a 6% increase in O.D. after 15 min of incubation with formaldehyde, which is about 15% of the maximum increase caused by the complete denaturation of the secondary structure by RNAse. These results correlated well with those obtained by Lodish (1970) with f2 RNA. Lodish (1970) had also found that partially denatured viral RNA was more

Figure 23. Effects of added salts on R17 RNA-directed *in vitro* protein synthesis by S-30 of *V. costicola*. Symbols: ammonium glutamate, ▲ ; potassium glutamate, ■ ; sodium glutamate, ● ; NaCl, ○ . Incubation time: 10 min.

Results are expressed as percentage of control (without added salt). 100% activity represents incorporation of 45 pmole of [^{14}C]valine into hot TCA-insoluble material per 10 µl of S-30.

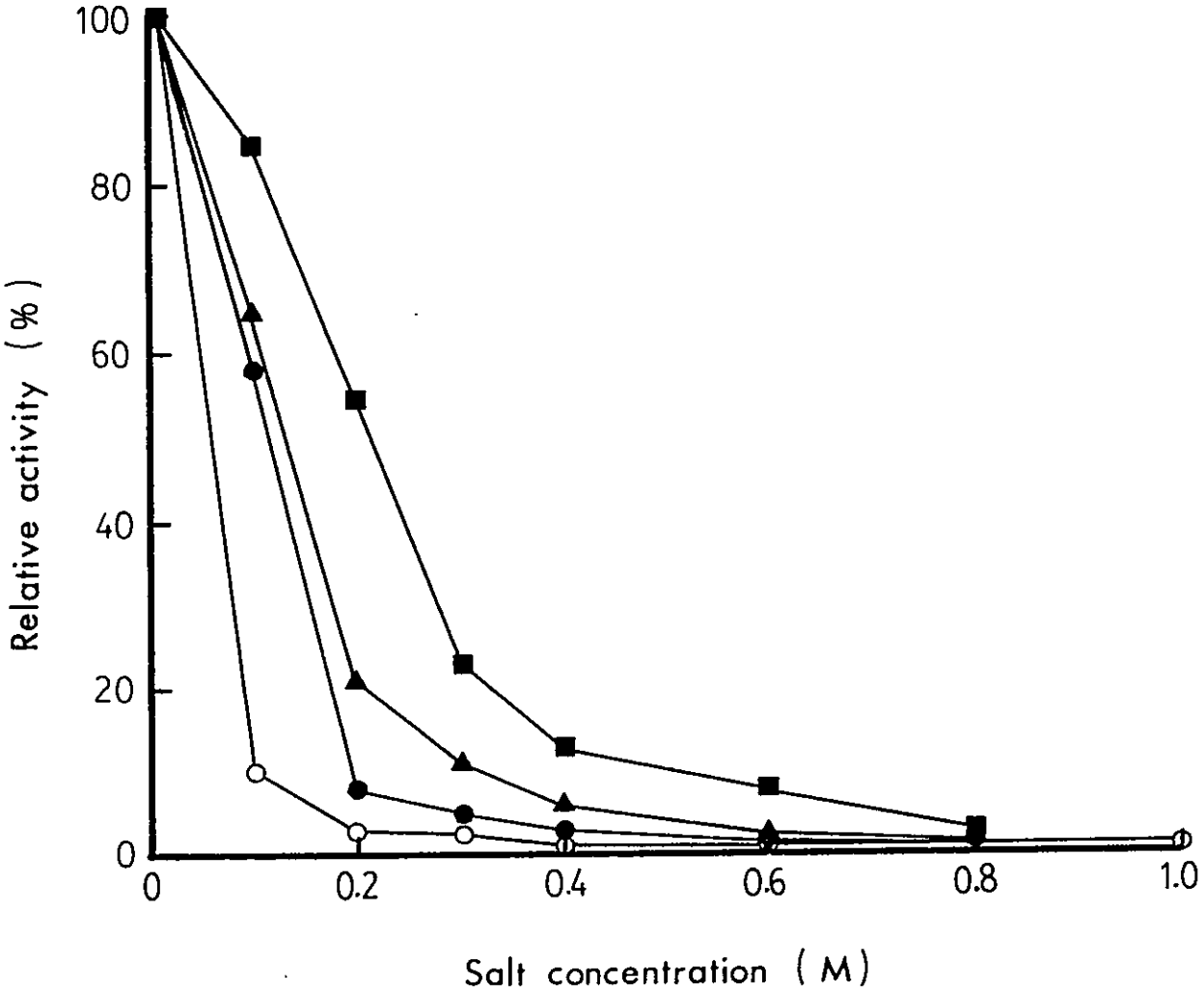

100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

Figure 24. Effects of added salts on the *in vitro* endogenous translation system of *V. costicola* (S-30). Symbols: ammonium glutamate, ▲ ; potassium glutamate, ■ ; sodium glutamate, ● ; NaCl, ○ . Incubation time: 10 min.

Results are expressed as percentage of control (without added salt), 100% being 13,128 DPM per 20 µl of S-30.

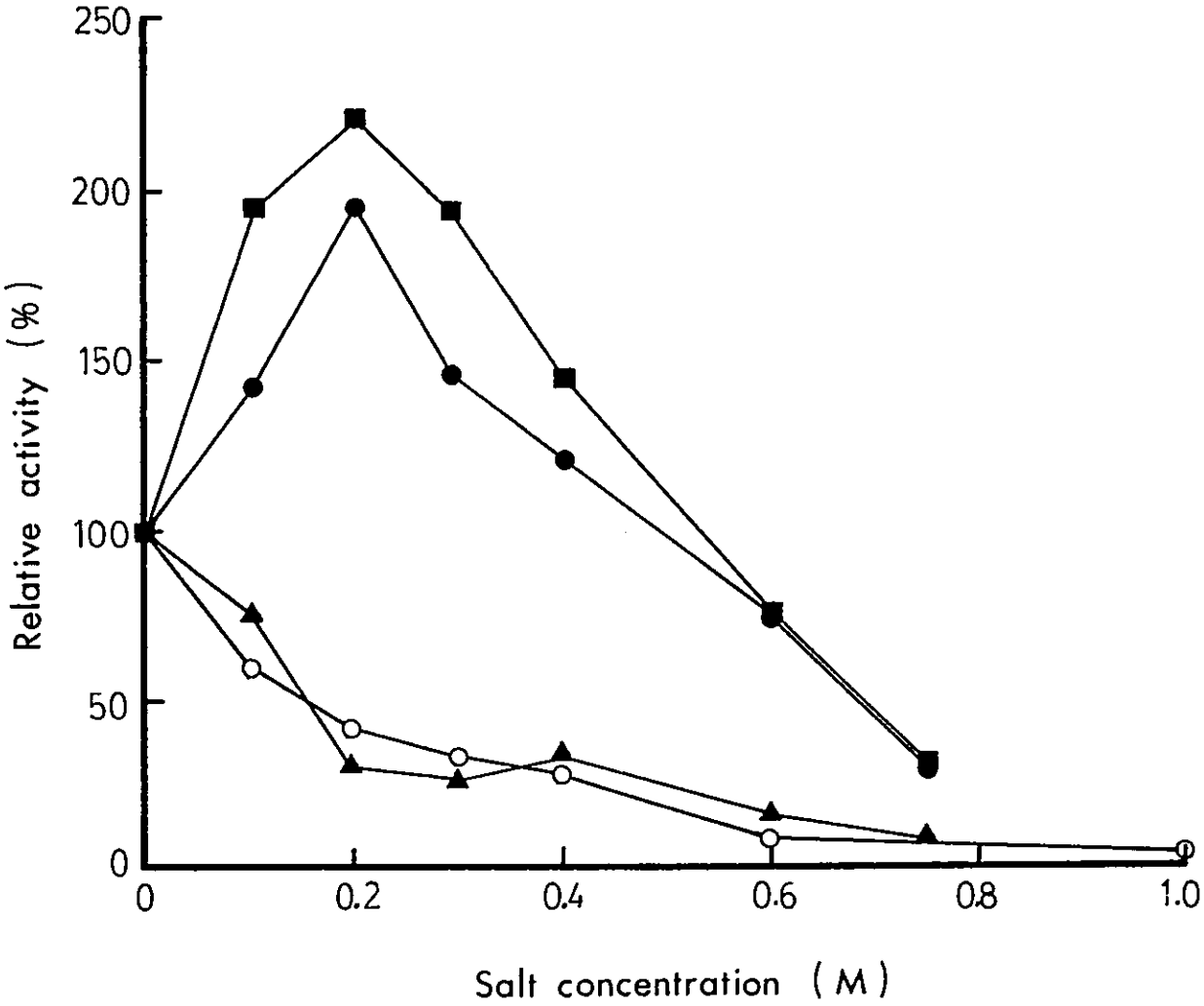
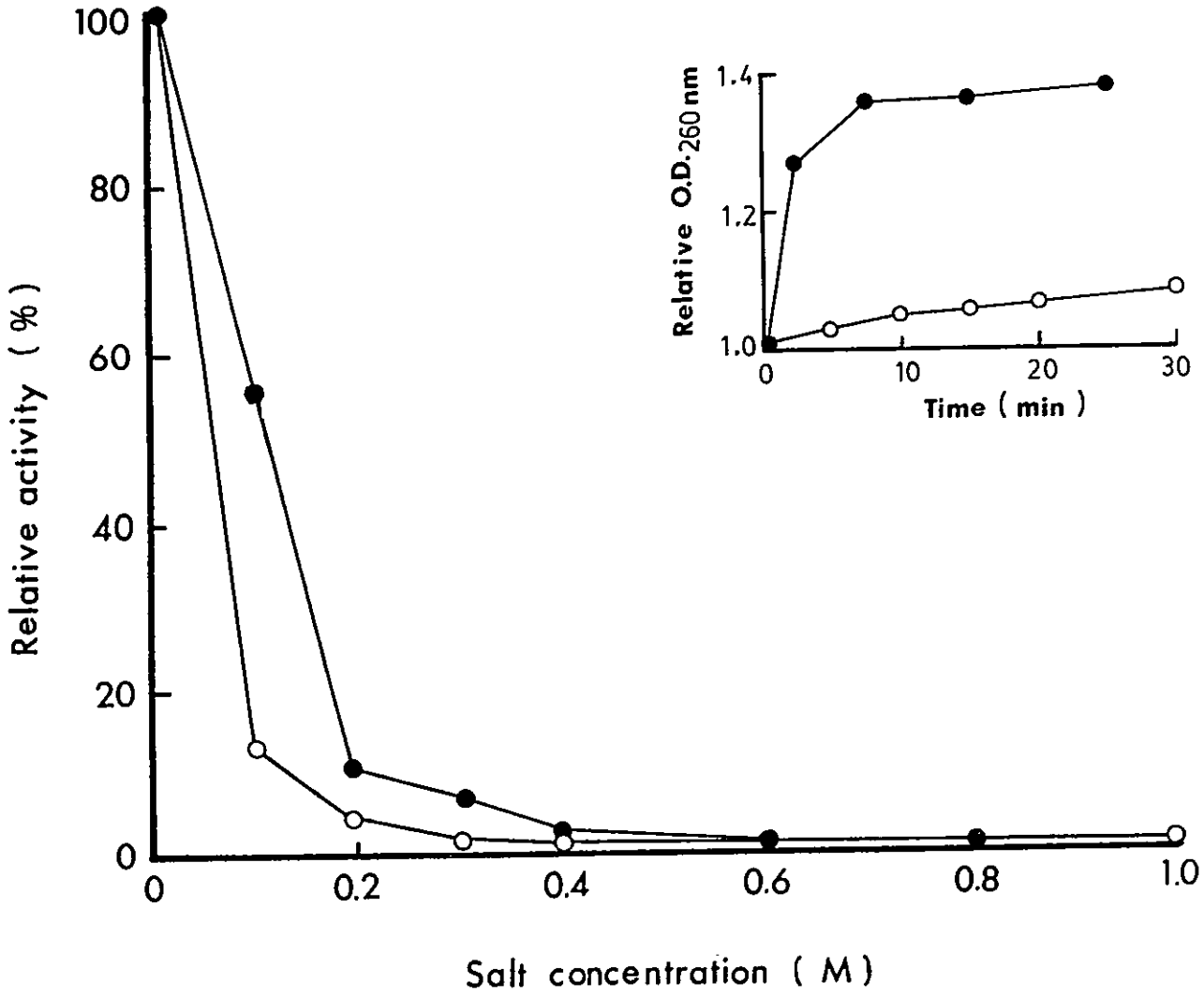

250
200
150
100
50
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

Figure 25. Effects of added salts on the *in vitro* protein synthesizing machinery of *V. costicola* (S-30) directed by formaldehyde-treated R17 RNA. Symbols; sodium glutamate, ● ; NaCl, ○ . Incubation time: 10 min.

Results are expressed as percentage of control (without added salt). 100% activity represents incorporation of 41 pmole of [^{14}C]valine into hot TCA-insoluble material per 10 µl of S-30.

Inserted figure: effects of RNAse (50 µg/ml) (●) and formaldehyde (1.0 M) (○) on the secondary structure of R17 RNA.


100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)
Relative O.D.260 nm
1.4
1.2
1.0
0
10
20
30
Time (min)

active in stimulating *in vitro* protein synthesis in *E. coli*. However, I did not observe such an increase in activity in the *V. costicola* system.

NaCl and sodium glutamate had similar inhibitory effects on protein synthesis directed by formaldehyde-treated R17 RNA as those obtained with untreated R17 RNA (Figure 25). Thus, salts probably did not act by changing RNA structure.

Betaine stimulated protein synthesis in both systems (Figure 26). Its presence also resulted in a higher total incorporation, in both systems, in the presence of NaCl (or KCl) (Figures 27 and 28). Therefore, it seems that betaine can afford some protection against the toxic action of Cl^- ions as was previously seen with the poly(U) system. Further addition of 0.2 M sodium or potassium glutamate reduced the stimulatory effect of betaine in the presence and absence of NaCl. This was especially notable in the R17-RNA directed system (Figures 27 and 28).

Formylmethionyl-tRNA synthetase

We investigated this enzyme as a possible site of action of Cl^- ions. However, the enzyme was only inhibited 20% by as much as 1.0 M NaCl (Figure 29).

Isolation of crude initiation factors of *V. costicola*

To further study the site of action of Cl^-, an attempt was made at isolating a preparation of crude initiation factors of

Figure 26. Effects of added betaine on R17 RNA-directed *in vitro* protein synthesis by S-30 of *V. costicola* and on the *in vitro* endogenous translation system of *V. costicola* (S-30). Symbols: ■ , R17 RNA-directed translation system; ● , endogenous translation system. Incubation time: 10 min.

Results are expressed as percentage of controls (without added betaine). 100% activity represents incorporation of 45 pmole of [^{14}C]valine per 10 µl of S-30 in the R17 RNA system and 13,128 DPM per 20 µl of S-30 in the endogenous system.

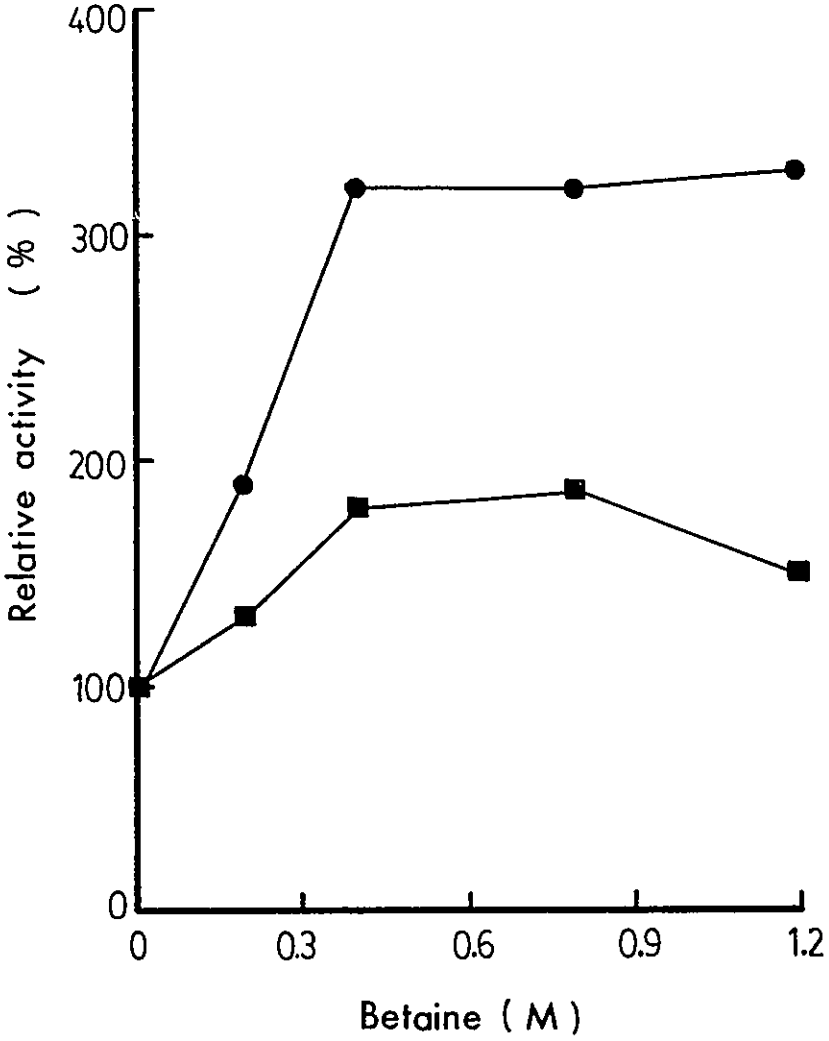

400
300
200
100
0
Relative activity (%)
0
0.3
0.6
0.9
1.2
Betaine (M)

Figure 27. Effects of betaine alone or with sodium (or potassium) glutamate on R17 RNA-directed *in vitro* protein synthesis by S-30 of *V. costicola* at increasing NaCl concentrations. Symbols: no addition, ○ ; 0.5 M betaine, ▲ ; 0.5 M betaine and 0.2 M potassium glutamate, ■ ; 0.5 M betaine and 0.2 M sodium glutamate, ● . Incubation time: 10 min.

Results are expressed as percentage of control (no added solute). 100% activity represents incorporation of 47 pmole of [^{14}C]valine per 10 µl of S-30.

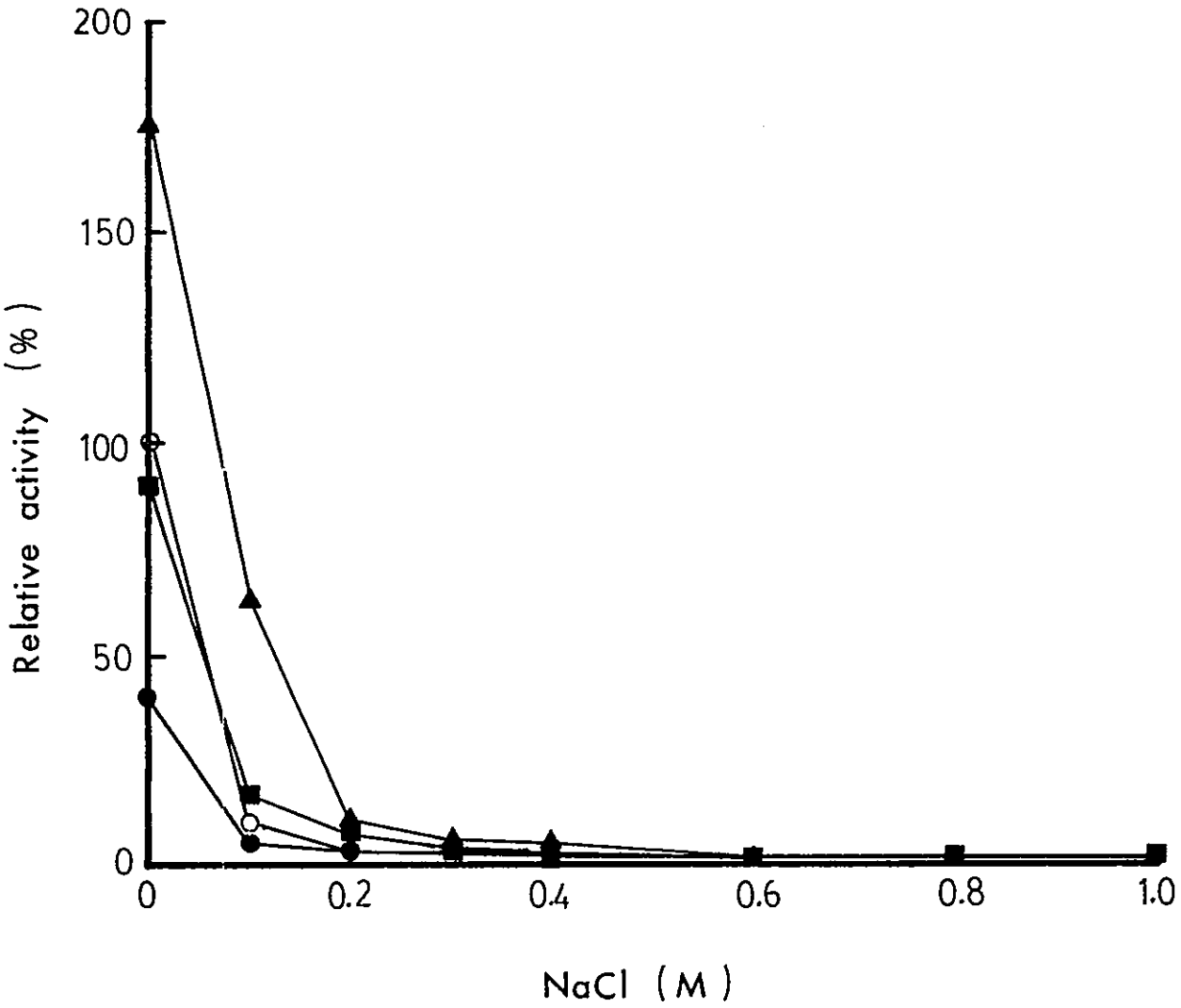

200
150
100
50
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)

Figure 28. Effects of betaine alone or with sodium (or potassium) glutamate on the *in vitro* endogenous translation system of *V. costicola* at increasing NaCl concentrations. Symbols: no addition, ○ ; 0.5 M betaine, ▲ ; 0.5 M betaine and 0.2 M potassium glutamate, ■ ; 0.5 M betaine and 0.2 M sodium glutamate, ● . Incubation time: 10 min.

Results are expressed as percentage of control (no added solute). 100% activity being 14,200 DPM per 20 µl of S-30.

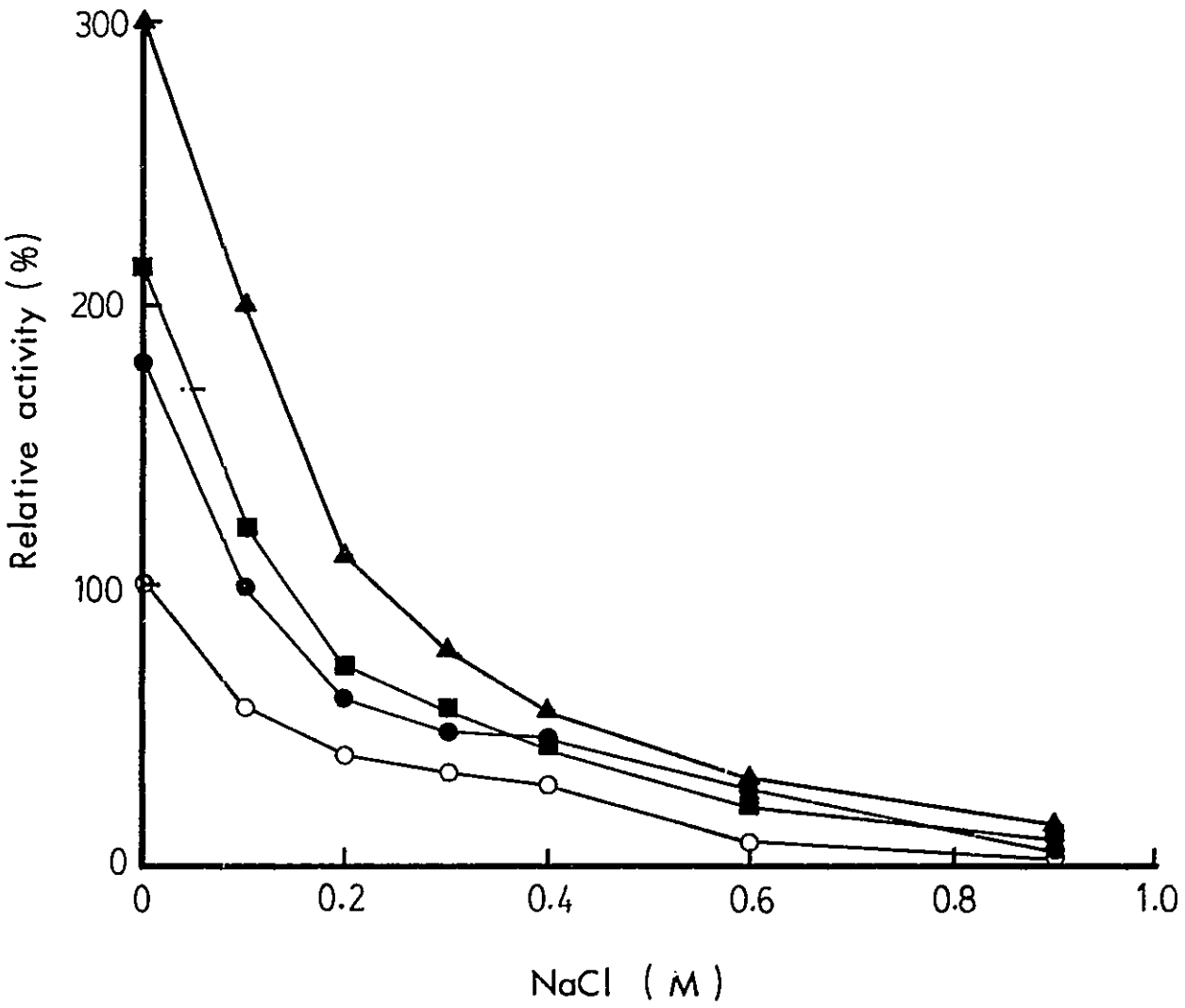
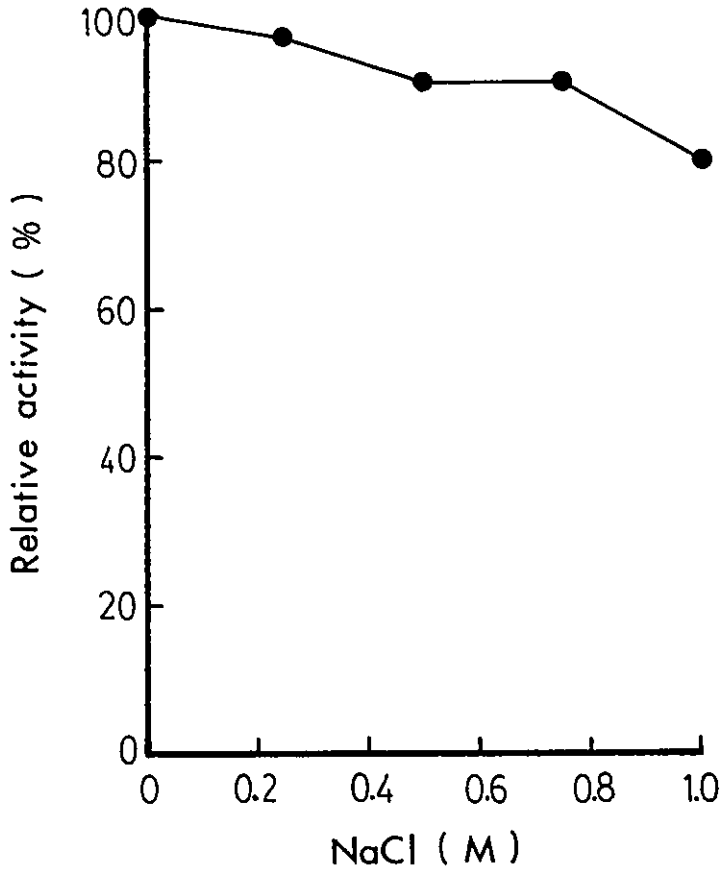

300
200
100
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)

Figure 29. Effect of NaCl concentrations on the enzyme formylmethionyl-tRNA synthetase of S-150 of V. costicola. Incubation time: 20 min.

Results are expressed as percentage of control (without NaCl). 100% activity represents 53 pmole of [^{14}C]methionine.


100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
NaCl (M)

V. costicola. The amount of NH_4Cl (1.0 M) normally used in the isolation of initiation factors from the ribosomes of E. coli (Hershey et al., 1977) did not yield a fraction on which R17 RNA-directed translation by V. costicola depended upon. Therefore, V. costicola ribosomes were washed at different NH_4Cl concentrations. To assess whether or not the initiation factors had been released, the ability of these washed-ribosomes to carry out in vitro protein synthesis was determined. Presumably, only the ribosomes that had not lost their initiation factors would carry out protein synthesis. The activity of ribosomes washed with 1.0 and 2.0 M NH_4Cl did not show decreased activity in comparison with low salt-washed ribosomes. However, ribosomes washed with solutions of 3.0, 3.5 and 4.0 M NH_4Cl had relative activity (compared to unwashed ribosomes) of 15%, 6% and 2%, respectively. Therefore, a concentration of at least 3.0 M was needed to significantly reduce the ability of ribosomes to participate in protein synthesis.

Ribosomes treated with 3.0 and 3.5 M NH_4Cl were only active if proteins obtained during this treatment (see materials and methods, p.43) were added again (Table 6). Presumably, these included the initiation factors. A much lower incorporation was observed when mixing 4.0 M NH_4Cl-washed ribosomes with the crude initiation factors isolated from them (Table 6). Hybridization studies between 3.5 and 4.0 M NH_4Cl-washed ribosomes and their respective initiation factors showed that this lack of

Table 6. R17 RNA-directed incorporation of [^{14}C]valine (DPM) at different concentrations of added crude initiation factors isolated from NH_4Cl-washed ribosomes of V. costicola.

initiation factors[1] (µg)	ribosomes (4 A_{260} units)		
	3.0 M NH_4Cl	3.5 M NH_4Cl	4.0 M NH_4Cl
0	4238	1746	721
13	8210	5855	953
26	11210	9775	1004
39	12097	10531	1253

[1] Crude initiation factors isolated from the ribosomes washed at each NH_4Cl concentration.

incorporation was mainly due to inactive 4.0 M NH_4Cl-washed ribosomes rather than inactive initiation factors (Table 7).

Binding of [^{14}C]fmet-tRNA or [^{3}H]R17 RNA by initiation factors

The retention of [^{14}C]fmet-tRNA or [^{3}H]R17 RNA on nitrocellulose filters was specifically dependent upon the addition of crude initiation factors (Table 8). Either retention was not due to unspecific binding of the radiolabeled compounds to proteins, since the sole addition of S-150 from V. costicola, which is rich in proteins, barely increased the amount of either radioactive material retained on the filters (in comparison with no addition). Presumably, the retention of [^{14}C]fmet-tRNA was caused by the formation of a complex between the tRNA and initiation factor-2 (IF_2) (Petersen et al., 1979; Van der Hofstad et al., 1979; Van der Laken et al., 1980) With IF_2 of E. coli, this complex is not stable and must be stabilized by fixing it with glutaraldehyde (Van der Hofstad et al., 1979; Van der Laken et al., 1980). However, with the initiation factors of V. costicola, fixing with glutaraldehyde (3.0%) did not increase the amount of [^{14}C]fmet-tRNA retained on the filters. The retention of [^{3}H]R17 RNA was presumably due to the formation of a complex between itself and initiation factor-3 (IF_3), as is the case with IF_3 of E. coli (Wahba et al., 1969; Sabol et al., 1970; Jay et al., 1974; Jonhson and Szekely, 1977, 1979).

Table 7. Hybridization studies between 3.5 M and 4.0 M NH_4Cl-washed ribosomes and the crude initiation factors isolated from them.

initiation factors (39 µg)	ribosomes (4 A_{260} units)	
	3.5 M NH_4Cl	4.0 M NH_4Cl
3.5 M NH_4Cl	10985	633
4.0 M NH_4Cl	7564	1098

Table 8. Retention of [^{14}C]fmet-tRNA and [^{3}H]R17 RNA on nitrocellulose filters.

Components added[a]	[^{14}C]fmet-tRNA (DPM)	[^{3}H]R17 RNA (DPM)
no addition	100	152
ribosomes (4 A_{260} units)	124	232
initiation factors (40 µg)	4828	6770
S-150 (350 µg proteins)	120	440
ribosomes (4 A_{260} units) + initiation factors (40 µg)	743	2736

[a] The standard reaction mixture contained 225 mM ammonium glutamate, 8 mM Mg^{++}, 82 mM Tris-acetate (pH 7.6), 7.5 mM reduced glutathione and 11 pmole [^{14}C]fmet-tRNA (550 DPM/pmole) or 100 µg [^{3}H]R17 RNA (900 DPM/µg).

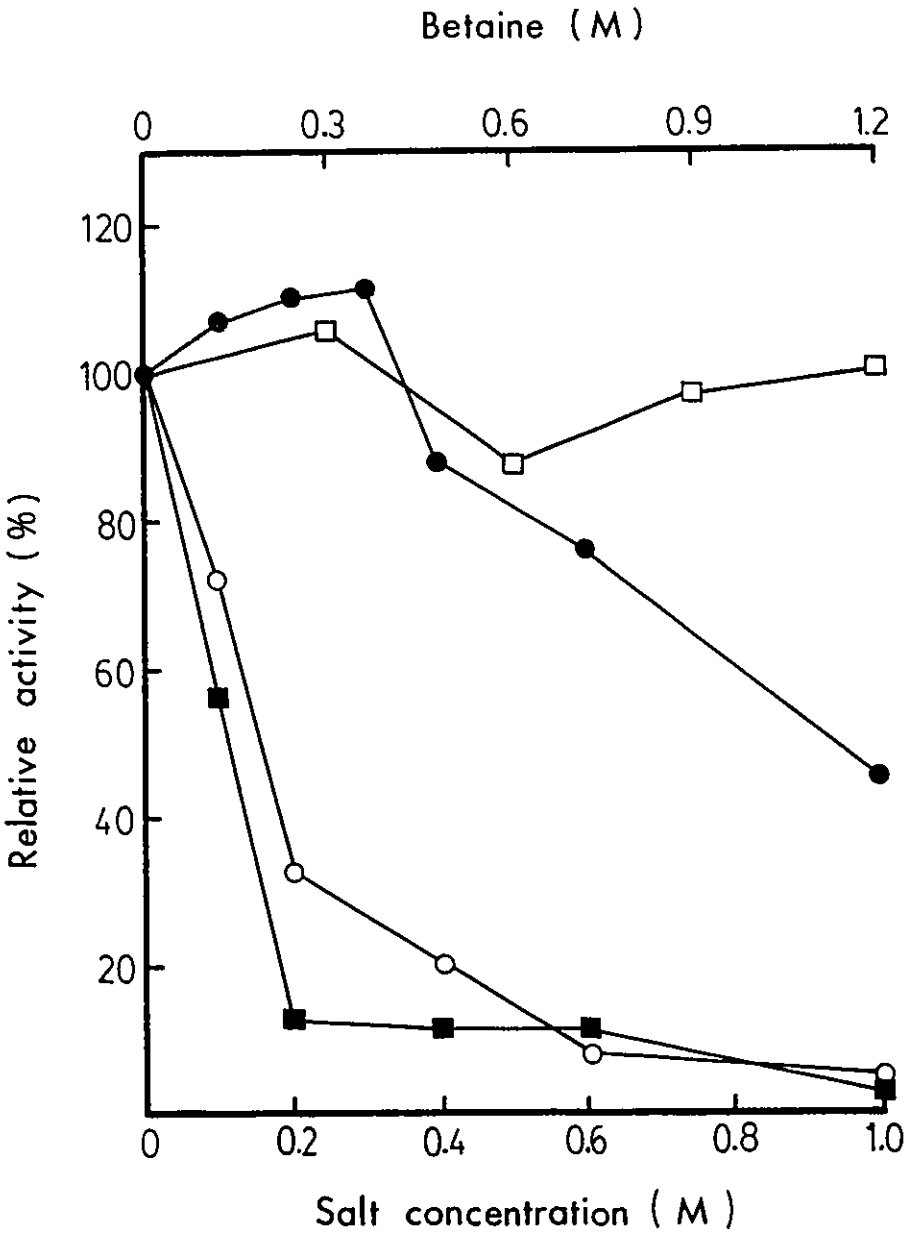
In the presence of initiation factors, ribosomes interfered with the retention of both radioactive molecules (Table 8). In E. coli, IF_3, which is required for mRNA binding, has a greater affinity for 30S ribosomal subunits than MS2 RNA (Vermeer et al., 1973). There is no data on the association constant of IF_2 with fmet-tRNA, but it is known that the association constant of IF_2 with 30S ribosomal subunits, in the presence of IF_3 and IF_1, is as high as that of IF_3 (Weiel and Hershey, 1982). This may also be true for V. costicola, so that the amount of initiation factors, available for the binding of fmet-tRNA (IF_2) and R17 RNA (IF_3) in the absence of ribosomes, could decrease in their presence due to the higher affinity of the initiation factors for the ribosomes.

These results (Table 8) show that the retention of [^{3}H]R17 RNA and [^{14}C]fmet-tRNA on nitrocellulose filters is a suitable method to study the effects of salts on the initiation factors of V. costicola. Such results could at least give some indication of the ability of the initiation factors to recognize their respective "ligand", namely fmet-tRNA for IF_2 and mRNA for IF_3, under different ionic conditions.

Cl^- ions had a strong inhibitory action on the binding of [^{14}C]fmet-tRNA by initiation factors: 0.2 M NaCl caused a 90% inhibition while 0.6 M sodium glutamate caused only a 25% inhibition (Figure 30). Betaine alone had no effect on binding over the whole concentration range studied, while its presence did not protect against the action of Cl^-. During the

Figure 30. Effects of added solutes on the retention of [^{14}C]fmet-tRNA filters, by crude initiation factors of V. costicola. Symbols: NaCl, ○ ; sodium glutamate, ● ; betaine, □ ; 0.6 M betaine at increasing NaCl concentrations, ■ . Incubation time: 15 min.

Results are expressed as percentage of controls (no added solute). 100% activity represents the retention of 8 pmole of [^{14}C]fmet-tRNA on nitrocellulose filters.


Betaine (M)
0
0.3
0.6
0.9
1.2
120
100
80
60
40
20
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

initiation of protein synthesis, the initiator tRNA is recognized by, and bound to, IF_2 (Petersen et al., 1979; Van der Hofstad et al., 1979; Van der Laken et al., 1980) and, therefore, it is likely that Cl^- may interfere with the function of this factor.

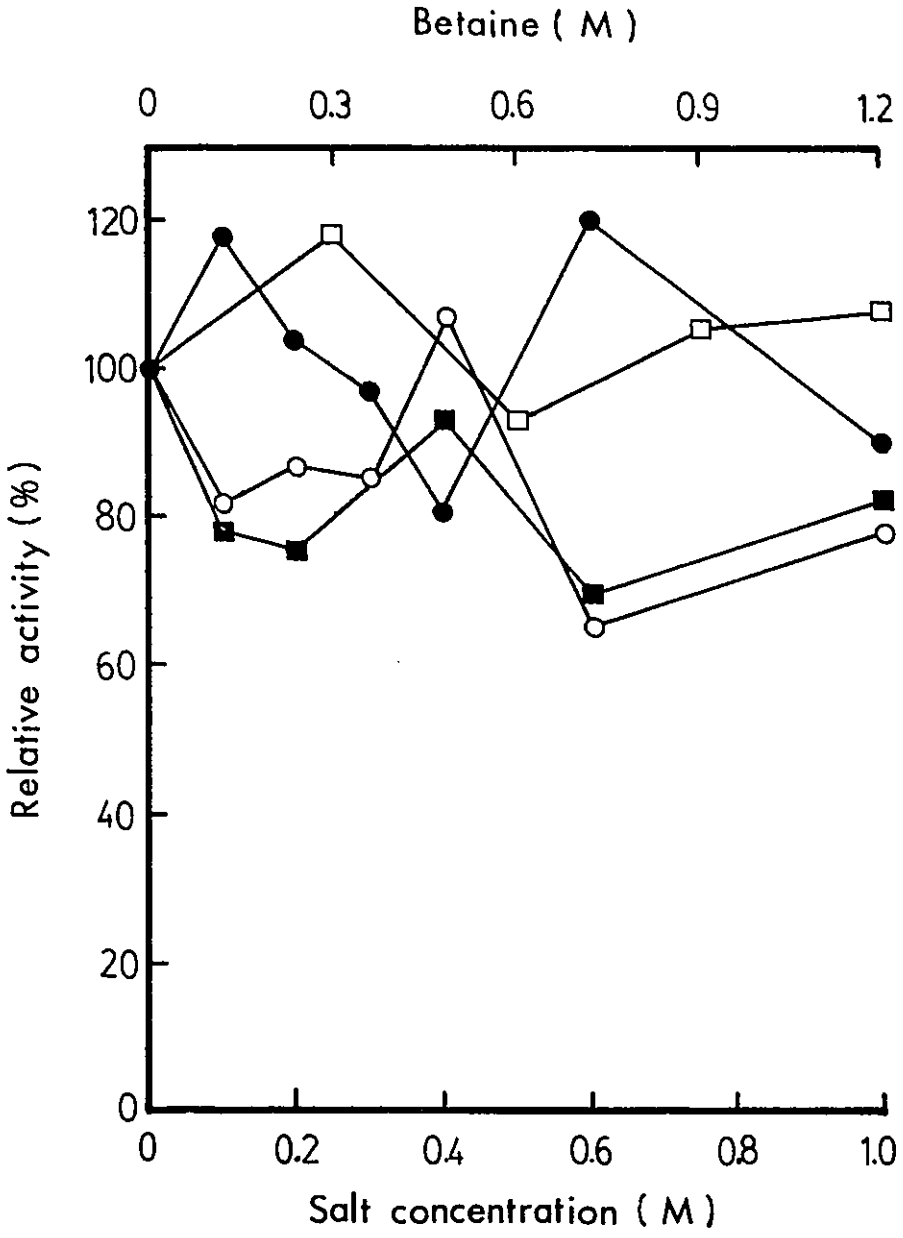
Contrary to the results obtained with [^{14}C]fmet-tRNA, the ones obtained on the retention of [^{3}H]R17 RNA were substantially scattered (Figure 31). Nevertheless, it was possible to establish that [^{3}H]R17 RNA, unlike [^{14}C]fmet-tRNA, was still retained on nitrocellulose filters in substantial amounts at higher NaCl concentrations. Similarly, sodium glutamate, betaine alone, and betaine in the presence of increasing NaCl, appeared to have no strong effects on the presumed binding of IF_3 to [^{3}H]R17 RNA (Figure 31).

OXIDATIVE PATHWAY OF CHOLINE TO BETAINE

As stated in the introduction, betaine plays an important role in osmoregulation. It also has a protective role against the inhibitory effects of high salt concentrations in several metabolic systems, including, as I have shown here, in vitro protein synthesis of V. costicola. In addition, betaine stimulated in vitro protein synthesis. For these reasons, we decided to investigate the possible synthesis of betaine, from choline, in V. costicola.

Figure 31. Effects of added solutes on the retention of [^{3}H]R17 RNA, on nitrocellulose filters, by crude initiation factors of V. costicola. Symbols: NaCl, ○ ; sodium glutamate, ● ; betaine, □ ; 0.6 M betaine at increasing NaCl concentrations, ■ . Incubation time: 15 min.

Results are expressed as percentage of controls (no added solute). 100% activity represents the retention of 7.8 µg of R17 RNA on nitrocellulose filters. The results were poorly reproducible: standard deviations (calculated from 4 replicates) varied between 9 and 22 % relative activity.


Betaine (M)
0
0.3
0.6
0.9
1.2
120
100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Salt concentration (M)

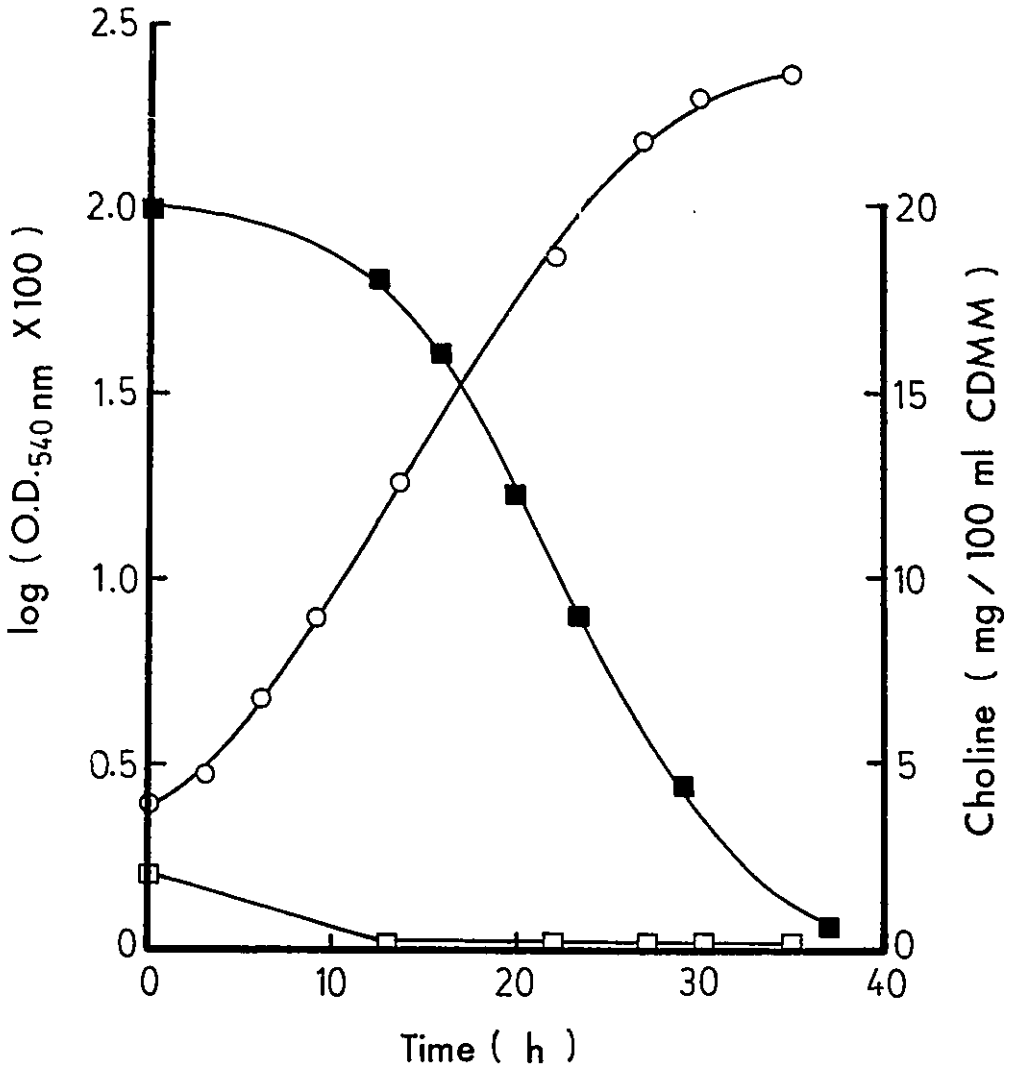
Uptake of choline

It was first established that V. costicola could indeed use choline as a precursor to betaine synthesis. Figure 32 shows that choline chloride was removed from the medium during the incubation of V. costicola in 1.0 M NaCl CDMM. Extraction of the cell pellets consistently resulted in the recovery of at least 80% of the radioactivity that had disappeared from the medium. Thus, V. costicola can transport exogenously supplied choline.

In all cases, analysis of the radioactive material recovered from the cell pellet revealed that at least 90% of it was betaine. There was never any betaine aldehyde or choline detected. We considered the possibility that choline could donate its methyl groups to a molecule, such as glycine, to yield betaine. In such a case, there should have been no radioactive betaine detected in cells grown in the presence of choline labeled in the C-2 position, $[(CH_3)_3\text{-}N\text{-}^{14}CH_2\text{-}CH_2OH]Cl$. However, the results were the same regardless of the position of the label in the choline molecule, thus indicating that choline was actually transformed into betaine as opposed to taking part in the reaction as a methyl donor.

Although the amount of choline chloride present in the medium had no effect on the growth of V. costicola (growth curves were the same regardless of the choline chloride concentration), it dictated the time it took for it to be depleted (Figure 32). At a concentration of 0.002%, it was

Figure 32. Growth curve and choline uptake of V. costicola grown in 1.0 M NaCl CDMM. Uptake of choline was studied in media containing 0.002% (□) and 0.02% (■) of choline chloride. The growth curves of V. costicola at the different choline chloride concentrations were identical (○).


2.5
2.0
1.5
1.0
0.5
0
log (O.D.540 nm X100)
20
15
10
5
0
Choline (mg / 100 ml CDMM)
0
10
20
30
40
Time (h)

depleted after only 13 hours of incubation, much before late exponential phase. However, in the presence of 0.02% choline chloride, more than half still remained after 24 hours of incubation. These results had important implications in the event that the enzymes involved in the oxidation of choline were only expressed in the presence of choline, that is, they could be hard to detect in cells grown, for 24 hours, in the presence of 0.002% choline chloride.

Localization of the enzymes

Enzymatic activity of choline dehydrogenase, which catalyses the oxidation of choline to betaine aldehyde, was only detected in membrane fractions (Table 9). This enzyme was also capable of oxidizing betaine aldehyde to betaine. It was consistently observed that while most of the reacted choline (70%) was recovered in the aldehyde form, the remaining 30% was recovered as betaine. Both activities were O_2-dependent, since they were both largely inhibited (85%) when incubated under N_2 (Table 9).

Betaine aldehyde dehydrogenase, which catalyses the NAD-dependent oxidation of betaine aldehyde to betaine, was found to be a cytoplasmic enzyme. It was easily detected in the S-150 extract (Table 9). No enzyme activity was observed with solubilized membranes providing further proof that the oxidation of betaine aldehyde to betaine, observed with membrane

Table 9. Localization of the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase of *V. costicola*[a].

cellular fraction	Enzyme activities (nmole/min/mg protein)	
	choline dehydrogenase	betaine aldehyde dehydrogenase
S-150	0.1	114
washed membrane fraction	4.7	N.D[b]
solubilized membranes	4.0	0
washed membranes under N_2	0.7	N.D.

[a] The cells were grown in 1.0 M NaCl CDMM containing 0.02% choline chloride.

[b] N.D., not determined.

fractions, was not due to the presence of contaminating NAD-dependent betaine aldehyde dehydrogenase.

Enzymes regulation

Enzyme activity of choline dehydrogenase (Table 10) was very hard to detect in cells grown, for 24 hours, in 1.0 M NaCl CDMM with 0.002% choline chloride. However, its activity was easily measured at a choline chloride concentration of 0.02%. A further increase in choline concentration, to 0.05%, did not significantly increase its specific activity. The specific activity of betaine aldehyde dehydrogenase (Table 10) also responded to increasing external choline concentration, but unlike choline dehydrogenase, its activity was easily measurable in cells grown at the lowest choline concentration.

It is not surprising that such low levels of choline dehydrogenase were measured in cells grown in the presence of only 0.002% choline, since after 24 hours of incubation choline had been depleted for a long time (Figure 32). In fact, enzyme activities in these cells were higher after only 13 hours of incubation. Furthermore, raising the choline concentration to 0.02% after 13 hours of incubation, increased enzyme activities to levels measured in cells grown, from the start, in the presence of 0.02% choline. These results suggest that choline dehydrogenase and, to a lesser extent, betaine aldehyde dehydrogenase are inducible enzymes.

Table 10. Effect of external choline concentrations on the activities of choline dehydrogenase and betaine aldehyde dehydrogenase of V. costicola.

choline chloride in CDMM (%)	incubation time (h)	Enzyme activities (nmole/min/mg protein)	
		choline dehydrogenase[a]	betaine aldehyde dehydrogenase
0.002	13	2.1	72
0.002	24	0.6	45
0.02	24	4.1	111
0.02[b]	24	4.6	118
0.05	24	4.7	138

[a] Assays were done with washed membrane fractions.

[b] The choline concentration was 0.002% for the first 13 hours of incubation and then raised to 0.02% for the remaining 11 hours of incubation.

The expression of these enzymes also appeared to be osmotically regulated (Table 11). Their activities increased in cells grown at increasing external NaCl concentrations, up to a concentration of 2.0 M. The lack of a further increase in cells grown in 3.0 M NaCl CDMM was not due to a depletion of available choline, as shown in uptake experiments with cells grown in media containing 2.0 and 3.0 M NaCl (Figure 33). With an initial choline concentration of 0.05%, around 0.02% choline remained in each media in the late stages of exponential phase (36 and 120 hours for for cells grown in 2.0 and 3.0 M NaCl, respectively).

Intracellular betaine concentrations

Direct intracellular betaine determinations showed that betaine concentration increased with increasing external choline and NaCl concentrations (Table 12). At each external NaCl concentration, betaine reached near maximum levels at a choline concentration of 0.02%, the same concentration at which near maximal enzymatic activities were observed (Table 10). This response to increasing external choline concentration provided further evidence that choline was the precursor to betaine synthesis. It is also interesting that, although the external choline concentration had a significant effect on the intracellular betaine concentration, only the growth of V. costicola in 3.0 M NaCl CDMM was stimulated by an increase in choline from 0.002% to 0.02% or higher (Figure 34). Growth

Table 11. Effect of the external NaCl concentration on the activities of choline dehydrogenase and betaine aldehyde dehydrogenase of V. costicola.

NaCl in CDMM[b] (M)	Enzyme activities (nmole/min/mg protein)	
	choline dehydrogenase[a]	betaine aldehyde dehydrogenase
0.5	1.4	56
1.0	4.7	138
2.0	6.5	160
3.0	6.9	154

[a] Assays were done with washed membrane fractions.

[b] Each medium contained 0.05% of choline chloride.

Figure 33. Growth curve and choline uptake of V. costicola grown in (A) 2.0 M NaCl CDMM and (B) 3.0 M NaCl CDMM. Symbols: ○ , O.D.; □ , choline, concentration in the medium.

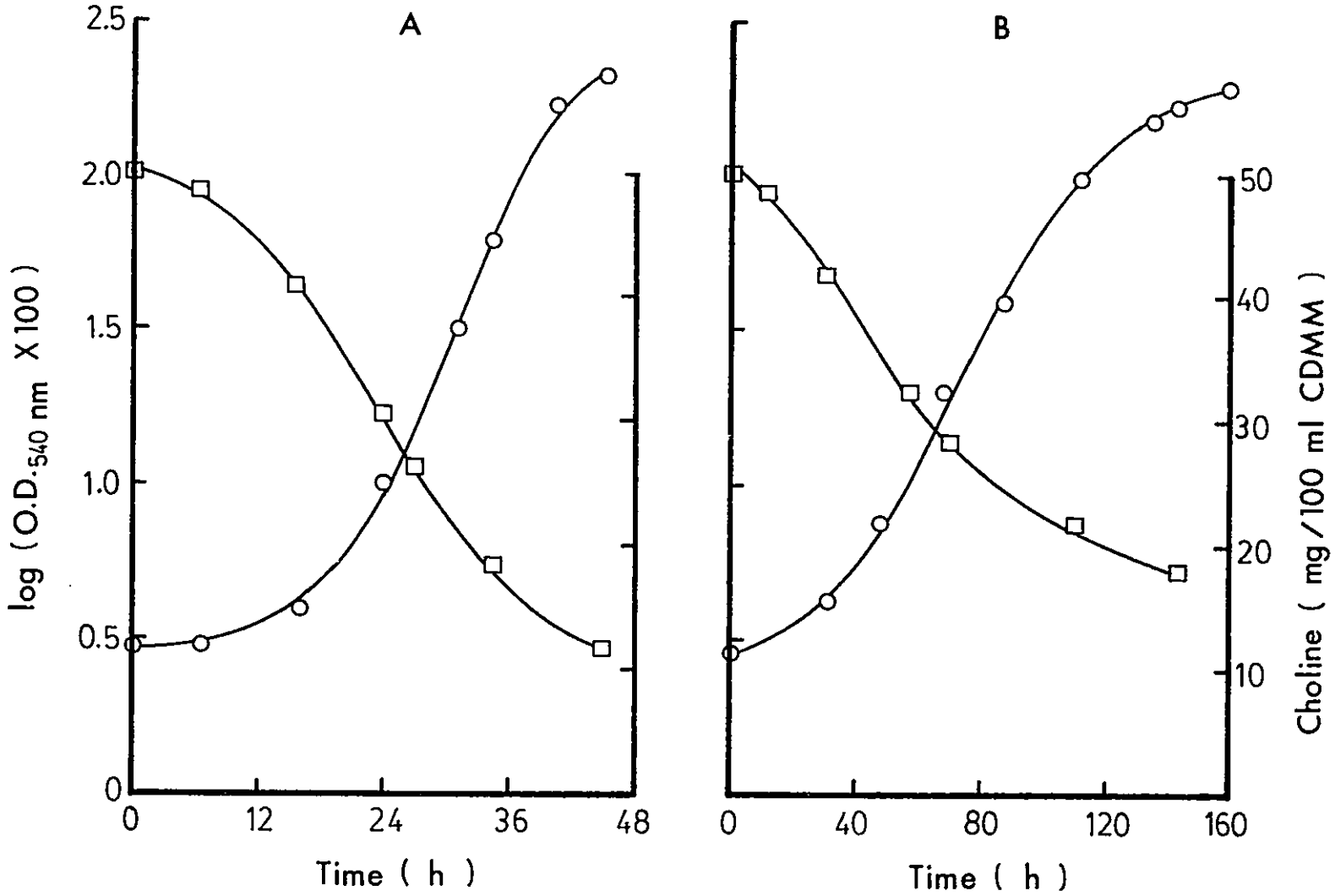

A
B
log (O.D.540 nm X100)
Choline (mg/100 ml CDMM)
Time (h)
Time (h)
0
0.5
1.0
1.5
2.0
2.5
0
12
24
36
48
0
40
80
120
160
10
20
30
40
50

Table 12. Intracellular betaine concentration[a] (M) of V. costicola grown at different choline and NaCl concentrations.

	choline chloride in medium (%)	NaCl concentration of medium			
		0.5 M	1.0 M	2.0 M	3.0 M
CDMM[b]	0.002	0.25	0.19	0.21	0.40
	0.020	0.25	0.45	0.90	1.28
	0.050	0.27	0.54	1.00	N.D.[e]
	0.200	0.25	0.65	0.94	1.20
PPT[c,d]	-----	0.15	0.27	0.66	1.1

[a] Betaine concentrations were calculated using the value of 1.8 µl/mg dry wt as the intracellular space of V. costicola (Hamaide, 1984).

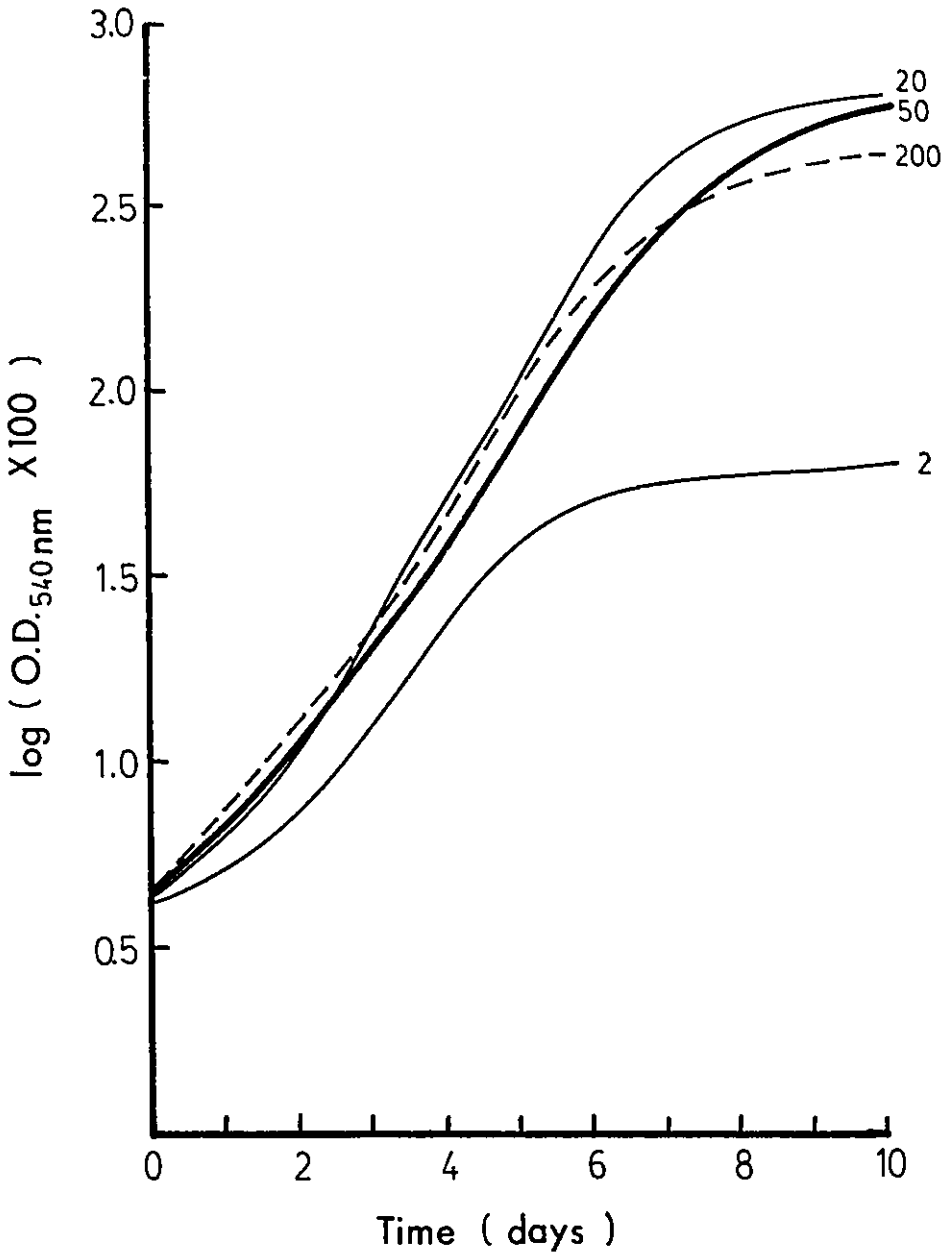
[b] CDMM, chemically defined minimal medium.

[c] PPT, complex medium containing, in addition to NaCl, 1% proteose peptone #3 and 1% tryptone. The choline concentration of this medium is unknown.

[d] The results were obtained from M. Klein and D.J. Kushner (unpublished data).

[e] N.D., not determined.

Figure 34. Growth curves of V. costicola in 3.0 M NaCl CDMM of different choline chloride concentrations (mg/100 ml of medium).


3.0
2.5
2.0
1.5
1.0
0.5
log (O.D.540nm X100)
0
2
4
6
8
10
Time (days)
20
50
200
2

curves of V. costicola 1.0 NaCl CDMM were the same regardless of the amount of choline present in the medium. The same was true for V. costicola grown in 0.5 and 2.0 M NaCl CDMM.

V. costicola grown in the complex medium PPT also accumulated betaine in response to increasing NaCl concentrations in the medium (Table 12). However, this accumulation is not necessarily the result of choline oxidation. V. costicola could obtain betaine directly from the medium, as this rich medium may well contain some. Betaine accumulation in these cells depends on protein synthesis since, in an upshock experiment where the NaCl concentration was suddenly raised from 0.5 M to 1.0 M NaCl, chloramphenicol inhibited the accumulation of betaine by more than 90% (Table 13).

Effects of salts on the activity of choline dehydrogenase and betaine aldehyde dehydrogenase

The enzyme choline dehydrogenase was inhibited by all chloride salts and by ammonium glutamate (Figure 35). However, potassium glutamate and sodium glutamate, even at concentrations of 1.0 M, had no inhibitory effects (Figure 35). Thus, the inhibition by NaCl and KCl was due to Cl^- rather than to Na^+ or K^+. NH_4^+ also had a toxic action on this enzyme since both its chloride and glutamate salts were inhibitory.

Different results were obtained for the enzyme betaine aldehyde dehydrogenase. There was no strong inhibition at KCl, NaCl or NH_4Cl concentrations lower than 1.0 M (Figure 36). Even

Table 13. Effect of chloramphenicol on the accumulation of betaine in V. costicola following an osmotic upshock (M. Klein and D.J. Kushner, unpublished data).[a]

Incubation medium	Intracellular betaine concentration[b] (M)	
	0 h[c]	4 h[c]
0.5 M NaCl PPT	0.02	0.04
0.5 M NaCl PPT + chloramphenicol	0.03	0.04
1.0 M NaCl PPT	0.07	0.62
1.0 M NaCl PPT + chloramphenicol	0.07	0.11

[a] Cells grown to late exponential phase at 30°C in 0.5 M NaCl PPT were diluted with an equal volume of either 0.5 M NaCl PPT (no change in NaCl concentration) or 1.5 M NaCl PPT (osmotic upshock, final concentration 1.0 M NaCl). The cells were then incubated at 30°C. At the end of the incubation, the intracellular betaine concentration (M) was determined as described in Materials and Methods, p. 59.

[b] Betaine concentrations were calculated using the value of 1.8 µl/mg dry wt as the intracellular space of V. costicola (Hamaide, 1984).

[c] Incubation time following dilution.

Figure 35. Effects of salts and betaine on the activity of the enzyme choline dehydrogenase of V. costicola. Symbols: NaCl, ○ ; KCl, □ ; NH_4Cl, △ ; sodium glutamate, ● ; potassium glutamate, ■ ; ammonium glutamate, ▲ ; and betaine, ⬡ .

Results are expressed as percentage of controls (no added solute). 100% enzymatic activity represents 4.5 nmole/min/mg protein.

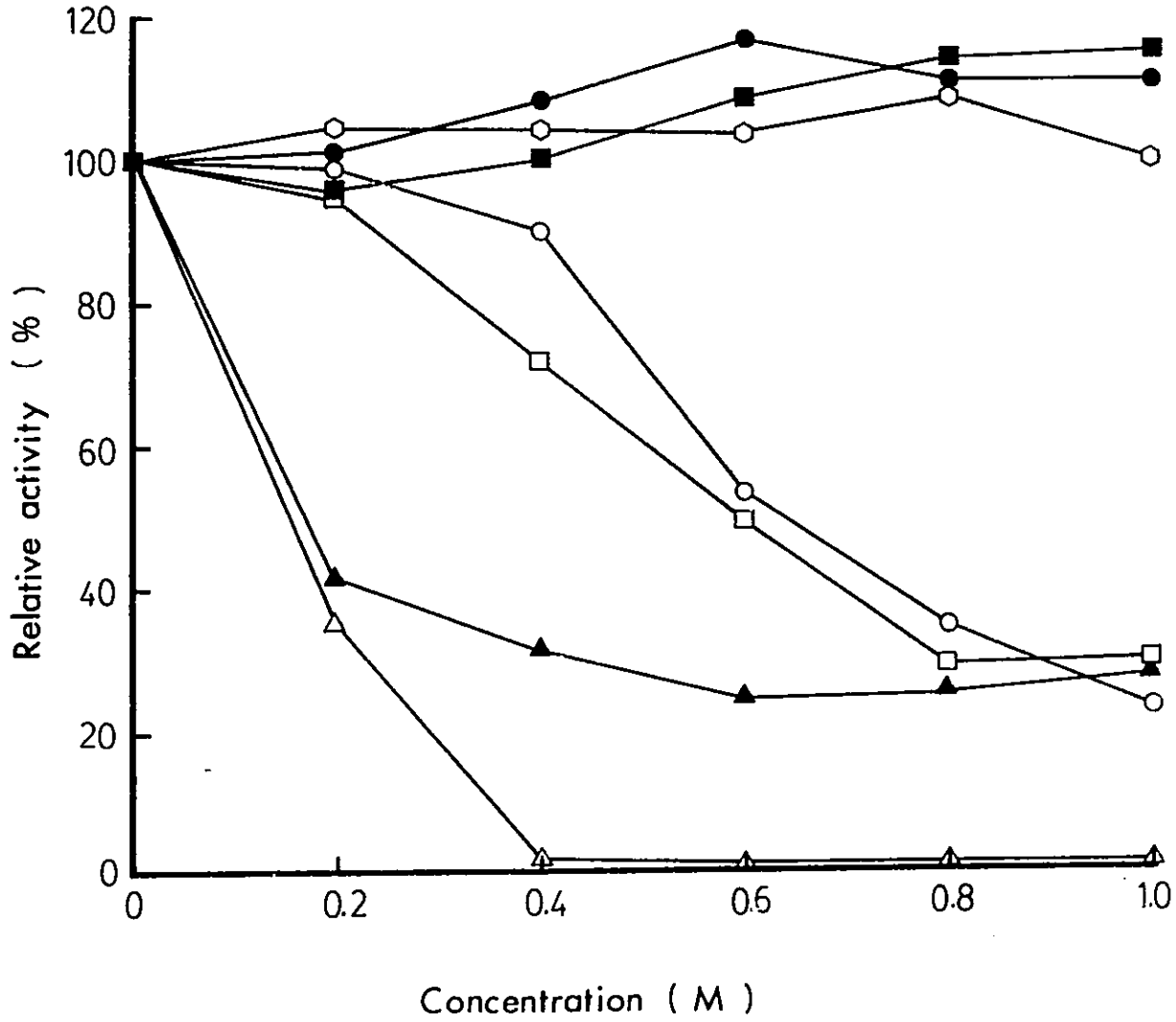
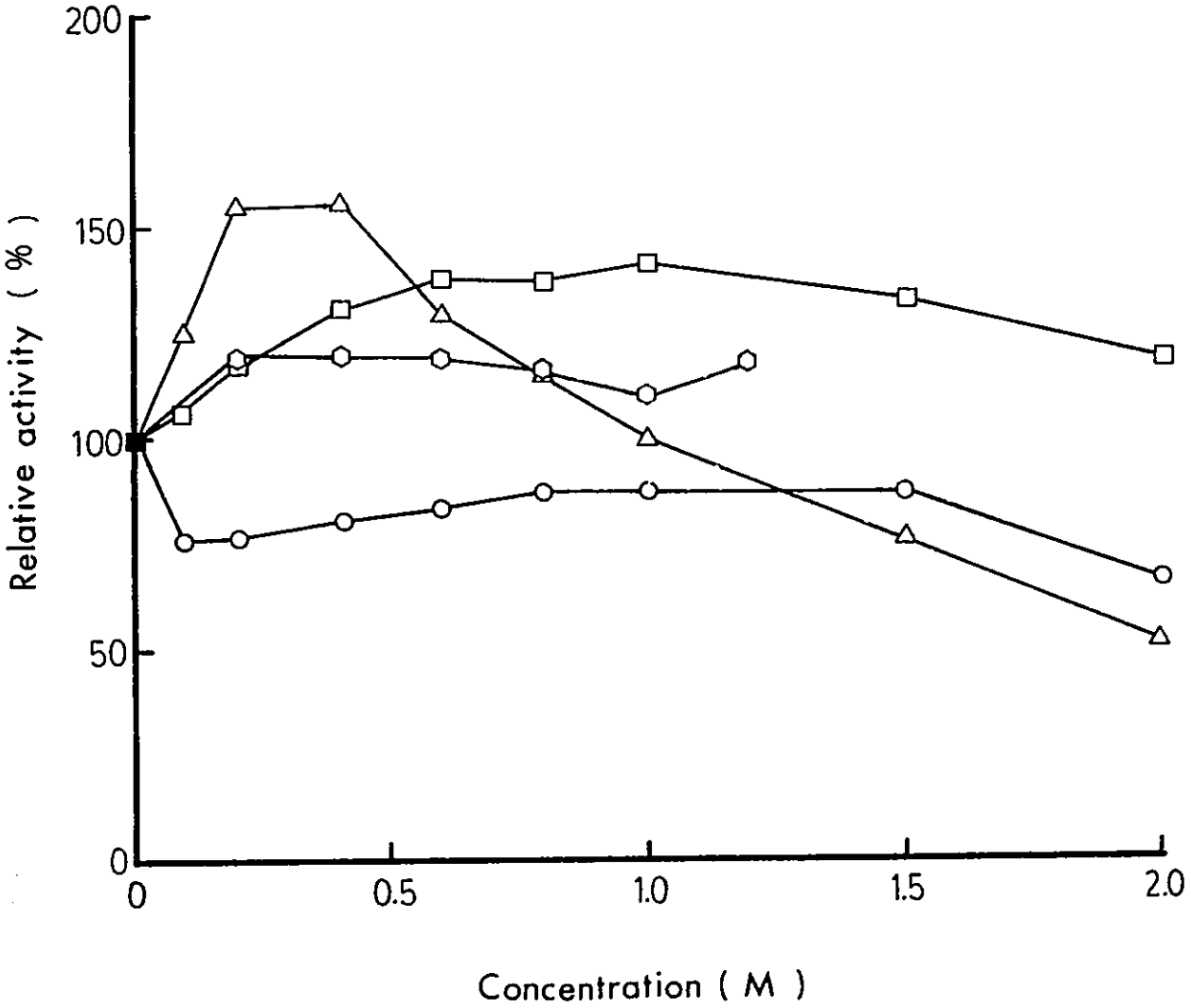

120
100
80
60
40
20
0
Relative activity (%)
0
0.2
0.4
0.6
0.8
1.0
Concentration (M)

Figure 36. Effects of salts and betaine on the activity of the enzyme betaine aldehyde dehydrogenase of V. costicola. Symbols: NaCl, ○ ; KCl, □ ; NH_4Cl, △ ; and betaine, ⬡ .

Results are expressed as percentage of controls (no added solute). 100% enzymatic activity represents 120 nmole/min/mg protein.


200
150
100
50
0
Relative activity (%)
0
0.5
1.0
1.5
2.0
Concentration (M)

at a concentration of 2.0 M, KCl had no inhibitory effect while NaCl and NH_4Cl did not inhibit the enzyme by more than 50%. Not only was the inhibition of these salts not as pronounced as with choline dehydrogenase, but both KCl and NH_4Cl actually stimulated its activity (Figure 36). The stimulation observed with NH_4Cl occurred over a narrower range (0-1.0 M) than with KCl (0-2.0 M).

Betaine, up to a concentration of 1.2 M, had no strong effects, either inhibitory or stimulatory, on the activity of both enzymes (Figures 35 and 36).

DISCUSSION

IN VITRO PROTEIN SYNTHESIS BY THE MODERATE HALOPHILE V. COSTICOLA

The poly(U)-directed incorporation of phenylalanine by extracts of V. costicola is rapid at first but slows and stops within 15 min. The number of ribosomes present seems to be the limiting factor (Figure 5). Some of my experiments (Figure 11) have suggested that, after the runoff of the 70S ribosome from the artificial messenger, the 50S subunit resulting from the dissociation of the ribosome cannot reattach to the 30S-mRNA complex. Whether this is due to the 50S or the 30S-mRNA complex is not known. However, major proportions of inactive ribosomes have been reported for other cell-free systems carrying out poly(U)-directed protein synthesis, including those of E. coli (Bilgin et al., 1988) and H. halobium (Saruyama and Nierhaus, 1985). It is also known that "ribosomes (of E. coli) that reach the end of poly(U) cease synthesizing polyphenylalanine" (Bilgin et al., 1988).

My results confirm the earlier finding that Cl^- is inhibitory to poly(U)-directed in vitro protein synthesis by V. costicola (Kamekura and Kushner, 1984). However, Cl^- has no effect on the fidelity of translation of this particular system (Table 4). This may be a reflection of the ability of V. costicola to grow at a wide range of NaCl concentrations;

although the concentration of Cl^- in cells grown in the presence of 1.0 M NaCl is only 0.2 M (Kamekura and Kushner, 1984), cells grown in the presence of 3.0 M NaCl contain 1.5 M Cl^- (Kushner and Kamekura, 1988). In contrast, fidelity of translation in cell-free protein synthesizing system from the extremely halophilic archaebacterium H. halobium, which requires almost saturated solutions for growth, is increased by the presence of NaCl (Bayley and Griffiths, 1967).

It was shown previously that the phenylalanyl-tRNA synthetase was not responsible for Cl^- inhibition of poly(U)-directed in vitro protein synthesis by V. costicola (Kamekura and Kushner, 1984). I have now shown that Cl^- can prevent attachment of an artificial mRNA [poly(U)] to both complete 70S ribosomes and the ribosomal subunits (Figure 12). A similar inhibition by Cl^- has also been demonstrated for the in vitro protein synthesis system of reticulocyte lysates (Weber et al., 1977). In this eukaryotic cell-free system, 200 mM Cl^- inhibited the binding of mRNA to reticulocyte ribosomal particles (80S and 40S).

There are a number of major differences between the translation system directed by poly(U) employed here, and one directed by a natural mRNA. Poly(U) has no Shine-Delgarno sequence, which normally plays an important role in the initial attachment of natural mRNAs to the ribosomes. At high Mg^{++} ion concentration (18 mM), the initiation factors are not required in the poly(U) system of E. coli (Lucas-Lenard and Lipmann,

1967). Finally, the initiator tRNA is not involved, since poly(U) has no initiation codon. Therefore, the initiation process of protein synthesis is not as well represented in an *in vitro* translation system directed by poly(U) as in one directed by a natural mRNA.

I wanted to study the effects of Cl^- ions on protein synthesis in *V. costicola* under more physiological conditions, i.e. using a natural mRNA. Unfortunately, none have been isolated from this, or any other, moderate halophile, though some studies had been done with a mixture of unidentified messengers present in the cell (Wydro *et al.*, 1977). This system has now been studied in more detail and has proven to be a useful tool for studying protein synthesis in *V. costicola*.

I have also shown that a natural mRNA which functions well in the cell-free translation system of *E. coli* (Boedtker and Stumpp, 1966; Tai *et al*, 1973; Kaempfer and Jay, 1979; Ellis and Conway, 1984), that of its phage R17, can also function *in vitro* with *V. costicola*.

When developing a new cell-free translation system, it is necessary to show that the incorporation of the radiolabeled amino acid(s) is indeed *de novo* synthesis and not unspecific absorption of the amino acid(s) to proteins. The R17 RNA-directed incorporation of [^{14}C]valine was dependent upon the addition of exogenous mRNA, an energy source, cellular extract and ribosomes (Figure 17). It was time-dependent, increased with the addition of $tRNA_f^{met}$ and was inhibited by different

protein synthesis inhibitors (Figures 17 and 18; Table 4). These are all characteristics of an *in vitro* protein-synthesizing system carrying out *de novo* synthesis. I also showed that this system synthesized the expected protein, i.e. the coat protein of the R17 bacteriophage (Figure 19). This proved that the natural mRNA of R17 could properly direct *in vitro* protein synthesis by the moderate halophile *V. costicola*. To my knowledge, this is the first example of *in vitro* synthesis of a specific protein by a halophilic bacterium, even though it is not of a protein it normally makes.

The translation of endogenous mRNA was also *de novo* protein synthesis, as it too was time and cellular extract-dependent (Figure 20) and was inhibited, partially or completely, by the different protein synthesis inhibitors (Table 5).

Both systems are more physiological than the poly(U) system; however, there is an important inherent difference between the R17 RNA system and the endogenous system. In the R17 protein-synthesizing system, every ribosome that becomes involved in protein synthesis must first go through normal initiation. In contrast, the translation of endogenous mRNAs involves, to a large extent, the completion of nascent polypeptides. This situation also exists in cell free translation systems directed by endogenous mRNAs of *E. coli* (Kucan and Lipmann, 1964; Grollman and Stewart, 1968). The preattached ribosomes can carry out elongation immediately, without having first to go through initiation.

The action of the different protein synthesis inhibitors on each system illustrates this difference (Table 5). Chloramphenicol and neomycin, which inhibit polypeptide elongation, strongly inhibited R17 RNA-directed translation and endogenous translation by V. costicola. Kasugamycin and ATA, which act on initiation of protein synthesis, strongly inhibited the R17 RNA-directed protein-synthesizing system, but they had a much weaker effect on endogenous translation. The presence of preattached ribosomes probably explains why the endogenous system did not respond to $tRNA_f^{met}$; incorporation will not increase if $tRNA_f^{met}$ does not significantly increase the amount of initiating ribosomes relative to preattached ribosomes. It is also possible that the amount of $tRNA_f^{met}$ present in the S-30 extract was enough to saturate the initiating ribosomes.

In the process of optimizing both systems, a number of interesting features of the in vitro protein synthesizing machinery of V. costicola were revealed. The low Mg^{++} ion requirement (8 mM) of both systems (Figures 21 and 22) is typical of translation systems directed by natural mRNAs (Modelell and Davis, 1968; Lucas-Lenard and Lipmann, 1967). In contrast, the poly(U) in vitro system required more than twice as much Mg^{++} (Wydro et al., 1977). This reinforced the notion that I was dealing with more physiological systems.

The NH_4^+ requirement was very similar in both systems, around 250 mM (Figures 21 and 22). This is lower than the 400 mM that has been reported in V. costicola grown in the presence

of 1.0 M NaCl (Wydro et al., 1977). In comparison with other cell-free translation systems, E. coli, a non-halophile, requires less NH_4^+ ions (60 mM) (Ellis and Conway, 1984; Tai et al., 1973), while halophilic archaebacteria may require as much as 3 M NH_4^+ (Sanz et al., 1988; Saruyama and Nierhaus, 1985) though, in fact, these organisms only contain 10-30 mM NH_4^+ (Kushner, 1988). The NH_4^+ ion requirement of V. costicola may be a reflection of its ability to grow best at moderate salt concentrations.

As in other eubacterial in vitro translation systems (Wydro et al., 1977; Boedtker and Stumpp, 1966; Conway, 1964), NH_4^+ ions were found to be more efficient than K^+ ions in supporting incorporation (Figures 21 and 22). This dependence on certain monovalent cations is not surprising since they are believed to be necessary for the binding of the aminoacyl tRNA to the ribosome-mRNA complex (Spyrides, 1964; Conway, 1964; Lubin 1963). However, what is quite unusual is that the NH_4^+ had to be supplied in the form of ammonium glutamate, NH_4Cl did not support high levels of activity. This is even more significant since I have found that R17 RNA-directed protein synthesis, by cellular extract of the non-halophile E. coli, was only slightly less efficient with 60 mM NH_4Cl (95%) than with 60 mM ammonium glutamate (data not shown).

The lack of incorporation in the presence of NH_4Cl could be interpreted as an inhibitory action by Cl^- ions on protein synthesis. We already know that Cl^- ions inhibit poly(U)-

directed protein synthesis (Kamekura and Kushner, 1984), and it is now clear that they also inhibit the R17 RNA and endogenous systems described here: added NaCl was much more inhibitory than added sodium glutamate (Figures 23 and 24). However, we cannot exclude the possibility that an actual need for glutamate anions exists in these systems. In contrast, it has been noted that in cell-free protein synthesis system of halobacteria (which have high internal Cl^- concentration) the anion requirement cannot be fulfilled by organic anions (Sanz et al., 1988). From these results, Sanz et al. (1988) implied that anions and cations play different roles in protein synthesis.

The responses of the two systems to added salts were quite different (Figures 23 and 24). The inhibition by Cl^- ions was much stronger in the R17-RNA protein-synthesizing system. This system was also inhibited by sodium glutamate and potassium glutamate, while endogenous translation was stimulated by them. These differences may be a reflection of the state of the ribosomes in each system (free or preattached) and would suggest different sites of action. The stronger inhibition by Cl^- ions on R17 RNA-directed incorporation would indicate that these ions are more harmful to the initiation of protein synthesis than to elongation. On the other hand, sodium or potassium glutamate appear to stimulate translation by preattached ribosomes (elongation), while inhibiting initiation (probably because of increased ionic strength).

The poly(U) system used here and in previous experiments (Kamekura and Kushner, 1984) reacted the same way as the endogenous system towards the addition of NaCl (Figure 6) and sodium glutamate (Figure 7). This may be because there is also no true initiation of protein synthesis in the poly(U) system. Cl^- ions in the poly(U) system prevented binding of the mRNA to the ribosomes. This may also be true for the inhibition of the endogenous system. However, Cl^- ions have a much more profound effect on "true" initiation of protein synthesis. This is apparently not due to absence of fmet-tRNA: the enzyme formylmethionyl-tRNA synthetase functions well even at high NaCl concentrations (Figure 29). Similarly, it had been shown that the enzyme phenylalanyl-tRNA synthetase was not strongly inhibited, less than 20%, by high NaCl concentrations (Kamekura and Kushner, 1984).

Other possible sites of action for Cl^- during initiation of protein synthesis include the attachment of the initiator-tRNA and of the mRNA to the ribosome. These two reactions are dependent upon the presence of initiation factors, which themselves could be a target of choice for Cl^- ions. In fact, Peumans et al. (1982) and Weber et al. (1977) have shown that in vitro eucaryotic translation systems were inhibited by Cl^- ions due to their action on one or more of the initiation factors.

Even though low amounts of ammonium glutamate were needed for maximum endogenous translation, higher amounts inhibited protein synthesis, while sodium or potassium glutamate

stimulated translation, presumably because of the effect of Na^+ and K^+. Since high concentrations of these ions are found in V. costicola (Shindler et al., 1977), it would be expected that they play an important role in protein synthesis.

So, how could Cl^- inhibit protein synthesis? Cl^-, being a strong anion, might interfere with protein-protein and protein-nucleic acid associations which involve ionic interactions (Weber et al., 1977), as well as induce intramolecular changes of proteins by interfering with the internal ionic interactions (Peumans et al., 1982). It is possible that Cl^- interfered with the protein-nucleic acid association involving the ribosomes and the mRNA, especially since it was shown that Cl^- could cause the release of already bound poly(U). Cl^- could also have an inhibitory effect on the initiation factors themselves or on their association with the ribosome, the initiator tRNA (Figure 31) or the mRNA (Figure 32).

The proteins and the RNA molecules that constitute the ribosomes are held together by protein-protein and protein-nucleic acid associations. I considered the possibility that Cl^-, by removing one or more ribosomal protein, could prevent the binding of poly(U) to the ribosomes. But this does not occur. For reasons not yet clear, ribosomes washed in 0.6 M NaCl are more active than those not washed at high salt concentration (Figure 15).

There may well be other sites of action of Cl^- since these and other cells (Kamekura and Kushner, 1984; Kushner, 1988) are

so concerned with keeping Cl^- out, presumably at some cost of energy. Therefore, it may be suspected that Cl^- can have many toxic sites of action. Futhermore, Cl^- sensitivity in halophilic eubacteria, and probably marine bacteria, is not restricted to protein synthesis. Although information on this subject is scarce, preliminary studies have shown that Cl^- can have an inhibitory effect on some cellular enzymes (reviewed in Kushner and Kamekura, 1988). Whether it can also affect other vital processes such as DNA replication or transcription is still unknown.

Our work has emphasized the importance of betaine in the physiology of halophilic eubacteria. This substance accumulates in every moderate halophile so far examined, as well as some non halophiles under osmotic stress (Galinski and Truper, 1982; Imhoff and Rodriguez-Valera, 1984), suggesting that it can act as a compatible solute in these bacteria. It has also been shown to relieve the effects of high NaCl concentrations on active transport in V. costicola (Kushner et al., 1983); to relieve the inhibition of respiration caused by high NaCl concentrations in the moderate halophile Ba_1 (Rafaeli-Eshkol and Avi-Dor, 1968); to protect the enzyme glutamine synthetase from inhibition by high salt concentrations in a halotolerant and a marine strain of cyanobacteria (Warr et al., 1984); to relieve the inhibition of nitrogen fixation by high salt concentrations in K. pneumoniae (Bouillard and Le Rudulier, 1983); and to

reverse the effects of osmotic stress on DNA replication and cellular division of E. coli (Meury, 1988).

Glutamate may also play an important role in V. costicola. Synthesis of glutamate can be osmotically induced in a number of non-halophilic and marine bacteria (Makenson and Hastings, 1979; Imhoff, 1986). In V. costicola itself, glutamate is the major known organic anion (Kamekura and Kushner, 1984).

Paradoxical results were obtained with betaine and glutamate regarding their potential role in protecting the cellular protein machinary against the toxic action of Cl^- in V. costicola. Results obtained with the poly(U) system would suggest that both substances could play such an important role. They were both able to reverse, at least partially, the inhibitory effect of Cl^- on poly(U)-directed phenylalanine incorporation (Figures 7 and 8) and the inhibitory effect of Cl^- on binding of poly(U) to the ribosomes (Figures 13 and 14).

Glutamate could counteract the action of Cl^- by occupying some of the Cl^- binding sites. Betaine, however, is a zwitterion at physiological pH and, therefore, could not act as a competitive anion to Cl^-. It would probably act in a different manner than glutamate, perhaps by stabilizing the poly(U)-ribosome complex at high Cl^- ion concentrations. There is evidence that certain osmolytes may act on enzymes via changes in the solvent properties of water rather than (or as well as) via a direct interaction with the enzymes (Pollard and Wyn Jones, 1979; Yancey et al., 1982). In the presence of Cl^-,

poly(U) may be prevented from binding to the ribosomes because of such indirect solvent-mediated forces, and protection by betaine (or glutamate) could then be the result of complex NaCl-betaine-protein/ribosome-water interactions (Pollard and Wyn Jones, 1979) and not due to direct binding of betaine to the ribosomes. Alternatively, the counteractions of betaine and glutamate could simply be the result of the stimulatory effects that both substances were shown to have on protein synthesis; i.e. higher incorporation of radiolabeled amino acids, even in the presence of Cl^-, due to the activation of protein synthesis by betaine or glutamate. This would imply that glutamate and betaine activate protein synthesis at sites other than the Cl^- toxic site.

Regardless of the manner in which betaine and glutamate protect the *in vitro* poly(U) directed protein synthesizing system, their combined presence is much more beneficial than the presence of either alone (Figure 9).

This protective action is not as evident in the more physiological systems (R17 RNA and endogenous systems) used here: although betaine increased incorporation, it did not protect very well against the toxic effects of Cl^- ions (Figures 27 and 28). Taken with the facts that glutamate seems to play a major role in these *in vitro* systems and that betaine stimulated protein synthesis in both systems, those results would support the hypothesis that betaine and glutamate may merely activate protein synthesis at sites other than the toxic Cl^- site. This,

by no means, reduces the importance of betaine in V. costicola, just its potential to protect protein synthesis against the inhibitory action of Cl^- ions.

V. costicola grown in a medium containing 3.0 M NaCl has a cell-associated Cl^- concentration of 1.5 M (Kushner and Kamekura, 1988), a concentration too high to permit in vitro protein synthesis. Kamekura and Kushner (unpublished data) have found that S-30 extracts of these cells are much less active in in vitro protein synthesis than S-30 extracts of cells grown in a medium containing 1.0 M NaCl. In hybridization experiments, using the ribosomal and soluble (S-150) fractions of cells grown in the presence of 1.0 and 3.0 M NaCl, they showed that the difference in protein synthesizing activity was due to the soluble fraction rather than the ribosomal fraction of these cells.

In their experiments, Kamekura and Kushner extracted the cells grown at 3.0 M NaCl with the same buffer used for the extraction of cells grown at 1.0 M NaCl. This buffer has a low ionic composition (124 mM NH_4Cl, 82 mM Tris-HCl and 20 mM $MgCl_2$) which probably represents more closely the intracellular environment of cells grown at the lower salt concentration than that of cells grown in 3.0 M NaCl PPT. Thus, these results may suggest that the soluble proteins are folded differently in the two intracellular environments. This, in turn, may imply that the biochemical properties of the protein synthesizing machinery of cells grown in 1.0 M NaCl are different than those of cells

grown at 3.0 M NaCl. Therefore, protein synthesis in cells grown at higher salt concentrations could involve proteins that are better adapted to high intracellular salt concentrations.

The observation of Kamekura and Kushner, that the ribosomes of V. costicola isolated from cells grown at 1.0 and 3.0 M NaCl functioned equally well in in vitro protein synthesis, is in agreement with the fact that the ribosomes of V. costicola are stable at low and high salt concentrations (Wydro et al, 1975). This suggests that the same ribosomes would take part in protein synthesis regardless of the intracellular ionic composition. This stability of the ribosomes at high salt concentrations is also reflected in the fact that the initiation factors of V. costicola are released from the ribosomes only at much higher NH_4Cl concentrations than that required to release initiation factors from the ribosomes of E. coli. All those properties may be necessary adaptations for bacteria that grow over a wide range of salt concentrations.

It is sometimes assumed that the conditions under which an in vitro system works best are those prevailing in the cell. Thus, the observed toxicity of Cl^- on in vitro protein synthesis by V. costicola would suggest that the intracellular Cl^- found in these cells [0.2 M (Kamekura and Kushner, 1984) and 1.5 M (Kushner and Kamekura, 1988) Cl^- in cells grown in media containing 1.0 M and 3.0 M NaCl, respectively] would not be, to a large extent, in a "free" ionic state, but rather complexed with organic molecules or structural components of the cell. In

Bacillus subtilis, for example, 96% of its cell-associated Cl^- is bound to the envelope (Coleman, 1974). Therefore, V. costicola could protect its protein synthesizing machinery by sequestering the toxic Cl^-.

It is not yet clear which one of these protective mechanisms is the most likely to be used by V. costicola. However, it is clear that they are not necessarily mutually exclusive and that all of them could play an important part in protecting the protein synthesizing machinery of these cells. Additional studies are needed to fully understand the conditions under which protein synthesis takes place in V. costicola. For one, in vitro protein synthesis, with extracts of cells grown in the presence of 3.0 M NaCl, should be studied with extracts prepared with buffers representing more closely the intracellular ionic environment of these cells. Furthermore, the purification and characterization of the initiation factors of V. costicola would allow a more detailed study of the effects of salts on their respective functions. For these studies, the R17 RNA protein synthesis system developed here would be very useful. These studies could also reveal additional characteristics of the protein synthesizing machinery of moderate halophiles that may be necessary for them to grow over such a wide range of salt concentrations.

OXIDATIVE PATHWAY OF CHOLINE TO BETAINE

V. costicola grown in a chemically defined minimal medium (CDMM) containing choline accumulates betaine in response to increasing external salt concentrations (Table 11). In this medium, exogenous choline is transported inside the cells (Figures 32 and 33) and serves as the precursor for betaine synthesis. With increasing NaCl concentrations, betaine is also accumulated in V. costicola grown in the complex medium PPT (Table 13). However, in this rich media, betaine accumulation, as in E. coli, is probably the result of uptake of betaine and/or choline present in the proteolytic and tryptic digests used in the medium preparation rather than complete de novo synthesis (Imhoff, 1986; Landfald and Strom, 1986). Uptake is less energy-demanding than complete de novo synthesis (Imhoff, 1986).

In V. costicola grown in PPT medium, the accumulation of betaine, in response to a sudden osmotic upshock, is dependent on protein synthesis, since the accumulation is inhibited by chloramphenicol. It is not possible, at this time, to say exactly what are the proteins that need to be synthesized. They could be proteins involved in the transport of exogenous betaine (such as the proU system in E. coli) or choline, as well as enzymes involved in the choline oxidation pathway. In any event, they would probably be regulated at the level of

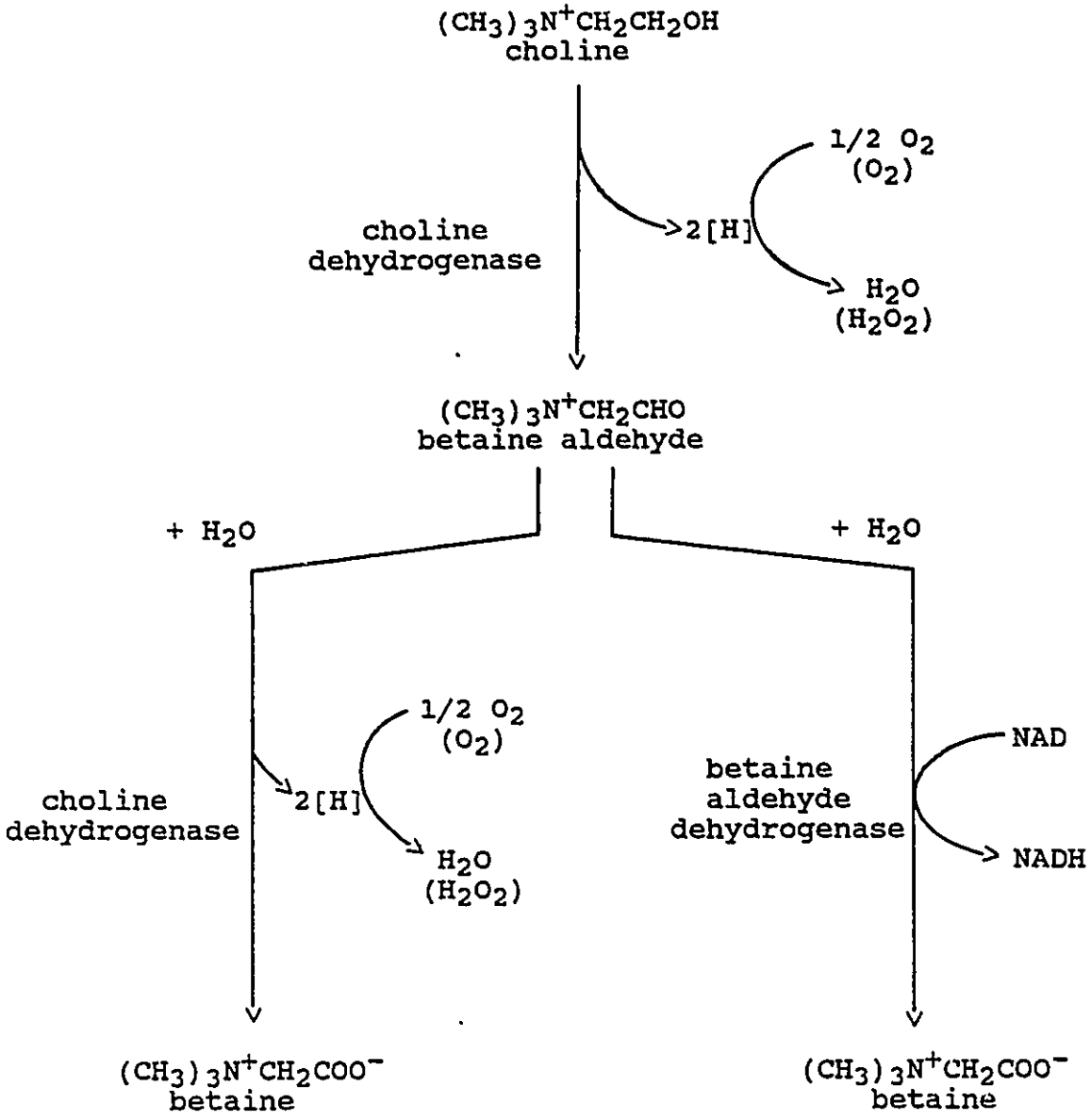
transcription. This is believed to be the case in E. coli (Landfald and Strom, 1986; Higgins et al., 1988).

Although they have not been tested in V. costicola, it is interesting that the precursors of betaine, namely choline and betaine aldehyde, have no osmoprotective effect in E. coli mutants defective in the choline oxidation pathway (Styrvold et al., 1986). This suggests that the dipolar character of betaine that allows it to be accumulated in cells without counterions is critical for its osmoprotective properties (Styrvold et al., 1986).

Oxidation of choline to betaine is a two-step process in V. costicola (Figure 37): (1) the oxidation of choline to betaine aldehyde catalyzed by the O_2-dependent membrane bound choline dehydrogenase (Table 8) and (2) the oxidation of betaine aldehyde to betaine catalyzed by the soluble NAD-dependent betaine aldehyde dehydrogenase (Table 8). These enzymes resemble the ones identified in E. coli (Landfald and Strom, 1986), P. aeruginosa (Nagasawa et al., 1975, 1976), A. globiformis (Ikuta et al., 1977) and R. melitoti (Tombras Smith et al., 1988). They are also similar to the ones found in mammalian liver cells (Wilken et al., 1970): a mitochondrial electron transfer-linked dehydrogenase and a soluble NAD-dependent betaine aldehyde dehydrogenase.

In V. costicola, choline dehydrogenase can also oxidize betaine aldehyde to betaine at at least 30% the speed of choline oxidation. Similar observations were made in E. coli: its

Figure 37. Choline-betaine oxidation pathway in V. costicola.


$(CH_3)_3N^+CH_2CH_2OH$
choline
1/2 O_2
(O_2)
choline
dehydrogenase
2[H]
H_2O
(H_2O_2)
$(CH_3)_3N^+CH_2CHO$
betaine aldehyde
+ H_2O
+ H_2O
1/2 O_2
(O_2)
NAD
choline
dehydrogenase
2[H]
betaine
aldehyde
dehydrogenase
H_2O
(H_2O_2)
NADH
$(CH_3)_3N^+CH_2COO^-$
betaine
$(CH_3)_3N^+CH_2COO^-$
betaine

choline dehydrogenase can oxidize choline and betaine aldehyde equally well (Landfald and Strom, 1986). In A. globiformis (Ikuta et al., 1977) and in an Alcaligenes sp. (Ohta-Fukuyama et al., 1980), there is a soluble, H_2O_2-forming choline oxidase, which oxidizes both choline and betaine aldehyde. It has not yet been determined if the reactions involving choline dehydrogenase in V. costicola produce H_2O or H_2O_2.

As in E. coli (Landfald and Strom, 1986), P. aeruginosa (Nagasawa et al., 1975, 1976), A. globiformis (Ikuta et al., 1977) and R. melitoti (Tombras Smith et al., 1988), choline dehydrogenase and betaine aldehyde dehydrogenase of V. costicola are probably formed inducibly in the presence of choline (Table 9). Their syntheses also seem to be osmoregulated, as their activities increased in cells grown at increasing salt concentrations (Table 10). It still has to be determined if, in effect, these increases occur in response to changes in the osmotic pressure of the external medium or if they are specific for NaCl. Landfald and Strom (1986) have shown that the dehydrogenases of E. coli are osmoregulated since increases in their activity were the same in cells grown in media containing NaCl, KCl or sucrose added to the same osmotic strength. In R. melitoti, the activity of its betaine aldehyde dehydrogenase increased when the cells where grown in the presence of 0.5 M NaCl but the activity of its choline oxidase (dehydrogenase) remained the same.

Enzymatic regulation of the choline-betaine oxidative pathway could first involve the induction of transport systems of the precursor choline in response to changes in the external osmotic pressure followed by enzymatic expression, presumably by an activation at the transcriptional level, with choline acting as an effector (Landfald and Strom, 1986). A membrane bound protein that sensed a change in external osmotic pressure could be responsible for triggering this system. Examples of such proteins do exist. The expression of the high affinity K^+ uptake system in E. coli is osmoregulated by the membrane protein KdpD (Laimins et al., 1981). The relative amounts of the 2 outer membrane proteins of E. coli, OmpF and OmpC, are osmoregulated by the EnvZ protein (Hall and Silhavy, 1981a, 1981b).

The activity of choline dehydrogenase and betaine aldehyde dehydrogenase are affected quite differently by salts. Activity of choline dehydrogenase is inhibited by Cl^- ions (Figure 35), while that of betaine aldehyde dehydrogenase is stimulated by KCl and NH_4Cl (Figure 36). In fact, betaine aldehyde dehydrogenase is inhibited no more than 50% by chloride salts at concentrations as high as 2.0 M. Most intracellular enzymes of V. costicola, and other moderate halophiles, are severely inhibited by chloride salts at concentrations less than 0.6 M (Kushner and Kamekura, 1988).

The different responses of betaine aldehyde dehydrogenase to each salt may indicate the presence of isomeric forms of the

enzyme. In spinach, barley and sugar beet, there are two such isomeric forms, a major chloroplastic form and a minor cytosolic form (Weretilnyk and Hanson, 1988). No such isomers have been identified in eubacteria.

Although choline dehydrogenase is inhibited by chloride salts, it is not inhibited by sodium glutamate and potassium glutamate (Figure 35) and, therefore, could function well at high Na^+ and K^+ concentrations normally found in V. costicola (Shindler et al, 1977). As suggested by Kushner and Kamekura (1988), these results emphasize the importance of doing enzymatic studies of halophilic bacteria with salts other than chloride salts, since inhibition by these may merely reflect Cl^- toxicity and not the halophilic or halotolerant characteristics of the enzyme(s).

Betaine had no effect on the activity of both enzymes which is in agreement with its role as a compatible solute. Assuming that, as in E. coli, betaine is a metabolically inert compound in V. costicola, then its regulatory mechanism could be a reflection of its osmoregulatory and protective functions (Landfald and Strom, 1986). Is is also interesting that, in contrast to the enzymes of betaine synthesis, in vitro protein synthesis by cellular extracts of V. costicola, regardless of the system used, is stimulated by betaine (Figures 8 and 26). Taken together with the fact that choline dehydrogenase and betaine aldehyde dehydrogenase are probably regulated at the level of transcription, it would seem possible for betaine to

stimulate its own synthesis; increasing amounts of betaine could stimulate translation of the newly-formed mRNAs encoding the enzymes of the choline oxidation pathway, resulting in an increase in the amount of these enzymes in the cell and, therefore, in an increase in the rate of betaine synthesis.

The intracellular betaine concentration of V. costicola is about half the external NaCl concentration (Table 12). Although there is no doubt that betaine is an important compatible solute in V. costicola, it is probably not the only one; it is not present in sufficient amount to maintain the positive turgor pressure within the cell. At a concentration half that of the external NaCl, betaine supplies only 25% of the needed osmotic strength since it is a single molecule, while NaCl dissociates into Na^+ and Cl^-, both of which contribute equally to the external osmotic pressure. Therefore, other substances must be involved in maintaining the turgor pressure.

These other substances may not necessarily be classified as compatible solutes; that is, accumulated in response to increasing external NaCl concentrations. For example, glutamate is always present at relatively high concentrations in V. costicola, ca. 0.3 M (Imhoff and Rodriguez-Valera, 1984; Kamekura and Kushner, 1984). This much glutamate would certainly contribute to the internal osmotic pressure. In addition, the different ions found in these cells (if they are present in their "free" ionic state) would also contribute significantly to the internal osmotic pressure.

There is some evidence that other structural components of eubacterial cells, such as membranes and nucleic acids, bind water. The water retained by these structures can be released by exposing them to an osmotically stressing molecule (Rau et al., 1984; Parsegian et al., 1984). Therefore, these structures, by holding some of the intracellular water, could also increase the internal osmotic pressure.

Macromolecules, because of their large size and low intracellular concentrations, have never been seriously considered as osmotically active molecules. However, Kushner (1988) points out that they should probably not be ignored. He refers to a study done by Parsegian et al. (1986) that showed that a number of polymers, such as dextrans and polyethylene glycol, at concentrations between 20-40%, gave osmotic pressure equivalent to that produced by 0.5 to 5.0 M solutions of an ideal solute. There is also data that suggest that the osmotic pressure of the periplasmic space of E. coli could be maintained by the oligosaccharides present in it (Kennedy, 1982).

The fact that betaine concentrations did not increase in V. costicola grown in CDMM at the lowest choline concentration until the external concentration of NaCl reached 3.0 M NaCl (Table 12), suggests that betaine is only required to maintain turgor pressure at the higher NaCl concentrations. This could also explain why the addition of exogenous choline only enhanced the growth of V. costicola in 3.0 M NaCl CDMM (Figure 34).

Finally, we cannot exclude the possibility that compatible solutes, other than betaine, do exist in V. costicola. New substances that accumulate under osmotic stress are constantly being identified. Ectoine, a new amino acid, was found in large amounts in the halophilic E. halochloris and in a moderately halophilic Micrococcus sp. (Imhoff, 1986). Recently, Cayley et al. (1989) have shown that in the absence of betaine, E. coli accumulates 3-(N-morpholino)propane-sulfonate (MOPS) when grown at high osmolarity (1.1 osM). Therefore, it is evident that more research is necessary in order to unravel the mystery of the "missing osmoticum" in V. costicola.

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