Dedicated to my tireless, understanding and beautiful mother

Kay,

to my beloved son

Daniel

and

to the sacred memory of my fine, generous and intelligent father

Irving Isadore Shindler.

WE SHALL NEVER CEASE FROM EXPLORATION
AND THE END OF ALL OUR EXPLORING
WILL BE TO ARRIVE WHERE WE STARTED
AND KNOW THE PLACE FOR THE FIRST TIME.

- T. S. ELIOT

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Abstract

Vibrio costicola, a moderately halophilic bacterium, originally isolated from bacon curing brines, grows over a range of medium salt concentrations from 0.2M to 4.0M NaCl. It is best adapted to growth in 1-2M NaCl in both complex and minimal salts-glucose media. Its closest relatives are probably slightly halophilic marine Vibrios (Ch. II p. 25).

Actively metabolizing V. costicola contained about 0.7M K^+ and an Na^+ concentration which brought the total $Na^+ + K^+$ to levels almost equivalent to that of the medium NaCl. An energy source was required to maintain high intracellular K^+/Na^+ levels; poisoning reduced this ratio markedly. Permeabilization resulted in a striking increase in cell bound Na^+, indicating that ions may be cell bound and not be free in the cytoplasm; the cellular location of the ions is still unknown.

Maximum V. costicola aspartate transcarbamylase activity was dependent upon 1.8M Na^+, yet the biosynthetic threonine deaminase (TDase) activity was inhibited 70% 1-3M NaCl. Both enzymes, however, were unstable in the absence of salts. Only the threonine deaminase activity was subject to regulation by feedback inhibition, isoleucine inhibition could be partially reversed by valine or high threonine substrate concentrations. The inhibition was of the "K" type with sigmoidal threonine saturation curves and Hill coefficient values more than unity in presence of isoleucine. The sensitivity of the enzyme to the inhibitor was decreased 50% when the Na^+ concentration was raised from 0.2M to 1-3M; salt also acted as a pseudocompetitive

inhibitor of activity. In spite of the salt inhibition, TDase retained good enzymatic and regulatory activity in the 1-3M range of measured intracellular Na^+ and K^+. The TDase activity could also be repressed at any medium NaCl concentration by inclusion of amino acids in the medium. The repression was not multivalent, as it required more than the three branched chain amino acids to effect a decreased enzyme synthesis.

Almost half of the enzymes from V. costicola and other moderate halophiles are salt-dependent. This may indicate that the intracellular distribution of ions is not uniform. If the cell-envelopes bind most of the Na^+ and K^+, then only membrane-associated enzymes may be halophilic, while the cytoplasmic "soluble" enzymes may not have high salt dependencies.

Résumé

Une bactérie halophile modérée: Vibrio costicola, isolée originellement des saumures de lard salé, peut croître sur un milieu contenant de 0.2M à 4M de NaCl. Elle est mieux adaptée à croître avec une concentration de 1 à 2M de NaCl ajoutée soit au milieu complexe soit au milieu minéral glucosé. Ses plus proches parents sont probablement des Vibrio marins légèrement halophiliques.

En pleine activité métabolique: Vibrio costicola contient environ .7M d'ions K^+ et des ions Na^+, ces deux types ions ajoutés donnent une concentration saline presque équivalente à celle du milieu de croissance avec NaCl. Une source d'énergie est nécessaire pour maintenir une concentration intracellulaire élevée de K^+ et de Na^+. La perméabilisation des cellules entraîne une forte augmentation du Na^+ lié aux cellules, ce qui semble indiquer que les ions ne sont pas libres à l'intérieur du cytoplasme, mais plutôt liés aux cellules. La localisation cellulaire des ions est toujours inconnue.

L'activité maximale de l'aspartate transcarbamylase est dépendante d'une concentration de Na^+ de 1.8M, alors que 70% de l'activité biosynthétique de la thréonine déaminase est inhibée par 1 à 3M de NaCl. Cependant, les deux enzymes sont instables en absence de sels. Seule, l'activité de la thréonine déaminase est assujettie au contrôle par la rétro-inhibition; l'inhibition

par l'isoleucine peut être partiellement levée par la valine ou par de fortes concentrations du substrat de la thréonine. L'inhibition est de type "K", les courbes de saturation de la thréonine sont de forme sigmoïdale, et le coefficient de Hill d'ordre supérieur à l'unité en présence d'isoleucine. La sensibilité de l'enzyme vis-à-vis d'un inhibiteur est diminuée de 50% si la concentration de Na^+ est augmentée de 0.2M à 1.3M. Le sel agit aussi comme un inhibiteur pseudo-compétitif de l'activité. En dépit de l'inhibition par le sel, la TDase conserve une bonne activité enzymatique et une bonne activité régulatrice même si la concentration intracellulaire de Na^+ et de K^+ est de l'ordre de 1M à 3M. La répression de l'activité de la TDase peut être obtenue par l'addition d'amino-acides au milieu contenant n'importe quelle concentration de NaCl. La répression n'est pas multivalente puisqu'elle nécessite plus que trois amino-acides ramifiés pour entraîner une diminution de la synthèse enzymatique. Presque la moitié des enzymes de V. costicola et d'autres halophiles modérées ont besoin de sel. Ceci peut indiquer que la distribution intracellulaire des ions n'est pas uniforme. Si les membranes lient la plupart du Na^+ et du K^+, alors seules les enzymes associées aux membranes sont peut être halophiliques; alors que les enzymes "solubles" dans le cytoplasme ne requièrent pas de fortes concentrations en sels.

CHAPTER 1

General Introduction to the Physiology of Halophilic Bacteria

Bacteria both require and tolerate inorganic ions. The extent of the requirement and degree of tolerance depend upon the individual characteristics of each strain. Ion concentrations can be an important factor in determining the kinds of micro-organisms present in a particular circumstance. Microbiological examination of saline environments such as salted fish, meat curing brines, soy sauce ferment and hyper-saline lakes and seas reveals that many organisms are inhibited by high salt concentrations; only those organisms which require or tolerate high salinities are present. Such organisms are described as halophilic (Gr. salt-loving) or halotolerant respectively.

Salts and brines have been used empirically for thousands of years to inhibit organisms which would otherwise certainly spoil foods and animal hides. Sometimes the conditions of saline preservation resulted in the growth of halophilic bacteria which themselves caused a spoilage of the "preserved" product. The economic importance of damage to salted fish and hides first attracted the attention of scientists around the turn of the century (see Flannery 1956).

Although coloration and spoilage of salted products by halophilic bacteria is no longer an important problem, the halophilic bacteria continue to attract the interest of researchers from a variety of fields. Because of their unusual characteristics, extremely halophilic bacteria have been the subject of considerable recent examination. But the moderately halophilic bacteria, which occupy environments intermediate in

salinity ranging from that of sea water (3% NaCl) to about half-saturated NaCl (18%), have been relatively neglected. Because much of the knowledge of physiology of halophilic bacteria comes from research on the extreme halophiles, a brief examination of their characteristics precedes the examination and research on the moderate halophiles.

The Extremely Halophilic Bacteria

The extreme halophiles require at least 2.5M salt* (15% NaCl) and grow best at salinities approaching saturation (30% NaCl). They have aroused interest, originally because of their involvement in the spoilage of salted fish and hides, and recently because of their unusual ability to live in extreme conditions which are detrimental to most other organisms. Past research has included comparative physiological investigations on their basic cellular processes, components, and structure, and the mechanisms of the salt requirement and tolerance. There has also been interest in their ecological and evolutionary relations. Several excellent reviews concerned with halophiles and related topics have been published (Flannery 1956, Ingram 1957, Scott 1957, Larsen 1962, 1967,

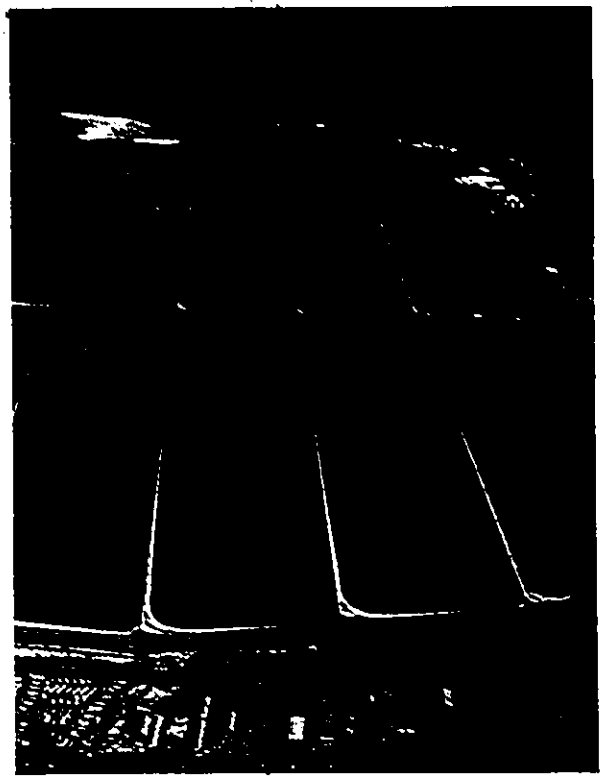
* "Salt" usually refers to NaCl but can also denote a mixture of inorganic substances appropriate to the circumstances. Where a specific substance is refered to, it is specified. "Salinity", an oceanographic term (Stanley & Morita 1968), denotes the weight of inorganic salts per kg of water. An equivalent amount of NaCl/kg water is a good approximation of the salinity level.

Brown 1964, Kushner 1968, 1971a,b, Gibbons 1969).

Only two genera of extremely halophilic bacteria appear to be substantiated on taxonomic grounds, the Gram-positive Halococci and the Gram-negative Halobacterium species (R. R. Colwell & N. E. Gibbons, unpublished work). Most of the research has been carried out on the red-pigmented rods belonging to the second group, while the cocci have been comparatively neglected.

The basic life processes of the extremely halophilic bacteria are similar to those of other kinds of bacteria. For instance, their energy production, biosynthetic mechanisms, nutritional requirements and reproduction are not radically different (except for the salt-requirement!) from other more "normal" bacteria (Larsen 1967). Halobacterium cellular components such as proteins, nucleic acids, membranes, cell envelopes and ribosomes are not superficially unusual. It is clear, however, that there are differences in cellular structures and ionic relations which permit function in an extremely saline milieu; an environment which precludes function and often destroys analogous structures in non-halophilic bacteria.

The extreme halophiles occur naturally in saline water bodies such as salt lakes (Baas-Becking 1928, Smith & ZoBell 1937, Brisou et al. 1974) and the Dead Sea (Volcani 1940). Halobacterium blooms along with other halophilic organisms can impart a distinct red color to sea water evaporation ponds as the water evaporates and the NaCl concentration approaches saturation (plates 1,2). Lower but substantial amounts of KCl and Mg^{+2} salts may also accumulate in these environments.



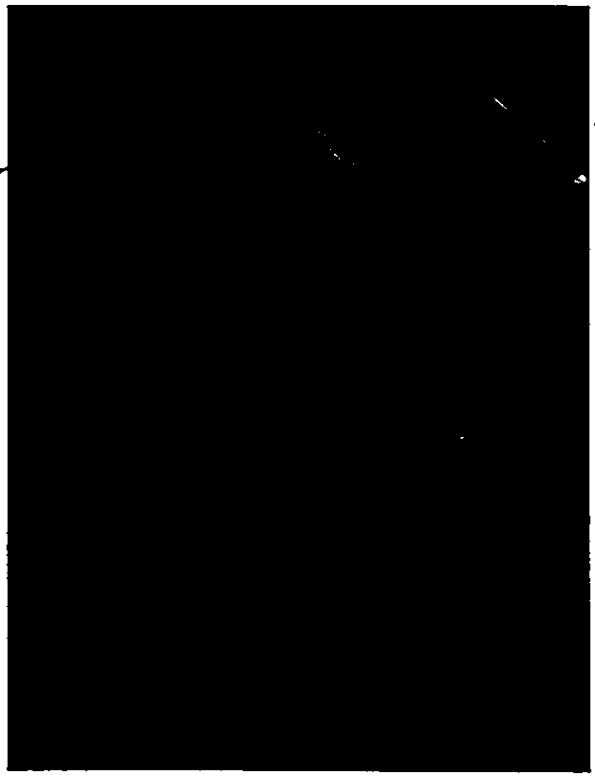


Plate 1 (left)
Plate 2 (right): The seawater is evaporated "until the color becomes red" - From an ancient Chinese treatise, 2500 B.C., on salt manufacture (Baas-Becking 1931). Aerial photos of the San Francisco salt evaporation ponds used for the collection of solar salt. The red-orange coloration is caused by the carotenoid pigments of extremely halophilic microorganisms including Halobacterium. (Courtesy Dr. Janos Lanyi, N.A.S.A., by permission).

Unrefined salt from solar evaporation ponds and from natural surface salt deposits are good sources of halophilic isolates (Larsen 1962, Eimhjellen 1964, Ishida & Fujii 1970). The unusual ability of these organisms to survive storage in dried salt has resulted in contamination of salt "preserved" food products, first reported by Farlow (1880).

Halobacterium growth is easily recognized as red coloration or slime. In eastern Canada the reddening of salted fish was sometimes called "pink" or "pinkeye" (Gibbons as quoted in Flannery 1956). One function of the pigments of the extreme halophiles is protection from the effects of solar radiation in their natural habitats, warm brines exposed to bright sunlight (Larsen 1962, Hescox & Carlberg 1972).

Halobacterium membranes contain unique isoprenoid pigments and lipids. The red pigment is bacterioruberein (Kelly et al. 1970). Under nutritional conditions which inhibit the production of bacterioruberein (Gochnauer et al. 1972), the visual pigment retinal in bacteriorhodopsin (Oesterhelt & Steckenius 1971, Blaurock & Stoeckenius 1971) confers a purple color on the cells (see below). The glycerol di-ether phospholipids of Halobacterium contain isoprenoid phytanyl hydrocarbon chains exclusively. Both the pigment and lipid isoprenoids are probably synthesized via a common mevalonate intermediate (Kates et al. 1968, Gochnauer et al. 1972). It is of great taxonomic importance that glycerol di-ether isoprenoid phospholipids have been found only in the extremely halophilic bacteria and may be considered a trademark (for references see Kushner 1968).

Halobacterium membranes contain discrete areas or patches called "purple membranes" which contain the retinal-protein complex bacteriorhodopsin (Oesterhelt & Stoechenius 1971, Blaurock & Stoeckenius 1971). The purple membrane contains only a single protein moiety complexed with retinal in a lipid matrix, mostly phosphatidyl glycerophosphate and glycolipids (Kushwaha et al. 1975). The fascinating discovery that the purple membrane may serve as a proton pump with the resulting synthesis of adenosine tri-phosphate (ATP) (Oesterhelt & Stoeckenius 1973) has caused considerable excitment.

Purple membrane energy generation appears to be important under conditions where oxygen is limited and respiration cannot supply the cellular energy requirement. Oxygen limitations could occur during natural blooms of Halobacterium. Such an alternate energy generating mechanism, which is not dependent upon chlorophyll, has not been found previously and has potential importance in terms of evolution of photoreactive and photosensitive organisms and ecological relations in saline environments (Danon & Stoeckenius 1974).

Studies of the purple membrane pump both in vivo (Oesterhelt & Stoeckenius 1973, Danon & Steockenius 1974, Oesterhelt et al. 1974) and in reconstituted membrane vesicle systems (Racker & Stoeckenius 1973, Racker 1973, Kanner & Racker 1975) have supported the concept of a chemiosmotically-linked energy generating proton pump. Moreover, since the pump appears simple it is being used as a photophosphorylation model system. The proposed role of bacteriorhodopsin in translocation of protons has resulted in a membrane model based on X-ray

diffraction data in which the α-helical region of the bacteriorhodopsin protein is perpendicular to the membrane, oriented through the lipid bi-layer (Henderson 1975, Blaurock 1975). It is fortunate for these studies that halophilic structures are able to withstand desiccation (e.g. halobacteria can survive in crystalline NaCl), thus their structures are probably preserved rather than destroyed during preparations for X-ray diffraction analyses. Such clear examples of the relation between structure and function in biological systems are rare.

The substances in the cell envelopes of Halobacterium differ chemically and structurally from those typical of Gram-negative and Gram-positive bacteria (Kushner et al. 1964, Kushner 1968). Only small amounts of hexose sugars, hexosamines and nucleic acids are present in the outer layers of halobacteria. Notably absent are muramic and diaminopimelic acids (Brown & Shorey 1963). In most bacteria these form a rigid polymeric mucopeptide structure. Halobacterium is sensitive to mild mechanical and osmotic disruption, probably because it lacks a rigid cell wall. It has been suggested that Halobacterium does not require a rigid polymeric network because of its osmotic relations, i.e. lack of high intracellular osmotic pressure within its normal range of salt concentrations in the environment (Stanier et al. 1970).

The Halococcus species, about which very little is known, also lack muramic acid (Brown & Cho 1970, Forsyth 1971), yet these cells are extremely rigid and refractory to mechanical rupture. Alternative mechanisms for structural maintenance

must be present. The amounts and kinds of stabilizing components may be very different when compared with Halobacterium. A recently identified constituent of a Halococcus sp., 2-amino-2-deoxyguluronic acid, may be important in maintenance of the strong cell wall (Reistad 1974).

Inorganic ions exert a major stabilizing effect on the Halobacterium cell envelope. When suspended in less than 1-2M NaCl at neutral pH the envelope dissolves (Kushner & Bayley 1963). The envelope residue of lipid and carbohydrate remaining after peptic digestion requires only small amounts of salts for stability. It appears that the envelope proteins are stabilized by salts. When the salinity is reduced changes in the proteins promote the dissolution of the envelope structure (Kushner & Onishi 1966). Studies on vesicles prepared from H. cutirubrum polar lipids suggest that Mg^{+2} or Ca^{+2} have squalene-mediated access to phospholipid polar groups deep in the membrane which stabilize lipid-protein interactions (Lanyi 1971, Lanyi et al. 1974).

The external layers of Halobacterium, especially the envelope proteins, are stablized by extremely saline environmental conditions. The intracellular proteins and structures also have a requirement for salts. Lanyi (1974) has recently reviewed the properties of proteins from halophilic bacteria and documents several dozen proteins, many of which are intracellular. All of the proteins were at least stabilized, if not activated by salts such as 2M or more NaCl or KCl. These proteins can be described as halophilic.

The regulatory enzyme aspartate transcarbamylase from H. cutirubrum is an example of an intracellular halophilic enzyme. It requires about 4M NaCl or KCl for maximal activity and also retroinhibition by CTP (Liebl et al. 1969). In comparison, the enzyme of non-halophilic Saccharomyces cerevisiae is almost totally inactive and insensitive to feedback inhibition at 1 M KCl. The Halobacterium enzyme also requires salt for stability. It is extremely rapidly inactivated at low salt concentrations with a half life of a few seconds at 1M NaCl, and less than one minute at 2M NaCl. At high salt concentrations, 4-5M NaCl, the enzyme was stable with a half-life measured in days (Norberg et al. 1973).

Halobacterium ribosomes also require high salt concentrations for maintenance of the 70s form which is associated with physiological function (Bayley and Kushner 1964). Protein synthesis as measured in cell-free extracts was maximal in 3.8M KCl, 1.0M NaCl, 0.4M NH_4Cl and 0.02 - 0.04M Mg^{+2} (Bayley & Griffiths 1968a).

That proteins and ribosomes from halophilic bacteria need high salt concentrations for structural stability, and often for maximal activity is not surprising since the intracellular milieu is also saline. The intracellular cation content of Halobacterium equals or exceeds that of the medium. But the distribution is different: K^+ is the predominant internal cation (3.7-5.5M) along with less Na^+ (0.5-2.1M) and probably moderate amounts of Mg^{+2} (Christian & Waltho 1962, Ginzburg et al. 1970, Gochnauer & Kushner 1971, Lanyi & Silverman 1972, see Ch. 3). Non-halophilic Escherichia coli, in contrast, has

an intracellular content of about 0.2M K^+ and 0.05M Na^+ during logarithmic growth in a defined salts-glucose-citrate medium (0.115M Na^+, 0.005M K^+) (Schultz & Solomon 1961).

Although the halophilic character of enzymes, ribosomes and cell envelopes is striking, there is no reason to believe that basic physiological processes in halophiles such as oxidative and energy yielding metabolism, biosynthesis of amino acids, proteins and nucleic acids etc. are unique. What is unusual is their absolute dependence upon salts. Halophilic counterparts of many enzymes from non-halophilic bacteria indicate that though the halophile metabolic pathways may have some unique features, they are basically similar to those of various non-halophilic bacteria. Halobacterium nutritional requirements are extensive and complex, probably indicating insufficient enzymatic machinery for growth on simple substrates alone. A synthetic medium for extreme halophiles includes 5 amino acids, adenylic and uridylic acid, glycerol, asparagine and various salts (Onishi et al. 1965). Until recently extreme halophiles were thought to lack enzymes for glucose metobolism and pathways for utilization of carbolydrates generally (Larsen 1962). At least some extremely halophilic isolates from salterns seem to be able to metabolize glucose via a modified Entner-Doudoroff pathway (Tomlinson & Hochstein 1972, Tomlinson et al. 1974). Regulation of Halobacterium metabolic pathways on the level of feedback inhibition has been demonstrated, e.g. in the pyrimidive pathway's aspartate transcarbamylase (Liebl et al. 1969), and in the catabolic threomine deaminase (Lieberman & Lanyi 1972). Whether metabolic regulation of enzyme synthesis (i.e. repression)

operates in extreme halophiles is unknown and deserves attention.

One line of evidence which could be indicative of alien structures and processes is the lack of sensitivity of extreme halophiles to most standard antibiotics (Moore & McCarthy 1969b). One interpretation might be that halophiles have structures and metabolic processes fundamentally different from other bacteria, and thus are immune to the action of antibiotics. Certainly, part of the explanation for this effect is due to unusual structures (e.g. cell walls). Also likely, however, is that the presence of salts interferes with physio-chemical actions (Moore & McCarthy 1969b).

Perhaps the most compelling evidence for "normal" physiological function in halophiles is the data on the translation of DNA codons. Cell-free extracts from Halobacterium were used to study the incorporation of amino acids into polypeptides directed by synthetic polyribonucleotides. Only incorporation of amino acids predicted on the basis of the universal genetic code were observed (White & Bayley 1972). The conditions for minimizing incorporation errors were identical to those for maximum cell-free amino-acid incorporation activity (Bayley & Griffiths 1968a,b). Extremely halophilic bacteria appear to be similar to all cells in terms of the fundamental processes of DNA transcription and RNA translation into protein.

It is important, however, not to dismiss extreme halophiles as ordinary bacteria with a salt requirement. Attempts to convert non-halophiles into obligate halophiles have been unsuccessful (Kushner 1968). The content of amino acids in halophilic proteins as opposed to non-halophilic proteins (e.g. see Lanyi 1974)

indicates appreciable evolutionary divergence. Also, existence of a large extra-chromosomal DNA segment (Joshi et al. 1963, Moore & McCarthy 1969a,b) which may be intimately related to the genetics of the halophilic character or perhaps to the presence of salt-dependent bacteriophages (Wais et al. 1975), along with unusual cell envelopes, pigments, lipids and membrane pumps argues for their grouping as separate from most other members of the microbial world; a group of unusual organisms of unknown evolutionary affiliation.

The existence of the extremely halophilic bacteria illustrates the fantastic flexibility of the evolutionary process. Appropriate selection for cellular structures and functions must have occurred in other bacterial groups as they have in halophiles tailoring them to life processes and exploitation of other extreme environments (for example see Brock & Darland 1970, Kushner 1971b). Defining the limits, beyond which life precesses are prohibited is a difficult task, considering the tenacity of bacteria adapted to extreme environments. It has thus been deemed reasonable to conduct experiments designed to detect life on the moon and on other planets, even though they look forbidding indeed. Perhaps earth was also a forbidding place when the first primordial cells formed.

Moderately Halophilic Bacteria

The moderate halophiles can be isolated from saline environments ranging from sea water to crude solar salt. They have salt requirements for growth between those of the non-halophiles and some marine bacteria on one hand, and those of the

extreme halophiles on the other. Unlike the well-studied extremely halophilic group, the moderate halophiles are a relatively poorly understood diverse assemblage of microorganisms.

Most of the available information on the moderately halophilic bacteria comes from a handful of publications over the past 25 years or so on the Canadian strains of Vibrio costicola, and Paracoccus halodenitrificans (originally called Micrococcus halodenitrificans), and on the Japanese strains of Micrococcus 203 and Pseudomonas 101. Table 1-1 shows characteristics of these and some other recently studied moderate halophiles. Others are listed by Larsen (1962). It is significant that no review article dealing mainly with moderate halophiles has been published, although they are given some attention in several reviews concerned with halophilism (Flannery 1956, Ingram 1957, Larsen 1962, Brown 1964, Kushner 1968, Gibbons 1969). No more will be said here about moderately halophilic bacteria which are dealt with in the rest of this dissertation.

Classification of Halophilic Bacteria

Classification of bacteria on the basis of their salt requirements for growth have been considered and suggested by Flannery (1956), Ingram (1957) and Larsen (1962). Extremely halophilic bacteria have posed little classification problems because of the few taxonomic types and their characteristic of having the ability to grow at high salt concentrations where few other organisms can survive. Moderately halophilic bacteria, on the other hand, pose more difficult classification problems. In their reviews, the three authors mentioned above discuss the

TABLE I-1

Moderately halophilic bacteria under recent or current investigation[1]

organism	growth range (M NaCl)	best growth (M NaCl)	source	reference
Vibrio costicola NRC 37001, NCMB 701	0.2-4.0	0.7-2.0	bacon curing brines	Smith 1938, Robinson 1950
V. alginolyticus 138-2	0.1-1.7	0.5	marine fish	Unemoto & Hayashi 1969
Paracoccus halodenitrificans[2]	0.4-4.0	0.5-2.0	bacon curing brines	Robinson 1950, Robinson & Gibbons 1952
Micrococcus 203	0.5-3.4	1.9-2.1	salted whale meat	Hiwatashi et al 1958
Pseudomonas[3] 101	0.5-4.3	2.1	crude salt imported into Japan	Hiwatashi et al 1958
Pseudomonas Ba_1	0-4.0	0.5-1.0	unrefined Dead Sea solar salt	Rafaeli-Eshkol 1968a
Micrococcus halobius	0.5-4.0	1-2	unrefined solar salt	Onishi 1972, Onishi & Kamekura 1972
M. varians	0-4	1-3	soy sauce mash	Kamekura & Onishi 1974a
Planococcus halophilus[4] NRC 14033	0-5	0.7-1.5	possible contaminant of halophile stock culture	Novitski & Kushner 197
Bacillus 21-1	0-4	1-2	unrefined solar salt	Kamekura & Onishi 1974b

1. Not an exhaustive listing.
2. Originally called Micrococcus halodenitrificans but reclassified on basis of new taxonomic evidence.
3. In reference given it is referred to as possibly an Achromobacter, but in most papers it is referred to as Pseudomonas.
4. Novitski and Kushner (1976) use the term "Facultative halophile" to describe this strain.

problems associated with classification schemes.

All cells require at least traces of salts in their environments; therefore, to that extent all bacteria might be called halophilic. The original intent, however, in using the term halophile was to describe organisms which had requirements for salts above trace levels. Two schools exist with respect to use of the term halophile in its original context. Some authors prefer to use it to denote a cellular salt requirement for minimal growth; others use it to denote a salt requirement for best growth, regardless of the minimal requirement (see Ingram 1957).

It is difficult to justify choosing either definition on physiological and ecological grounds. Survival of a strain in a circumstance may depend upon the minimal requirement for salts; exploitation of the environment may depend upon the salt requirements for rapid growth. Both survival and growth may be important selective parameters in natural environments.

A recent study by Novitski & Kushner (1975) illustrates a difficulty in classifying halophilic bacteria, especially moderately halophilic strains, on the basis of minimal requirement for salts. A halophilic coccus, now called Planococcus halophilus (Novitski & Kushner 1976), is capable of growth over a wider range of NaCl concentrations than any other bacterium previously described. Growth was possible in the presence of 5.5M NaCl and at almost no salt, 0.09M NaCl, at 20 or 30C. At 37C, a commonly used temperature for growing halophiles, the NaCl requirement for growth increased to 0.5M. Thus, this organism could be classed as either an extremely halotolerant or a

moderately halophilic organism. The authors have called it a "facultative halophile" using Flannery's (1956) definition. They suggest that organisms previously classified as moderate halophiles might exhibit growth over wider NaCl concentrations if growth response at incubation temperatures other than the "optimum" were checked.

The only systematic study of the effects of temperature on the salt requirement of moderately halophilic bacteria was carried out by Ishida (1970). Isolates from solar salt (Ishida & Fujii 1970) were divided into two groups: obligate halophiles not able to grow in the absence of added salts and facultative halophiles able to grow without added salts, (the extreme halophiles were excluded from this study). 25 of 27 strains of obligate halophiles belonging to the genera Flavobacterium and Brevibacterium were able to grow at lower salt concentrations if the temperature was lowered from 35C to 27 or 22C. Most of the 21 facultative halophiles, predominately Gram-positive cocci, were able to grow well, but suboptimally, in the absence of added NaCl (the "no salt" medium actually contained less than 0.1M NaCl) at all temperatures tested, 22,27 or 35C.

The obligate group's NaCl requirement for optimum growth changed less than the minimum NaCl requirement for growth as the temperature was decreased. In about half of the strains the NaCl optimum for growth dropped as the temperature was decreased from 37 to 22C. Most of the remaining obligate halophiles were insensitive to temperature changes. The facultative group clustered around a 5% NaCl optimum for maximum growth with a slight tendency toward an

increase in the NaCl concentration for maximum growth as the temperature was lowered (7/21 strains).

Ishida's data indicate that there may be no consistent patterns of temperature - NaCl relations in halophiles in general. But underlying physiological differences governing salt response between the Gram-positive cocci and the Gram-negative rods deserve more research effort. Ishida's Gram-negative halophilic isolates with NaCl optima at 10-20% NaCl required less salt at lower temperatures while the facultative Gram-positive cocci were generally less sensitive to temperature changes. Only about 15% of the bacteria isolated from the oceans are Gram-positive cocci which seem to have minimum requirements for NaCl similar to those of terrestrial strains and lower that most of the Gram-negative marine organisms (MacLeod 1968).

Examination of other references gives a fragmented picture of the importance of temperature and other environmental factors on salt requirements and tolerances. Data of Christian (unpublished, see Ingram 1957) shows that the NaCl requirement of the extreme halophile H. halobium decreases as the incubation temperature is lowered from 45C to 37C. An extremely halophilic coccus, Sarcina morruhuae, requires 12% NaCl at 37C but can grow at 9% NaCl at 30C (Forsyth & Shindler unpublished observation). Some bacteria from marine sources show a decreased salinity requirement when the temperature is lowered (Stanley & Morita 1968, Ishida et al. 1974, Morita 1975). The type of oxidizable substrates available to marine bacteria can effect the minimum requirements for Na^+ (see MacLeod 1965, 1968). Several marine bacteria can grow under higher hydrostatic pressures if the salt concentration is

increased (Albright & Hanigman 1971). Non-halophilic bacteria are individualistic in their responses to salinity and temperature. Escherichia coli and typhoid bacteria have been reported by Dumesh in the 1930's to grow in 25% NaCl at 5-8C but not in 10% at 37C (see Ingram 1957). A recent study of Salmonella strains demonstrated an increased salt tolerance up to about 8% NaCl as the temperature was raised to 37C. The overall picture indicates a tendency, by no means universal, for bacteria to require or tolerate higher concentrations of salts as the temperature is raised (and vice-versa).

In view of the possible physiological variability governing minimal salt requirements it seems reasonable to devise a classification scheme for the halophiles which is based on more stable physiological characteristics. A classification based on the NaCl concentration range for optimum growth which appears to meet the requirement is proposed in Table 1-2. Although nutrition, temperature, the presence of other salts, and the methods used to measure bacterial growth (i.e. yield vs. growth rate) might shift, expand or contract the range slightly, it is doubtful that this would require an organism to be reclassified from one group to another, unless the organism in question was already on the borderline between two groups. The groupings are not complex; excessive complexity only obscures the observation of true physiological differences between or within classes. Moreover, the groupings are similar enough to previous classifications that bacteria previously classed as moderately or extremely halophilic remain in the same category.

The rationale for including a group which is intermediate between the non-halophiles and the moderate halophiles deserves

TABLE I-2

Classification of Bacteria by Salt Response[1]

Category	Reaction
Non-halophile	Grows best in media containing less than 0.2 M salt[2].
Slight-halophile	Grows best in media containing 0.2-0.5M salt.
Moderate-halophile	Grows best in media containing 0.5-2.5M salt. If a moderate halophile is able to grow in presence of less than 0.09M salt it is classed as a "facultative" strain.
Extreme-halophile	Grows best in media containing 2.5-5.0+M salt.
Halotolerant	Non-halophile which is able to tolerate salt. If the growth of a halotolerant bacterium extends into the range of best growth of a moderate halophile it is classed as "moderately" halotolerant; if the growth extends above 2.5M salt it is classed as "extremely" halotolerant.

1. This classification is an extended and modified version of that presented by Larsen (1962).

2. "Salt" usually refers to NaCl but can be an appropriate mixture of other ions in addition to a minimum amount of NaCl.

discussion. At first it was felt that there was no necessity for including the slightly halophilic category. But examination of recent literature on bacteria isolated from marine environments indicated that a potentially large number of strains exist with salt optima for best growth around 0.2-0.3M (MacLeod 1965, 1968, Hidaka & Sakai 1968, Kakimoto et al. 1974, Pratt & Tedder 1974). Not all bacteria from marine sources fall into this category, of course, because of the contiguity of marine environments with terrestrial, fresh water and airborne bacterial flora and also because of the variability of salinity, temperature, pressure etc. in the oceans themselves.

It goes without saying that the classification scheme presented here (Table 1-2) or any one for that matter, are not intended to be applied extremely rigidly. As new physiolocial information becomes available, more suitable classifications may become obvious, in which case this one, although presently useful, should be discarded. One very promising area of investigation toward a better physiological basis for classification schemes is the examination of little known halophilic cocci. Preliminary evidence gives support for the possible future separation of moderate halophiles into two distince groups; the Gram-positive cocci and the Gram-negative rods.

Goals of Thesis

The goal of this thesis is to present work on certain physiological aspects of Vibrio costicola, a moderate halophile. V. costicola is one of the better known "typical" halophilic bacteria. A few substantial biochemical and physiological

studies have provided a background of information upon which the present work is based. The available information on V. costicola is reviewed in the following chapters of the thesis.

Chapter II deals with studies on the growth and behaviour of V. costicola in synthetic salts-glucose media along with a discussion on its occurrence and ecology in relation to other moderately halophilic bacteria. The intracellular ionic composition is investigated in Chapter III. Understanding of the ionic relations of V. costicola is crucial to the analyses of the salt responses and characteristics of the biosynthetic enzymes aspartate transcarbamylase and threonine deaminase which follow in Chapters IV and V. Data is included on the feedback inhibition and repression of the threonine deaminase activity. It is hoped that the present studies will contribute to a better understanding of the physiology of moderately halophilic bacteria, and be helpful to other investigators working with V. costicola.

Chapter II

Vibrio costicola: aspects of growth, taxonomy, and ecology.

The first detailed study of moderately halophilic bacteria was carried out by Smith in Australia in 1938. As part of the processing of bacon, pork ribs are marinated in a saline brine. A "taint" sometimes developed in the rib tissues resulting in a degradation of the meat around the bones and "malodour". During Smith's investigation of this economically annoying taint, he isolated two halophilic bacteria implicated in the spoilage which he named Vibrio costicolus and Vibrio halonitrificans. He found that if the salinity of the curing brine were kept above 24% (w/v NaCl) these halophilic bacteria did not grow in the animal tissue. The organisms were adapted to growth in a moderately saline environment but were inhibited under extremely saline conditions. Although Smith's isolates apparently have been lost, many recent studies on V. costicola, including those in this dissertation, have utilized the strain isolated from bacon curing brine in Canada (Robinson 1950).

The decision to use V. costicola for this work stems from several considerations. The organism chosen is a genuine moderately halophilic bacterium using the definitions of several workers; its halophilic status is not in doubt. Background knowledge and experience is available on V. costicola strains from studies dating back to those of Smith (1938). Moreover, the organism has attributes which make it suitable for physiological studies; it is nutritionally versatile and

has a reasonably rapid growth rate. It is able to grow in a minimal medium (NaCl-salts-glucose, Forsyth & Kushner 1970). A stationary phase culture can be harvested from 1M NaCl minimal medium after about 48 hours. Good growth in minimal medium makes V. costicola suitable for experiments on metabolic regulation such as enzymatic regulation and repression (see Chapter 5).

This chapter begins with a discussion of the history and taxonomy of V. costicola. Before physiological studies were undertaken it was also necessary to characterize the growth properties of this organism further. The growth media, methods and results are presented here; they will be referred to in later chapters. Finally, comments on the occurrence and ecology of V. costicola are presented in relation to information on growth and taxonomy.

History and taxonomy of the strain of V. costicola used in this dissertation.

Isolation and characteristics

The strain of V. costicola used in this work was isolated around 1950 by John Robinson from bacon curing brines at the Canada Packers Ltd. plant in Hull, Quebec, Canada. It was originally deposited in the National Research Council of Canada Culture Collection, Ottawa, as Vibrio strain #75. It was later redesignated as NRC #508, then around 1970 once again redesignated as NRC #37001. Some characteristics of the strain were presented in Robinson's doctoral thesis (1950). Flannery et al. (1952) stated that most of the characteristics of the strain

did not differ greatly from the strain described by Smith (1938). Recently NRC personnel have rechecked the characteristics of Robinson's strain (NRC #37001). The results are shown in comparison to other V. costicola strains in Table II-1. With one major exception, motility, there appear no major differences between Smith's strain, Robinson's description and the recent tests, in spite of many possible methodological variations between tests done in different eras, let alone in different laboratories.

Our strain of V. costicola was not motile. This loss of motility has also been confirmed by NRC personnel (L. Stryde, R. Latta personal communications). As far as is possible to recall, active motility was never observed with NRC #508 or NRC #37001. Possibly during the many stock transfers a non-motile variant was transferred. Changes such as motility loss are not uncommon in strains stored over long periods.

Gibbons (1969) pointed out that both V. costicola taxonomy and nomenclature were in doubt. He stated that Robinson's strain, then called V. costicolus, was not a Vibrio because it was not sensitive to vibriostatic agent 0/129 (Shewan et al. 1954, Bain & Shewan 1968), and that it moreover should have the species name costicola (L. rib dweller). Sensitivity to vibriostatic agent 0/129 is, indeed, an important criterion for inclusion in the genus Vibrio (Park 1962, Colwell & Gochnauer 1968). My results (below) indicate that V. costicola is vibriostat sensitive. The nomenclature question was settled when, in the 8th edition of Bergey's manual (Buchanan & Gibbons 1974), the specific name of this strain was changed to costicola.

I reexamined the sensitivity of V. costicola (NRC #37001) to vibriostatic agent 0/129. For the test 1mg/ml 2,4-diamino-6,7-diisopropyl pteridine (B-grade, Calbiochem Co., California) dissolved in acetone was applied drop-wise to 12.7mm diameter paper filter discs (type 740-E Schleicher & Schuell, Keene, N. H.) to achieve a concentration of 500µg per disc. Discs containing the pteridine as well as control discs with same volume of acetone applied were placed on the surface of 1M NaCl proteose-peptone and trytone (PPT) agar medium which had been seeded with V. costicola. After a 30C 2 day incubation a clear zone, indicating complete growth inhibition, extended 3-5mm from the edges of the discs containing the pteridine. The control discs had no inhibitory effects; the lawn of V. costicola grew up to the discs' edges. As a comparison, Vibrio metchnikovii (ATCC #7708) seeded on trypticase soy agar (Baltimore Biological Laboratories, Inc.) was subjected to the same test. After a 37C 2 day incubation a 2mm zone of complete inhibition was evident which was surrounded by a further 8mm zone of partial inhibition.

Although Gibbons (1969) did not state the conditions under which his test was run, it is probable that he did not observe inhibition because the action of agent 0/129 is inhibitied by salts in the medium (Merkel 1972). If the contents of the active vibriostatic ingredient in the filter paper disc is less than 100µg, a medium NaCl level of several percent will render it ineffective. I used 500µg per disc. In another independent test, discs soaked in saturated 0/129 in acetone and then placed on seeded plates resulted in a zone of partial

inhibition up to 11mm from the disc's edge (L. Stryde, personal communication).

Morphology of V. costicola

V. costicola was comma shaped and grew singly and in chains. At salt concentrations near the lower extreme permitting growth, the chains were tightly coiled and the cells smaller than when grown near or above the optimum salt concentration. Large round swollen cell structures, two to five times the average diameter of normal cells, often at the ends of chains of cells, occurred in 1-5% of the cells at low medium salt concentrations or in stationary phase cultures. Except for the observations on the swollen cells, which Robinson (1950) did not report, my observations on the morphological characteristics of V. costicola are in basic agreement with his.

The presence of such "round bodies" is probably a common trait in many Vibrio strains (Christian & Ingram 1959, Kennedy et al. 1970, Colwell 1974). Christian and Ingram (1959) concluded that such V. costicola structures were cells in which the cell walls had ruptured with the extrusion of membrane bound cytoplasm. Thin-section electron micrographs of a marine Vibrio indicated that the "round bodies" were surrounded by an apparently intact cell wall (Kennedy et al. 1970). The authors concluded that plasticity and accelerated local growth of the cell wall was responsible for the swollen structures. Colwell (1974) called the "round bodies" sphaeroplasts.

The causes of sphaeroplast or round body formation in V. costicola may be related to the role of ions in stabilization of the cell wall structure combined with intracellular osmotic pressure effects. In Halobacterium sodium and magnesium are required to maintain the structural integrity of the cell envelope (see Ch. 1, p.8). In the moderate halophile Paracoccus halodenitrificans if the cell walls were stabilized by the presence of Ca^{+2} or Mg^{+2} ions, the lower limiting NaCl concentration for growth was lowered from 0.55M to 0.3M (Takahashi & Gibbons 1959). Leaky membranes, especially at low salt concentrations, were probably responsible for the escape of DNA which formed a slime layer coat on V. costicola and P. halodenitrificans (Smithies & Gibbons 1955, Takahashi & Gibbons 1957). MacLeod (1968) and co-workers have demonstrated that decreasing the concentration of sea water salts in which a marine bacterium was suspended, resulted in release of cell wall material followed by osmotic lysis. Possibly, the round bodies of V. costicola grown at low salt concentrations resulted from decreases in cell wall strength with consequent osmotic swelling.

Relationship of V. costicola NRC #37001 to other Vibrio strains.

It is unclear if the NRC strain of V. costicola is the type strain of the species. The origin of the type strain of V. costicola listed in Bergey's Manual as NCMB* #701 (Buchanan & Gibbons 1974), and the strain listed in the Handbook of Microbiology (Colwell 1974), presumably the same one as in Bergey's, is presently uncertain. The type strain originated

from N. E. Gibbons at NRC Ottawa, Canada (N. E. Gibbons personal communication, M. S. Hendrie, NCMB personal communication). It was likely Robinson's strain. But apparently NCMB lost the strain and requested another culture from M. Ingram who may have been in possession of both Smith's (1938) original V. costicola strain and Robinson's strain. Records are presently being checked carefully to determine the origin of the strain which Ingram supplied to NCMB. Characteristics of the type strain are shown in the last two columns of Table II-1. Some of the differences in characteristics between the different strains may be due to methodological differences and may not be real. Of particular importance may be such factors as salt concentration in the test media, composition of sugar oxidation media, sensitivity of the H_2S production test and incubation temperature. It has been suggested that the NCMB and NRC strains ought to be checked side by side both in Canada and NCMB, Scotland to see if they are identical (S. Martin, NRC, personal communication, M. S. Hendrie, NCMB, personal communication). The origin and taxonomy of V. costicola strains is important to investigators who want to compare their results with those of other workers. It is encouraging that the strains do not exhibit great differences.

The relationships of V. costicola to other moderately halophilic bacteria are practically unknown. Colwell and Gibbons (unpublished) have done a numerical taxonomy study on halophiles and point out that the NRC strain of V. costicola

* National Collection of Marine Bacteria, Aberdeen, Scotland

Table II-1

Some taxonomic features of Vibrio costicola strains.

	origin or location of strain				
characteristic	Smith (1938)	Robinson (1950)	NRC 37001[a]	NCMB 701[b]	Listed in "Handbook of Microbiology"[c]
motility	+	+	-	+	+
DNA %(G + C)				50	50
indole reaction	-	-	-	-	-
methyl red reaction			(+)	-	-
Voges-Proskauer reaction	-	+	(+)	v	+
citrate utilization			-	-	-
sensitivity:					
0/129			+	v	v
novobiocin			+	+	-
terramycin			+		
growth:					
1% NaCl	-	-	-	-	-
7% NaCl	+	+	+	+	+
10% NaCl	+	+	+	+	+

Table II-1 (Con't)

characteristic	Smith	Robinson	NRC	NCMB	"Handbook"
acid production from:					
arabinose	-	-		-	-
inositol				-	-
mannitol		+	+	v	v
mannose	+	-		+	+
salicin	-			-	-
sucrose	+	+	+	+	+
glucose	+	+	+	+	+
glycerol			+		
gelatin liquefaction	v	+	+	v	v
hydrolysis of:					
casein	-	-	-	-	-
starch	-	-	-	-	-
tween 80®	-	-	-	v	+
H_2S production	+	+	(+)	-	-

KEY: + = positive, - = negative, (+) = weak positive, v = variable.

a. The strain used in this dissertation. Originally isolated and described by Robinson (1950) (preceeding column). Now known as National Research Council of Canada culture collection #37001. Data from S. Martin, L. Stryde and R. Latta (NRC, personal communications) and from this dissertation.

b. National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland strain #701. Data from Buchanan & Gibbons - "Bergeys Manual" (1974). Listed as the type strain of V. costicola.

c. Data from Colwell (1974) in "Handbook of Microbiology". The characteristics are presumably those of NCMB 701.

is probably identical to Pseudomonas beijerinckii (ATCC #19372 but distinct from yet another strain of V. costicola held by the Institut Pasteur, Lille, France.

Information on the taxonomic relationship of V. costicola to other Vibrio species is also scarce. Data of Hanaoka et al. (1969) from DNA hybridization experiments showed that V. costicola NCMB #701, was most closely related to V. anguillarum NCMB #829, V. ichthyodermis NCMB #1291, V. piscium NCMB #571 and V. metchnikovii IAM #1039. All of these strains had DNA with at least 80% homology to V. costicola DNA. V. parahemolyticus strains and V. cholera El Tor were only 20-30% homologous. The DNA reassociation data of Staley and Colwell (1973) are in substantial agreement.

Anderson and Ordal (1972) proposed a formula to calculate the percentage of mispaired bases between marine Vibrio strains based on DNA homology data:

$$\text{mispaired base \%} = \frac{(100 - \text{\% DNA homology})}{6.8}$$

The formula is used here as an interesting approximation of DNA differences. Using the data of Hanaoka et al. (1969); compared to V. costicola (NCMB #701), V. metchnikovii (95% homology) had only 1% mispaired bases, while V. anguilarum and V. ichthyodermis (80-82%) had about 3% mispaired bases. V. costicola appears quite closely related to a strain which causes a cholera-like disease in animals (V. metchnikovii) and to strains which are associated with diseased states of fresh water and marine fish. V. costicola has not, however, been

shown to be a disease-causing organism, nor is it closely related to the well studied pathogen, V. parahemolyticus.

V. costicola growth

Methods and Materials.

Media Table II-2 shows the reagent grade constituents of the synthetic media. A solution containing the medium salts without glucose was made up and the pH adjusted with NaOH using a low sodium error Corning 476050 combination electrode to ensure a final pH 7.6 ± 0.1 (the amount of additional Na^+ ion added as hydroxide was 0.01M). A separate 10% (w/v) D-glucose solution was added to the salts solution after both solutions were steam sterilized at 121C for 15 min. An alternate method of sterilization involved filtration of complete medium through 0.2μ membrane filters.

One of the complex media used was PPT: 1% (w/v) Difco proteose peptone plus 1% Difco tryptone in addition to the specified amount of NaCl, pH 7.6 ± 0.1, autoclaved at 121C for 15 minutes. With the addition of 15g/l Difco agar this medium was used for storage of V. costicola in slant cultures and for plating experiments. Other complex media used were CAA: 1% (w/v) Difco vitamin-free casamino acids, 0.1 g/l $MgSO_4.7H_2O$; SGS + CAA; the SGS formulation (Tab. II-2) supplemented with 0.5% (w/v) Difco vitamin-free casamino acids; and Difco 2216 marine broth. Each medium included an appropriate amount of NaCl, was adjusted to pH 7.6 ± 0.1 and autoclaved at 121C for 15 minutes.

Table II-2

Constituents and ionic contents of synthetic medium used for growth of V. costicola

Constituent[a]	MEDIA BSG	SGP	SGS
NaCl	58.44 g/l[b]	58.44 g/l[b]	58.44 g/l[b]
K_2HPO_4	3.12	12.48	
KH_2PO_4	0.28	1.12	1.12
Na_2HPO_4			10.17
$MgSO_4$	0.049	0.049	0.049
NH_4Cl	2.0	2.0	2.0
$(NH_4)_2SO4$	2.0	2.0	2.0
glucose	10.0[c] (pH 7.6±1)	10.0[c] (pH 7.6±1)	10.0[c] (pH 7.6±1)
Major ions			
Na^+	1.0 M	1.0 M	1.14 M
K^+	0.038	0.152	0.008
Mg^{+2}	0.00041	0.00041	0.00041
NH_4^+	0.068	0.068	0.068
Cl^-	1.04	1.04	1.04
PO_4^{-3}	0.02	0.08	0.08
SO_4^{-2}	0.016	0.016	0.016

a Anhydrous chemicals normally used; hydrated substances can be used if amounts are increased appropriately.

b Amounts of NaCl stated are for 1M NaCl media.

c Glucose concentration: 0.056M.

Abbreviations: BSG, basal salts-glucose; SGP, salts-glucose-potassium phosphate buffer; SGS, salts-glucoss-sodium phosphate buffer.

Cultures V. costicola stock cultures were stored on 1M NaCl PPT agar slants at 4C. Every 20 weeks a fresh PPT slant was loop-inoculated from the previous stock culture, incubated 4 days at 30C, then refrigerated.

When a culture was required, 10ml 1M NaCl PPT medium contained in a 16mm culture tube was inoculated from a stock slant, then incubated at 30C for 24-36 hours on a reciprocal shaker at a rate of 100 strokes/min with the tube mounted 30° from the horizontal. The PPT culture (0.1 ml) was inoculated into a 50 or 100ml flask preculture (the size of the Erlenmeyer flask was either 250 or 500ml respectively) of the medium desired in the final culture. The preculture was incubated at 30C on a reciprocal shaker (100 strokes/min, excursion 30mm) until late exponential phase was reached. The preculture was checked for purity using a Wild M-20 phase-contrast microscope then a 1% (v/v) inoculum was generally taken for the final culture which was usually incubated and agitated at 30C. The rate of shaking was 100 strokes/min for 50 or 100ml cultures, but somewhat slower (about 50 strokes/min) for 1ℓ cultures in 2.8ℓ Fernbach flasks to achieve good aeration while preventing excessive splashing.

Measurement of growth Growth was monitored on a Coleman Jr. II 6/20 spectrophotometer at 660nm using round 18mm path-length cuvettes. In the growth experiments special 250 or 500ml culture flasks with attached 18mm path-length "side-arm" cuvettes were used for incubation and repeated determinations of the culture absorbance. The sealed side-

arm flask could simply be tilted so that the medium ran into the cuvette which was then inserted into the spectrophotometer for absorbance measurement. Use of the side-arm culture flasks avoided repeated sampling of cultures which would have resulted in culture volume changes and increased chances of contamination. Cuvettes containing the appropriate media were used as blanks.

Growth rates were determined from repeated measurements of 660nm culture absorbance. Semi-log plots of absorbance vs. time allowed determination of the beginning and end of exponential growth. The 5-10 exponential phase absorbance readings were converted to $\log_2$ values and regressed vs. time on a Hewlett-Packard 9810A calculator yielding the growth rate as generations per hour.

To obtain information on the relationship between culture 660nm absorbance and cell numbers near the point where cultures were usually harvested for physiological experiments, counts were carried out using a Petroff-Hausser counting chamber mounted on a Wild M-20 phase-contrast microscope. 20 independent counts were used to determine the following relationship:

At a 660nm absorbance of 0.5 with 1M NaCl SGS grown cells (mid exponential phase) there were 13×10^8 cells/ml, 2.0 mg cell wet weight/ml and 0.65 mg cell dry weight/ml.

Results of growth studies

Although studies on the growth of V. costicola had been carried out (Flannery & Kennedy 1962, Forsyth & Kushner

1970) it was necessary to further characterize the growth response. Measurements of an important growth parameter, exponential phase growth rate as a function of medium NaCl concentration had not been done and are reported here. Modifications to make synthetic media more suitable for physiological studies on intracellular ionic content were also carried out and the growth response examined in the modified media.

Other aspects of V. costicola growth presented in this section include experiments on the lowest and highest salt concentrations which allow growth and some thoughts on the natural habit and ecology of V. costicola.

Growth of V. costicola in defined minimal media.

Flannery and Kennedy (1962) designed a simple synthetic medium for V. costicola containing glucose, salts and several amino acids. Forsyth and Kushner (1970) found that the amino acids were not essential for good growth if a source of ammonium is included (instead of nitrate in Flannery and Kennedy's medium) in addition to the glucose, NaCl and other salts. The resulting basal-salts-glucose formulation (BSG, Tab. II-2) is a minimal medium with glucose as the sole carbon and engery source. The buffer in BSG is 0.02M phosphate (Table. II-2). Forsyth and Kushner (1970) also reported that a substantial improvement in cellular growth yield resulted from increasing the phosphate concentration in BSG from 0.02 M to 0.08 M. The exponential growth rate of V. costicola in the resulting medium, salts-glucose-

potassium phosphate (SGP, Table II-2), was not examined by Forsyth and Kushner (1970). My experiments show that increasing the potassium phosphate concentration to 0.08 M did not significantly affect the exponential growth rate but did result in a doubling of both the exponential phase duration and stationary phase absorbance (Tab. II-3). The final pH level was about 0.6 unit higher compared to BSG.

Since addition of extra potassium phosphate to BSG resulted in a large increase in the medium K^+ concentration as well as in phosphate concentration, it was important to determine if BSG medium contained a K^+ concentration which was growth-limiting. Addition to KCl to BSG did not result in any significant growth stimulation (Table II-3). Thus the increase in growth was most likely due to the increased buffering capacity of the extra phosphate. The pH values at the end of growth indicate also that tris was an ineffective substitute buffer.

Another possibility, much less likely, is that a phosphate growth limitation was overcome by the addition of extra phosphate to BSG. Although normal phosphorous requirements of cells are well below the levels in BSG, it is not known whether halophilic bacteria have a higher requirement. All experiments referred to in this dissertation which utilized minimal medium formulations had 0.08 M phosphate concentrations (i.e. the SGP and SGS formulations, Tab. II-2) to avoid possible phosphate growth limitations, and as well, to take advantage of the better buffering power and the consequent increased growth yield.

Table II-3

Effect of buffers and potassium on growth of Vibrio costicola in salts-glucose media

addition to salts-glucose (BSG) medium[a]	buffer	$[Na^+]M$	$[K^+]M$	lag,hr	R gen hr^{-1}	absorbance 660nm end log phase	absorbance 660nm station.ph.	station. phase pH
none	0.02M PO_4^{-3}	1.0	0.038	10.8[b]	0.36[c]	0.45	0.56	5.4
0.06M PO_4^{-3}[d]	0.08M PO_4^{-3}	1.0	0.15	11.9	0.40	0.86	1.25	6.0
0.1125M KCl	0.02M PO_4^{-3}	1.0	0.15	11.2	0.39	0.58	0.69	5.2
0.1M tris	0.02M PO_4^{-3} 0.10M tris	1.0	0.038	10.7	0.40	0.51	0.83	5.3
0.1M tris 0.1M KCl	0.02M PO_4^{-3} 0.10M tris	1.0	0.15	10.5	0.40	0.48	0.75	5.3

a. BSG medium components g/l: NaCl 58.44, K_2HPO_4 3.12, KH_2PO_4 0.28, $MgSO_4$ 0.049, NH_4Cl 2.0, $(NH_4)_2SO_4$ 2.0, glucose 10, pH 7.6 $\pm$ 1. Incubation at 29 $\pm$ 1°C.

b. Interval before resumption of exponential growth; 0.2% v/v inoculum was mid-exponential phase cells growing in SGP medium.

c. Growth rate during expoential (log) phase, mean of two determinations.

d. Addition of 0.06M PO_4^{-3} as potassium salt to BSG medium produces a medium identical to the SGP formulation.

The better growth in the medium with extra potassium phosphate (Tab. II-2, SGP) was desirable. But the SGP formulation had an unusually high potassium concentration. It was necessary for some experiments to utilize a defined medium with a lower K^+ level, similar to that of other media used for V. costicola cultures. The proteose-peptone and tryptone (PPT) medium used by Christian and Waltho (1962) for their work on the salt content of V. costicola, for instance, contained 0.004 M K^+, compared with 0.152 M K^+ in SGP. The simplest way to lower the medium K^+ content without adversely affecting the phosphate buffer system was to substitute an equimolar quanitity of Na_2HPO_4 for the K_2HPO_4 of SGP.

The resulting salts-glucose-sodium phosphate (SGS) formulation differed from SGP in having more Na^+ and less K^+ (Tab. II-2). The low level of K^+ (0.008M) is SGS did not appear to radically modify the exponential phase growth rates of V. costicola compared to growth in SGP. The SGS medium may have caused slight changes in the duration of exponential growth at various salt concentrations compared to SGP (Fig. II-2), but the growth yields were similar (not shown). The durations of the lag phase in both SGS and SGP are also similar.

Effects of salt concentration on growth in various media.

Exponential growth rates of V. costicola in proteose-peptone and tryptone (PPT) complex medium and in SGS and SGP synthetic media are shown in Fig. II-1. Both the growth rate and the range of salt concentration which allowed growth were greater in the complex media (data on maximum growth (Forsyth & Kushner 1970) show the same pattern).* In the synthetic media the optimum salt concentration for growth was lower (near 1M) than in the complex medium (about 1.25M).

Fig. II-2 shows measurements of other growth parameters in SGP and SGS media. The two synthetic media were similar in lag phase duration, showing minima at 1-1.2M. In SGP medium the culture absorbance at the end of exponential growth was slightly higher than in SGS medium. The different Na^+/K^+ ratio of the media may have affected growth parameters slightly, although no major differences were ever observed between SGP and SGS media. The maximal growth yield curve for SGP medium shows that the yield of cells was not dependent upon the rate of growth. The yield was maximal at 1.25-2M NaCl, yet the range of peak exponential growth occurred around 1M NaCl. Figs. II-3 and II-4 shown typical growth curves and semi-log exponential growth plots of SGS growth data.

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* Water activity (a_w), a measure of the amount of water which is available or free after solute interactions are taken into account, has been used by some authors instead of salt concentration. The range of NaCl concentration for growth in PPT, 0.2 - 4.0M NaCl, corresponds to a_w 0.99 - 0.85. (For discussions of a_w see Scott 1957, Ingram 1957, Kushner 1971).

Fig. II-1. *V. costicola* exponential growth rates as a function of medium salt concentration in complex and synthetic media. 1% proteose peptone + 1% tryptone medium (PPT, top) salts-glucose-sodium phosphate medium (SGS, middle), salts-glucose-potassium phosphate medium (SGP, bottom).

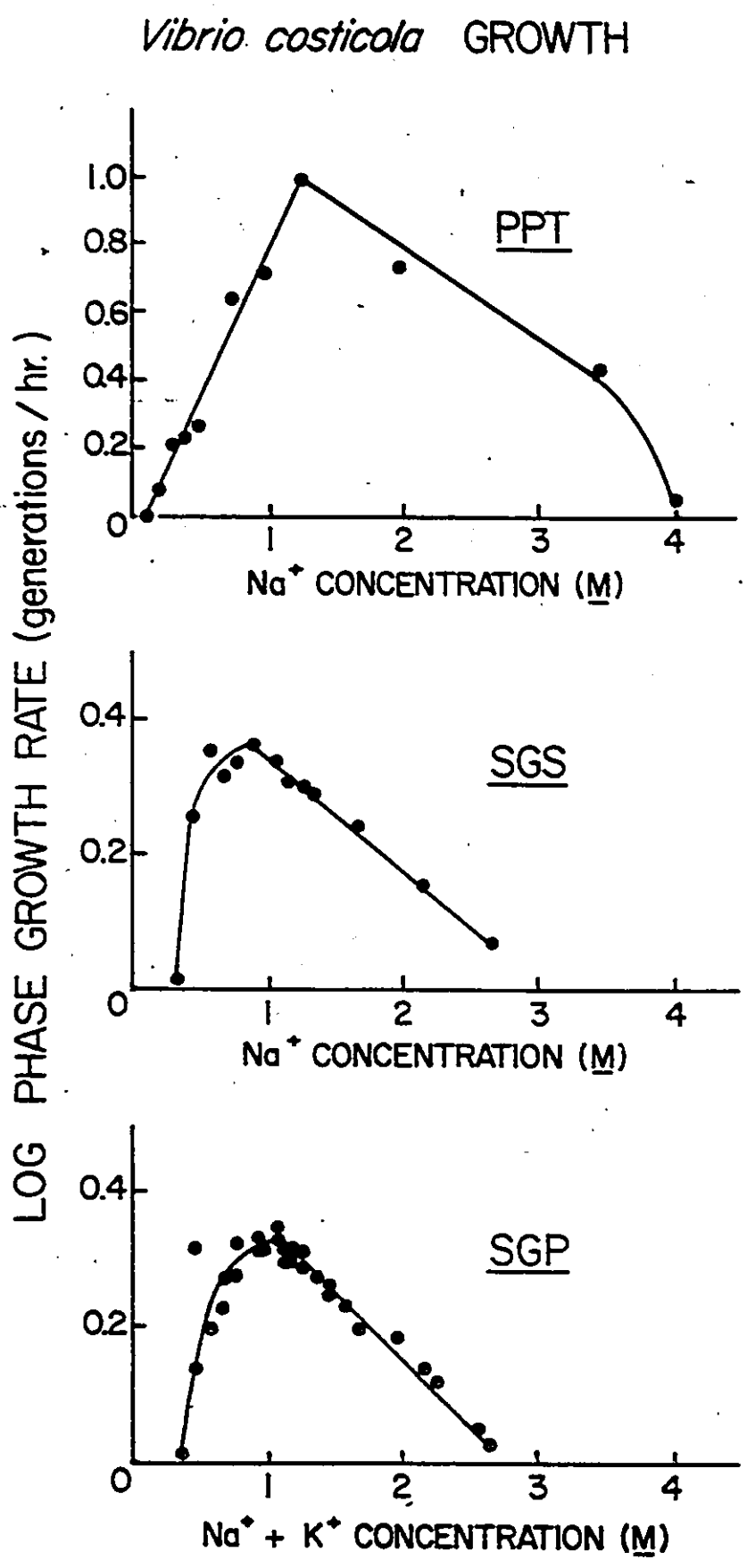

Vibrio costicola GROWTH
LOG PHASE GROWTH RATE (generations / hr.)
PPT
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
Na⁺ CONCENTRATION (M)
SGS
0.4
0.2
0
1
2
3
4
Na⁺ CONCENTRATION (M)
SGP
0.4
0.2
0
1
2
3
4
Na⁺ + K⁺ CONCENTRATION (M)

Fig. II-2. *V. costicola* growth as a function of medium salt concentration in synthetic media. Upper plots: maximum 660nm absorbance in SGP medium and absorbance reached at end of exponential growth in SGP and SGS media. Bottom: Duration of log phase in SGS and SGP media.

Vibrio costicola GROWTH

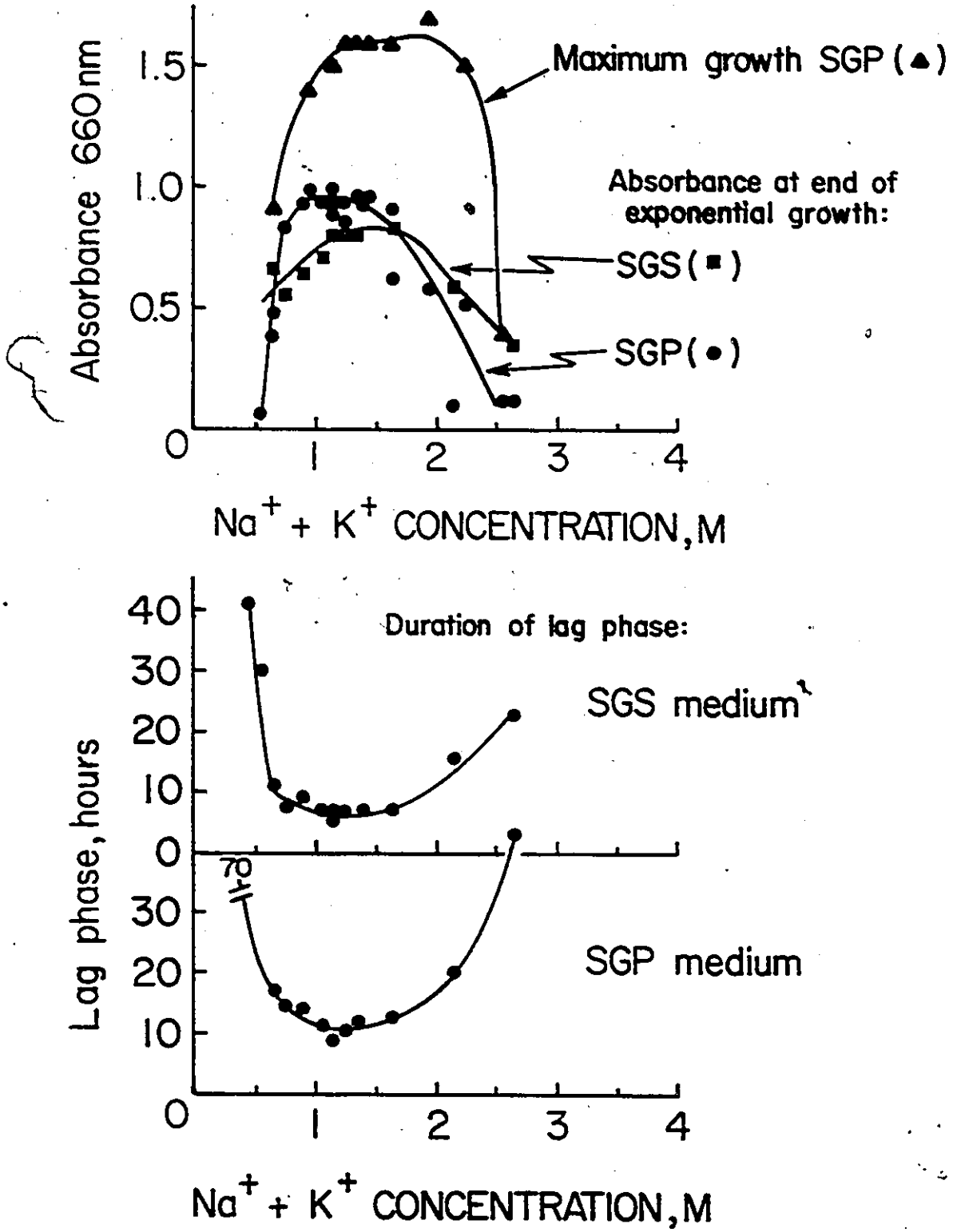


Fig. II-3. *V. costicola* growth as a function of time in SGS medium at several medium sodium concentrations. Stationary phase pH values given in parenthesis.

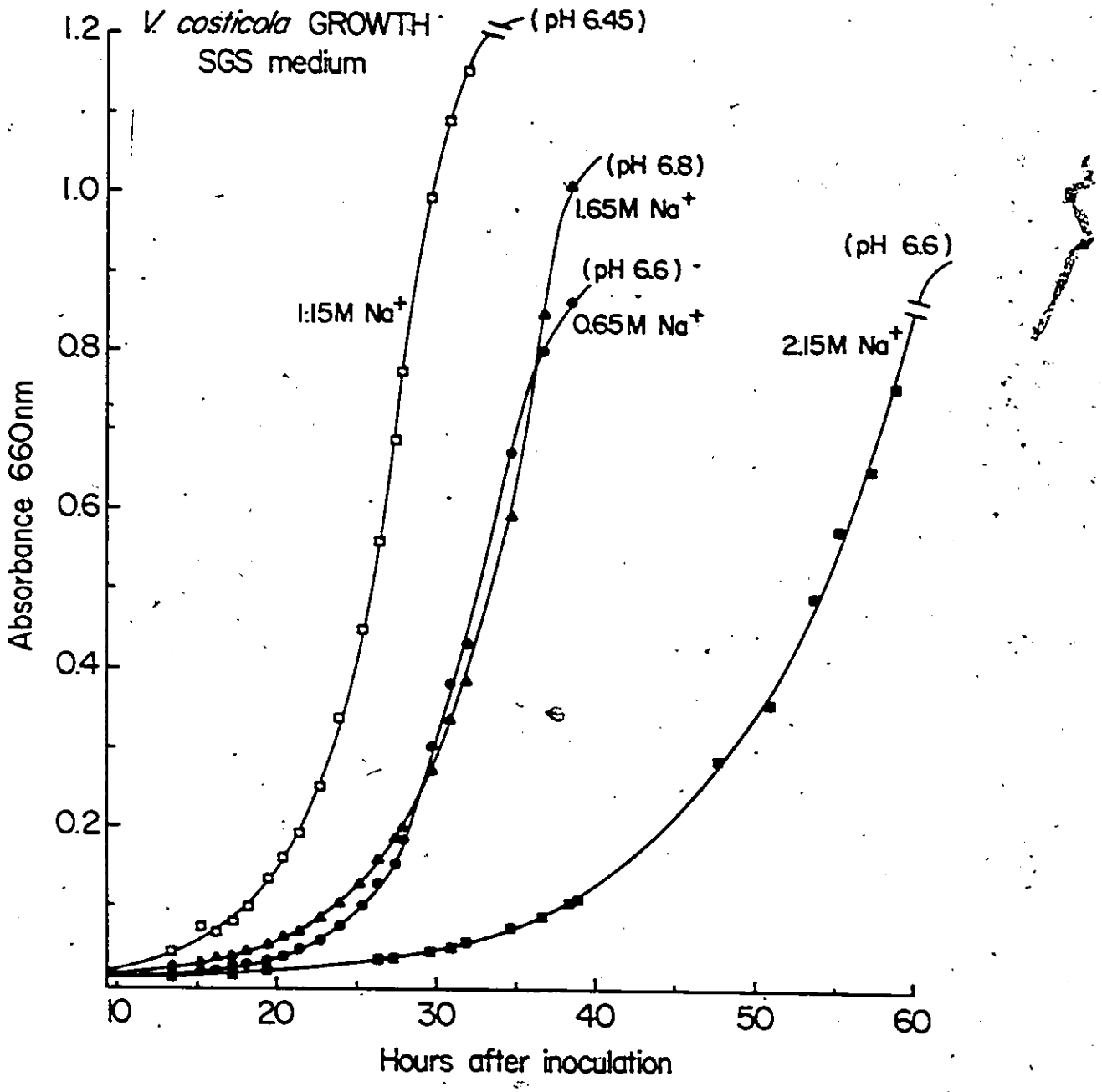
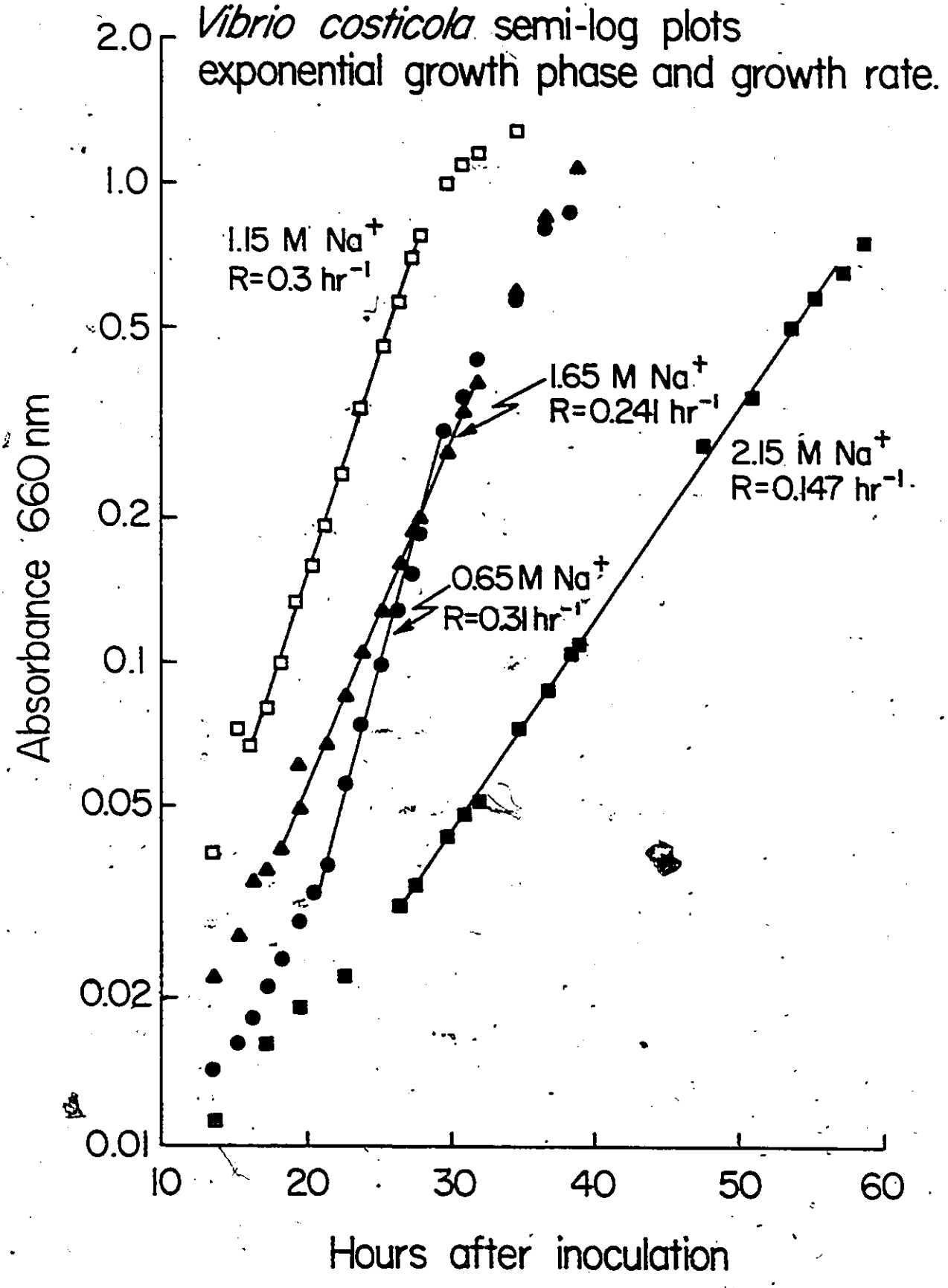

V. costicola GROWTH
SGS medium
(pH 6.45)
(pH 6.8)
1.65M Na+
(pH 6.6)
0.65M Na+
1.15M Na+
(pH 6.6)
2.15M Na+
Absorbance 660nm
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
Hours after inoculation

Fig. II-4. Data from Fig. II-3 replotted as log absorbance vs. time. Slope of linear portion of each semi-log plot yields growth rate (R) expressed as doublings per hour at the SGS medium Na^+ concentration indicated. R values given have three significant figures although zeros are not shown.


Vibrio costicola semi-log plots
exponential growth phase and growth rate.
1.15 M Na+
R=0.3 hr-1
1.65 M Na+
R=0.241 hr-1
2.15 M Na+
R=0.147 hr-1
0.65 M Na+
R=0.31 hr-1
Absorbance 660 nm
2.0
1.0
0.5
0.2
0.1
0.05
0.02
0.01
10
20
30
40
50
60
Hours after inoculation

Minimum and maxium salt concentrations which allowed growth.

The maximum salt concentration allowing growth increased from about 2.7M in the SGP and SGS media to nearly 4M in PPT medium. The lower salt concentration limit for growth also appears extended when complex media were used (Fig. II-1, Forsyth & Kushner 1970). Recognition of the "osmotic lysis" phenomenon reported by Christian and Ingram (1959) necessitated a reexamination of growth at low salt concentrations. Christian and Ingram showed that V. costicola cells grown in a proteose-peptone (0.5% w/v) and tryptone (0.5% w/v) medium at a particular NaCl concentration lysed if resuspended in a medium with an NaCl concentration one-third or less of that present during growth. Lysis was presumably caused by sudden osmotic pressure changes upon rapid lowering of the extracellular salt concentration.

Cells grown in 1M NaCl SGP or SGS media (Tab. II-1) were normally used as inocula for the experiments which yielded the data on the salt ranges of growth in SGS and SGP shown in Fig. II-1. Some cultures were thus inoculated into cultures which were below the critical one-third of growth NaCl concentration. Experiments using cells grown in lower salt concentrations e.g. 0.5M NaCl, as inocula showed that the lower limit for growth in SGS and SGP media was extended to 0.2M medium $Na^+ + K^+$. The growth rate of V. costicola at that concentration is about 0.01 gen. hr^{-1} compared to a rate of 0.1 gen. hr^{-1} at 0.5M NaCl. 0.2M NaCl was also the lower limit for growth in PPT medium (see below).

A test of the hypothesis that lowering the growth temperature can decrease the ionic requirements for growth.

Novitsky and Kushner (1975) showed that a halophilic coccus strain (Planococcus halophilus) could grow at very low salt concentrations at 20C but not at 35C. They suggested that other halophiles might respond in a similar manner. To test this possibility with V. costicola, precultures were grown in both 0.2M NaCl SGP and 0.3M NaCl PPT media. Inocula were then introduced into 0.1M NaCl PPT cultures at 5, 19 and 30C and subsequently incubated at the respective temperatures. The only changes in culture absorbances at 660nm were slight decreases during the first few hours after inocultaion. No growth was evident up to 11 days post-inoculation. Other experiments showed that growth always occurred in 0.2M NaCl PPT medium. Cultures containing low salt concentrations were routinely checked to make sure that growth was not a result of contamination. It appears that the lower limiting salt concentration allowing growth of V. costicola, 0.2M NaCl, is not significantly affected by reduction of the temperature.

Ability of V. costicola to survive in salt concentrations approaching saturation and in solid salt.

Experiments were carried out to determine if V. costicola although incapable of growth above 4M NaCl (Fig. II-1), might have the ability to survive in salt concentrations near saturation or in solid NaCl. In one experiment one ml quanti-

ties of 2M NaCl grown V. costicola cultures were put into sterilized flasks each of which contained several grams of sterile NaCl crystals. The added culture fluid was allowed to flow gradually into the salt crystals over a period of an hour so that no sudden changes in salt concentration occurred. In a second experiment about 0.25 ml of a 2M NaCl PPT culture was added dropwise to a sterile tube containing the sterile dry ingredients for 10ml 1M NaCl Difco 2216 marine broth. After one week at 30C 1M NaCl sterile PPT medium was added aseptically to the flasks and 10ml sterile water added aseptically to the tubes; the final NaCl concentration was approximately 1M. Flasks and tubes were incubated at 30C. In neither case did the V. costicola survive, the cultures did not develop turbidity.

Discussion

Salt requirement of V. costicola

The minimum NaCl concentration which supported V. costicola growth, in either complex (proteose-peptone and tryptone) or salts-glucose (SGS or SGP) media was 0.2M. Lowering the incubation temperature reduced the salt requirement of a halophilic coccus but did not affect the V. costicola requirement. But the growth requirement is not exclusively for 0.2M NaCl. Robinson (1950) reported that 0.2M NaBr, $Na_2S_2O_3$, Na_2SO_4, LiCl and $MgCl_2$ could also support growth. It is not clear, however, how much and which salts were contained in the nutrient broth to which the other salts were added. Using trypticase soy broth Flannery et al. (1952)

found that the growth began at about 0.4M NaCl or at equivalent ionic strengths of Na_2SO_4 or other salts. In both studies the limiting concentrations for growth appeared to depend more on the ionic strength of the non-toxic salts above the trace levels contained in the complex media ingredients, rather than on the particular salts themselves. According to Christian (1956, see MacLeod 1965) V. costicola had only a non-specific requirement of about 0.4M salt in the medium but a specific requirement for only 0.017M Na^+. Research on marine bacteria indicated that specific Na^+ requirement is related to envelope stabilization and physiological functions such as transport of amino acids and other substances (see MacLeod 1968 for a review) and protein synthesis (Webb & Payne 1971). It is possible that the absolute NaCl requirement of V. costicola depends upon similar factors.

The exact minimum salt or Na^+ concentration allowing growth of V. costicola may vary with experimental conditions and is perhaps not as important as the observation that this strain and some other moderately halophilic bacteria can grow at low salt concentrations substantially below the optimum growth concentrations (see Tab. I-1). This has importance in considering the habitats and ecology of moderately halophilic bacteria (see below).

Salt tolerance of V. costicola

The rich PPT medium extended the maximum NaCl concentration tolerated from 2.5M NaCl (salts-glucose media) to

almost 4M. It has been suggested that certain enzymes in cells growing in minimal media responsible for the formation of amino acids and growth factors might be inhibited at high salt concentrations (Forsyth & Kushner 1970). An exogenous supply of amino acids etc. contained in ingredients of complex media might allow growth under conditions where the cell's synthetic machinery was inhibited. Taken together, the results of the growth studies indicate that V. costicola is best adapted for growth in environments containing 1-2M salt.

The natural habitat and ecology of V. costicola.

Bergey's Manual (Buchanan & Gibbons 1974) listed the habitat of V. costicola as cured meats and brines, probably because they are commonly found in bacon curing brines (Smith 1931, Robinson 1950) and because such brines were the sources of V. costicola strains. But what is the natural habitat and how is V. costicola introduced into commercial brines? The physiological and taxonomic traits give some indications.

V. costicola, in spite of being best adapted to growth and function within the 1-2M NaCl range, could grow well at lower salt concentrations. In Difco marine broth 2216 (ZoBell 1941) or PPT, growth was excellent at 0.5M NaCl; good growth also occurred in 0.5M NaCl SGS or SGP (Fig. II-1). Thus growth was possible at salt concentrations approximating those in marine environments in both rich complex and minimal media.

Taxonomically, V. costicola is closely related to other Vibrio strains known to have salt requirements such as V. metchnikovii and V. anguillarum (see above p. 27). Although V. costicola is adapted to more saline environments than its close relatives, it is probably quite successful in marine environments. The well studied pathogen V. parahemolyticus is a marine organism, slightly halophilic, yet its occurrence is not confined to areas of marine salinity. It is found in sea water, fish and shell-fish and also in less saline estuarine waters. V. costicola probably has a special marine micro-habitat or niche which has yet to be identified.

Slight and moderate halophiles have been isolated from widely differing natural environments. Shah and de Sa (1964) isolated 22 slightly halophilic Vibrio strains and 4 moderately halophilic strains (including 3 V. costicola strains) from sea water, salt and fish. Many other strains of moderately halophilic bacteria were isolated from these sources and from saline soil also. In a study of intertidal marine bacteria from the Bay of Fundy we isolated many halo-tolerant, slightly and moderately halophilic bacteria from tidal pools which were often slightly less saline than the ocean water (Forsyth et al., 1971). When V. costicola was tested for growth on the "ocean salts" media used in the intertidal study it showed a growth pattern in various salt concentrations which made it indistinguishable from many of the marine isolates (Tab. II-4), which were unexpectedly halotolerant. Previous studies (Brown & Turner 1963, Tyler et al. 1960) under different isolation and culture conditions had not indicated the presence of many

Table II-4

Salt response of intertidal marine isolates and *V. costicola* in "ocean salts"* medium

% NaCl in isolation medium	% NaCl in test medium: 0	3	10	20	25	30	No. strains showing growth pattern indicated
	Growth patterns						
3	+++	+++	+++	0	0	0	3
	NT	+++	+++	0	0	0	3
	0	+++	++	0	0	0	1
	0	+++	+++	0	0	0	4
	+++	+++	+++	±	0	0	1
	NT	+++	+++	++	0	0	1
	+++	+++	+++	++	0	0	2
	+++	+++	+++	+++	0	0	3
	±	+++	+++	++	0	0	1
	0	+++	+++	+++	0	0	2
							Total = 21
10	+++	+++	+++	0	0	0	4
	NT	+++	+++	0	0	0	1
	0	+++	+++	0	0	0	2
	+++	+++	+++	±	0	0	2
	+++	+++	+++	++	0	0	3
	+++	+++	+++	+++	0	0	5
	0	+++	+++	++	0	0	1
	0	+++	+++	+++	0	0	1
	+++	++	+++	++	++	0	1
	+++	+++	+++	+++	++	0	1
	0	++	+++	+++	±	0	1
	NT	+++	+++	+++	++	0	3
	+++	+++	+++	+++	++	+	1
							Total = 26
20	++	+++	+++	++	0	0	1
	+++	+++	++	++	0	0	1
	NT	+++	+++	++	0	0	4
	+++	+++	+++	++	0	0	2
	NT	+++	+++	+++	NT	0	5
	+++	+++	+++	+++	0	0	3
	+	+++	+++	+++	0	0	1
	0	+++	+++	++	0	0	1
	0	+++	+++	+++	0	0	2
	+++	+++	+++	++	++	0	1
	0	+++	+++	+++	++	+	1
							Total = 22
							Grand total = 69
V. costicola	0	+++	+++	±	0	0	

*Salt response: +++ growth before 8 days (usually 1-2 days), ++ growth in 9-14 days, + growth in 15-21 days, ± growth in 22-28 days, 0 no growth in 4-5 weeks, NT not tested. Incubations at 30C in "ocean-salts" medium: 0.1% Difco yeast extract, 0.4 % Difco casamino acids, and regular strength "Instant Ocean", an artificial seawater mixture supplied by Aquarium Systems, Inc., Wickliffe, Ohio, U.S.A., described in Segedi & Kelley 1964. This solution contains NaCl, 2.65% (w/v); $MgSO_4$, 0.66%; $MgCl_2$, 0.52%; $CaCl_2$, 0.13%; KCl, 0.071%; $NaHCO_3$, 0.02%, and traces of other ions. The final NaCl concentrations in the selective media were adjusted to 3, 10, 20,25, and 30%. The medium for testing growth in "0%" NaCl contained $KHCO_3$, 0.025% (w/v); $CaSO_4 \cdot 2H_2O$, 0.0069%; $MgCl_2 \cdot 6H_2O$, 0.0305%; $MgSO_4$, 0.012%; $Ca(NO_3)_2 \cdot 4H_2O$, 0.00006%, in addition to yeast extract and casamino acids as above. The last two components contributed about 0.06% NaCl. Final pH of all media after autoclaving at 15 lb pressure for 15 min was 7.6 ± 0.1. Marine isolate data were previously published (Forsyth, Shindler, Gochnauer and Kushner, 1971).

salt tolerant isolates. Extremely saline environments such as the Dead Sea (Volcani 1940), hypersaline lakes (Brisou 1974), Liman mud (Larsen 1968) and solar salt (Ishida & Fujii 1970) also harbor populations of moderately halophilic bacteria. Thus, the moderate halophiles are taxonomically and environmentally diverse.

If V. costicola was originally a marine organism, it is likely that it was introduced into the bacon curing brines via the crude solar salt (Ingram 1957) often used. Moderately halophilic bacteria are found in solar salt (Ishida & Fujii 1970) and other food related brines to which salt is added such as soya sauce (Ueno 1964, Yoshii 1967, Kitahara & Ishizuka 1968, 1969), salted beans and anchovies, and fish curing brines (Larsen 1962, Tab. I-1). Although an attempt to isolate V. costicola from a 1M SGS enrichment culture using an inoculum of crude solar salt resulted only in detection of strains, one of them vibriod, which did not match the characteristics of V. costicola, it is possible that under natural circumstances V. costicola could survive the slow dehydration during solar salt production. Alternatively, V. costicola might be inoculated into brines via airborne transmission of cells directly from the oceans.

Chapter III

Intracellular sodium, potassium and magnesium concentrations of V. costicola.

Moderately halophilic bacteria have the ability to grow over wide ranges of environmental salt concentrations, often from the low salt concentrations where the non-halophiles grow to the high salt concentrations where the extremely halophilic bacteria thrive. The structures and functions of the non-halophiles such as E. coli are adapted to low salt concentrations, those of the extreme halophile H. cutirubrum are adapted to high salt concentrations, but what about those of the moderate halophiles? Do they resemble the non-halophiles or the extreme halophiles? Or do they change their halophilic character according to the concentration of salt in the growth medium? To interpret experiments on this subject it is essential to measure the internal ionic contents of moderate halophilic bacteria and the ways in which these change with changing salt concentrations in the growth medium.

There is abundant evidence that, in general, internal water activities of bacteria are equivalent to or lower than the water activities of the media in which they are grown (Christian & Ingram 1959b, Christian & Waltho 1962), that is, the internal solute concentrations are equivalent to or higher than those of the medium. If the internal solute content of halophiles were significantly lower than that of the medium it would be difficult to sediment cells by centrifugation (Ingram 1957), which is not the case; moreover,

to maintain the concentration difference would require a water barrier or a water pump, neither of which have been shown to exist in bacteria (Brown 1964). Measurements of the ionic contents of cells shows that the total intracellular cation concentration often exceeds that of the medium, e.g.; Halobacterium strains grown in 4M NaCl media contained 4-5M $Na^+ + K^+$ (Christian & Waltho 1962, Ginzburg et al. 1970, Gochnauer & Kushner 1971, Lanyi & Silverman 1972). Escherichia coli during exponential growth in 0.11M Na^+ contained about 0.25M $Na^+ + K^+$ (Schultz & Solomon 1961). Over the salt concentration range of growth of a moderate halophile it would be advantageous to know if the total ionic content of the cell increases with increasing medium salt concentration.

Some data is available on internal ion concentrations of V. costicola and Paracoccus halodenitrificans. When grown in 1M NaCl media, V. costicola contained 0.9M $Na^+ + K^+$, P. halodenitrificans contained 0.8M $Na^+ + K^+$, in addition to other solutes (Christian & Waltho 1962). Few other moderate halophiles' internal ion concentrations have been analysed. A moderately halophilic pseudomonad #101, cultured at its 2M NaCl optimum, contained 1.15M Na^+ and 0.9M K^+; these concentrations changed little when grown at 1M or 3M NaCl (Masui & Wada 1973). The ionic content of bacteria is not simply a function of the medium NaCl concentration; in a marine pseudomonad it was shown that the amounts of Na^+ and K^+ depend upon the metabolic state of the cells. Freshly aerated, actively metabolizing cells had much higher

concentrations of K^+ and lower ones of Na^+ than the medium ions, while in non-metabolizing cells Na^+ entered and K^+ left (Thompson & MacLeod 1973).

This chapter describes an examination of the cell associated Na^+, K^+ and Mg^{+2} in V. costicola and of the effect on these of media composition and cell metabolic status.

Experimental methods

Outline of general procedure for intracellular ion determinations

V. costicola cells were grown, harvested, washed once in a buffer solution isotonic to the growth medium, then resuspended in a small amount of the isotonic buffer solution to make a thick suspension (20-40mg wet weight/ml). The cell suspension was aerated by vigorous shaking for 30 minutes, then sets of three identical 10ml samples removed. The first sample of each set was centrifuged, and the pellet extracted with hot trichloroacetic acid. The pellet Na^+, K^+, and Mg^{+2} were determined using a flame spectrophotometer. To the second sample of each set a small amount of radioactive solute which could not penetrate into the cells was added then the cells were pelleted. The amount of non-penetrating radioactive solute which was trapped between the cells was used to determine the intercellular or interstitial volume. The third sample was centrifuged and used to determine the wet and dry pellet weights and, by difference, the pellet water. Calculations on the basis of these determinations yielded the amount of cell-associated ions and cell water

weight and thus the intracellular ion molality. Details of the procedures, relevant references, calculations and checks on the methods are given in this section below and in the results section. Where significant variations in the techniques were used in experiments, the changes are mentioned in the results section.

Growth of cells, washing and thick suspension preparation procedures.

Media and incubation conditions for cultures were given in Chapter 2 (see p. 31-34). Cells were usually grown to mid- or late exponential phase. The culture volumes were large, usually from 2-4 l., in order to obtain adequate amounts of cells for the thick suspensions. Cells were centrifuged for 5-10 min. at 4C in 250-300ml bottles, resuspended in several hundred ml of the appropriate buffer (below) in order to wash them free of culture media, and centrifuged again. The centrifugal force was about 5000 x g to avoid producing very hard pellets which would be difficult to resuspend. The cells were then resuspended in buffer to form a thick suspension (after Takacs et al. 1964) such that a 10ml sample yielded a pellet of 200-400 mg cell wet weight or about 65-135mg dry weight.

The buffer used for washing and resuspending was isotonic to the media in which the cells were grown. 1M NaCl salts-glucose-potassium phosphate (SGP) grown cells were generally suspended in "complete salts buffer" (CS), 1M NaCl, 0.15M KCl, 0.41 mM $MgSO_4$, 0.001M $(NH_4)_2HPO_4$, 0.05M tris, pH7.5.

1M NaCl salt-glucose-sodium phosphate (SGS) grown cells were suspended in "low potassium isotonic buffer" (LK), 1.2M NaCl, 0.008M KCl, 0.41mM $MgSO_4$, 0.05M tris, pH7.5. Cells grown in complex media such as 1M NaCl, SGS + 0.5% w/v casamino acids (SGS + CAA) or 1M NaCl PPT were suspended in either fresh culture media or in LK buffer. The NaCl content of the buffers was adjusted appropriately for use in experiments where cells were grown in other NaCl concentrations. Table III-1 shows flame spectrophotometric ion analyses and calculated ion concentrations of various media and buffers.

Effects of pellet resuspension procedure on cells. V. costicola cells are sticky and sediment in a packed cell mass which is difficult to disperse by mild agitation procedures such as shaking or "vortexing". It was thus necessary to develop a technique for resuspending V. costicola pellets which was rapid yet did not damage the cells. For this plastic syringes (syringe volume was approximately equivalent to suspension volume) with attached large bore 14 gauge 10cm cannulae were used. After adding resuspension solution the cannula tip was used as a stirring rod to break up the pellet, then 10-20 syringe strokes were used to take up and force out the resuspension solution and pellet clumps. Visual and microscopic examination of the suspensions produced from the syringing technique showed that the method yielded a completely homogenous cell suspension in a short time.

To check for the possiblity of cell damage, release of 260nm absorbing material for cells after different resuspension

Table III-1

Ionic content of media, components and buffers.

Solution	Na^+		K^+		Mg^{+2}	
	Anal[a].	Calc.	Anal.	Calc.	Anal.	Calc.
SGP medium	1.04M	1.0M	0.159M	0.15M	0.45mM	0.41mM
SGS medium	1.28	1.2	0.011[b]	0.008	0.41	0.41
1M NaCl PPT	0.96	1.0	0.009	-	0.39	-
1% w/v proteose peptone + 1% w/v tryptone	0.05	-	0.004	-	0.40	-
0.5% w/v casamino acids	0.10	-	0.0014	-	0.015	-
CS isotonic buffer	0.89	1.0	0.16	0.15	0.41	0.41
LK isotonic buffer	1.26	1.2	0.012[b]	0.008	0.35	0.41

a Analyses done by flame spectrophotometry.

b Value significantly different from calculated value due to extra K^+ added as contaminants of other substances, especially NaCl.

techniques was measured (Tab. III-2). The results show that no significant release of 260nm material occurred if 16 syringing strokes were used. 64 strokes released material almost equivalent to that released from a brief sonication at low power. V. costicola cells did not seem to be greatly affected by the 10-20 syringe strokes which made resuspensions convenient and rapid.

Thick suspension incubation and sampling. Cell suspensions containing 30-40mg wet cells per ml were prepared as described above in 350ml (60 x 140mm) wide-mouth polycarbonate centrifuge bottles. To some suspensions 25mM glucose, 25mM ethanol or poison (10mM NaCN + 10mM iodoacetate) were added. The bottles were capped and agitated in a New Brunswick Scientific Co. G-76 gyrotory water bath shaker at 250 r.p.m. to ensure vigorous aeration during the usual 30 min. 30C incubation. In some experiments N_2 or O_2 gas was passed through the headspace of the bottles above the suspension. At appropriate intervals, sets of three 10ml samples were removed for the various determinations.

Determination of the ionic contents of the pellets. Each 10ml thick suspension sample for ionic analysis was pipetted into a 50ml (29 x 104mm) polycarbonate centrifuge tube, in which the extractions were to be carried out. The tubes were centrifuged at 25,000 x g for 10 min. in an International B-20 centrifuge at 20C (in some early experiments the temperature was 4C). The resulting supernatants were discarded and the

Table III-2

Release of 260nm absorbing material from V. costicola cells as a result of various resuspension procedures.

pellet[1] treatment		260nm absorbance
shaken manually until resuspended		0.009[2]
shaken mechanically, "vortexed" until resuspended		0
syringed:	8 strokes	0
	16 strokes	0.009
	32 strokes	0.051
	64 strokes	0.097
sonicated[3]	4 sec., low power	0.14
sonicated	4 sec., high power	1.8

1. Cells grown in SGP to end of exponential phase, washed and resuspended in CS buffer.
2. Absorbance 260nm with buffer from previous wash step used as the reference blank, absolute absorbance 0.15.
3. Disruption of cell suspension using Branson S-125 ultrasonic oscillator.

inside walls of the tubes carefully rinsed with distilled-deionized water (R>5 megohm-cm) from a squeeze bottle to remove residual ions left from the supernatant. Care was taken so that the water did not contact the pellet. The inside walls of the tubes were then wiped with tissue paper (Kimwipes type 900-S). A volume of deionized water equivalent to 24-fold pellet wet weight was added to each pellet which was then resuspended by extensive mechanical "vortex" agitation until no large pellet clumps remained. A 25-fold wet weight volume of 10% (w/v) trichloroacetic acid was added to each resuspended pellet (the final extraction solution contained 5% trichloracetic acid), the polycarbonate tubes shaken briefly then immersed in a 95-99C water bath for 5 min. The tubes containing the extract were centrifuged at 25,000 x g for 10 min. at 4C and the supernatants decanted into plastic or polycarbonate containers for later dilution and analysis. The small amount of floccular precipitate did not contain significant amounts of the ions measured indicating that the extraction procedure solubilized the cellular ions (Tab. III-3). Care was taken during the procedures that all trichloracetic acid solutions, including the extracts, did not contact glass or other materials which might add contaminating ions.

Extracts were diluted appropriately with distilled-deionized water using Gilson Pipetman adjustable pipettes (volume calibrations checked before use) with plastic tips and 1ml Fisher Scientific Co. plastic mini beakers and caps. A Perkin-Elmer 403 Atomic absorption spectrophotometer was

Table III-3

A check on the effectiveness of the cell ion extraction proceedure: Ionic contents of solid residue* material after 5% trichloroacetic acid 5 min. 95C extractions.

	Na^+	K^+	Mg^{+2}
Duplicate determinations on residues from cell extracts:	6µg/ml	3µg/ml	0.7µg/ml
	3	0	0.7
Duplicate determinations on control crucibles without cell residues:	1.5	2.5	0.075
	4.5	1	0.06

* 0.038g duplicate dry precipitates from extractions of cell pellets were ashed at 900C in porcelain crucibles along with control crucibles containing no precipitates. 2ml 5% trichloroacetic acid was added to each crucible and ions determined by flame spectrophotometric analyses. The ion contents of the cell extract, calculated assuming 0.1g extract was diluted 200 times was 0.42µg/ml Na^+, 0.24µg/ml K^+ and 0.013µg/ml Mg^{+2}. The pellet residue values less the control crucible values were not significantly different from zero. The high readings were caused by contaminating ions extracted from the crucibles by the 5% trichloroacetic acid.

used for flame emission analyses of Na^+ and K^+, and for flame absorption analysis of Mg^{+2}. The gas was acetylene/air, the wavelengths were 5893Å, 7665Å and 2852Å for concentration ranges of 0-1mg/ml, 0-1mg/ml and 0-0.5mg/ml for Na^+, K^+ and Mg^{+2} respectively. Although the flame spectrophotometer was operated within the linear concentration ranges in which the emission or absorption was proportional to the output display adjusted to read µg/ml directly, series of standards were always prepared and standard curves drawn to check accuracy (Figs. III-1, III-2, III-3). To attain a composition similar to the samples, standards for Na^+ and K^+ were made up from a stock solution containing equal amounts of both ions, Mg^{+2} standard had a background of 10-fold excess Na^+ and K^+. Several samples were also analysed using Orion Na^+ or K^+ - specific electrodes as a check of the flame analysis data. The electrode data was in substantial agreement with the flame analysis figures.

Determinations of pellet inter-cellular space. The 10ml thick suspension samples for intercellular volume determinations were pipetted into Corex #8441 15ml centrifuge tubes which contained 1ml of a solution of a radioactive solute which could not penetrate into cells. ^{14}C-inulin was usually used as the non-penetrating solute, though labelled sucrose and ATP were used in some experiments. Solutes were made up in the same buffer and salt concentration as the thick suspension. The amount of labelled non-penetrating solute, (with extra non-labelled carrier solute added when the

Fig. III-1

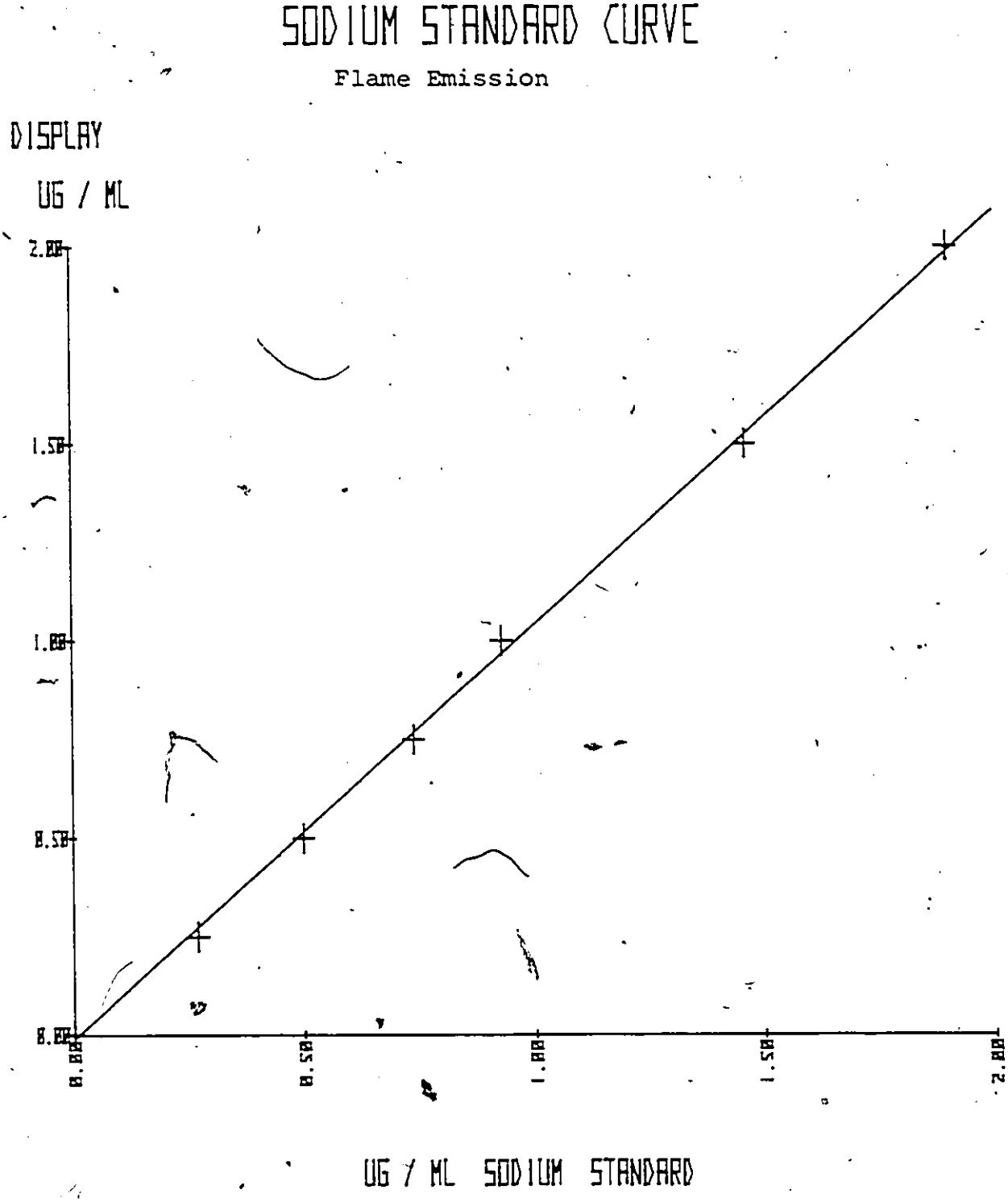

SODIUM STANDARD CURVE
Flame Emission
DISPLAY
UG / ML
2.00
1.50
1.00
0.50
0.00
0.00
0.50
1.00
1.50
2.00
UG / ML SODIUM STANDARD

Fig. III-2

POTASSIUM STANDARD CURVE

Flame Emission

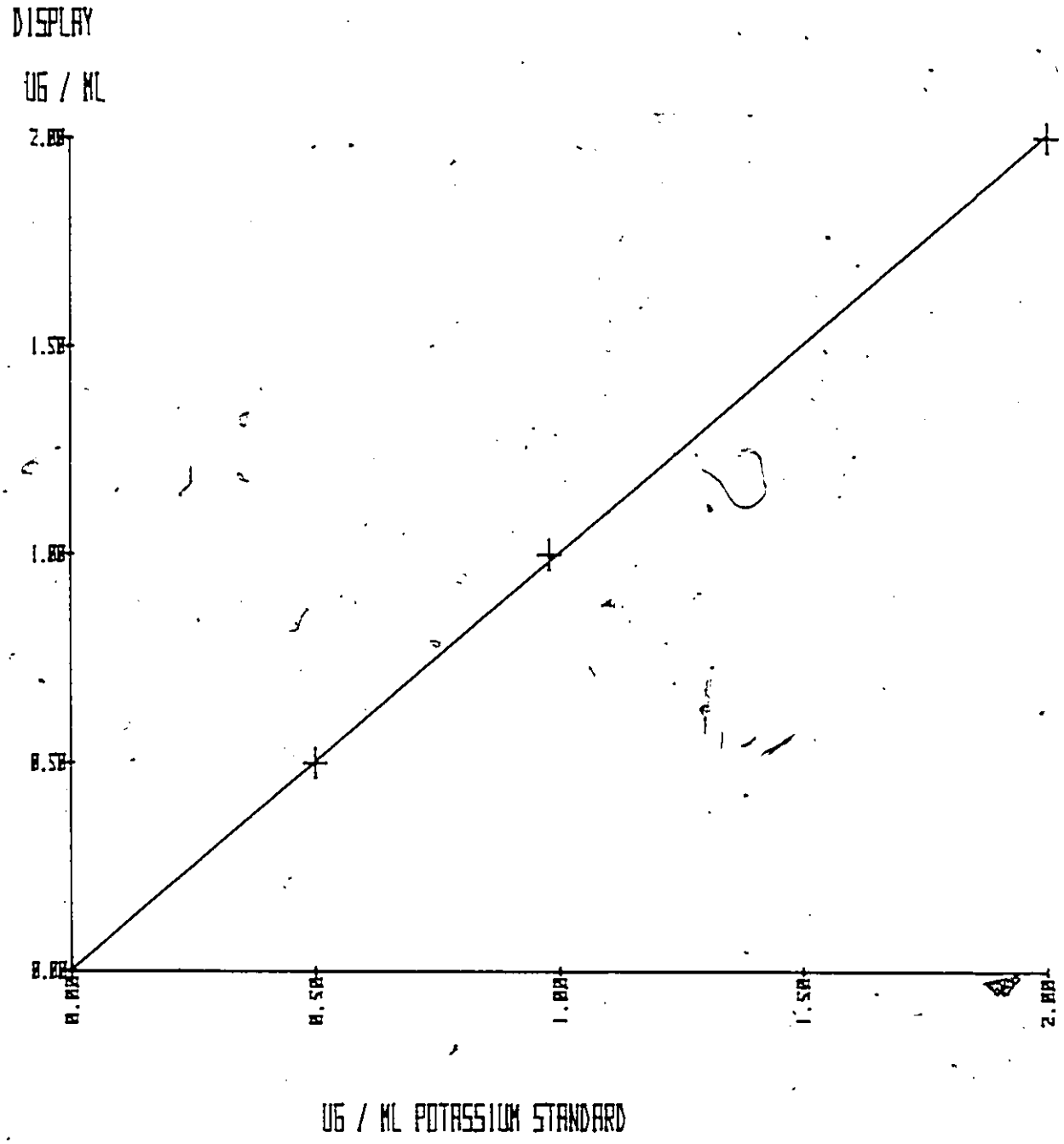
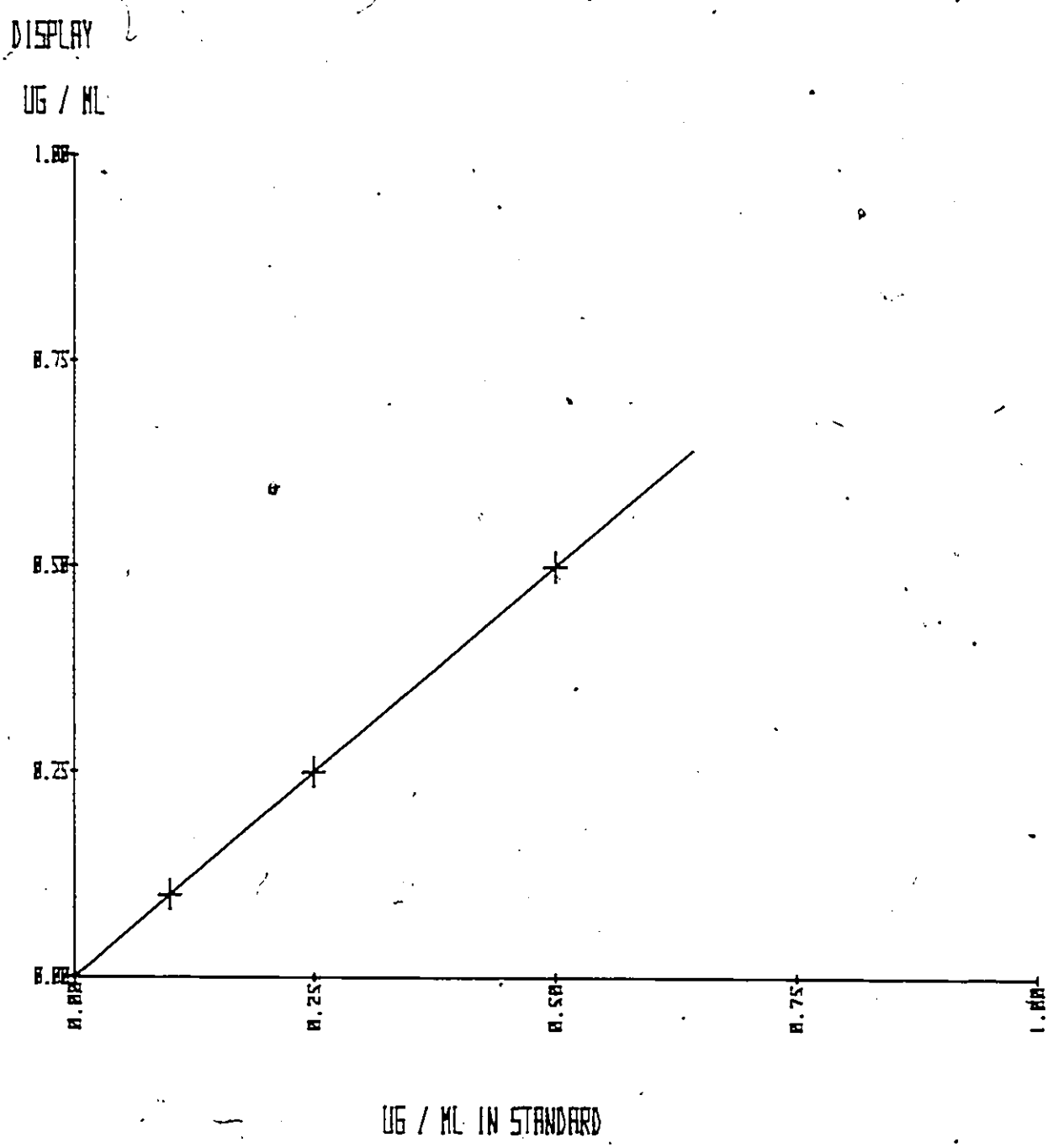


Fig. III-3

STANDARD CURVE FOR MAGNESIUM

Atomic Absorption



concentrations of labelled substance were low, e.g. carrier adenosine 5' triphosphate was added to Amersham/Searle CFA.426 [8-^{14}C]ATP to achieve 10^{-4}M in the cell suspension; New England Nuclear NEC 164P inulin carboxy-^{14}C was used at 0.03mg/ml without carrier, uniformly labelled Amersham/Searle CFB.4 sucrose-^{14}C was prepared for use at 0.01mg/ml without addition of carrier), was adjusted to give adequate counts, about 1000 c.p.m., from the Z_2 supernatant aliquot (see below). The tubes containing the suspension and labelled substance were mixed by vortexing, then centrifuged at 25,000 x g for 10 min. at 20C. The resulting supernatant, Z_1, was decanted and saved, the inside tube walls rinsed with deionized water using a squeeze bottle to remove adhering radioactive substance, and the walls with tissue paper. Several ml (volume V_2) of the same buffer solution, except without the labelled substance, was added to each pellet then the pellets resuspended using disposable 10ml plastic syringes with attached cannulae. The suspensions were centrifuged at 25,000 x g for 10 min. at 20C and supernatant Z_2 decanted and retained. Aliquots (0.2ml) of Z_1 and Z_2 were pipetted into scintillation vials to which 10ml New England Nuclear Aquasol cocktail plus 1ml H_2O had been added. Counting was done in a Beckman LS-230 liquid scintillation system. The intercellular volume, i.e. the volume of solution trapped in the first pellet per g cell wet weight was determined using the formula:

$$S = \frac{V_2 Z_2}{(Z_1 - Z_2)P}$$

where S = inter-cellular space, ml/g wet pellet.

P = wet weight of pellet, g.

Z_1 = c.p.m. (background corrected) in 0.2ml aliquot of the first supernatant.

Z_2 = c.p.m. (background corrected) in 0.2ml aliquot of the second supernatant.

V_2 = volume, ml, of the buffer solution added to the first pellet (equivalent to the volume of the second supernatant).

Typical values for S were 0.26 - 0.32 ml/g wet cells using inulin with 1M SGS grown cells.

The formula was derived from the relationship of volume (v) to concetration (c), i.e. a given solute concentration decreases proportionally with the volume of solvent added:

$$v_1 c_1 = v_2 c_2$$

In the case of the pelleted cells the concentration of non-penetrating solute trapped between the cells in the first pellet is expressed as Z_1, the volume of the solution + intercellular space into which the trapped solute is liberated upon resuspension is $S + V_2$, and the concentration of solute in the second supernatant as a result of resuspension is Z_2.

Thus:

$$S.Z_1 = (S + V_2)\ Z_2$$

$$S.Z_1 = S.Z_2 + V_2 Z_2$$

$$S.Z_1 - S.Z_2 = V_2 Z_2$$

$$S(Z_1 - Z_2) = V_2 Z_2$$

$$S = \frac{V_2 Z_2}{Z_1 - Z_2}$$

Dividing by the wet weight of the pellet yields the intercellular space per gram wet pellet.

Pellet wet and dry weight determinations. 10ml samples for pellet weights were centrifuged at 25,000 x g for 10 min. at 20C in predried (105C), preweighed corex #8441 15ml centrifuge tubes. The supernatants were discarded, the inside tube walls rinsed with deionized water and wiped with Kimwipes. A jet of gently flowing dry air was directed inside the tubes until the walls were dry (1-3 min.) The tubes were weighed and the wet weights of the pellets determined. After 24-36 hours at 105C the tubes attained constant weight and were reweighed to determine the dry weights of the pellets. The ratio of dry to wet pellet weight was usually about 0.3.

Derivation of the formula for and calculation of the cell-associated ion molalities.

All the formulas which have been used to calculate intercellular ion concentrations (Schultz & Solomon 1961, Christian & Waltho 1962, Takacs et al. 1964, Ginzburg et al. 1970, Lanyi & Silverman 1972, Unemoto et al. 1973) can be reduced to:

$$\frac{\text{(pellet ion amount)} - \text{(intercellular ion amount)}}{\text{(cell } H_2O \text{ amount)}}$$

From this basic expression a formula was designed for this work as follows:

The numerator was defined as

$A-(S \cdot B)$ where: A = pellet ion concentration mg/g wet pellet

S = inter-cellular space, ml/g wet pellet

B = ion, mg/ml in intercellular solution.

The denominator contains an expression used to calculate the amount of water associated with the cells in the pellet.

$(1-D)-(W \cdot S)$ where: D = g dry weight/g wet weight of pellet

W = H_2O content of thick suspension buffer solution, g/ml, estimated from tables in Weast (1973).

(1-D) is pellet water per gram wet pellet and (W•S) is an estimate of the gram inter-cellular water per gram wet pellet.

The final formula used to calculate C, the molal cell-associated ion concentration (mol/kg cell H_2O),

$$C = \frac{A - (S \cdot B)}{([1-D]-[W \cdot S])F}$$

where F = molecular weight of ion,

was incorporated into a computer program for the Hewlett-Packard 9830A calculator and used for routine automated calculations.

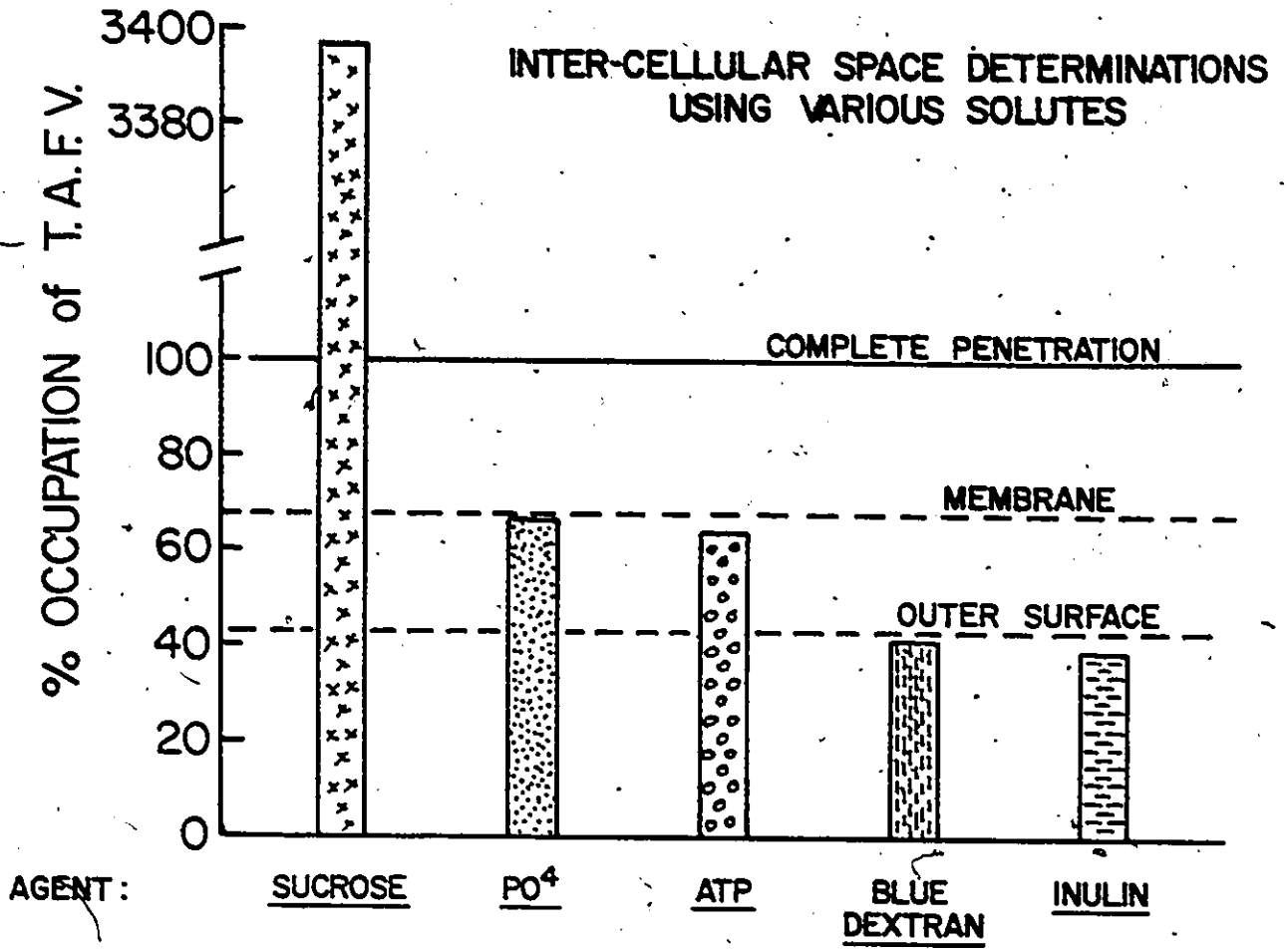
Results

Determination of pellet inter-cellular space.

Estimates of cell-associated ions depend on accurate determinations of the extra-cellular volume in the packed cell pellet. Several types of solutes have been used to measure the volume between the cells. Large molecules such as albumin (Ginzburg 1969, Schultz & Solomon 1961), dextrans (Mitchell & Moyle 1965, Ginzburg et al. 1970, Lanyi & Silverman 1972) and inulin (Schultz & Solomon 1961, Takacs et al. 1964) probably cannot penetrate further than the outside wall surface of most cells (Mitchell & Moyle 1956, Buckmire & MacLeod 1970). (Extremely halophilic organisms are an interesting exception in that they appear permeable to large molecules such as inulin (Ginzburg 1969)). Small molecules such as sucrose (Buckmire & MacLeod 1970) or phosphate (Mitchell & Moyle 1956, Christian & Waltho 1962) probably penetrate through the outer cell layers to the cell membrane (Mitchell 1953).

Fig. III-4 shows the penetrability of five solutes which were used for preliminary estimates of V. costicola pellet inter-cellular space. Sucrose, which had been used successfully with a marine pseudomonad for estimates of extra-cellular volume (Thompson & MacLeod 1971, 1973), penetrated and was concentrated in 1M NaCl grown V. costicola cells. Phosphate and adenosine-5' - triphosphate (ATP) occupied about 65% of the total available fluid volume (estimated as 1-D) in the pellet, penetrating to the cell membrane. Blue dextran and inulin occupied about 21% less of the total availa-

Fig. III-4.



INTER-SPACE(S), ml/g wet pellet:	23.1	0.452	0.428	0.277	0.271
(standard error):	(2.9)	(0.057)	(0.026)	(0.007)	(0.032)
for (n determinations):	(4)	(2)	(4)	(2)	(4)

* Total available fluid volume of pellet estimated as (1-D). See text.

ble fluid volume, perhaps penetrating only to the cell surface. The pattern of penetration shown in Fig. III-4 is consistent with previous findings with a marine pseudomonad (Buckmire & MacLeod 1970, Thompson & MacLeod 1971).

Assuming that V. costicola cells can be considered as spheres 1.9μm in diameter with a cell volume of 3.5μm^3 (equivalent to the volume of a cylinder of the approximate 1 x 4.5μm dimensions of V. costicola cells (Robinson, 1950)), a rough calculation indicated that phosphate and ATP penetrated 0.074μm deeper into the cell than inulin or blue dextran. 74nm is not an unrealistic estimate of the thickness of the cell layers external to the cell membrane. Schultz and Solomon (1961) gave the thickness of the E. coli cell wall as 15nm, which accounts for 13% of the cell volume (cell dimensions: 0.5μm diameter, 2μm length, cell volume of 0.4μm^3).

For the work with V. costicola the use of ^{14}C-ATP or ^{14}C-inulin as the non-penetrating solutes permitted the use of rapid liquid scintillation methods instead of chemical or colormetric determinations for phosphate or blue dextran. Besides, phosphate was considered undesirable because of the possibility that V. costicola under some conditions might concentrate it. ATP, although at first thought to be as close as possible to an ideal non-penetrating solute because of its reputation of not entering cells, was subsequently found to penetrate V. costicola cells grown at 0.8M or 1.8M NaCl (giving inter-cellular space values of 1.23 and 3.6ml/g wet pellet respectively). In fact ATP has been reported to penetrate eukaryotic red cells (Elford, 1975) and muscle fibres (Chaudry & Gould 1970). Thus it was decided to use inulin for most routine measurements of pellet

inter-cellular volume.

Table III-4 shows the different values obtained for cell-associated ions of 1M NaCl SGS grown cells using ATP and inulin to estimate inter-cellular volume. The use of ATP resulted in a higher estimate of inter-cellular volume than inulin, and a corresponding lower cell volume, and therefore cell water, estimate. K^+ and Mg^{+2} were in low concentrations in the inter-cellular buffer, thus their cellular concentrations increased due to the lower cell water estimate. On the other hand, there was a substantial amount of Na^+ in the inter-cellular buffer. The higher inter-cellular volume estimate caused a large amount of Na^+ to be subtracted from the pellet Na^+ amount, more than overcoming the effect of less cell water, yielding a lower cell-associated Na^+ concentration with ATP.

The agent used to measure pellet intercellular space must not penetrate cells and, moreover, must be freely diffusable and not bind to the cell surface, so that it is free to be released into the Z_2 buffer used to resuspend the cells. In order to check this, the radioactivity associated with 1M NaCl grown cells was estimated by filtering the Z_2 cell suspension through Millipore^R HA 0.45µm pore size filters, followed by several 10ml washes with 1M NaCl unlabelled buffer. Table III-5 shows that only a few counts, less than 10% of the actual Z_2 counts, were associated with the cells using inulin or ATP, while the labelled sucrose was not removed from the cells. At 1M NaCl, ATP and inulin were not significantly bound or taken up by the cells. Sucrose, as pointed out earlier, was taken up and probably metabolized.

Table III-4

Effect of use of ATP vs. inulin on the estimates of cell-associated ions[1].

Agent (S-value)[2]	Na^+	K^+	$Na^+ + K^+$	K^+/Na^+	Mg^{+2}	cell water[3]
ATP (S=0.428)	0.30 ± 0.05[4]	1.30 ± 0.01	1.60	4.3	0.081 ± 0.001	0.27
inulin (S=0.271)	0.64 ± 0.03	0.82 ± 0.04	1.44	1.28	0.051 ± 0.001	0.42

1. 1M NaCl SGS grown late log cells, aerated 30 min. in thick suspension.
2. S = inter-cellular space / ml wet pellet.
3. Cell H_2O g/ml wet pellet
4. Molal cell associated ion ± standard error for 4 determinations.

Table III-5

Retention of ATP, inulin and sucrose by cells on filters.

	agent		
	ATP	inulin	sucrose
0.1ml cell suspension[1]	55[2]	28	26,000
0.1ml buffer[3]	5	7	13
counts in 0.1ml Z_2 buffer[4]	713	220	7,900

1. Cells from second resuspension in inter-cellular volume procedure filtered through Millipore^R HA 0.4 µm filters, then a further 20ml isotonic cold buffer washed through filter.
2. Counts per minute, background corrected.
3. Buffer filtered and filter washed as per note 1.
4. Counts in an aliquot of unfiltered Z_2 supernatant. used in determination of inter-cellular volume.

The inter-cellular space values obtained in experiments using inulin were consistent. Values for intercellular space were; for mid-log 1M NaCl SGS grown cells, 0.315 ± 0.006 mg/g wet pellet (± standard error, n=7 determinations); for late log SGS cells, 0.265 ± 0.006 (n=18). In only one case did the inulin space exceed the normal range of values: a pellet of cells grown and resuspended in 0.8M NaCl buffer was inadvertently resuspended in 1.6M NaCl buffer. The duplicate tube which was resuspended in the proper buffer yielded a value for inulin space of 0.37ml/g wet pellet while the tube in which the error was made gave a value of 0.99ml/g wet pellet. V. costicola cells exhibit sensitivity to rapid changes in medium salt concentrations (Christian & Ingram 1959a) and in this case changed their permeability characteristics.

Although different experimental techniques were used, it is instructive to compare pellet inter-cellular space values obtained in these experiments with those of other workers. Mitchell (1953) referred to a theoretical figure for the interstitial space of closely packed spheres as 26% of the total volume of the spheres plus the interstitial space (i.e. equivalent to approximately 0.26ml/g wet pellet). Experiments with Halobacterium strains gave albumin and dextran spaces of 0.3-0.4ml/g wet pellet (Ginzburg 1969, Lanyi & Silverman 1972). Schultz & Solomon (1961) found the albumin and inulin space to be 0.19ml/g wet pellet with E. coli. Unemoto (1973) found the inulin space of Vibrio alginolyticus to be 0.23ml/g wet pellet. The estimates given above for V. costicola are comparable to and within the range of the other estimates.

The intercellular space value determined for V. costicola was not greatly affected by the physiological state of the cells, whether poisoned (not metabolizing) or metabolizing with or without an energy source (Tab. III-6 and other data not presented here at different salt concentrations). The medium salt concentration did alter the pellet inter-cellular space with a consistent decrease from 0.8 to 1.8M NaCl (Tab. III-7). Such changes may be due to differences in pellet packing, cell density and boyancy.

Effects of V. costicola metabolic state on cell-associated sodium and potassium.

It was clear from work on a marine bacterium (Thompson & MacLeod 1973) that the amounts of cell-associated sodium and potassium ions are greatly affected by the metabolic status of cell suspensions. Cells washed several times and then pelleted for ion determinations may not have ion distributions resembling those of active cells in culture. To examine this, the ionic contents of V. costicola cells were determined after incubation in thick suspensions (Takacs et al. 1964) under several conditions including aeration, aeration with energy source, aeration with metabolic poisons, and incubation under O_2 and N_2.

Table III-8 shows cell-associated ion results typical of V. costicola grown in 1M NaCl salts-glucose-potassium phosphate (SGP) medium. The Na^+ values hovered around 1 molal, while the K^+ values increased slightly from the zero time

Table III-6

Effects of aeration, aeration with glucose and poisoning on pellet inter-cellular space[1].

cell suspension	S, inter-cellular space	95% confidence interval, (n)
no treatment	0.253	± 0.019 (4)
aeration	0.273	± 0.048 (4)
aeration + 25mM glucose	0.248	± 0.013 (2)
aeration + poison	0.251	± 0.025 (2)

1. 1M NaCl SGS cells grown to late log phase, treatments of 30 min. duration. n= no. of determinations.

Table III-7

Effects of growth NaCl concentration on inter-cellular space and % occupation of pellet total available fluid volume (T.A.F.V.).

NaCl M	inulin S[1]	pellet fluid volume[2]	% occupation T.A.F.V.[3]
0.8[4]	0.38 ± 0.016	0.72	53.0 ± 2.20
1.2	0.31 ± 0.006	0.67	46.6 ± 0.86
1.8	0.25 ± 0.005	0.66	37.7 ± 0.79
2.2	0.26 ± 0.001	0.64	40.0 ± 0.16

1. Inter-cellular space, ml/g wet pellet, ± standard error for n = 6 Determinations at each salt concentration.
2. ml, estimated as (1-D), D = dry weight/g wet pellet.
3. ± standard error for n = 6 determinations at each salt concentration.
4. SGS late-log cells, at NaCl concentration given.

values to about 0.7 Molal as a result of the aeration or O_2 treatments. When the cell suspension was poisoned or allowed to stand at 30C, the Na^+ increased about 25% and the K^+ fell by about 50%. Mg^{+2} consistently remained, in this experiment as well as most others, in the range 0.05 - 0.08 molal. At the medium and buffer ion values used in this experiment the ion amounts and distributions i.e. K^+/Na^+ ratio, did not change greatly unless the suspensions were allowed to sit for long periods or poisoned. It was thought possible that cells were affected by the unusually high K^+ level (0.15M) in the medium and buffer.

Several experiments were carried out to check if cells might be affected by lower buffer K^+ concentrations comparable with those used by other workers (see Ch. II p. 38). Cells grown in SGP medium were washed and resuspended in a low potassium (LK) buffer solution isotonic to the growth medium, but containing less K^+ and more Na^+ than the growth medium. Cells suspended in the LK buffer (0.008M K^+ and 1.2M Na^+) became more depleted in K^+ after washing than those suspended in CS buffer. Before incubation cell-associated K^+ in two experiments using LK buffer (Figs. III-5, III-6) was 0.4molal, similar to unshaken or poisoned CS buffer suspensions (Tab. III-8).

Cells aerated in LK buffer were able to accumulate K^+ over a period of 20-30 min. (Figs. III-5, III-6). The final cellular K^+ concentration after aeration was similar to that in cells aerated in CS buffer (Tab. III-8), about 0.7 molal.

Fig. III-5. Effect of aeration and incubation under N_2 gas on V. costicola cell-associated Na^+ and K^+. 1M NaCl SGP late-log phase cells washed and suspended in LK isotonic buffer.

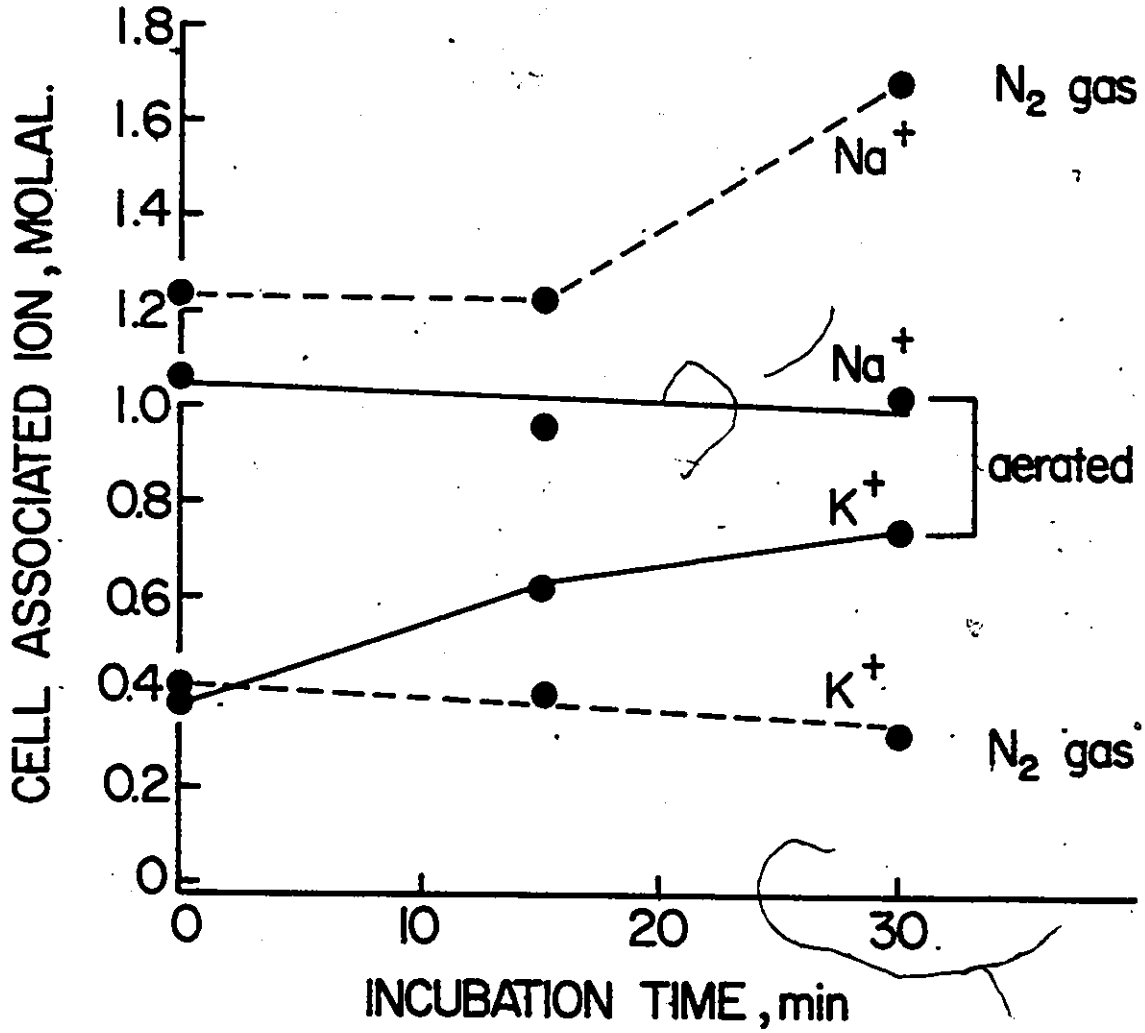
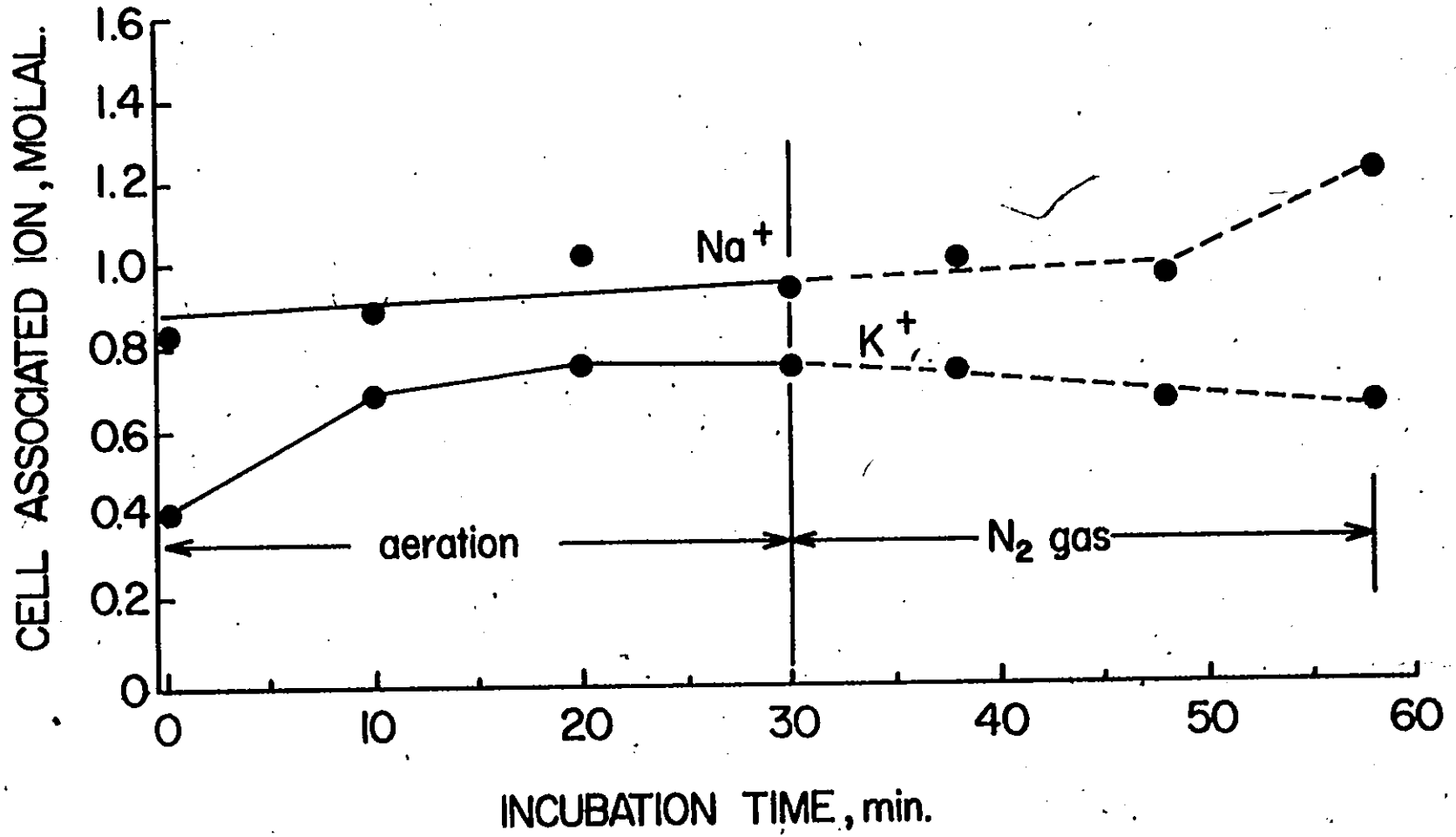

CELL ASSOCIATED ION, MOLAL.
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
INCUBATION TIME, min
N_2 gas
Na^+
Na^+
aerated
K^+
K^+
N_2 gas

Fig. III-6. Effects of aeration and subsequent incubation under N_2 gas on *V. costicola* cell-associated Na^+ and K^+. 1M NaCl SGP late-log phase cells washed and suspended in LK isotonic buffer.


CELL ASSOCIATED ION, MOLAL.
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Na+
K+
aeration
N2 gas
0
10
20
30
40
50
60
INCUBATION TIME, min.

Na^+ concentrations in the time-course experiments (Fig. III-5, III-6) again were around 1 molal and under aeration only changed slightly from the pre-incubation values.

The increase in K^+ upon aeration appears to be due to metabolic events because the increase can be prevented by incubation under N_2. Cellular K^+ concentrations in cells gassed with N_2 decreased slightly from the pre-incubation value over the 30 min. period (Figs. III-5, III-6). The cellular Na^+ increased after about 18 minutes. In the experiments shown in Figs. III-5 and III-6 about 30 min. of N_2 incubation increased the total $Na^+ + K^+$ to about 2 molal, mostly as a result of the marked Na^+ increase (Table III-8).

The time course of change in cellular ion concentrations as a result of incubation under N_2 (Figs. III-5, III-6) indicates that there probably were no major changes in ion distribution during the 10 min. centrifugation required to pellet the cells. The changes due to N_2 treatment took about 15 minutes to begin.

Metabolic experiments with 1M NaCl salts-glucose-sodium phosphate (SGS) grown cells. The SGS medium was formulated to attain a lower medium K^+ concentration. Experiments with SGS grown cells which were washed and suspended in LK buffer yielded results quantitatively and qualitatively similar to those of SGP grown cells (e.g. Tab. III-8, Figs. III-5, III-6). Table III-9 shows that aeration increased the K^+/Na^+ ratio while poisoning allowed the K^+ to leave the cells. With late-log cells, Na^+ also increased on poisoning. Mg^{+2} levels were

Table III-8

Effects of incubation conditions on V. costicola cell-associated ions.

Condition	incubation period, min.	Na^+	K^+	Mg^{+2}	$Na^+ + K^+$	K^+/Na^+
suspension[1] before incubation	0	0.95[2]	0.60	0.080	1.55	0.63
aerated[3]	30	0.86	0.57	0.059	1.43	0.66
aerated[3]	60	1.04	0.68	0.055	1.72	0.65
aer. + ethanol[4]	30	1.07	0.78	0.077	1.85	0.73
aer. + ethanol[4]	60	1.19	0.78	0.055	1.97	0.66
0_2 gas + ethanol	30	1.00	0.68	0.071	1.68	0.68
0_2 gas + ethanol	60	1.23	0.65	0.048	1.88	0.53
aer. + poison[5]	60	1.23	0.35	0.080	1.58	0.29
no addition, not shaken	60	1.23	0.42	0.064	1.65	0.34

1. SGP (1M NaCl, 0.15M K^+) mid-log grown cells suspended in CS buffer (1M Na^+, 0.15M K^+).
2. Molal values.
3. Shaken at 30C at 250 r.p.m.
4. 25mM ethanol.
5. 10mM iodoacetate + 10mM NaCN.

Table III-9

Effects of incubation conditions on cell-associated ions of 1M NaCl SGS grown V. costicola.

Cells	Condition	Na^+	K^+	Mg^{+2}	$Na^+ + K^+$	K^+/Na^+
mid-log phase:	suspension[1] before incubation	1.20[2]	0.66	0.074	1.86	0.55
	aerated[3]	1.19	0.81	0.064	2.00	0.68
	aer. + ethanol[4]	1.27	0.85	0.073	2.12	0.67
	poisoned[5]	1.12	0.20	0.073	1.32	0.18
late-log phase:	suspension before incubation	1.16	0.49	0.063	1.65	0.42
	aerated	1.15	0.79	0.052	1.94	0.69
	aer. + ethanol	1.21	0.75	0.049	1.96	0.62
	poisoned	1.47	0.18	0.066	1.65	0.12

1. SGS (1.15M Na^+, 0.008M K^+) grown cells suspended in LK buffer (1.2M Na^+, 0.008M K^+).
2. Molal values.
3. Shaken at 30C for 30 min. at 250 r.p.m.
4. 25mM ethanol.
5. 10mM iodoacetate + 10mM NaCN.

not significantly affected by any of the treatments. Aerated cells contained 1.2 molal Na^+ with 0.8molal K^+, yielding a K^+/Na^+ ratio of about 0.67, very close to the ratio for SGP grown cells (Tab. III-8).

Determinations of cell-associated ions on cells pelleted directly from cultures. Further experiments were carried out to determine if the results of thick-suspension experiments using CS or LK buffers were actually related to events occurring in culture. One approach involved attempts to pellet cells directly from cultures and weigh and analyse the small pellets formed. The data was calculated using pellet inter-cellular space data from other thick-suspension experiments. Although the errors were great, the data obtained from several experiments (Tab. III-10) supports the contention that the estimations of the ion contents of cells in thick suspensions were pertinent to the ionic contents of cells in culture. Generally, the cellular K^+ and Mg^{+2} ion values of cells taken directly from culture agreed with the aerated thick suspension values. Cellular Na^+ in cells taken directly from culture was much more variable, probably because the amount of medium which contained high Na^+ levels trapped in each small pellet varied. But the cellular Na^+ values of 1M NaCl grown cells still hovered around 1 molal. Most of the K^+/Na^+ ratios values were in the range 0.5 - 1.0, again in agreement with previous thick-suspension experimental results.

Table III-10

Cell-associated ions of cells pelleted directly from cultures.

Experiment	medium	cells[1]	Molal Na^+	K^+	Mg^{+2}	$Na^+ + K^+$	K^+/Na^+
1.	1M NaCl SGP	mid-log (n=2)[2]	0.95 ±0.04[3]	0.65 ±0.03	-	1.60 ±0.08	0.69 ±0.01
2.	1M NaCl SGS	mid-log (n=1)	0.91	0.62	0.066	1.53	0.68
		late-log (n=1)	0.65	0.62	0.074	1.27	0.95
3.	1M NaCl SGS	early-log (n=2)	0.75 ±0.25	0.58 ±0.02	0.057 ±0.002	1.33 ±0.26	0.86 ±0.27
		mid-log (n=3)	1.31 ±0.53	0.65 ±0.13	0.062 ±0.013	1.97 ±0.63	0.70 ±0.26
		late-log (n=1)	0.94	0.61	0.057	1.55	0.65
		stationary (n=1)	0.96	0.51	0.040	1.47	0.53
4.	0.6M NaCl SGS	late-log (n=2)	0.64 ±0.06	0.68 ±0.01	0.071 ±0.001	1.32 ±0.07	1.08 ±0.07
5.	1.6M NaCl SGS	late-log (n=1)	1.11	0.55	0.044	1.67	0.50
6.	2.0M NaCl SGS	late-log (n=1)	1.88	0.71	0.056	2.60	0.38

1. Growth phases of 1M NaCl media related to approximate 660nm absorption ranges as follows: early-log, 0.1-0.4; mid-log, 0.4-0.6; late-log, 0.6-0.8; stationary, 0.8-1.3.

2. n: number of determinations.

3. ± standard error of the mean.

Glucose effect. Another approach used to check if the thick suspension techniques gave relevant data involved the use of alternate buffers and energy sources. When glucose was added to the SGS-salts buffer, essentially making complete SGS medium, the cellular Na^+ level decreased after aeration to a value about half that of the value without glucose. The K^+ level after incubation with glucose was higher than the aerated value. The resulting K^+/Na^+ ratio had a value larger than unity (Tab. III-11).

If cells were washed and resuspended in complete SGS medium, including glucose, even without aeration, the values of cellular Na^+ were lower and those of K^+ were higher than in the absence of glucose (Tabs. III-11, III-12). With the mid-log cells, which grew during the 48 min. incubation period (pellet dry weights increased 12%), both Na^+ and K^+ increased slightly causing a decrease in the K^+/Na^+ ratio. Late-log cells which did not grow, lost Na^+ during incubation, thus showed an increase in the K^+/Na^+ ratio.

Effects of the medium NaCl content on cellular Na^+, K^+ and Mg^{+2}.

An important aspect of the salt relations of V. costicola involves the cell-associated ion concentrations at various medium NaCl concentrations. An experiment with SGP grown cells (Tab. III-13) showed that suspensions which were not aerated contained more total $Na^+ + K^+$ than the medium. The ratio in the last column of Table III-13 indicates, however, that the cellular ions did not increase proportionally with the medium salts. Data with cells pelleted directly from cultures grown

Table III-11

A comparison of washing and thick-suspension buffers:

Cell-associated ions of cells[1] in LK buffer vs. "SGS-salts" buffer[2].

thick-suspension 1M NaCl isotonic buffer	incubation condition	molal Na^+	K^+	Mg^{+2}	$Na^+ + K^+$	K^+/Na^+
LK buffer	suspension before incubation	1.52	0.48	0.061	2.00	0.32
	aerated 30 min.	0.87	0.53	0.043	1.40	0.61
	poisoned[3]	1.32	0.26	0.050	1.58	0.20
"SGS-salts buffer"[2]	suspension before incubation	0.98	0.14	0.051	1.11	0.14
	aerated 30 min.	0.95	0.54	0.035	1.48	0.57
	aerated+glucose[4]	0.50	0.78	0.049	1.28	1.55

1. 1M NaCl SGS grown late-log cells.
2. SGS-salts buffer: 1M NaCl SGS medium without glucose.
3. 10mM iodoacetate + 10mM NaCN.
4. 25mM glucose.

Table III-12

Effects of use of complete 1M NaCl SGS medium[1] as washing and thick-suspension buffer on V. costicola cell-associated ions.

cells	aeration time, min.	cellular ions, molal				
		Na^+	K^+	Mg^{+2}	$Na^+ + K^+$	K^+/Na^+
mid-log[2]	0	0.40	0.53	0.054	0.93	1.33
	24	0.42	0.60	0.054	1.02	1.42
	48	0.59	0.61	0.057	1.19	1.04
late-log[3]	0	0.47	0.57	0.056	1.04	1.21
	24	0.37	0.59	0.057	0.96	1.59
	48	0.31	0.53	0.055	0.84	1.71

1. Including 56mM glucose.
2. During 48 min. incubation at 30C cells increased dry weight by 12%.
3. During 48 min. incubation at 30C cells increased dry weight by 2%.

Table III-13

Effect of SGP medium NaCl concentration on *V. costicola* cell-associated Na^+ and K^+.[1]

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

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

at various salt concentrations corroborate these results (Tab. III-10).

Experiments using SGS grown cells in thick suspensions gave more information on the effects of medium Na^+ content on cellular ions (Tabs. III-14, III-15). Except at the highest medium salt concentration, 2.2M, aerated cells maintained a total $Na^+ + K^+$ content at least equivalent to that of the medium. The ionic contents of cells aerated with glucose were somewhat lower, because of the lower cellular Na^+ content. The K^+/Na^+ ratios decreased with increasing medium salt content in all three treatments. Poisoned cells showed the lowest K^+/Na^+ ratios, less than half that of aerated cells. Actively metabolising V. costicola cells, on the other hand, maintained 0.5 - 0.6 molal cellular K^+ in addition to an amount of Na^+ which resulted in the cellular ion concentration being almost as high as the medium Na^+ content. At the highest medium NaCl concentration tested, where growth in culture was slow, the K^+/Na^+ ratios were lowest and the greatest cellular ion deficit relative to the medium existed.

Table III-15 shows that cellular Mg^{+2} concentrations were not greatly affected by the treatments or medium NaCl concentrations. Poisoned cells may have slightly higher Mg^{+2} contents than aerated cells. Increasing medium NaCl appeared to cause a decrease in cellular Mg^{+2}. The Mg^{+2} values, on the basis of many experiments seem to be roughly constant and did not show changes like those of Na^+ and K^+. This suggests that Mg^{+2} is bound to a fixed number of sites in V. costicola and is not displaced by metabolic changes.

Table III-14

Effect of medium Na^+ concentration on *V. costicola* cell-associated ions.

Medium		Cells[1] aerated 30min				Cells aerated+glucose[2]				Cells poisoned[3]			
Na^+	K^+	Na^+	K^+	Na+K	K/Na	Na^+	K^+	Na+K	K/Na	Na^+	K^+	Na+K	K/Na
0.8M[4]	8mM	0.650[5]	0.716	1.37	1.10	0.505	0.524	1.03	1.04	0.936	0.398	1.33	0.43
1.2M	8mM	0.889	0.821	1.71	0.92	0.584	0.661	1.24	1.13	0.965	0.327	1.29	0.34
1.8M	8mM	1.39	0.565	1.96	0.41	1.09	0.594	1.68	0.54	1.36	0.237	1.60	0.17
2.2M	8mM	1.29	0.548	1.84	0.42	0.898	0.567	1.46	0.63	1.52	0.278	1.80	0.18

1. SGS cells grown to late exponential phase at each salt concentration then harvested, washed and resuspended in the respective LK isotonic buffer.
2. 25mM glucose.
3. 10mM NaCN + 10mM iodoacetate.
4. Growth medium had 0.05M less Na^+, concentration given is for incubation isotonic buffer(LK).
5. Value is molal, calculated on the basis of the parameters given in Table III-15, mean of duplicate samples.

Table III-15

Effect of medium Na^+ concentration on *V. costicola* cell-associated Mg^{+2}, and parameters used in calculations of cell-associated ions*.

Medium Na^+	Medium K^+	Medium Mg^{+2}	aerated Mg^{+2}	+glucose Mg^{+2}	poisoned Mg^{+2}	inter-space S	dry/wet D	buffer H_2O W
0.8M	8mM	0.4mM	0.053	0.051	0.062	0.38 ± 0.016	0.28 ± 0.005	0.984
1.2M	8mM	0.4mM	0.048	0.056	0.056	0.31 ± 0.006	0.33 ± 0.005	0.976
1.8M	8mM	0.4mM	0.037	0.038	0.040	0.25 ± 0.005	0.34 ± 0.002	0.964
2.2M	8mM	0.4mM	0.038	0.042	0.046	0.26 ± 0.001	0.36 ± 0.006	0.956

* S, D and W parameters used to obtain the molal ion values for Mg^{+2} above, and for the values of Table III-14. Cells and conditions as per footnotes of Table III-14. Units are: S, inter-cellular space pellet ml/g wet pellet; D, ratio of dry/wet weight of pellet; W, g H_2O per ml inter-cellular isotonic buffer. Parameter values given with standard errors of means of 6 determinations at each salt concentration.

Intra-cellular ions of cells grown in complex media.

When cellular ion determinations were attempted with V. costicola cells grown in complex media, a serious problem occurred. During the thick suspension procedure in six of ten experiments the cells exhibited signs of lysis. The high inulin intercellular space values of 0.6-0.8ml/g wet cells (approaching 100% penetration of the total available pellet fluid space, estimated as 1-D) and low pellet dry to wet weight ratios obtained in the six experiments probably indicated that the cells had lost an effective permeability barrier (also see discussion). In the remaining four experiments (Tab. III-16) pellet parameters seemed to be more realistic and closer to the results of many experiments using cells from defined salts-glucose media. The causes of such lysis effects were not further investigated.

With cells grown in complex media, the tentative data of Table III-16 indicates that, as with SGS and SGP grown cells, the total $Na^+ + K^+$ increased with the medium salt concentration. At higher salt concentrations the cellular $Na^+ + K^+$ was less than that of the medium. A considerable amount of K^+ was contained in cells grown at medium NaCl concentrations of 1.3 - 1.8M; cells had less K^+ at lower and higher medium salt concentrations. These data are not inconsistent with data on the effect of medium NaCl on SGS or SGP grown cells (Tabs. III-10, III-13, III-14, III-15).

Table III-16

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

medium & medium ion concentrations	molal Na^+	molal K^+	molal Mg^{+2}	K^+/Na^+	$Na^+ + K^+$	cell $Na^+ + K^+$ / medium $Na^+ + K^+$	D^2	S^3
SGS + CAA[4]								
0.5M NaCl (0.8M Na^+, 0.008M K^+)	0.65	0.39	0.046	0.61	1.04	1.29	0.41	0.24
1.0M NaCl (1.3M Na^+, 0.008M K^+)	0.39	1.02	0.021	2.6	1.41	1.08	0.31	0.50
1.5M NaCl (1.8M Na^+, 0.008M K^+)	0.518	1.01	0.098	1.9	1.52	0.84	0.35	0.40
PPT[5]								
3.0M NaCl (3.1M Na^+, 0.009M K^+)	1.78	0.37	0.028	0.21	2.15	0.69	0.33[6]	0.26

1. The results given here are those experiments in which the cells did not show evidence of lysis, i.e. $D > 0.24$, $S < 0.50$. Cells washed and aerated in thick-suspension in fresh growth medium.
2. D = pellet dry weight/wet weight.
3. S = inulin inter-cellular space, ml/g wet pellet.
4. 0.5% w/v casamino acids with the SGS formulation.
5. 1% w/v proteose-peptone + 1% w/v tryptone.
6. estimated value.

Effects of CETAB treatment on V. costicola cell-associated ions.

V. costicola cells were treated with 1mM cETAB (cetyltrimethylammonium bromide) in order to "permeabilize"* the cells, i.e. make their membranes permeable to small molecules. Large molecules such as proteins are presumably retained by the cells. On the basis of equivalent 10ml thick-suspension samples the CETAB treated cells had more than twice the amount of Na^+, about the same amount of K^+ and one half the Mg^{+2} content of healthy V. costicola cells (Tab. III-17). The treated cells when pelleted had half the dry weight of untreated cells, and a dry/wet ratio (D) if 0.16, indicating a loss of material. In experiments with cells grown in complex media at various salt concentrations, when lysis occurred the intercellular volume (S) values were equivalent to the total available fluid volume (0.6-0.8ml/g wet pellet, i.e. complete penetration) and the D values were low (0.16-0.21). The calculated values for the cellular ions of the lysed cells (not shown) indicated that, as with the SGP grown cells, instead of the cellular ions attaining concentrations in equilibrium with the buffer, more were cell bound. If a defect in the permeability barrier allowed

* "Permeabilization", a term now in common usage, denotes the destruction of the cell's selective permeability barriers, rendering the cells freely permeable to small molecules and the unmasking, but not the release, of certain cellular enzymes (Jackson & Demoss 1965). The term arose from work on toluene-treated bacteria; CETAB treatment has similar effects (R. McDonald, personal communication). The permeability characteristics of CETAB-treated V. costicola are assumed to be similar to those of other bacteria so treated, i.e. freely permeable to inorganic ions, freely permeable to nucleotide tri-phosphates but not permeable to most proteins.

Table III-17

Effects of CETAB permeabilization on *V. costicola* cellular ions*

	Na^+		K^+		Mg^{+2}		dry weight	
	mg/g wet pellet	molal	mg/g wet pellet	molal	mg/g wet pellet	molal	of 10ml suspension	D
aerated cells (n=6)	17.02 ± 0.53	1.06 ± 0.06	13.1 ± 0.55	0.69 ± 0.03	0.63 ± 0.04	0.062 ± 0.003	0.092	0.28
CETAB treated cells (n=3)	49.57 ± 2.61	3.37 ± 0.21	14.63 ± 0.99	0.60 ± 0.05	0.25 ± 0.03	0.019 ± 0.03	0.046	0.16
poisoned cells (n=1)	18.6	1.23	7.5	0.35	0.82	0.080	0.092	0.28

* 1M NaCl SGP grown cells, aerated in CS buffer, treated with 1mM cetyl trimethylammoniumbromide (CETAB) or poisoned with NaCN and IAA. n= the number of determinations for the standard errors, D= dry weight/wet weight. The molal values were calculated assuming that the permeabilized cells had the same intercellular space (S) value, 0.28 ml/g wet pellet, as the aerated and poisoned cells.

ions to flow freely in and out of the cells the calculated equilibrium value for pellet ions would have been 19.2mg Na^+/g wet pellet and 4.91mg K^+/g wet pellet (equal to 1M Na^+ and 0.15M K^+ occupying the total available fluid volume of the pellet). The CETAB treated cells, which were not metabolically active, concentrated Na^+ and K^+ together to about 3 times the extracellular level.

Discussion

Effect of growth phase on cell-associated ions.

The effect of growth phase on cellular ions of V. costicola is difficult to ascertain. Washed cells in thick suspension may not respond as if they had remained in the same growth phase at which they were harvested. Three sources of information were used to obtain some indications on effects of growth phase: First, data on cells spun directly from cultures; second, consistent effects in thick-suspension experiments due to the growth phase of cells when harvested; and, third, examination of data from thick suspension experiments where cells were able to continue their growth patterns.

Data on cells pelleted directly from cultures for ionic analyses are given in Tab. III-10. No growth phase trends are clearly evident in the data. The results of many thick-suspension experiments using mid- and late-log cells also show no distinct differences between cells within the exponential growth phase. It may be tentatively concluded that if differences

do exist, they are probably not major.

Cells resuspended in culture medium and aerated showed an interesting growth related effect. When mid-log phase cells were resuspended and aerated in fresh culture medium the cells continued to grow, and while doing so increased their Na^+ contents (Tab. III-12). On the other hand, cells harvested from a late-log culture did not grow and showed a decrease in cellular Na^+. It is possible that the rate of growth may have an effect on the amounts of cellular Na^+ and K^+. This effect, however, was not corroborated by the erratic data on cells spun directly from culture (Tab. III-10).

Possible errors in and interpretation of cell-associated ion data.

Because there are several interrelated parameters which are involved in estimations of cell-associated ions, errors in any one may lead to an inaccurate result. As pointed out previously an increase in inter-cellular space (S) results in a decrease of the cellular water estimate and an increase in the amount of inter-cellular buffer ion subtracted from the pellet ion content, and vice-versa. The value of S depends upon the type of impenetrable solute chosen, either small molecules which probably penetrate to the cell membrane, or large molecules which penetrate to a lesser extent. The estimation of cellular ions based on the use of small molecules such as ATP or PO_4^{-2} involves the implicit assumption that all the cell ions are contained in the space bounded by the permeability barrier, i.e. the membrane. But the distribution of ions in the cell is not known; in fact, it is probable that a significant portion

of ions are associated with the cell wall matrix external to the cell membrane. Estimates of S based on larger molecules, such as inulin, which was used in this study, probably result in a more accurate estimation of the effective cell space enclosing the ions. Besides, the estimates based on inulin are conservative; if an error exists it is probably toward a general underestimation of cellular ions. The term "cell-associated ions" (or the abridged form, "cellular ions") is used to infer that not all the ions are necessarily intracellular in the classical sense of being internal to the membrane, i.e. their distribution in or on the cell is presently unknown. Both S and the ratio of pellet dry to pellet wet weight, D, will determine the calculated amount of cell water, and thus the estimated concentration of cellular ions. Cell water is a quantity which should be consistent for a particular type of cell under similar conditions. In fact Christian & Waltho (1961) found that four Gram-positive cocci, one Gram-positive and one Gram-negative rod all gave an identical value for cell water, 1.5g H_2O/g dry weight! Table III-18 shows comparative cell water data for halophilic bacteria. The values obtained for V. costicola agree well with Christian & Waltho's (1962) estimate and also are in substantial agreement with the data on P. halodentrificans and Pseudomonas #101. The trend toward slightly higher cell water values as the growth medium salt concentration increased was also evident in the Pseudomonas #101 data. But the treatments given to V. alginolyticus and the marine pseudomonad caused on opposite trend. In general, strains which have growth optima at higher salt concentrations had the

Table III-18

Cellular water estimates of halophilic bacteria.

strain	medium or conditions	cell H_2O g/g dry weight	reference
V. costicola (NRC 37001)	0.6M NaCl SGS med.	1.24	this work
	1.0M NaCl SGS med.	1.1-1.4 (range)	
	1.6M NaCl SGS med.	1.68	
	2.0M NaCl SGS med.	1.51	
V. costicola	1M NaCl PPT med.	1.40±0.16 (s.e.m.)	Christian & Waltho 1962
P. halodenitrificans	1M NaCl PPT med.	1.59±0.10	Christian & Waltho 1962
Pseudomonas #101	1M NaCl defined med.	1.31±0.07 (s.e.m.)	Masui & Wada 1973
	2M NaCl defined med.	1.40±0.08	
	3M NaCl defined med.	1.52±0.10	
V. alginolyticus	washed with 0.2M NaCl	2.5	Unemoto et al. 1973
	washed with 0.6M NaCl	2.2	
	washed with 1.0M NaCl	1.8	
Marine pseudomonad B-16 (ATCC 19855)	suspended in 0.24mM NaCl	1.79	Takacs et al. 1964
	suspended in 211mM NaCl	1.43	
	suspended in 1,157mM NaCl	1.38	
Halobacterium	4M NaCl complex med.	1.00±0.02	Christian & Waltho 1962
E. coli (non-halophilic)	minimal salts-glucose	2.55	Schultz & Solomon 1961

least cell water (see also data of Christian & Waltho 1962), which is consistent with higher internal solute concentrations in the halophilic strains.

Tables III-19 and III-20 show calculated values of cell-associated Na^+, K^+ and cell water based on hypothetical deviations in steps of 10% of S and D parameters from actual experimentally determined values. With 0.8M Na^+ SGS grown cells (Table III-19), if the precision of measurement of S and D were no better than ±10% then the possible range of ion values could be 0.45-0.55 molal Na^+ and 0.46-0.69 molal K^+ yielding $Na^+ + K^+$ values of 0.91-1.2 molal. For the 2.2M Na^+ SGS grown cells (Table III-20), the ranges would be 0.71-1.1 molal Na^+, 0.50-0.71 molal K^+ and 1.2 -1.8 molal $Na^+ + K^+$. The results of the studies, then, would not be invalidated by a moderate degree of imprecision in estimates of S and D parameters, up to approximately ±10%.

Maintenance of cellular K^+ and Na^+ concentrations.

Actively metabolizing V. costicola maintained a 75-100-fold concentration gradient of K^+ with respect to the medium. The cellular K^+ concentration was independent of the medium NaCl content. Cellular Na^+, on the other hand, was greatly affected by the medium NaCl content, assuming a concentration in metabolizing cells which was enough or nearly enough when added to the cellular K^+ content to make the cells roughly iso-osmotic to the medium (Table III-14). At medium NaCl concentrations above 1.2M, considerably more than half of the cellular monovalent cation content was Na^+. Substantial amounts of cellular Na^+ are not usually found in actively metabolizing non-halophilic bacteria such as E. coli

Table III-19

Effects of varying the inter-cellular space (S) and dry/wet weight ratio (D) parameters on the estimated cell-associated Na^+ and K^+ concentrations and cellular water of 0.8M Na^+ SGS grown V. costicola[a].

	D=0.25		D=0.28*		D=0.31		D=0.34	
	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
S=0.30	0.54[b]	0.42	0.58	0.45	0.63	0.49	0.68	0.53
	(0.455)[c]		(0.425)		(0.394)		(0.365)	
S=0.34	0.52	0.46	0.56	0.50	0.61	0.54	0.66	0.59
	(0.415)		(0.385)		(0.355)		(0.325)	
S=0.38*	0.49	0.51	0.53	0.55	0.58	0.61	0.64	0.67
	(0.376)		(0.346)		(0.316)		(0.286)	
S=0.42	0.45	0.57	0.49	0.62	0.55	0.69	0.61	0.77
	(0.337)		(0.307)		(0.277)		(0.247)	

* Actual measured S & D values.
a Cells aerated with glucose in LK buffer.
b Molal.
c Calculated g cellular water/g wet pellet.

Table III-20

Effects of varying the inter-cellular space(s) and dry/wet weight ration (D) parameters on the estimated cell-associated Na^+ and K^+ concentrations and cellular water of 2.2M Na^+ SGS grown V. costicola[a].

	D=0.28		D=0.32		D=0.36*		D=0.40	
	Na^+	K^+	Na^+	K^+	Na^+	K^+	Na^+	K^+
S=0.23	0.83[b]	0.46	0.90	0.50	0.99	0.54	1.09	0.60
	(0.500)[c]		(0.460)		(0.420)		(0.380)	
s=0.26*	0.74	0.48	0.81	0.53	0.89	0.58	1.00	0.65
	(0.471)		(0.431)		(0.391)		(0.351)	
s=0.29	0.64	0.51	0.71	0.57	0.78	0.63	0.88	0.71
	(0.443)		(0.403)		(0.363)		(0.323)	
s=0.32	0.53	0.55	0.58	0.61	0.65	0.68	0.74	0.77
	(0.414)		(0.374)		(0.334)		(0.294)	

* Actual measured S and D values.
a Cells aerated with glucose in LK buffer.
b Molal.
c Calculated g cellular water/g wet pellet.

(Schultz and Solomon 1961) or Streptococcus fecaelis (Harold & Altendorf 1974) but have been noted in most of the halophilic bacteria analysed (see below).

The cellular K^+ gradient was reduced markedly when the cells were poisoned indicating that metabolic energy was essential for the processes which concentrate K^+ in the cells. The decrease in cellular K^+ was more than compensated for by increases in Na^+ in poisoned cells. Although, in general there was an exchange of cellular Na^+ for K^+, the Na^+ and K^+ translocation mechanisms were not directly linked to each other because cellular K^+ could increase without a corresponding decrease in cellular Na^+ (e.g. data in Tabs. III-9, III-11, Fig. III-6). Studies on ion transport in S. fecaelis (Harold & Altendorf 1974), in E. coli (Schultz & Solomon 1961) and in a marine pseudomonad (Thompson & MacLeod 1973) have indicated that bacterial transport of Na^+ and K^+ are not tightly coupled.

The ion transport mechanisms of bacterial cells are not well understood. Harold and Altendorf (1974) have outlined the current knowledge of bacterial ionic relations in a recent review. The authors used P. Mitchell's chemiosmotic hypothesis (Mitchell 1966) as a framework for the consideration of the available data and phenomena. The central tenets of Mitchell's hypothesis, an ion impermeable cell membrane across which an electrical potential difference (cell interior negative) and pH gradient (cell interior alkaline) as established by active vectoral outward proton transport by metabolically and ATPase mediated pumps, incorporate the elements which appear to be universally related to bacterial ion transport.

The potential gradient and pH difference established allows work to be done by the cell, including inward transport of K^+ perhaps with other essential substances and extrusion of Na^+. It is only possible to relate my own findings in a most general way to Mitchell's hypothesis and data on other bacteria. But it is significant to note that the dependence upon metabolic energy, i.e. active glucose metabolism, in V. costicola for maintenance of the highest cellular K^+/Na^+ ratios is similar to that of other bacteria studied and might be considered in relation to the probable existence of a proton gradient.

The apparently universal requirement of a high intracellular K^+/Na^+ ratio in order for adequate active cell function has not been fully rationalized. One aspect, in agreement with chemiosmotic concepts, involves the finding that transport of the amino acid analog, α-aminoisobutyric acid requires high K^+ concentrations inside a marine pseudomonad as well as Na^+ in the medium (Thompson & MacLeod 1971). K^+ depleted cells could not transport the amino acid analog. More recent work has demonstrated a relation between both α-AIB and K^+ transport and electron transport chain carriers located between cytochrome c and cytochrome oxidase (MacLeod, personal communications). Other aspects of cellular function reviewed by Harold and Altendorf (1974) thought to be dependent upon cellular K^+ include protein-synthesis, internal pH regulation, and osmotic adaptation.

Relationship between medium osmolarity or salt content and ionic content of bacteria.

K^+ ions in non-halophilic bacteria probably play an important role in maintaining osmotic equilibrium. In studies with Serratia species (Gale et al. 1970), Aerobacter aerogenes (Tempest & Meers 1968) and Staphylococcus aureus (Christian & Waltho 1964), the amount of intracellular K^+ was directly related to the various levels of NaCl included in the growth media. Salt tolerance in non-halophiles has been related to the ability to accumulate potassium within the cells (Christian & Waltho 1961).

Direct and indirect measurements indicate that halophilic bacteria maintain high internal solute concentrations. Centrifugation experiments (data unpublished) carried out as part of this work indicated that 1M SGP grown V. costicola had a density of 1.21, which is only possible if the cells contained a substantial amount of small dense molecules such as sodium or potassium. The freezing points of halophilic cells, including V. costicola were close to those of the medium in which they were grown (Christian & Ingram 1959b). Lysis experiments with V. costicola and slightly halophilic V. metchnikovii showed that osmotic sensitivity was related to the salt concentration of the growth medium: The lysis point was always one-third of the NaCl concentration during growth (Christian & Ingram 1959). [Work with V. alginolytcus, on the other hand, showed that the lysis point was independent of the growth medium salt concentration (Unemoto el al. 1973). It can be argued that lysis phenomena were not related solely to osmotic events but possibly also to envelope destabilization as salts are removed and to other unknown factors (MacLeod & Matula 1962)].

Measurements of cellular ions show directly that halophilic bacteria maintain high internal solute concentrations. K^+ and Na^+ ions are important osmotic solutes in moderately halophilic bacteria (Table III-21). It is not clear from the few studies completed, if an increase in medium NaCl content in which a moderately halophilic bacterial strain is growing results in a corresponding increase in intracellular salt content. V. costicola, V. alginolyticus and the marine pseudomonad (Table III-21) were able to maintain an internal salt content roughly equivalent to the medium NaCl content. On the other hand Pseudomonas #101 and the unidentified Gram-negative rod did not maintain internal salt concentrations equivalent to the medium. Cations, of course, may not necessarily be the only osmotically active substances in the cell. Sugar-tolerant yeasts are known to contain osmotically active polyhydric alcohols (Brown & Simpson 1972, Brown 1974). The halophilic algae Dunaliella parva maintains osmotic equilibrium with glycerol (Ben-Amotz 1974). A recent report underlines the potential importance of amino acids in osmoregulation, especially in organisms where the amount of cellular salts may not be adequate to attain osmotic pressure equilibrium (Measures 1975). The possible roles of amino acids or other small molecules in osmoregulation deserves further attention in view of the relatively low ion contents of at least one moderate halophile (Tab. III-21).

Magnesium content of V. costicola

Unlike the labile Na^+ and K^+ ions, cell Mg^{+2} content

Table III-21

Intracellular cation concentrations of various moderately halophilic bacteria.

strain	growth medium	condition	molal cellular Na^+	molal cellular K^+	$Na^+ + K^+$	K^+/Na^+	Cell $Na^+ + K^+$ / medium $Na^+ + K^+$	reference
V. costicola (NRC 37001)	0.6M NaCl SGS	aerated with glucose	0.505	0.524	1.03	1.04	1.36	this work
	1.0M NaCl SGS	"	0.584	0.661	1.24	1.13	1.07	
	1.6M NaCl SGS	"	1.09	0.594	1.68	0.54	0.95	
	2.0M NaCl SGS	"	0.898	0.567	1.46	0.63	0.65	
V. costicola	1M NaCl PPT	stationary phase	0.684 ± 0.090	0.221 ± 0.054	0.905	0.323	0.901	Christian & Waltho 1962
P. halodenitrificans	1M NaCl PPT	stationary phase	0.311 ± 0.016	0.474 ± 0.038	0.785	1.51	0.782	Christian & Waltho 1962
Pseudomonas #101	1M NaCl defined	early stationary phase	0.90 ± 0.36	0.71 ± 0.31	1.61	0.79	1.60	Masui & Wada 1973
	2M NaCl defined	"	1.15 ± 0.26	0.89 ± 0.14	2.04	0.77	1.02	
	3M NaCl defined	"	1.04 ± 0.25	0.67 ± 0.24	1.71	0.64	0.57	
V. alginolyticus	0.5M NaCl complex	washed with 0.2M NaCl	0.185	0.165	0.35	0.89	1.75	Unemoto et al. 1973
		washed with 0.6M NaCl	0.434	0.422	0.86	0.97	1.42	
		washed with 1.0M NaCl	0.698	0.453	1.15	0.65	1.15	
Unidentified Gram-negative rod	0.6M NaCl complex	logarithmic phase	0.05	0.34	0.39	6.8	0.58	Matheson et al. 1976
	4.4M NaCl "	" "	0.62	0.58	1.2	0.94	0.27	
	0.6M NaCl complex	stationary phase	0.29	0.32	0.61	1.10	0.98	
	4.5M NaCl "	"	1.01	0.66	1.67	0.65	0.37	
Marine pseudomonad B-16 (ATCC 19855)	0.3M NaCl complex	log. phase, aerated in: 0.3M NaCl-salts buffer	0.123	0.374	0.497	3.04	1.56	Thompson & MacLeod 1973
		0.5M NaCl-salts buffer	0.187	0.434	0.621	2.32	1.19	

was unaffected by metabolic changes in V. costicola. Likewise, under conditions where the cell K^+ increased in chemostat cultures of Aerobacter aerogenes, Mg^{+2} hardly varied (Tempest & Meers 1968). In V. alginolyticus, Mg^{+2} was not affected by washing with solutions containing various NaCl concentration, either (Unemoto et al. 1973). In V. costicola the maintenance of the 100-fold gradient of cellular Mg^{+2} with respect to the medium is consistent with tight binding to cell substances such as nucleic acids, ribosomes, and cell envelopes.

Distribution and state of cellular K^+ and Na^+.

A controversy exists on the state of intracellular Na^+ and K^+. Proponents of the "association-induction hypothesis" (Ling et al. 1973) in which membranes properties and pumps are discounted in favor of specific selective binding of ions by the macromolecular-aqueous complex system of the cell, argue that N.M.R. data shows that the Na^+ in muscle and the K^+ in Halobacterium are complexed to fixed sites (Ling & Cope 1969, Cope & Damadian 1970). On the other hand, Lanyi and Silverman (1972) showed that the K^+ of freeze-thawed halobacterium cells diffuses freely, thus in the cell it was probably in the free state but held in place by an impermeable membrane. Harold and Altendorf (1974) prefer the "middle approach" with statements to the effect that the ions are neither free nor bound but have reduced mobility.

Whether Na^+ and K^+ are bound or free has great potential importance to the understanding of the physiology of halophilic bacteria. When V. costicola cells lysed or were

permeabilized with CETAB, they did not attain equilibrium with the ions in the extracellular solution. Instead the amounts of cellular Na^+ increased several fold, as if some binding sites had been unmasked. Masui and Wada (1973) showed that the isolated cell envelope of the moderately halophilic pseudomad #101 also contained a large amount of Na^+ and somewhat less K^+. The distribution of ions in Bacillus amyloliquefaciens was studied by Coleman (1974). 80% of the cellular Na^+ and all of the Ca^{+2} and Cl^- were found bound to the envelope while most of the Mg^{+2} and K^+ were considered to be intracellular, but probably associated with phosphate groups of DNA and ribosomes. These analyses argue strongly against the concept that the cellular Na^+ and K^+ ions, or even most of them, are free in the cytoplasm.

If significant amounts of ions are found associated with the V. costicola cell envelope then the volume of the cell wall and membrane structures should be adequate to account for the proposed binding. A rough calculation using 100nm as the approximate thickness of the entire cell envelope (slightly larger than the difference between the ATP and inulin penetration estimations) indicates that the envelope structures occupy about 30% of the 3.59 μm^3 cell volume (cell radius 0.95 μm, considered as spherical). Thus the intra-cellular ions could be accomodated by halophilic cell envelope structures.

Recent data on the salt requirements and tolerances of V. costicola enzymes and ribosomes makes much more sense if the majority of ions were not free in the cytoplasm. In vitro protein synthesis (R. Wydro, T. Hiramatsu & D. J. Kushner,

unpublished) and many, but certainly not all, enzymes are inhibited by levels of Na^+ and K^+ far below the measured cellular concentrations. It seems sensible to consider that the salt sensitive structures of halophilic bacteria such as V. costicola must be somehow separated from the bulk of the cellular ions. In the next chapters the response of two enzymes to salt is presented along with an examination of similar work on other enzymes.

Chapter IV

The aspartate transcarbamylase of Vibrio costicola.

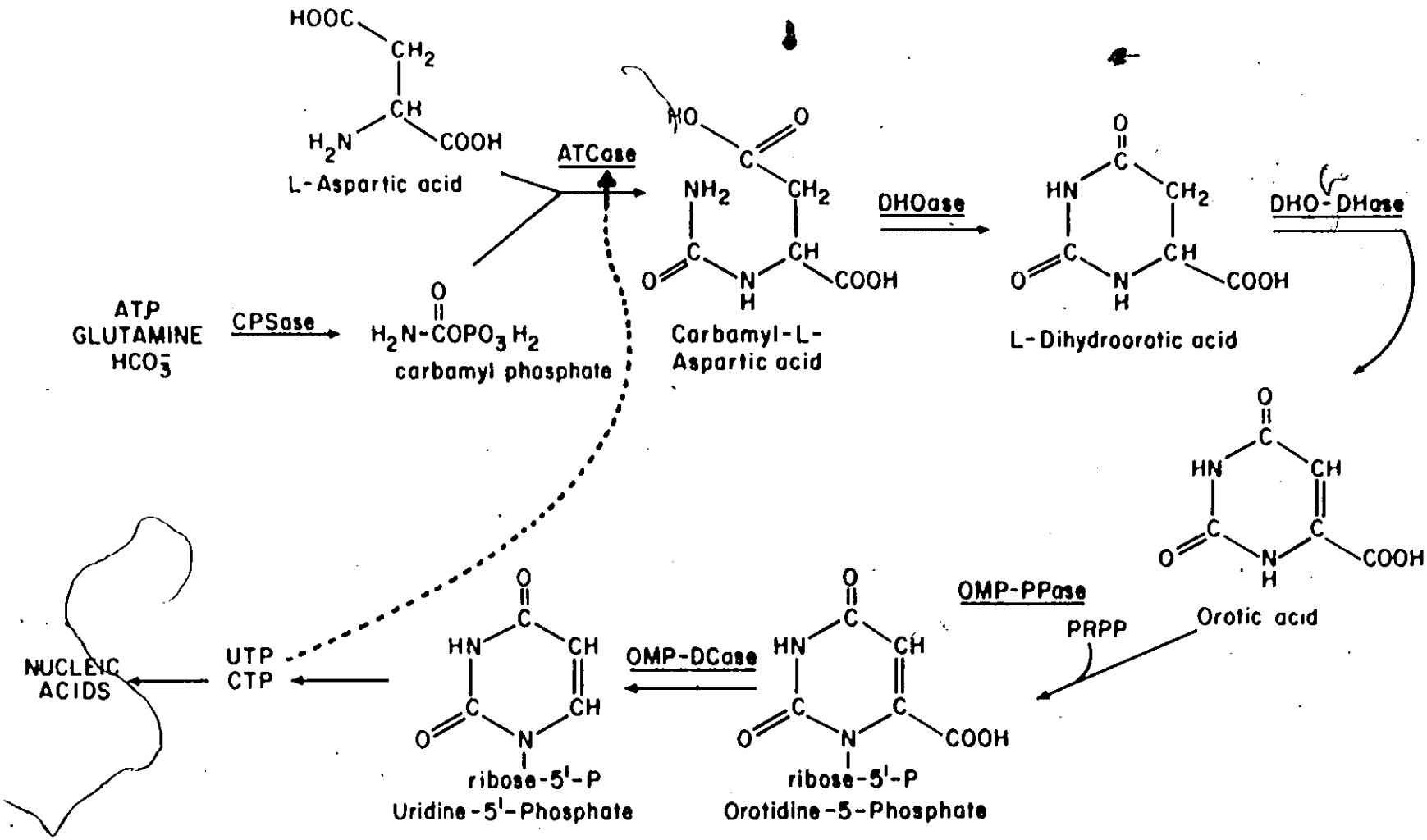
Introduction

A striking quality of many enzymes of the extremely halophilic bacteria is a pronounced salt requirement for activity and stability. The aspartate transcarbamylase (ATCase) from Halobacterium cutirubrum depended upon the presence of 3-4M NaCl or KCl for optimum activity, feedback inhibition (Liebl et al. 1969) and stability (Norberg et al. 1973). The work described in this chapter was designed to examine the salt response of the ATCase from the moderate halophile, V. costicola and to compare the properties of this enzyme with those from several non-halophilic bacteria and yeasts as well as from the extreme halophile. Possible regulatory functions of the V. costicola enzyme were of special interest.

In bacteria, aspartate transcarbamylase (ATCase, EC 2.1.3.2) catalyses the first committed step in the pyrimidine synthetic pathway (Fig. IV-1). The enzyme mediates the condensation of carbamyl phosphate (CP) and aspartic acid to form carbamyl aspartic acid (CAA) which then continues through the sequence via orotic acid forming the pyrimidine nucleotides; uridylic acid (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), cytidine triphosphate (CTP), and cytidine diphosphate (CDP), and then the deoxypyrimidine nucleotides. The enzyme's action was first described by Reichard and Hanshoff (1955, 1956). Shortly thereafter Yates and Pardee (1956)

Fig. IV-1

The de novo pyrimidine biosynthetic pathway. Dashed line represents regulatory action on aspartate transcarbamylase by end product feedback inhibition.


L-Aspartic acid
ATCase
Carbamyl-L-Aspartic acid
DHOase
L-Dihydroorotic acid
DHO-DHase
Orotic acid
OMP-PPase
PRPP
ribose-5'-P
Orotidine-5-Phosphate
OMP-DCase
ribose-5'-P
Uridine-5'-Phosphate
UTP
CTP
NUCLEIC ACIDS
ATP
GLUTAMINE
HCO3-
CPSase
carbamyl phosphate

discovered the regulatory significance of the activity with a report that an excess of a pathway end product, CTP, would inhibit the condensing activity of ATCase from Escherichia coli. The amount of nucleotides produced, then, was regulated by their cellular concentrations. Any buildup in CTP concentration would result in an inhibition in the activity of the pyrimidine pathway's first enzyme, thus effectively turning off the pathway until more CTP was required. ATCase, along with the biosynthetic threonine deaminase (Umbarger 1956) were the enzymes in which the important regulatory phenomenon, end product retroinhibition was first discovered.

When the work on the V. costicola ATCase was begun, preliminary experiments had indicated that the intracellular salt concentrations were substantial, Na^+ being the dominant cation. Most of the experiments in this and the next chapter involve enzymatic assays where NaCl was a major component. KCl was often present in substantial amounts during enzyme extraction but was only a minor component in the final reaction mixtures. Many halophilic enzymes studied are affected in roughly the same way by either Na^+ or K^+ ions. Since the cellular Na^+ content is often greater and more physiologically variable than the K^+ content (See Ch. III), the use of Na^+ for the enzyme studies was considered appropriate.

Methods

Cells. V. costicola cells were generally grown in the 1M NaCl, 1% (w/v) proteose-peptone, 1% (w/v) tryptone medium as described in ch. 2 (p. 31-34) and harvested at the mid-

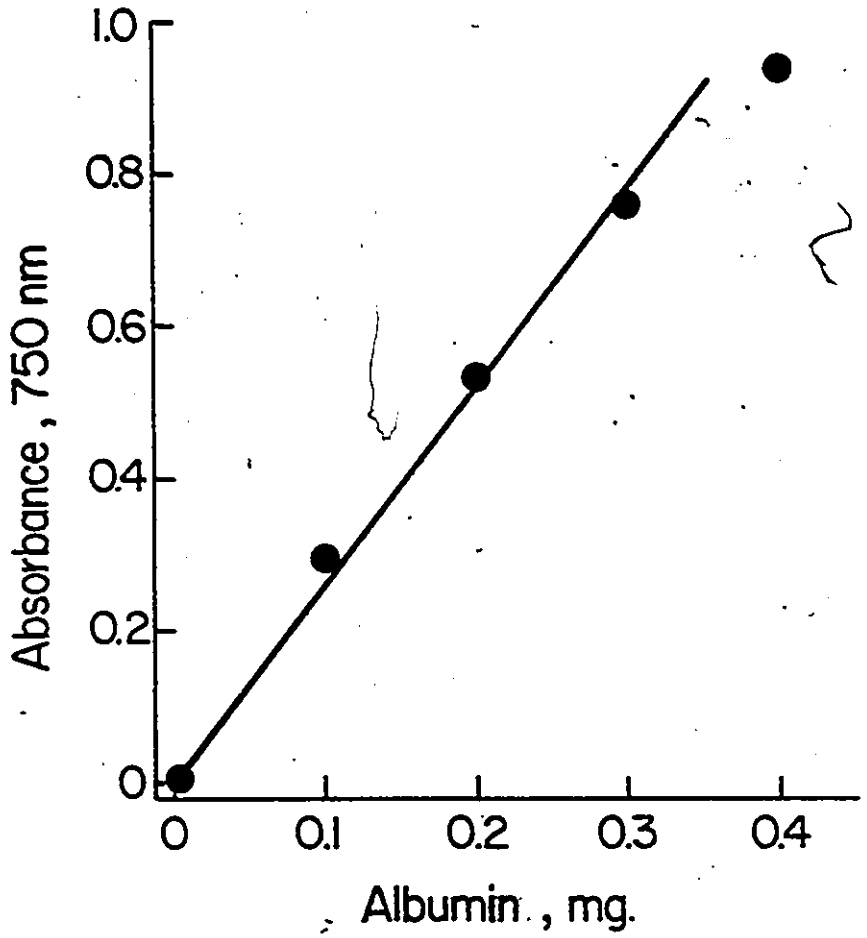
to late-exponential phase (approximately 24 hrs. growth). The cells were washed twice at 4C in 0.1M K_2HPO_4, 1M NaCl pH 7.5 then resuspended at 100-fold their culture concentration in the buffer, kept at 4C, in which the cells were to be broken.

Extraction of enzyme activity. The extraction buffer was either identical to the washing buffer (in some experiments, tris hydroxymethylamino methane or Na_2CO_3 was substituted for the K_2HPO_4) or more often, contained 1.5M NaCl, 10^{-3}M dithiothrietol, 10^{-4}M mono-magnesium di-sodium ethylenediamine-tetraacetate and 0.1M buffer substance. In some experiments devised to test for feedback inhibition, specified small quantities of cytidine triphosphate (CTP) were also included in the buffer. Cells were broken either by sonic disruption at 4C using a Branson sonifier or pressure disruption at 4C using an American Instrument Co. "French pressure cell". Four 30 sec. 8-10 amp. pulses with 30 sec. cooling intervals or two passes through the pressure cell were sufficient to attain virtually complete cell breakage as determined by phase contract microscopic observations. After disintegration the cell debris was separated from the straw-colored supernatant which contained all of the ATCase activity, by centrifuging for 20 min., 25,000 x G at 4C. The protein content of the extract was determined by the Lowry et al. (1951) method using bovine serum albumin standards (Fig. IV-2).

Reaction Mixtures. Reactions were begun with the addition

Fig. IV-2

Typical Lowry et al. (1951) protein standard curve using bovine serum albumin.


1.0
0.8
0.6
0.4
0.2
0
Absorbance, 750 nm
0
0.1
0.2
0.3
0.4
Albumin., mg.

of 0.5ml crude cell extract (usually without further purification) to 2.0ml of a solution containing NaCl, aspartate, carbamyl phosphate (freshly prepared Calbiochem B-grade used as supplied or after the recrystallization procedure of Davies et al. 1970) and buffer (e.g. tris hydroxymethylamino methane) at concentrations and pH specified in the results section. During the 30C incubation, five or six 0.2ml aliquots were removed from the reaction mixtures at regular 4-7 min. intervals each aliquot being added to a tube on ice containing 0.5ml 0.1M HCl in absolute ethanol, which stopped the reaction. The colorimetric assay for carbamyl aspartate was performed in the tubes containing the reaction mixture aliquot. After incubation the pH of the remaining reaction mixture solution was checked with a Corning 476050 low-sodium-error micro combination electrode. Because a significant amount of the carbamyl aspartate reaction product sometimes formed non-enzymatically (see below), identical control reaction mixtures except without enzyme were sampled along with the enzyme reaction mixtures.

Assay for the reaction product. Carbamyl aspartate was measured by either the colorimetric methods of Koritz and Cohen (1954) as modified by Gerhart and Pardee (1962) or by the method of Prescott and Jones (1969). With the latter method, the intensity and quality of light used for color development was important (L. M. Prescott, personal communication). The light source used consisted of two 40 watt Phillips F40T/CW cool white fluorescent tubes shielded by a medium

yellow acetate filter and 3/16" window glass. The light intensity was 40 ft.-candles read on a General Electric type 213 light meter (equivalent to 0.63 watt/m^2). Both colorimetric methods were sensitive to the presence of NaCl or KCl, however the amounts of salts in the reaction mixture aliquots had only small effects. Corrections for any salt effects were routinely accomplished by including the appropriate amount of salts in with carbamyl aspartate standards used for the standard curves (Figs. IV-3, IV-4). The curves remained linear although the slope or the range of the assays were reduced by the presence of high salt concentrations in the reaction mixture aliquots.

Enzyme reaction rates. Rates of enzyme reaction were measured by regression of time course curves based on 5 or 6 points over a 15-30 min. period. The enzyme reaction rate for a particular set of conditions were expressed in terms of specific activity: μmoles carbamyl aspartic acid produced per hour per mg protein. Time course curves were linear and the reaction rates were strictly proportional to enzyme concentration after necessary corrections for the non-enzymatic reaction (Fig. IV-5).

Corrections for the non-enzymatic reaction. Under certain conditions, especially at elevated temperatures and high substrate concentrations, a significant non-enzymatic formation of carbamyl aspartic acid occurred in aspartate transcarbamylase reaction mixtures. Several groups of

Fig. IV-3

Typical carbamyl aspartate standard curves, assay of Koritz and Cohen (1954) as modified by Gerhart and Pardee (1969). With this method salts affected the slope of the calibration curves but linearity was maintained. Standards were routinely run containing salt concentrations identical to those of the enzyme assay samples. The concentrations of salts given were those in the reaction mixture aliquots.

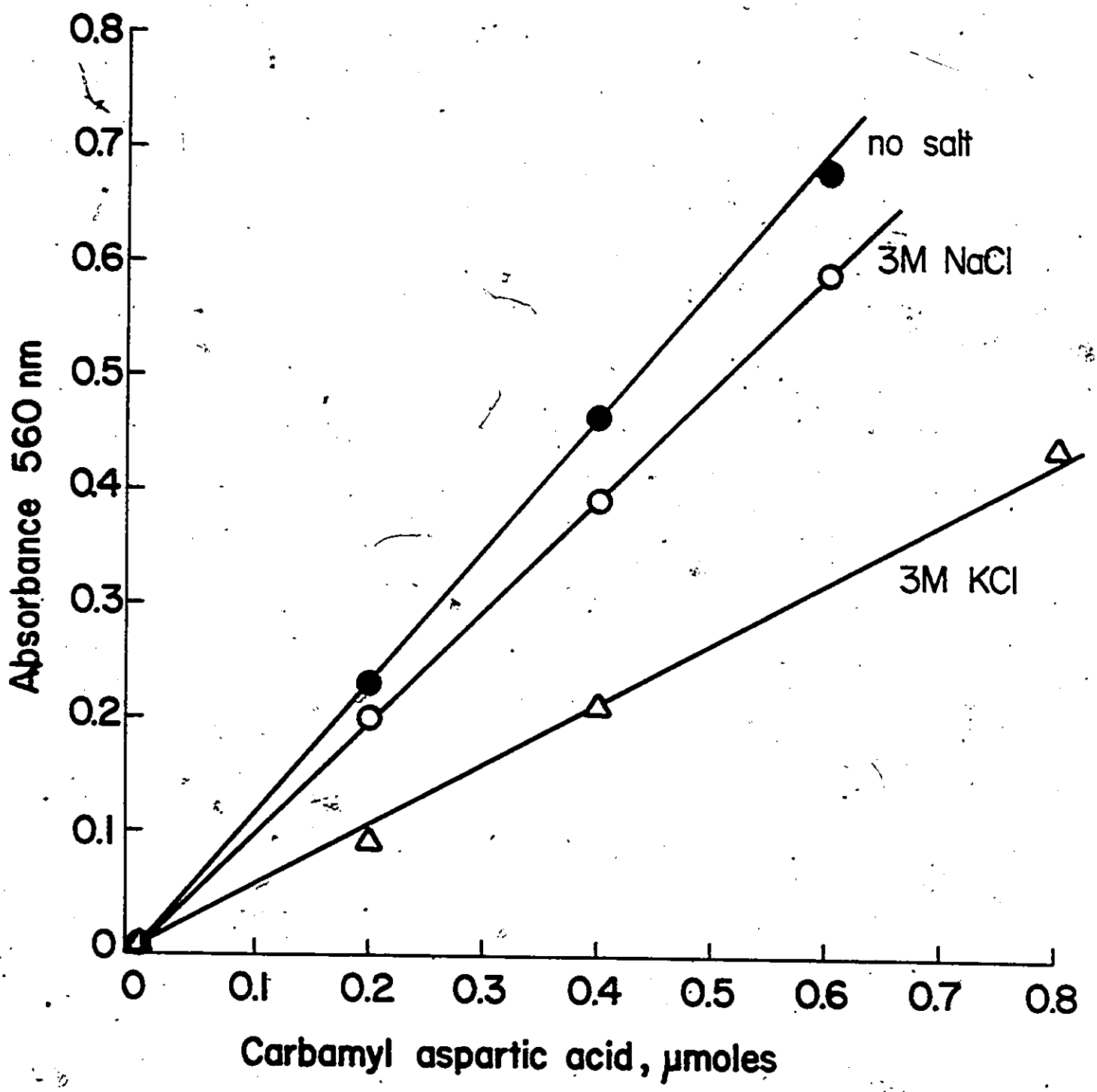

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance 560 nm
no salt
3M NaCl
3M KCl
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Carbamyl aspartic acid, µmoles

Fig. IV-4

Typical carbamyl aspartate standard curves, Prescott and Jones (1969) assay method. Using this method the standard curves were linear and unaffected by salt up to 0.15 µmoles carbamyl aspartic acid. The concentrations of salts given were those in the reaction mixture aliquots.

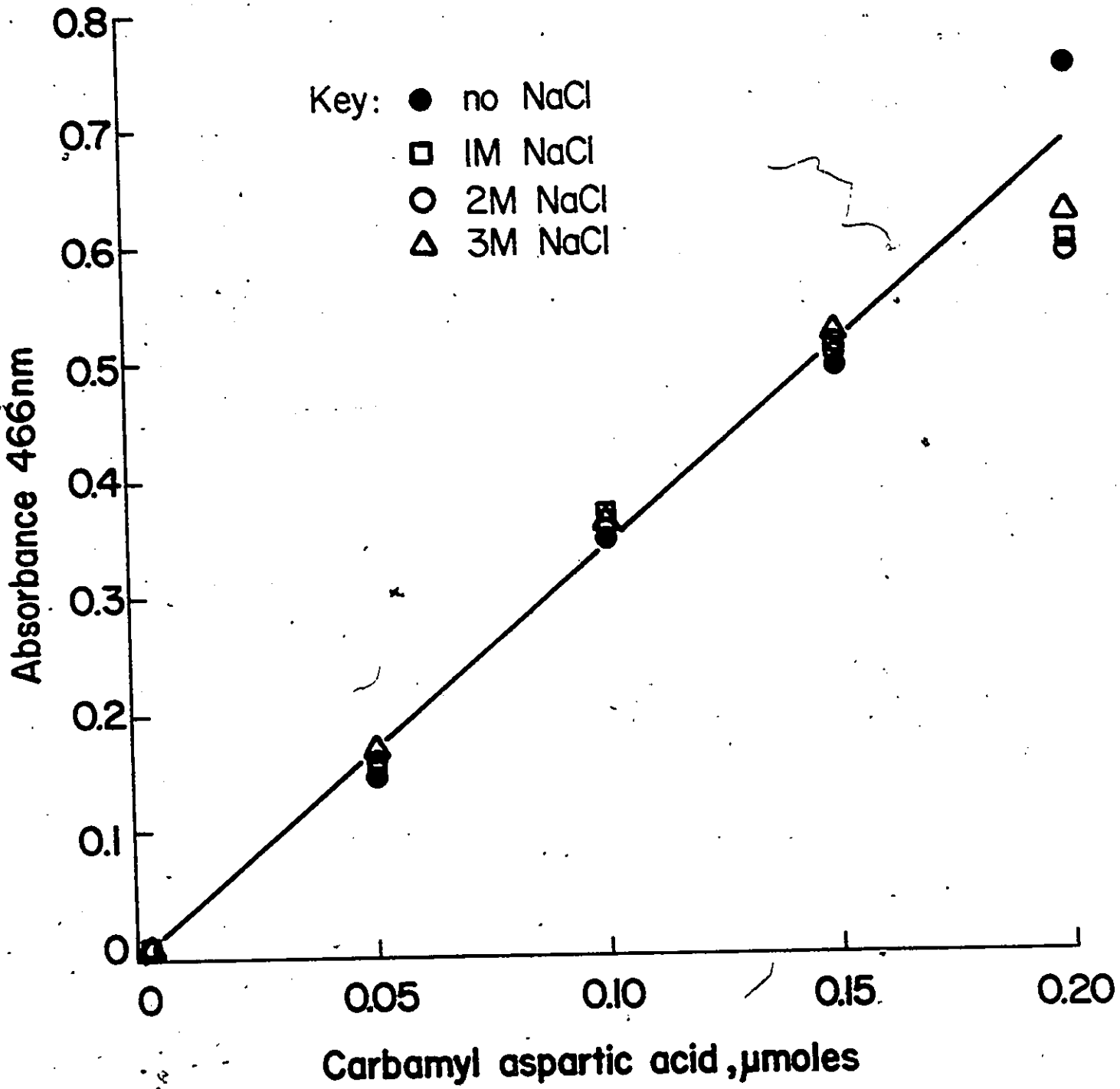
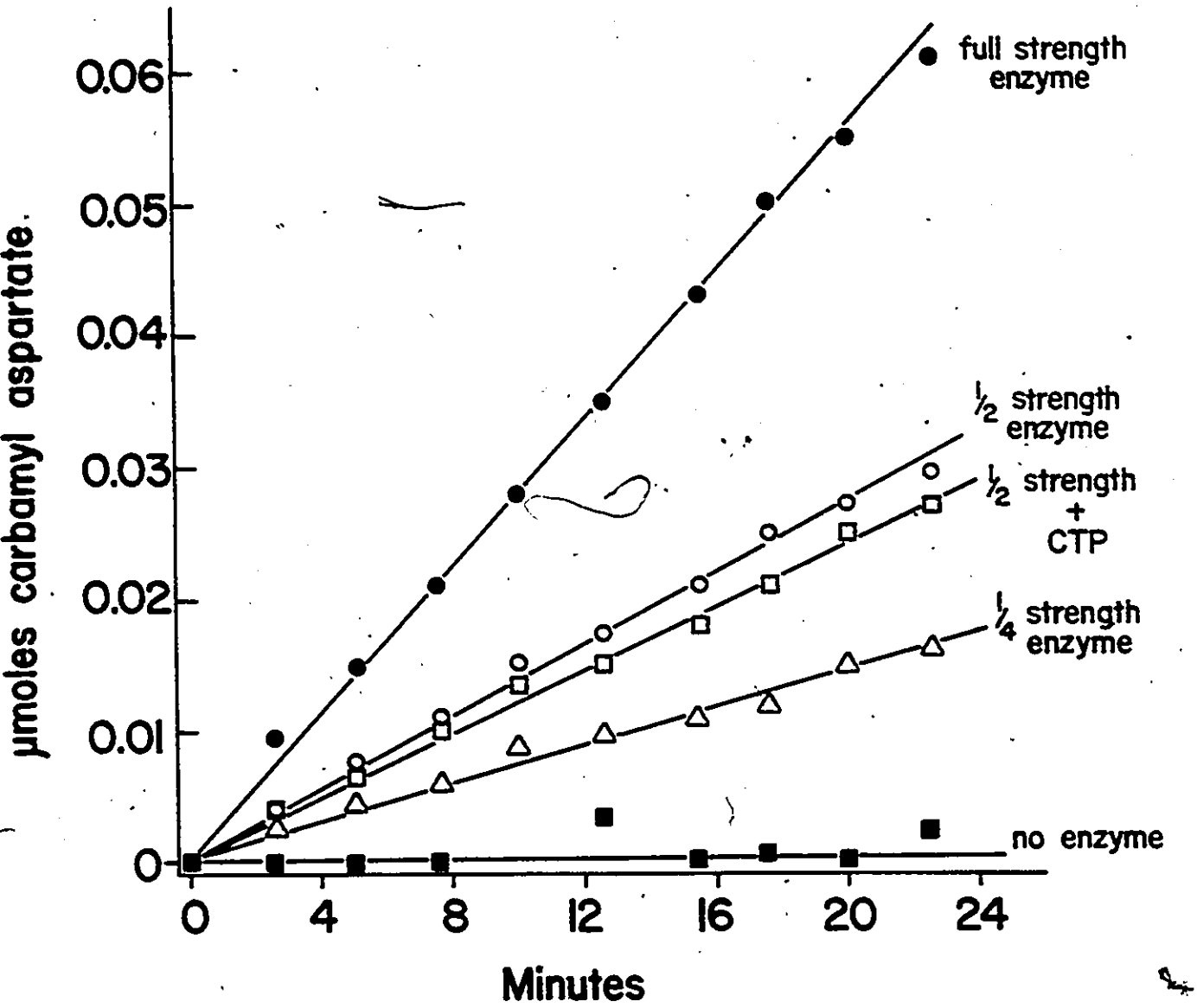

Key: ● no NaCl
□ 1M NaCl
○ 2M NaCl
△ 3M NaCl
Absorbance 466nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.05
0.10
0.15
0.20
Carbamyl aspartic acid, µmoles

Fig. IV-5

Effect of enzyme concentration on aspartate transcarbamylase activity. 1.5M NaCl reaction mixture contained 0.05M aspartate, 0.01M carbamyl phosphate, 0.10M tris, pH 7.4. Prescott and Jones (1969) colorimetric assay, specific activity of full strength enzyme 3 µmoles CAA/hr./mg prot. Relative rates; full strength enzyme 0.170 µmoles CAA/hr, ½ strength enzyme 0.083 µmoles CAA/hr, ½ strength enzyme in presence of potential feedback inhibitor 10^{-3}M CTP 0.080 µmoles CAA/hr, and ¼ strength enzyme 0.044 µmoles CAA/hr. Another experiment using a similar 2.0M reaction mixture with the Gerhart and Pardee (1962) colorimetric assay yielded reaction rates for the full strength enzyme 2.0 µmoles CAA/hr and for the ½ strength enzyme 0.9 µmoles CAA/hr.


0.06
0.05
0.04
0.03
0.02
0.01
0
μmoles carbamyl aspartate
0
4
8
12
16
20
24
Minutes
full strength enzyme
½ strength enzyme
½ strength + CTP
¼ strength enzyme
no enzyme

investigators have noted the non-enzymatic reaction (Lowenstein & Cohen 1955, Gallach 1959, Stein & Cohen 1965, Weitzman & Wilson 1966, Bethell et al. 1968, Bethell 1968, Prescott & Jones 1970) but did not show specific data on the reaction mechanism or the product formed. Some investigators used inappropriate correction methods for the enzyme "blank". Thus I carried out a brief experimental study on the non-enzymatic reaction.

It was important to identify the non-enzymatic reaction product. The substance formed (1) migrated at exactly the same rate as authentic carbamyl aspartate in a thin-layer electrophoresis procedure (Fig. IV-6), (2) was detected by colorimetric and radiotracer assay methods (Prescott & Jones 1969, Gerhart & Pardee 1962, Porter et al. 1969) specific for carbamylated compounds, and (3) was formed in a manner dependent upon the presence and concentrations of aspartic acid and carbamyl phosphate, the reactants, thus was identical to the enzymatic product (Fig. IV-7B). In enzymatic reaction mixtures the contribution of the non-enzymatic carbamylation reaction was subtracted yielding a true enzymatic carbamylation rate (Fig. IV-8).

The mechanism of the non-enzymatic reaction does not involve a direct reaction between carbamyl phosphate and aspartic acid. Carbamyl phosphate is probably not a good carbamylating agent (Stark 1965). Under alkaline conditions it is degraded to phosphate and cyanate (Halman et al. 1956, Jones & Lipmann 1960). Cyanate has been shown to readily react with amino groups of amino acids and proteins forming carbamylated derivatives (Stark et al. 1960, Stark 1965).

Fig. IV-6

Autoradiograph of thin-layer electrophoresis plate showing that the non-enzymatic product(B) and the product of the cyanate-aspartate reaction (C) had the same mobility as carbamyl aspartic acid (D).

Standards: (A) L-^{14}C aspartic acid, (D) carbamyl-^{14}C aspartic acid (prepared enzymatically from L- ^{14}C aspartic acid and carbamyl phosphate using Escherichia coli B crude extract under conditions where less than 0.5% of labelled non-enzymatic product was formed. Carbamyl-^{14}C aspartic acid was isolated using a Dowex 50 X 8-400 column at pH 3.2.) Reaction mixtures: (B) 0.05M L-^{14}C aspartic acid, 0.05M carbamyl phosphate and 0.10M tris-HCl pH 7.4 incubated 30 min at 60C, (C) 0.10M L- ^{14}C aspartic acid, 0.02M sodium cyanate and 0.10M tris-HCl pH 7.4 incubated 30 min at 37C.

Samples of reaction mixtures and standards were spotted on a thin-layer Kieselguhr-G coated glass plate which had been previously sprayed with the buffer (acetic acid:formic acid:H_2O 15:5:80, pH 2.0). 500V was applied for one hour at 4C. The plate was then dried at 37C. X-ray film was exposed to the plate in darkness for about one week and then developed. Amounts of labelled substances and reaction mixture conditions were calculated to yield dark spots on the film of approximately equal intensities. After autoradiography the plate was sprayed with alcoholic ninhydrin to confirm the positions of the spots.

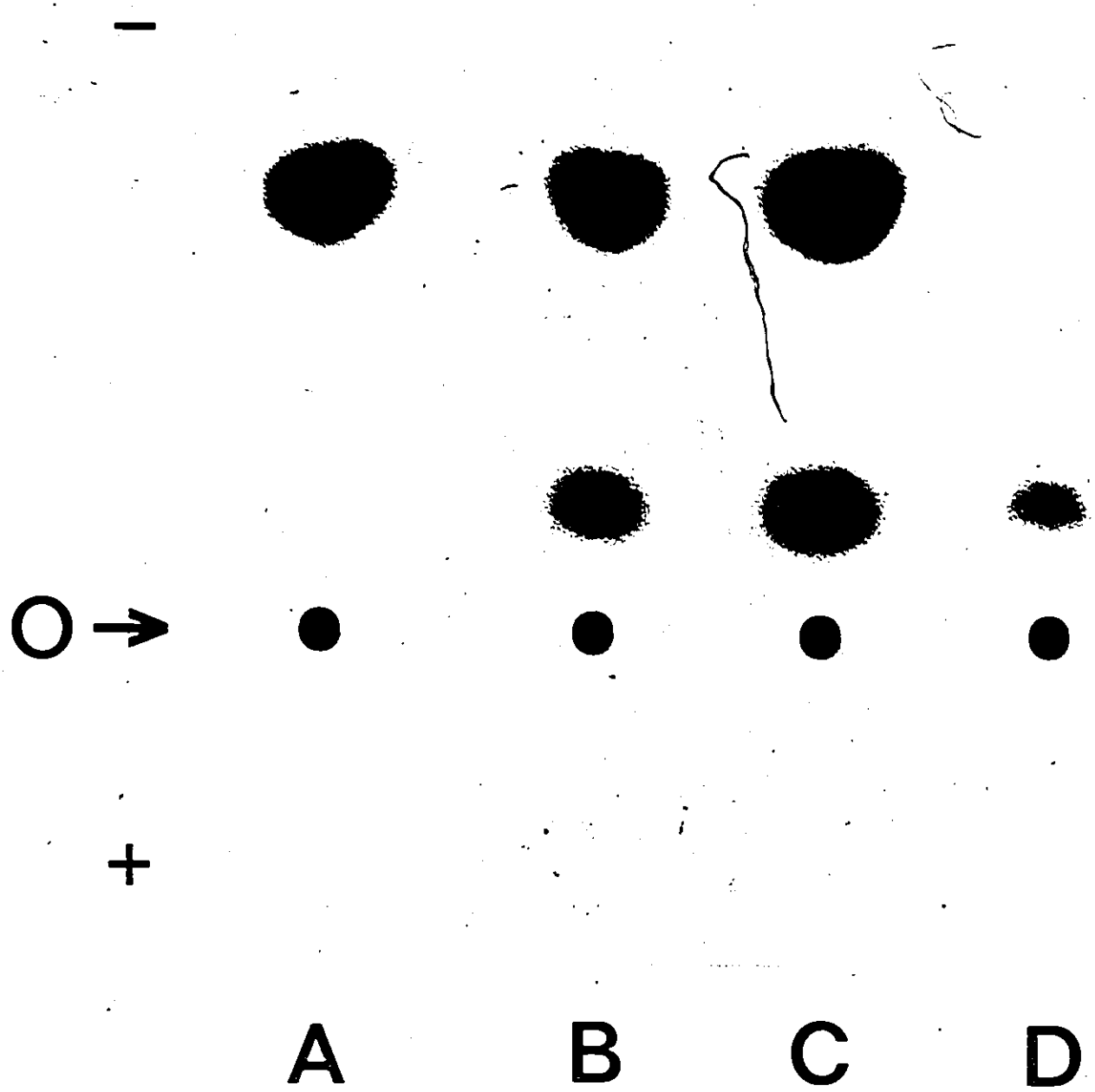

−
O →
+
A
B
C
D

Fig. IV-7

(A) Effect of temperature on the formation of carbamyl aspartic acid shows that the non-enzymatic reaction is highly temperature-dependent. Reaction mixtures contained 0.05M aspartic acid, 0.01M carbamyl phosphate and 0.10M phosphate buffer pH 7.5. Broken lines represent data obtained from the assay method using L-^{14}C-aspartic acid and counting the radioactive carbamyl aspartic acid produced. Only derivatives of aspartic acid which lack the free α-amino group are detected with this method (Porter et al. 1969). (B) Effect of reactant concentrations on the non-enzymatic formation of carbamyl aspartic acid. Reaction mixtures incubated at 37°C contained aspartic acid (ASP) and carbamyl phosphate (CP) at concentrations indicated in addition to 0.01M phosphate buffer pH 7.5. The amount of carbamyl aspartic acid formed per ml. after 30 min. reaction at 37C from 0.05M aspartic acid and 0.01M carbamyl phosphate was 0.05 ± 0.03 μmoles (± 95% confidence interval on the standard error of the mean); from 0.10M aspartic acid and 0.02M carbamyl phosphate was 0.19 ± 0.05 μmoles. If either reactant was omitted from the reaction mixture no detectable reaction occurred at 37C.

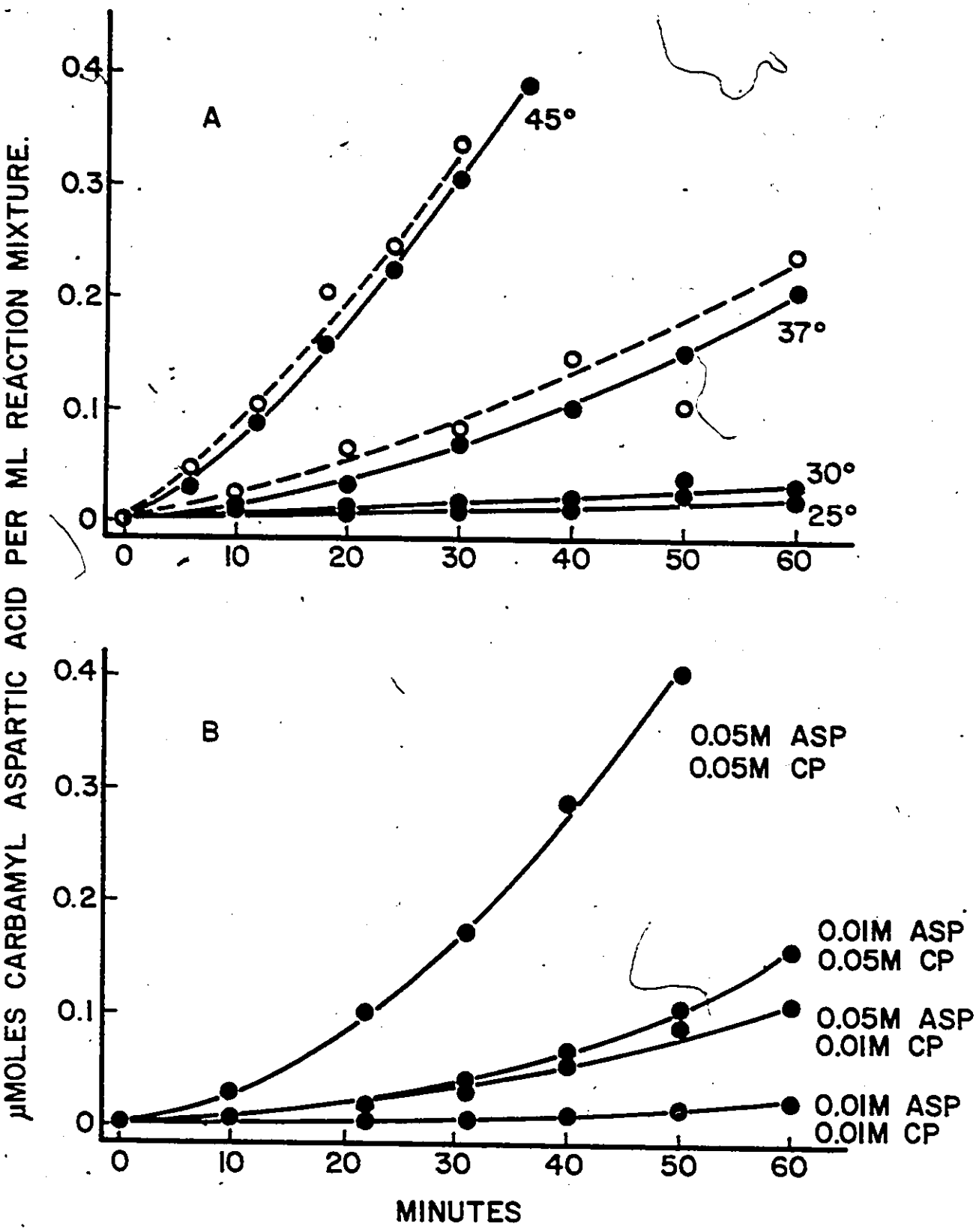
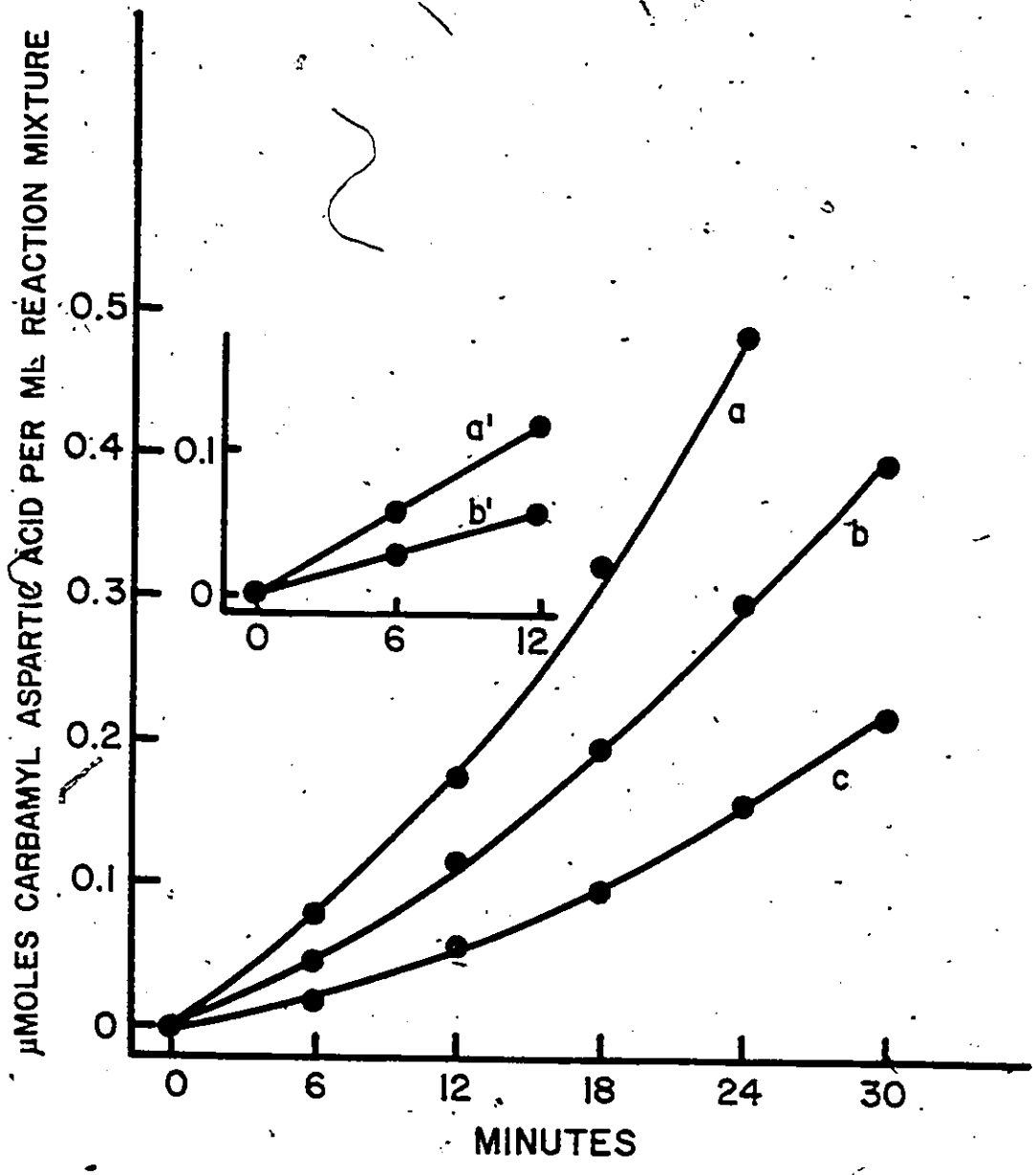

A
45°
37°
30°
25°
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
B
0.05M ASP
0.05M CP
0.01M ASP
0.05M CP
0.05M ASP
0.01M CP
0.01M ASP
0.01M CP
µMOLES CARBAMYL ASPARTIC ACID PER ML REACTION MIXTURE.
MINUTES

Fig. IV-8

Correction of initial rates of enzymatic carbamyl aspartic acid formation by subtracting the non-enzymatic reaction's contribution (c). 37 C reaction mixtures contained, in addition to 0.10M aspartic acid, 0.02M carbamyl phosphate and 0.10M tris pH 7.5, (a) undiluted Escherichia coli B crude extract, (b) extract diluted to one-half enzyme oncentration, and (c) no enzyme. Inset shows corrected linear initial rates of enzymatic reaction (a', b'). Several investigators have applied similar corrections (Sallach 1959, Weitzman & Wilsin 1966, Bethell et al. 1968, Prescott & Jones 1970). Under some conditions corrections may be impractical; it was very difficult to measure the activity of ATCase of thermophilic Bacillus stearothermophilis at 60C because the non-enzymatic reaction had a rate many times higher than the enzymatic rate.


µMOLES CARBAMYL ASPARTIC ACID PER ML REACTION MIXTURE
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
MINUTES
a
b
c
0.1
0
0
6
12
a'
b'

Indeed, carbamyl aspartic acid can be formed at room temperature from cyanate and aspartic acid (Nyc & Mitchell 1947), and was formed under reaction mixture conditions at 37C (Fig. IV-6C).

That a conversion of carbamyl phosphate to cyanate did occur was confirmed by experiments in which carbamyl phosphate was preincubated before reacting with aspartic acid. Above 30C reaction mixtures of carbamyl phosphate and aspartic acid showed hyperbolic time-course plots of production. Yet after carbamyl phosphate preincubation the reaction proceeded linearly (Fig. IV-9A) in a manner identical to the cyanate-aspartic acid reaction (Fig. IV-9B). Other experiments (not shown) indicated that the plots increased in rate as well as approached linearity as carbamyl phosphate was preincubated for varous periods up to two hours.

The spontaneous transformation of carbamyl phosphate into cyanate in reaction mixtures occurred at a temperature-dependent rate (Fig. IV-7A). Above 30C cyanate was formed faster than it could react with aspartic acid, thus it accumulated, increasing the rate of carbamyl aspartic acid production with time. At or below 30C the transformation was slower yielding a linear rate of carbamyl aspartic acid production.

Results and discussion, including comparisons with ATcase's from other sources. Salt effects on enzyme activity.

Fig. IV-10 shows that the V. costicola ATCase activity

Fig. IV-9

(A) Effect of preincubation of carbamyl phosphate (CP) on the rate of formation of carbamyl aspartic acid. Carbamyl phosphate in 0.25M tris-HCl buffer pH 7.5 was preincubated at 37C for 2 hours. Reaction mixtures contained 0.10M aspartic acid, 0.02M carbamyl phosphate either preincubated or fresh), and 0.20M tris-HCl buffer pH 7.5.

(B) Formation of carbamyl aspartic acid from reaction of 0.10M aspartic acid with cyanate at concentrations indicated. 37C reaction mixtures included 0.10M tris-HCl buffer pH 7.5.

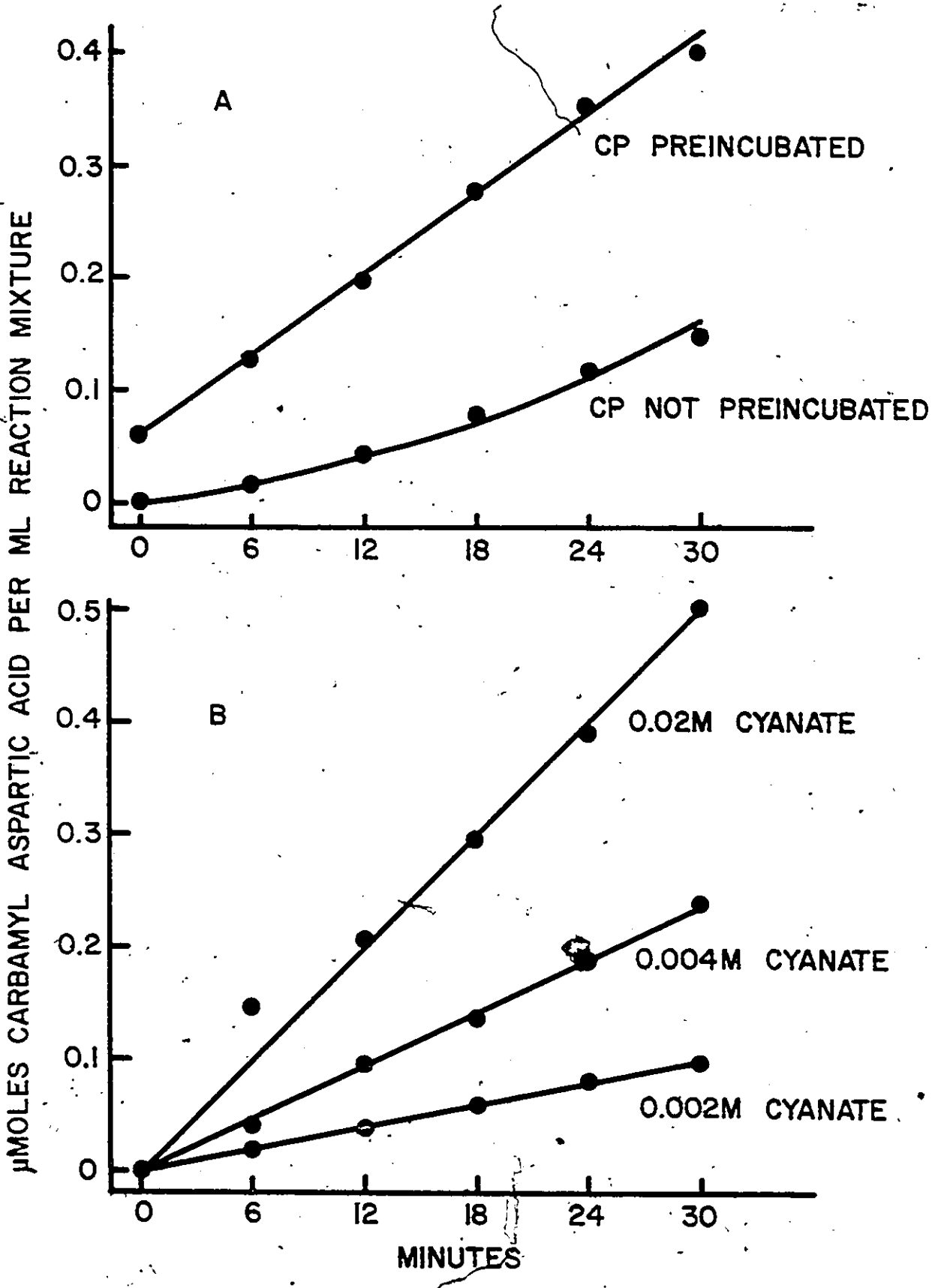
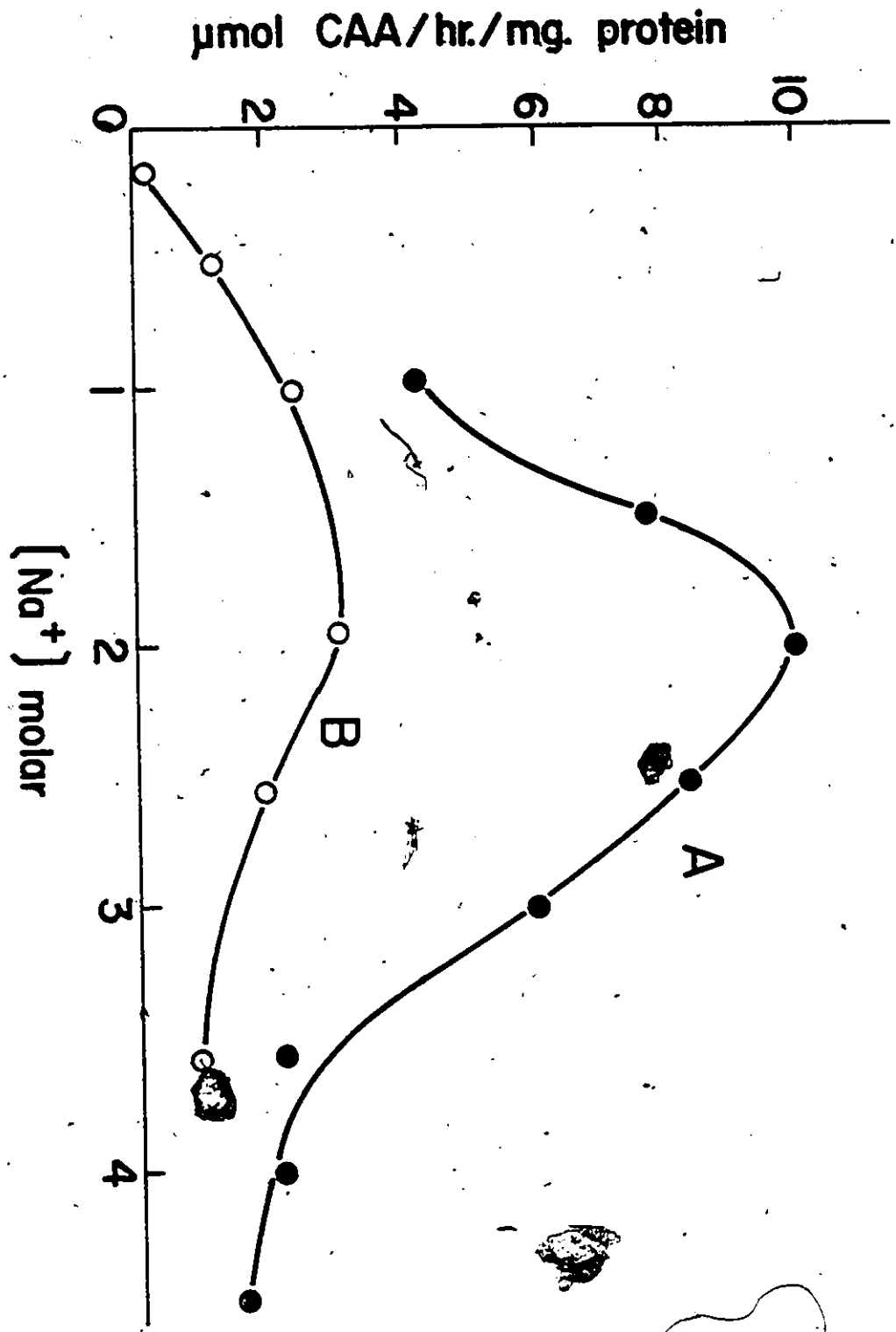

A
CP PREINCUBATED
CP NOT PREINCUBATED
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
B
0.02M CYANATE
0.004M CYANATE
0.002M CYANATE
0.5
0.4
0.3
0.2
0.1
0
0
6
12
18
24
30
MINUTES
μMOLES CARBAMYL ASPARTIC ACID PER ML REACTION MIXTURE

Fig. IV-10

Salt response of aspartate transcarbamylase activity. (A) Activity in presence of near-saturating substrate concentrations; 0.4M aspartate, 0.025M carbamyl phosphate, with 0.1M sodium carbonate buffer, pH 9.55 ± 0.05. Because of the Na^+ added as hydroxide required to neutralize the 0.4M aspartic acid in the reaction mixtures, the lowest Na^+ level tested was 1M. The extraction buffer contained 1M NaCl and 0.1M Na_2CO_3 buffer pH 9.5. (B) Activity in presence of half-saturing (at pH 7.5) 0.1 M aspartate and 0.01M carbamyl phosphate with 0.1M tris pH 7.5 ± 0.1. The extraction buffer contained 0.5M KCl, 0.5M NaCl, 0.01M $MgCl_2$, 10^{-4}M dithiothreitol, 10^{-5}M CTP, 0.01M aspartate and 0.1M tris pH 7.5.


µmol CAA/hr./mg. protein
0
2
4
6
8
10
1
2
3
4
[Na+] molar
A
B

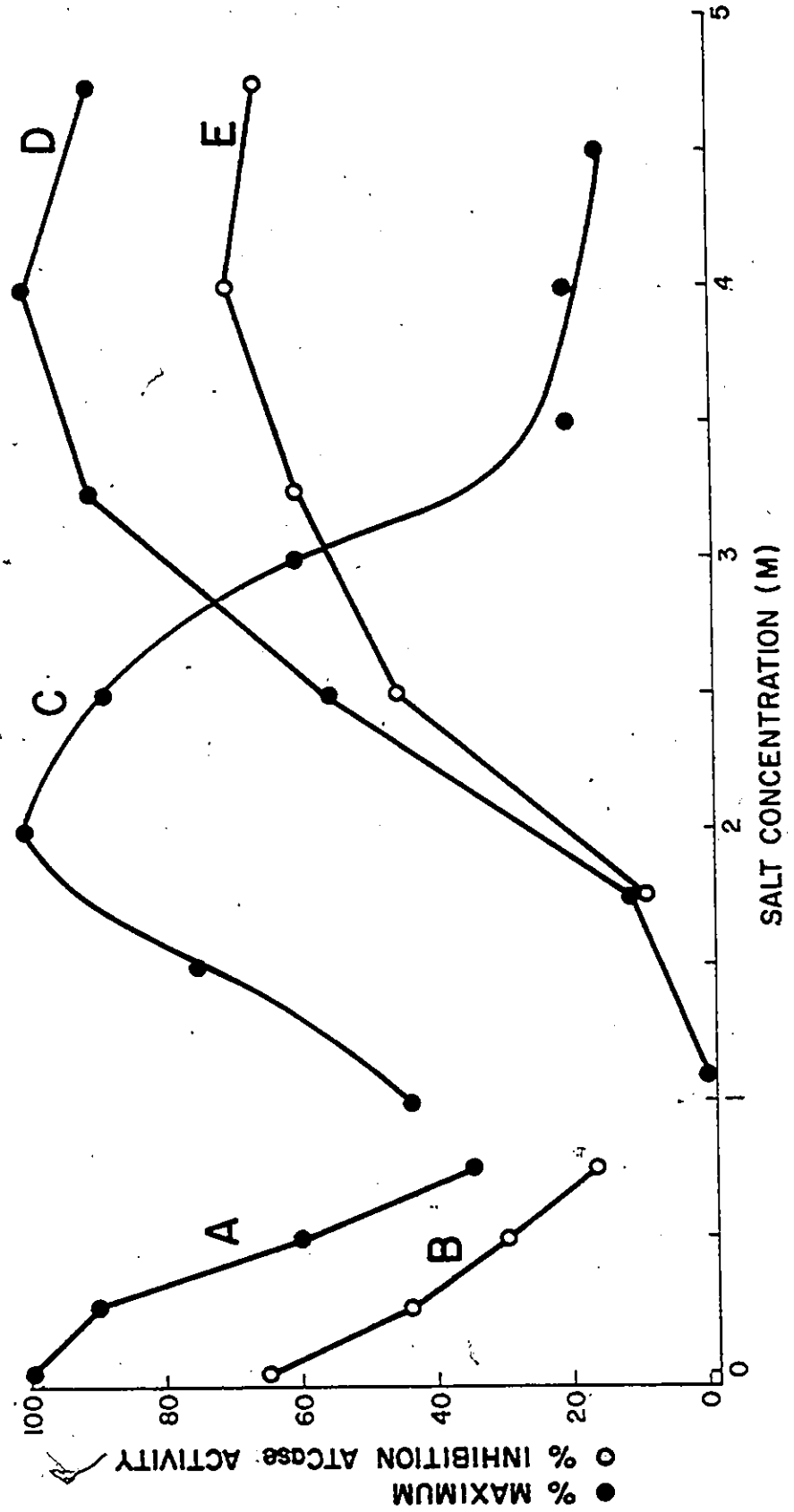
exhibited a definite halophilic character, with an optimum activity at 2M Na^+. The salt response did not change when the aspartate concentration in the reaction mixture was reduced to half the saturation value (i.e. to the K_m value at pH 7.5 for aspartate, Fig. IV-10 (B)). K^+ had a similar stimulatory effect on enzyme activity as the level was increased from low to moderate salt concentrations; in a separate experiment run under conditions similar to those of Fig. IV-10 (B) with KCl instead of NaCl, the ratio of activity at the optimum, 2M K^+, to the activity of 0.5M K^+ was 1.6, compared to a ratio of 2.0 for curve B of Fig. IV-10.

A comparison of the V. costicola enzyme's salt response with the ATCases from a non-halophilic yeast and an extremely halophilic bacterium is shown in Fig. IV-11. Unlike the other enzymes, the Vibrio ATCase was insensitive to the feedback inhibitors UTP and CTP (see below). Because of its dependence upon intermediate salt concentrations for maximum activity the V. costicola ATCase could well be classified as a moderately halophilic enzyme.

Of the three ATCases from non-halophilic microorganisms whose salt response has been tested, only the enzyme from S. fecaelis was stimulated by salt. Half-molar KCl almost doubled the activity over that in the absence of added salt (Bethell & Jones 1969); low NaCl concentrations also activated the enzyme (Chan & Jones 1974). The E. coli ATCase was found to be inhibited by NaCl or KCl (Bethell & Jones 1969) as well as by a variety of cations and anions

Fig. IV-11

Effect of salt concentration on activity and feedback inhibition of ATCases from the non-halophilic yeast *Saccharomyces cerevisiae* (A), and from the extremely halophilic bacterium *Halobacterium cutirubrum* (D), compared with the enzyme from the moderately halophilic bacterium, *Vibrio costicola* (C). % feedback inhibition of the yeast enzyme by UTP is shown in curve B; % inhibition of the *Halobacterium* enzyme by CTP is shown in curve E. Data on the *Vibrio* enzyme from Fig. IV-8, on the two other ATCases from Liebl et al. (1969). "Salt concentration" refers to either Na^+ or K^+ as used in the particular experiments for each organism tested.


● % MAXIMUM
○ % INHIBITION ATCase ACTIVITY
100
80
60
40
20
0
0
1
2
3
4
5
SALT CONCENTRATION (M)
A
B
C
D
E

(Kleppe 1966, Kleppe & Spaeren 1966). Both the activity and feedback inhibition of the S. cerevisiae enzyme were reduced about 50% by 0.5M salt (Liebl et al. 1969, Fig. IV-11). With the possible exception of the S. fecaelis ATCase, pronounced activations by moderate to high salt concentrations appear to be confined to the halophilic ATCases.

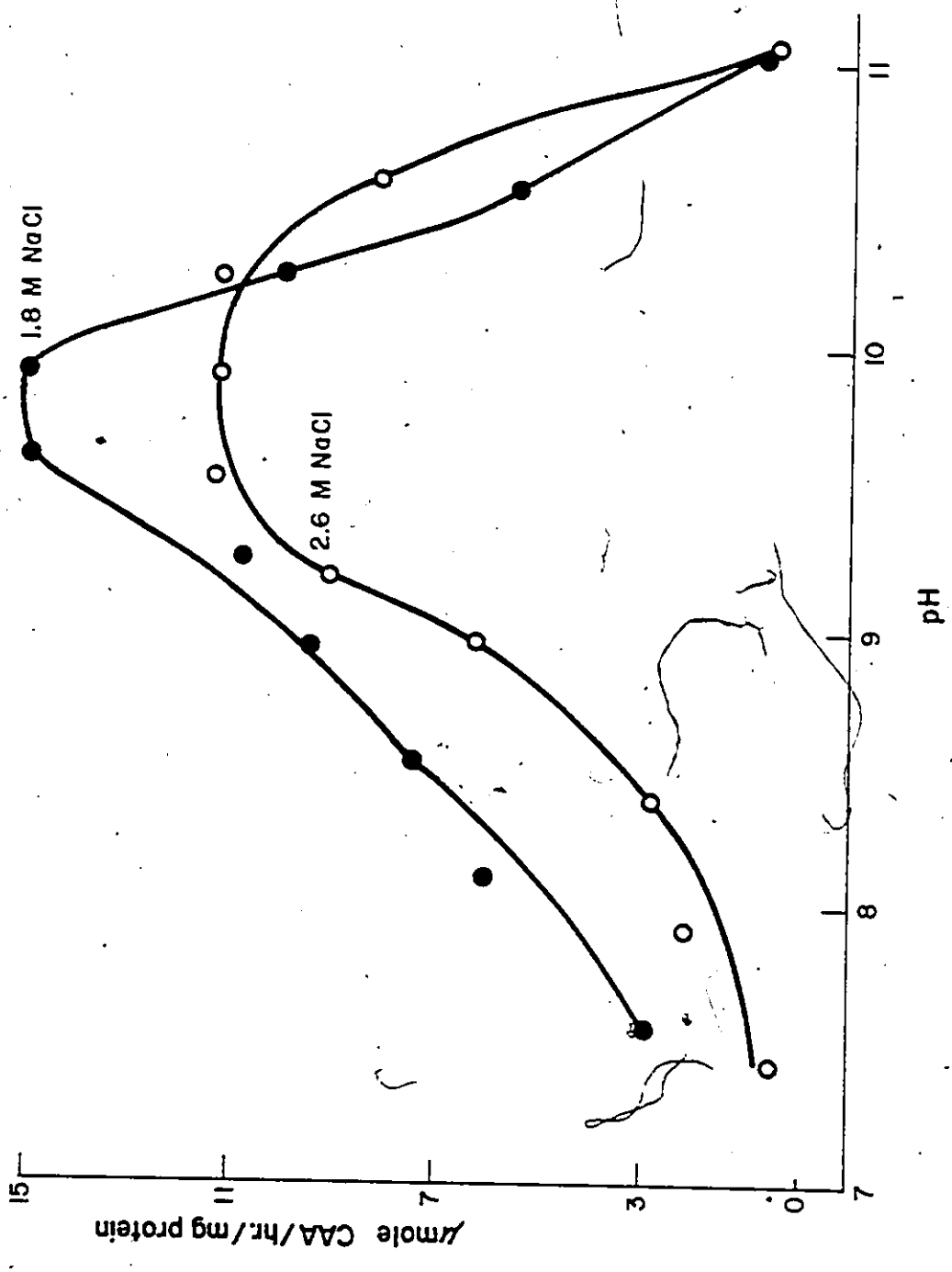
As an interesting comparison, several experiments on the ATCase from a halotolerant yeast, Saccharomyces rouxii, were carried out. The strain, isolated by Hiroshi Onishi from the 18% NaCl environment of Japanese Soya Sauce ferment, grew well in 0-3M NaCl, with slightly better growth at 0.1-1.0M NaCl. The ATCase was also halotolerant. Its best activity occurred in the absence of added salts (0-0.1M) but with 50-80% maximum activity at 3M NaCl or KCl. Although the responses of the enzyme to salts and nucleotides were not fully characterized, UTP and CTP appeared to inhibit activity at salt concentrations below 1M, while also having stabilizing effects. In contrast to the other ATCases of Fig. IV-11, the S. rouxii enzyme did not exhibit a pronounced dependence on a narrow range of salt concentrations for maximal activity.

Effect of pH on the V. costicola ATCase.

Fig. IV-12 shows the pH response curves at salt concentrations near and above the optimum salt concentration for enzyme activity. At the higher salt concentration the pH optimum was broader than at the lower salt concentration,

Fig. IV-12

Effect of pH on V. costicola ATCase activity. Enzyme extracted in 1.8M NaCl, 0.1M tris pH 7.5 was added to reaction mixtures with indicated final salt concentrations, 0.4M aspartate and 0.025M carbamyl phosphate. 0.1M tris, 0.1M K_2HPO_4 and 0.1M glycine buffers were used in all the reaction mixtures to cover the pH range of the experiment. The final pH of each reaction mixture was checked with a low sodium-error Fisher-92 micro combination electrode.


1.8 M NaCl
2.6 M NaCl
μmole CAA/hr./mg protein
pH
15
11
7
3
0
7
8
9
10
11

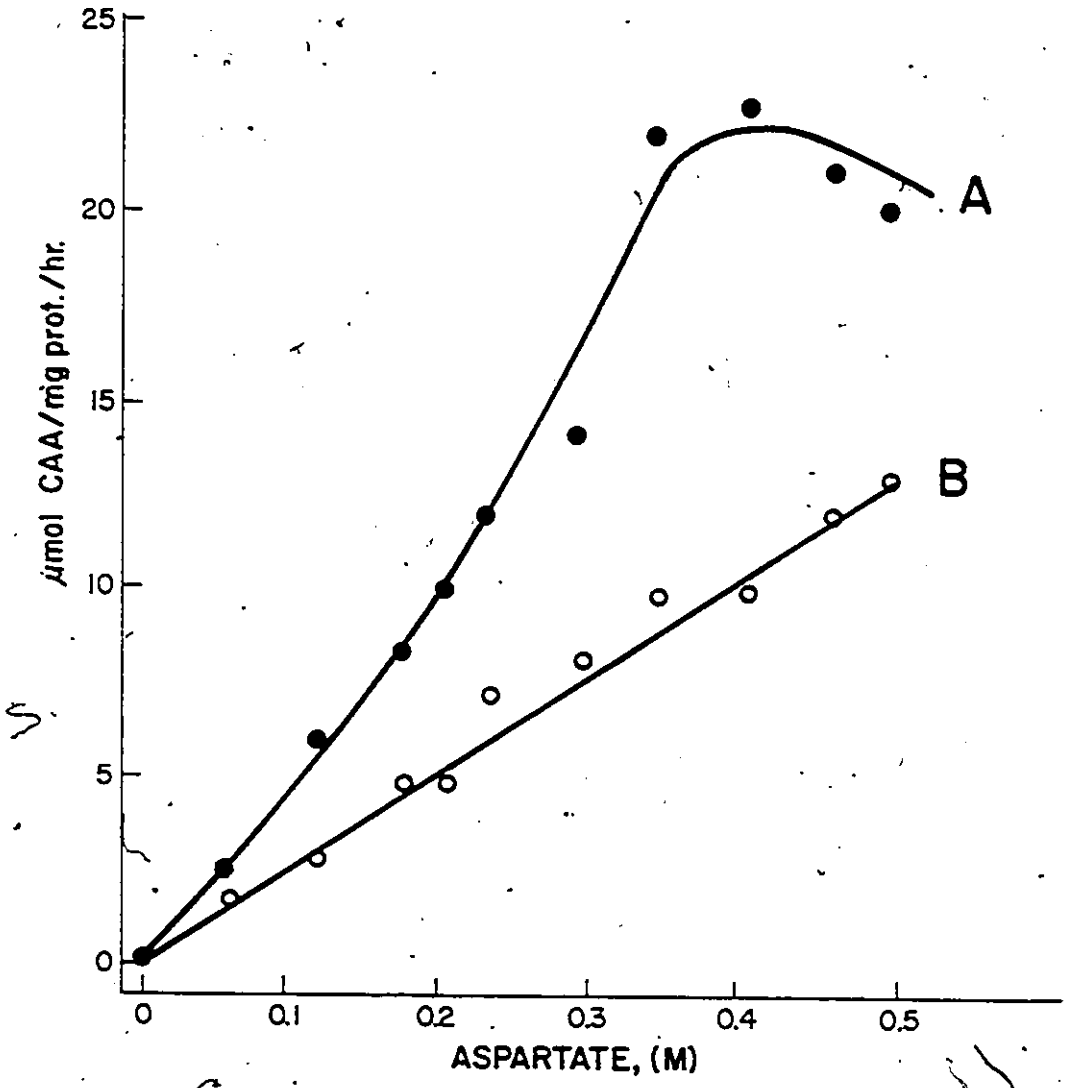
a not uncommon salt effect in halophilic enzymes (J. K. Lanyi, personal communication). The alkaline pH 9.5-10 activity optimum exhibited was most similar to that of the purified Streptococcus faecalis enzyme, which, like the V. costicola enzyme (discussed below), was also insensitive to nucleotide regulation (Prescott & Jones 1970). E. coli native ATCase has two pH optima, one around pH 8.5 (Gerhart & Pardee 1964, Porter et al. 1969) and another more pronounced optimum at pH 10.2 (Weitzman & Wilson 1966). The E. coli native enzyme was sensitive to CTP feedback inhibition at both optima, thus an alkaline optimum did not, per se, preclude sensitivity to inhibition by nucleotides. The ATCase from Halobacterium had a broad pH optimum around pH 7.5-8 but had a more specific optimum at pH 7.5 for maximum CTP regulatory inhibition (Liebl et al. 1969). A similar pH dependence pattern for activity and inhibition by UTP was also observed with the non-halophilic S. cerevisiae ATCase (Kaplan et al. 1967).

Substrate saturation kinetics.

Two aspects of the V. costicola aspartate saturation curves are striking (Fig. IV-13). First, the curve was sigmoid. Second, the concentrations of aspartate required to achieve V_{max} were inordinately large, around 0.4M! It was thought that a possible enzyme sensitivity to low temperatures might have caused the effects but an experiment using enzyme extracted at room temperature indicated

Fig. IV-13

Aspartate saturation curves. ATCase activity was assayed in 2.0M NaCl, 0.1M Na_2CO_3 pH 9.6 ± 0.1 with saturating 0.03M carbamyl phosphate. Curve A: enzyme extracted at 4C in 0.8M NaCl, 0.4M KCl, 5 X 10^{-4} dithiothreitol, 5 X 10^{-4}M Mg-EDTA, 0.1M Na_2CO_3 pH 9.8. Curve B:enzyme extracted as in curve A except extraction carried out at 25C.


25
20
15
10
5
0
μmol CAA/mg prot./hr.
A
B
0
0.1
0.2
0.3
0.4
0.5
ASPARTATE, (M)

only a loss of activity but the same high aspartate requirement. Saturation curves produced from experiments run at pH 7.5 showed similar shapes (Fig. IV-14A). The double reciprocal plot (Fig. IV-14B) was typical of enzymes having substrate reaction orders higher than unity. In contrast to the aspartate saturation curves, the carbamyl phosphate curves were hyperbolic (Fig. IV-15).

An experiment with permeabilized V. costicola.

The assumption that the enzyme is not altered in the extraction process is implicit in this work. Halophilic enzymes might be sensitive to removal from their intracellular environment. To test for this possibility with V. costicola experiment with 1mM cetyltrimethyl ammonium bromide (CETAB) permeabilized cells (R. McDonald, personal communication) was carried out. The destruction of the permeability barrier to small molecules while at the same time retention of most of the enzymes inside the cell (Jackson & DeMoss 1965, see also footnote p.97) provides a model system where enzymes are probably operating under conditions resembling those in vivo. The results of the experiment showed that although the permeabilized cells had slightly lower activity than a french press extract prepared from the same amount of cells run in a parallel experiment (9 vs. 5 arbitrary activity units), there was no effect of 2×10^{-3}M CTP on the activity of either preparation (reaction conditions: 1.2M NaCl, 0.25M aspartate, 0.025M carbamyl phosphate, pH 7.8). Extraction effects such as the Euler effect (Kaplan 1955) or use of un-physiological

Fig. IV-14

(A) Aspartate saturation curve at pH 7.45 ± 0.05. Enzyme extracted in 1.8M NaCl, 0.1M tris pH 7.5. Reaction mixtures contained saturating 0.04M carbamyl phosphate and 0.1M tris and NaCl. The reaction mixture Na^+ concentration in the absence of aspartate was 1.8M, in the presence of 0.25M aspartate was 2.1M. The increase was due to the extra Na^+ added as hydroxide necessary to neutralize the aspartate. In one set of reaction mixtures 10^{-3}M CTP was included to test for feedback inhibition.

(B) Double reciprocal plots of the data from curve A. Dashed line represents an ideal line required to approximate the K_m and relative Vmax values appropriate to the data, 0.1M aspartate and Vm=1 respectively. The line is approximately tangent to the points at the highest substrate concentration.

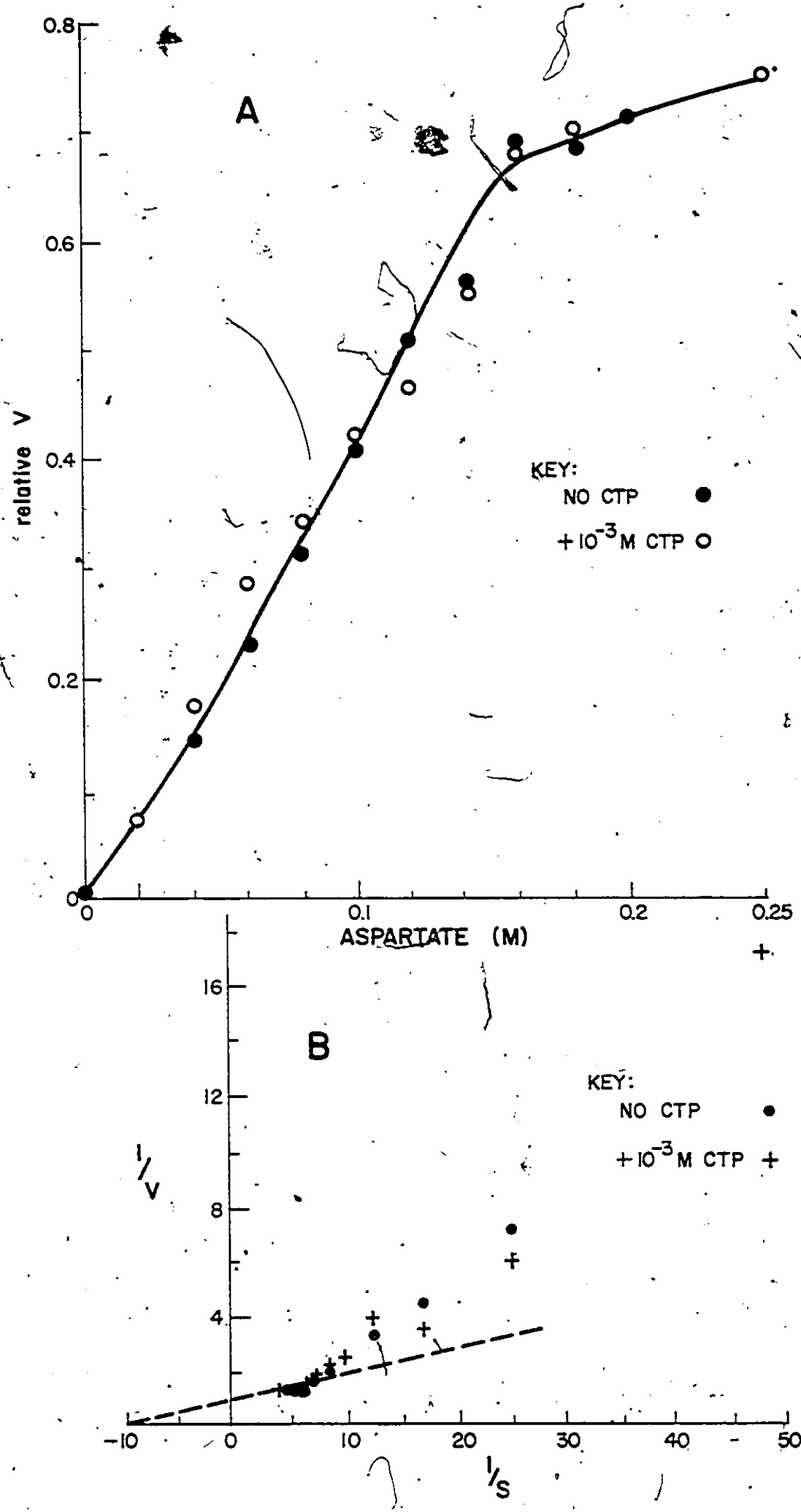
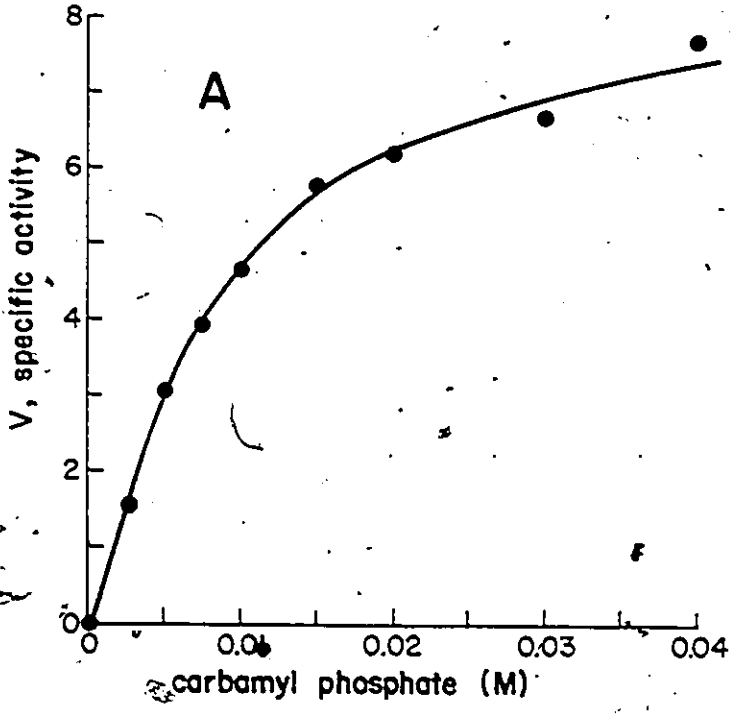
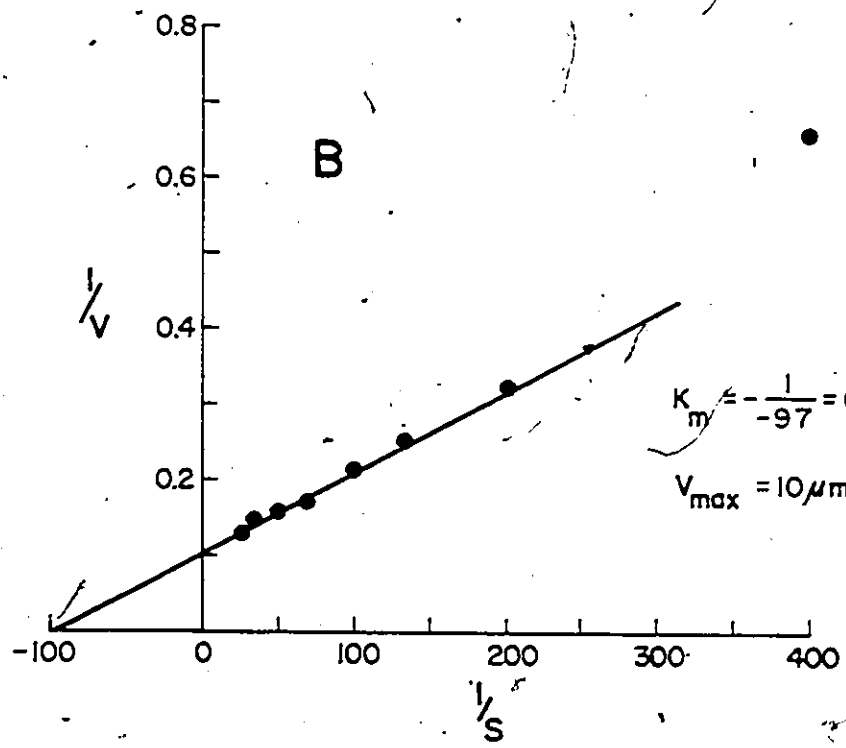

A
relative V
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.25
ASPARTATE (M)
KEY:
NO CTP
+ 10⁻³M CTP
B
1/V
16
12
8
4
-10
0
10
20
30
40
50
1/S
KEY:
NO CTP
+ 10⁻³M CTP

Fig. IV-15

(A) Carbamyl phosphate saturation curve at pH 7.5. Extracts prepared as per Fig. IV-12. Reaction mixtures contained 1.8M NaCl, 0.1M tris pH 7.34 ± 0.02 and near-saturating 0.2M aspartate.

(B) Double reciprocal plots of the data of curve A yielded the kinetic parameters shown.





$K_m = -\frac{1}{-97} = 0.01$ M CP

V_{max} = 10 μmoles CAA / hr/mg prot

reaction mixture conditions (Kornberg & Malcovati 1973) cannot be ruled out by this experiment, but at least they were not overt.

The search for feedback inhibition in V. costicola ATCase.

Since one aspect of this work was to examine regulatory phenomena in a moderately halophilic bacterium, it was important to determine if the V. costicola ATCase was involved in the regulation of the pyrimidine biosynthetic pathway.

Substrate concentrations have been implicated in feedback effects; if the E. coli ATCase's substrates were at high concentrations, the inhibition effects were overcome (Gerhart & Pardee 1962, Bethell et al. 1968). Fig. IV-14 shows that the V. costicola ATCase was not affected by 10^{-3}M CTP at any aspartate concentration up to saturation. A parallel experiment with a carbamyl phosphate saturation curve (not shown) demonstrated that CTP did not affect the enzyme at any CP concentration. Table IV-1 and other experiments at even lower substrate concentrations showed clearly that any absence of CTP or UTP effects was not due to high substrate concentrations. CTP and UTP also did not inhibit V. costicola ATCase at salt concentrations lower than the optimum.

Tests for feedback inhibition by 10^{-3}M CTP at pH values ranging from 6-11, and with UTP at pH 10 showed no significant effects. Table IV-2 shows that there were little significant effects of any nucleotide at pH 7.2-7.4 on enzyme activity. If any consistent feedback effects existed with V. cos-

Table IV-1

Tests of feedback inhibition of ATCase by CTP at various substrate concentrations.

substrate conc. M					
ASP	CP	2×10^{-3} M inhibitor	relative activity	95% confidence interval	pH
0.3	0.02	-	15.3	± 0.7	7.2
0.3	0.02	CTP	14.3	± 1.3	7.22
0.15	0.02	-	7.5	± 0.6	7.26
0.15	0.02	CTP	7.8	± 0.6	7.27
0.15	0.01	-	5.7	± 0.5	7.32
0.15	0.01	CTP	5.3	± 0.6	7.32
0.10	0.01	-	3.1	± 0.9	7.34
0.10	0.01	CTP	3.0	± 0.6	7.32

Cells extracted in 1M NaCl, 5×10^{-5} M CTP, 0.1M KH_2PO_4 pH 7.5, reactions run at 1.8M NaCl.

Table IV-2

Effect of various nucleotides on ATCase activity.

2×10^{-3}M nucleotide	enzyme specific activity*	95% confid. interval	pH
none	0.128**	± 0.008	7.42
CTP	0.073	± 0.012	7.20
CDP	0.095	± 0.005	7.35
CMP	0.078	± 0.010	7.20
UTP	0.087	± 0.018	7.33
UDP	0.100	± 0.005	7.37
UMP	0.100	± 0.009	7.50
ATP	0.104	± 0.010	7.24

* µmoles caa/hr/mg protein. Enzyme extracted in 1.0M NaCl, 5×10^{-5}M CTP, 0.1M KH_2PO_4 pH 7.5 using Braun cell disintegrator and 0.17 mm glass beads at 4C. Reaction mixtures contained 1.8M NaCl, 0.04M aspartate, 0.02M carbamyl phosphate, 0.08M tris pH 7.5.

** The control specific activity was higher in this experiment than in other similar experiments. In this case it appears that it was a falsely high determination.

ticola ATCase, presumably they would have appeared in some experiments.

Stability of V. costicola ATCase at low salt concentrations.

Halophilic enzymes often irreversibly lose activity when they are suspended in dilute salt solutions. V. costicola ATCase lost half its activity in the first thirty minutes of suspension in 0.04M NaCl (Tab. IV-3). The enzyme retained all its activity over the 1-3M NaCl range for 2 hrs. The Halobacterium enzyme, at a similar salt concentration lost half its activity within 30 seconds (Norberg et al. 1973). The Vibrio ATCase was also more stable at 4C than at room temperature (Fig. IV-13).

A molecular weight estimate for V. costicola ATCase.

Bethell and Jones (1969) classified bacterial ATCases by molecular size and feedback regulation characteristics. The characteristics of their class C enzymes, the smallest ATCases having molecular weights of 120-140,000 Daltons, show the greatest similarity to V. costicola ATCase. These enzymes include the B. subtilis ATCase, the S. fecaelis ATCase and the E. coli ATCase catalytic subunit. The aspects which these enzymes have in common with each other and with V. costicola ATCase were insensitivity to 10^{-3}M pyrimidine nucleotides and a pH optimum near or above 8.5. As mentioned previously, the pH optimum of the S. fecaelis ATCase was identical to that of V. costicola (Fig. IV-12). V. costicola ATCase, however, did not have a "typical Michaelis-Menten"

Table IV-3

Inactivation of V. costicola ATCase by the removal of salts.

treatment	% remaining activity
none, zerotime, 2.0M NaCl	100
30 min at 0.04M NaCl	53
4½ hr. at 0.04M NaCl	38

Extracted enzyme concentrated 25 fold by ultrafiltration. For the inactivations the enzyme was diluted into a low salt solution, final concentrations: NaCl 0.04M, 0.1M tris pH 7.5. The enzyme was held in the low salt solution for the inactivation period, then added to a 1M NaCl, 0.25M aspartate, 0.05M carbamyl phosphate, 0.1M tris pH 7.5 reaction mixture in which the activity remaining was measured.

aspartate saturation curve (Figs. IV-13, 14). The other class C enzymes showed hyperbolic saturation curves for both substrates.

A preliminary estimate of the molecular weight of V. costicola ATCase was carried out, partly to compare it with ATCases from other organisms. Since the Vibrio enzyme was not purified as part of this study, the molecular weight determination method had to be amenable to crude extracts. Agarose gel chromatography was chosen.

Agarose is reputed to be the chromatography medium least affected by high salt concentrations in terms of swelling and separation parameters. A first attempt using a Sepharose 4B, 175 ml, 33.5 X 2.5 cm, upward flow column at 4C with a buffer made up of 1.8M NaCl, 0.01M $MgCl_2$, 10^{-3}M mercapto-ethanol and 0.1M tris pH 8.5 resulted in an estimate of V. costicola molecular weight of 130-150,000 daltons. The void volume was determined with blue dextran and crude extract enzyme samples were chased with 10% sucrose. Fractions were assayed for ATCase activity using the Gerhart and Pardee (1962) method, and for protein spectrophotometrically with the aid of an LKB Uvicorder II. The molecular weight estimations were predicated upon some of the marker proteins with the characteristics shown in Table IV-4.

It was evident that the marker proteins chromatographed on agarose at 1-1.8M NaCl concentrations acted neither like they did at low salt concentrations nor like they did at high

salt concentrations. Bovine serum albumin, for instance, behaved as a dimer when chromatographed on agarose at 3.5M NaCl (Norberg et al. 1973) while at low salts usually behaved as a monomer. At the intermediate salt concentrations used in this study both forms of albumin appeared in the column effluent. Yet, when analysed in the analytical ultracentrifuge, bovine serum albumin gave similar S values under both low salt and 1M NaCl conditions. The $S_{20,w}$ values were: 4.04 ± 0.12S in 0.1M tris pH 7.5 (literature value 4.3S, Martin 1964) and 4.04 ± 0.068S in 1M NaCl, 0.1M tris pH 7.5. Bovine serum albumin, as well as other proteins, interacted with the column gel at 1 or 1.8M NaCl, resulting in poorly understood effects.

Using the assumption that the presence of salts caused changes in the subunit aggregations of the non-halophilic marker proteins (Gawronski & Westhead 1969, Norberg et al. 1973) the data in Fig. IV-16 was plotted. The small marker proteins fit the ideal curve well, as also reported by Louis et al. 1970. The column conditions in this experiment (Bio-Gel A-1.5m agarose 2.4 X 45cm column with a bed volume of 217ml and a blue dextran void volume of 88ml run upward at 4C with 1M NaCl, 0.M tris pH 7.8 buffer) resulted in associations and disaggregations in the intermediate and high molecular weight marker proteins (Tab. IV-4). The published range for the gel, 50,000 to 1.5 million Daltons (Bio Rad Inc., Richmond, Calif., U.S.A.) was reduced to 10,000-200,000 Daltons in the presence of 1M NaCl (Fig. IV-16). The molecular weight value determined for the V. costicola ATCase

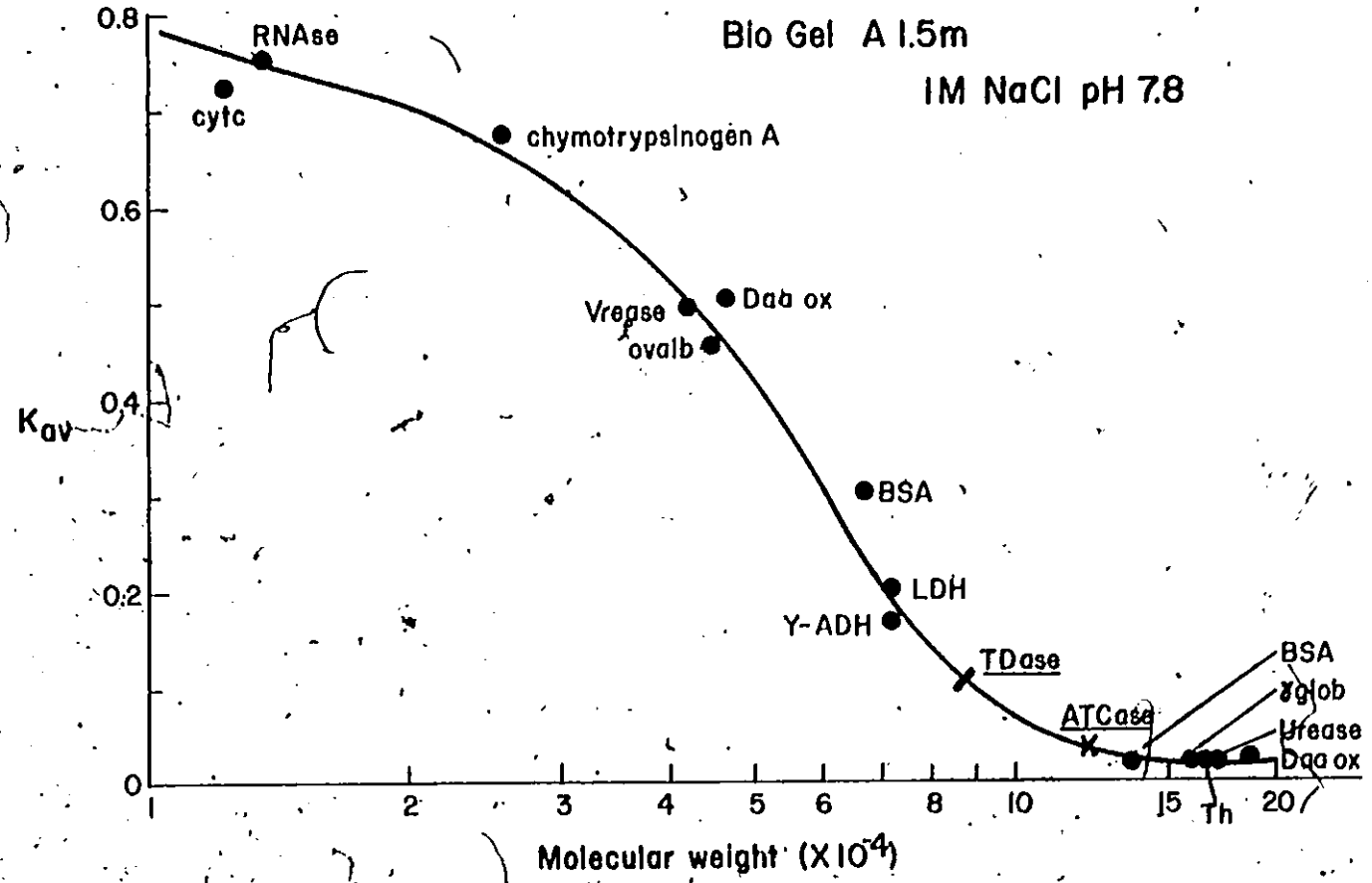
Table IV-4

Marker proteins used for molecular weight estimations by agarose gel chromatography.

marker protein	source	literature mol. wt.	subunits mol. wt.	Bio-Gel A 1M NaCl pH 7.8 effective mol. wt.	abbrev.
ribonuclense A	Sigma IA, bovine pancreas	13,700	-	13,700	RNAsc
cytochrome c	Sigma III, horse heart	12,400	-	12.400	cyt c
chymotrypsinogen a	Sigma II, bovine pancreas	25,000	-	25,000	-
D-amino acid oxidase	Nutritional Co., hog kidney	90,000	47K, 182K	47,000, 182,000	D aa ox
urease	Sigma III, jack bean	483,000	83K	42,000, 172,000	-
ovalbumin	Sigma type V, cryst.	45,000	-	45,000	ovalb
bovine serum albumin	General, fraction V or Calbiochem A grade	67,000	67K, 135K	67,000, 135,000	BSA
L-lactate dehydrogenase	Worthington, beef heart	140,000	72K, 34K	72,000	LDH
yeast alcohol dehydrogenase	Sigma, cryst.	150,000	37K	72,000	Y-ADH
gamma globulin	Nutritional, Human fr. II	160,000	50K, 23.5K	160,000	γ-glob

Literature and subunit values compiled from literature supplied by Sigma, worthington, Calbiochem, Mann, General Biochemicals, Nutritional Biochemicals, Pharmacia, and from literature references: Norbey et al. 1973, Louis et al. 1970, Weber & Osborn 1969, Klotz & Darnall 1969, and Batlle 1967.

Fig. IV-16. Agarose gel chromatography protein standard curve and molecular weight estimations of V. costicola ATCase and TDase.


Bio Gel A 1.5m
1M NaCl pH 7.8
RNAse
cytc
chymotrypsinogen A
Vregse
ovalb
Daa ox
BSA
LDH
Y-ADH
TDase
ATCase
BSA
γglob
Urease
Daa ox
Th
K_{av}
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
8
10
15
20
Molecular weight (X 10^{-4})

was 120,000 Daltons, in close agreement with the 130,000-150,000 figure obtained from the previously discussed Sepharose 4B column experiment. These estimates are preliminary because only one technique, gel chromatography, was used, and require future confirmation using other methods. Sucrose density gradient centrifugation is a promising method which could be used (Martin & Ames 1961), since it has been applied to enzyme molecular weight estimates at high salt concentrations (Louis et al. 1970).

Discussion: Lack of feedback regulation by nucleotides in V. costicola ATCase.

Experiments under a variety of conditions indicated the V. costicola ATCase was insensitive to pyrimidine nucleotide regulation. Moreover, the molecular weight of the enzyme corresponds to those of a class of ATCase which are not feedback regulated. Even though the sigmoidal aspartate saturation curves of the V. costicola were not modified by pH changes or the presence of nucleotides, as they were in the nucleotide sensitive ATCase of E. coli (Gerhart & Pardee 1963), unequivocal evidence for lack of feedback sensitivity must come from studies on a purified enzyme.

Organisms which lack ATCase inhibition sometimes exhibit alternate means of pyrimidine pathway regulation. Among the bacteria which have ATCases which are insensitive to feedback regulation are S. fecaelis (Prescott & Jones 1970),

Pseudomonas acidovorans (Kelln & Warren 1974), and Bacillus subtilis (Potvin et al. 1975, Brabson & Switzer 1975). In the Bacillus enzyme the pyrimidine pathway may be regulated by a multienzyme complex with UTP sensitive carbamyl phosphate synthetase and a somewhat less sensitive dihyroorotase (Potvin et al. 1975). If CPSase is the site of efficient pyrimidine pathway regulation, there must be tight coupling between the CPSase and the ATCase. Unless the carbamyl phosphate produced by the CPSase is channeled directly to the ATCase, regulation will be inefficient since carbamyl phosphate in bacteria is produced by a CPSase which is common to both the arginine and pyrimidine pathways (for instance see Issaly et al. 1969). Experiments have confirmed that such a channeling mechanism of eukaryotic cells operates in the multienzyme aggregate CPSase-ATCase complex of S. cerivisae (Lue & Kaplan 1970). Other multienzyme aggregates appear to be important in pyrimidine regulation in Neurospora crassa (Williams et al. 1970) and mouse spleen (Hoogenrad et al. 1971), for example.

Because the V. costicola ATCase enzyme did not allow experiments on the effects of salts on regulation, it was decided to proceed on an investigation of the regulatory biosynthetic threonine deaminase, presented in the next chapter. A discussion of the properties of halophilic enzymes with references to both the V. costicola ATCase and threonine deaminase follows the results of the threonine deaminase experiments.

Chapter V

Threonine deaminase activity and its regulation in Vibrio costicola

Introduction

Little information is available on metabolic regulation in halophilic bacteria. Feedback inhibition of a salt-dependent enzyme, aspartate transcarbamylase (ATCase) from Halobacterium was first demonstrated in 1969 (Liebl et al.). Since then, several other enzymes have been found to be regulated. In the moderately halophilic bacteria, there are only two studies which relate to control of enzymatic activity, one on V. costicola isocitrate dehydrogenase (Wydro 1974) and the other on V. costicola pyruvate kinase (E. de Medicis, personal communication). Since one of the aims of this dissertation was to study cellular and enzymatic regulation phenomena in a moderate halophile, and since the V. costicola ATCase was insensitive to feedback inhibition (Ch. IV), a study of the activity and feedback inhibition in the biosynthetic threonine deaminase (TDase, L-threonine hydrolase (deaminating), EC.4.2.1.16) was launched. The results of several experiments on TDase repression are also presented. Repression in halophiles has received virtually no attention; only one observation has been published. Ornithine transcarbamylase activity was reduced by about 1/3 in Halobacterium grown in synthetic medium supplemented with citrulline (Dundas & Halvorson 1966).

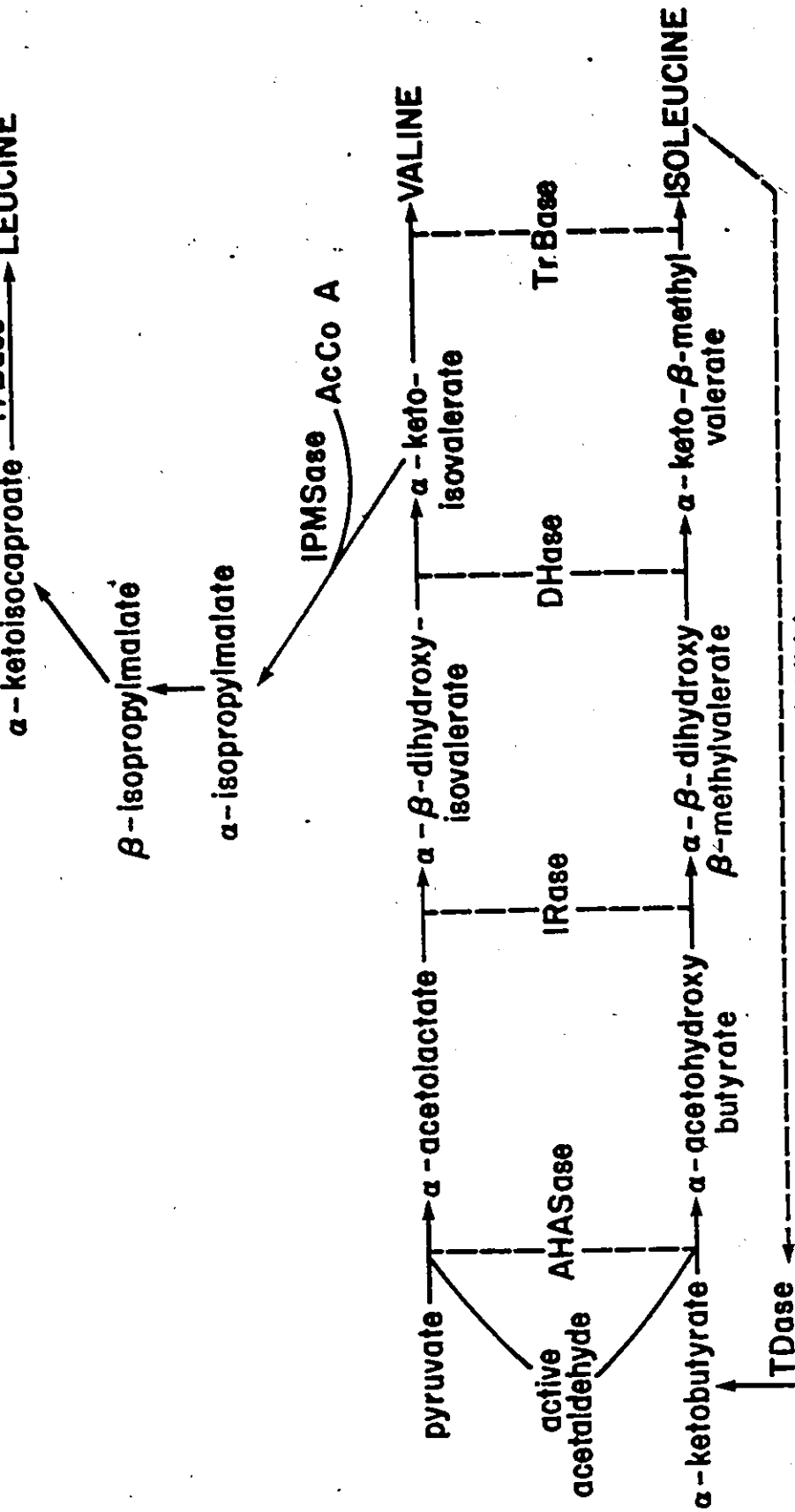
The regulatory inhibition of the first enzyme of a biosynthetic pathway by the end product(s) was simultaneously discovered in ATCase (Yates & Pardee 1956) and TDase. Umbarger (1956) recognized the possible metabolic significance of the competitive

inhibition of threonine deamination by isoleucine in E. coli. Since then a plethora of publications on feedback regulation has shown it to be a universal phenonenon. Umbarger's group and other groups have extensively studied the regulation of the biosynthesis of three branched-chain amino acids, isoleucine, leucine and valine, with the result that a complex regulatory model has been proposed. TDase is centrally important in the model proposed for Escherichia coli (Levinthal et al. 1973), Salmonella typhimurium (Calhoun & Hatfield 1973) and Saccharomyces cerevisiae (Bollon & Magee 1973): not only does it function to control the rate of isoleucine production by the fine control feedback inhibition mechanism; it is also involved in autoregulation (Hatfield & Burns 1970), i.e., control of its own synthesis.

According to the autoregulatory model, newly synthesized monomeric TDase polypeptide chains first condense into an inactive, apotetrameric enzyme. In the presence of pyridoxal phosphate, a co-factor for enzyme activity, the apotetramer is converted into an immature holotetramer which still lacks activity. In the presence of excess cellular isoleucyl-, valyl-, and leucyl-aminoacylated t-RNA species, which would only occur when all three amino acids were in excess, the holotetramer forms a repressor complex with the t-RNA species. This repressor complex presumably interacts with the ilv operons, preventing further synthesis of TDase and the other enzymes involved in branched chain amino acid synthesis (Fig. V-1). Alternatively, the holoenzyme can be converted into mature native TDase by interaction with isoleucine, valine or threonine. This model

Fig. V-1

Biosynthetic pathways of isoleucine, valine and leucine. Threonine deaminase (TDase) catalyses the first step in isoleucine synthesis and is feedback regulated (dashed line). All three branched chain amino acids share common enzymes: Acetohydroxy acid synthetase (AHASase), α-acetohydroxy acid isomeroreductase (IRase), dihydroxy acid dehydrase (DHase), and transaminase B (TrBase). The pathway branches at α-ketoisovalerate; the first committed step in leucine synthesis involves the leucine regulated α-isopropylmalate synthetase (IPMSase). The AHASase is also feedback regulated by valine.


α-ketoisocaproate
Tr.Base
LEUCINE
β-isopropylmalate
α-isopropylmalate
IPMSase
AcCo A
α-keto-isovalerate
VALINE
Tr.Base
ISOLEUCINE
DHase
α-keto-β-methyl valerate
α-β-dihydroxy-isovalerate
α-β-dihydroxy β-methylvalerate
IRase
α-acetolactate
α-acetohydroxy butyrate
AHASase
pyruvate
active acetaldehyde
α-ketobutyrate
TDase
threonine
feedback inhibition

explains the requirement for all three branched-chain amino acids for (multivalent) repression of the threonine deaminase. It is not presently possible to apply all aspects of this sophisticated model to the halophiles, but it does serve as a blueprint for interpretation of experimental results.

Methods

Cells. The SGP (salts-glucose-potassium phosphate buffer) medium (Ch. II p. 32) was used for most of the experiments. Various amino acid additions were filter sterilized and aseptically added to the autoclaved media used for the repression experiments. Mid-exponential cultures (660nm absorbance 0.4-0.7 for 1M NaCl cultures) were harvested and washed twice in a 0.1M tris buffer pH 7.8 with an NaCl concentration equivalent to that of the medium.

Extraction of enzyme activity. Resuspension of the cells and extraction methods were described in Ch. IV (p. 117). The extraction buffer for the TDase extractions generally contained 0.5M NaCl, 0.5M KCl, 10^{-2}M $MgCl_2$, 10^{-4}M dithiothreitol, 10^{-3}M mono-magesium di-sodium ethylenediaminetetraacetate and 0.1M tris, pH 7.8. The protein content of extracts or partially purified preparations was determined using the Lowry et al. (1951) method (Fig. IV-2, p. 118).

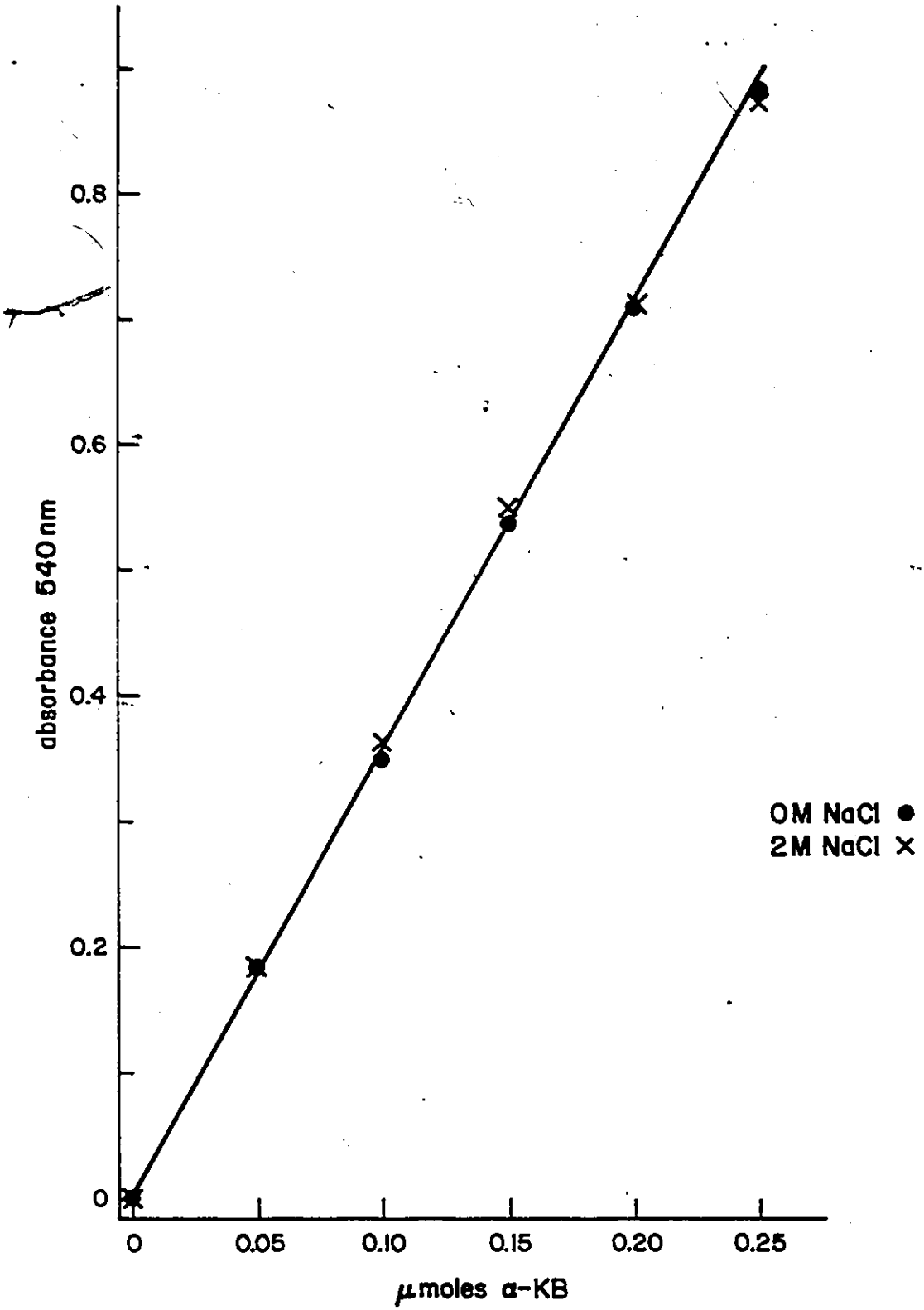
Reaction mixtures. Reactions were begun with the addition of 0.5ml crude or partially purified enzyme (see below) to 2.0ml of a solution containing NaCl at a specified concentration, 0.01M threonine, 10µg/ml pyridoxal phosphate and 0.1M tris pH 7.8.

Isoleucine was included in some reaction mixtures to test for feedback inhibition. During the 25C incubation, five or six 0.1ml aliquots were removed from the reaction mixtures at regular 4-7 min. intervals, each aliquot being added to a tube on ice containing 0.5ml 0.1N HCl which stopped the reaction. The colorimetric assay for α-ketobutyrate (KB) was performed in the tubes containing the aliquot. After incubation the pH of the remaining reaction mixtures solution was checked with a Corning 476050 low-sodium micro combination electrode.

Assay for the reaction product. α-ketobutyrate was measured by a modification of the method of Friedeman and Haugen (1943). To each tube 0.6ml DNP reagent (0.2% di-nitrophenylhydrazine in 2N HCl was stirred in the dark at room temperature for 1-1½ hr., filtered through #1 filter paper then stored at 4C in a tightly-stoppered brown glass bottle for up to 2 weeks. Just before use, 1 part of the 2N HCl solution was added to 5 parts 0.1N HCl) was added with a syringe pump, then the tubes were mixed and incubated for 10 min. at 25C. One ml 2.5N NaOH was then added with a syringe pump to each tube, which was immediately mixed and incubated for another 10 min. period at 25C. Color development was measured on a Beckman DB spectrophotometer at 540nm. α-ketobutyrate standards were made up to contain 0-0.25μmoles per 0.1ml aliquot. Although standard curves were not affected by reaction mixture salt concentrations up to 3M (Fig. V-2), the standards were prepared to match each reaction mixture's salt concentration.

Fig. V-2

Typical α-ketobutyrate standard curves, OM and 2M NaCl.


absorbance 540nm
0
0.2
0.4
0.6
0.8
0
0.05
0.10
0.15
0.20
0.25
μmoles α-KB
0M NaCl ●
2M NaCl ×

Enzyme reaction rates. Linear time-course curves were regressed and specific activity calculated (μ moles α ketobutyrate/ min/mg protein) for each reaction mixture. The reaction rates were strictly proportional to enzyme concentration (Fig. V-3).

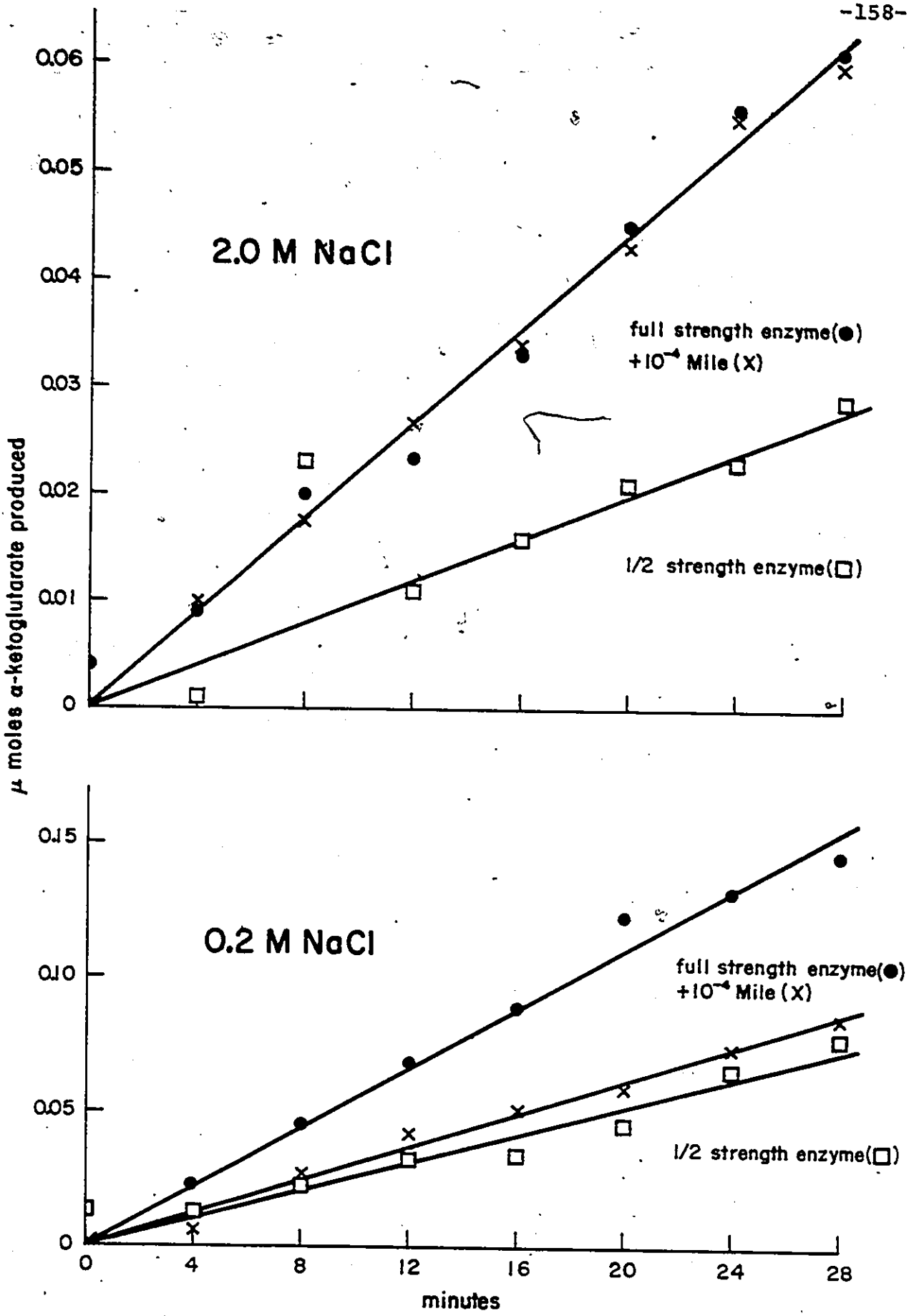
Partial purification of TDase. Solid $(NH_4)_2SO_4$ (enzyme grade, purified, low heavy metal content) was added slowly with stirring to cold crude extracts until 27.5% saturation was achieved. After 15 minutes more stirring the extract was centrifuged at 4C, 25,000 x g for 15 min. To the resulting supernatant more ammonium sulfate was added slowly to achieve a 37.5% saturation which precipitated most of the TDase activity. After centrifugation the pellet was resuspended in fresh extraction buffer. The fractionated enzyme typically had a 1.5-3 fold higher specific activity than the crude extract. About half of the activity of the crude extract was recovered (50% yield). The partially purified enzyme remained as sensitive to isoleucine feedback inhibition as the crude extract and could be stored frozen at -20C in 1M salt for at least one week without loss of activity or feedback inhibition.

Results: Properties of the V. costicola biosynthetic threonine deaminase.

Effect of salt on enzyme activity. Unlike the V. costicola ATCase, the threonine deaminase did not exhibit a salt requirement for maximum activity. The presence of 1M NaCl inhibited the activity of the enzyme 70%, but the activity remained constant, or decreased slightly in some experiments, between 1 and 3M NaCl

Fig. V-3

Effect of enzyme concentration on threonine deaminase activity. In addition to either 0.2M or 2M NaCl, reaction mixtures contained 0.01M threonine, 10µg/ml pyridoxal phosphate and crude extract (full strength enzyme, 3.0 mg/ml). Specific activities were: 0.2M NaCl full strengh enzyme enzyme 68 m µmoles αKB/min/mg prot., ½ strength enzyme 33; 2.0M NaCl full strength enzyme 28, ½ strength enzyme 12. 10^{-4}M isoleucine (ile) in the 0.2M NaCl reaction mixture caused 41% inhibition of activity but was not effective in the 2.0M case at that concentration. Note the differences in the ordinate scales.


2.0 M NaCl
full strength enzyme(●)
+10⁻⁴ Mile (X)
1/2 strength enzyme(□)
0.2 M NaCl
full strength enzyme(●)
+10⁻⁴ Mile (X)
1/2 strength enzyme(□)
μ moles α-ketoglutarate produced
minutes
0
0.01
0.02
0.03
0.04
0.05
0.06
0.10
0.15
4
8
12
16
20
24
28

(Fig. V-4). The Escherichia coli B biosynthetic TDase activity was also inhibited about 70% at 1M NaCl, but increasing the salt concentration caused a further inhibition; by 2M NaCl only about 10% of the maximum activity remained.

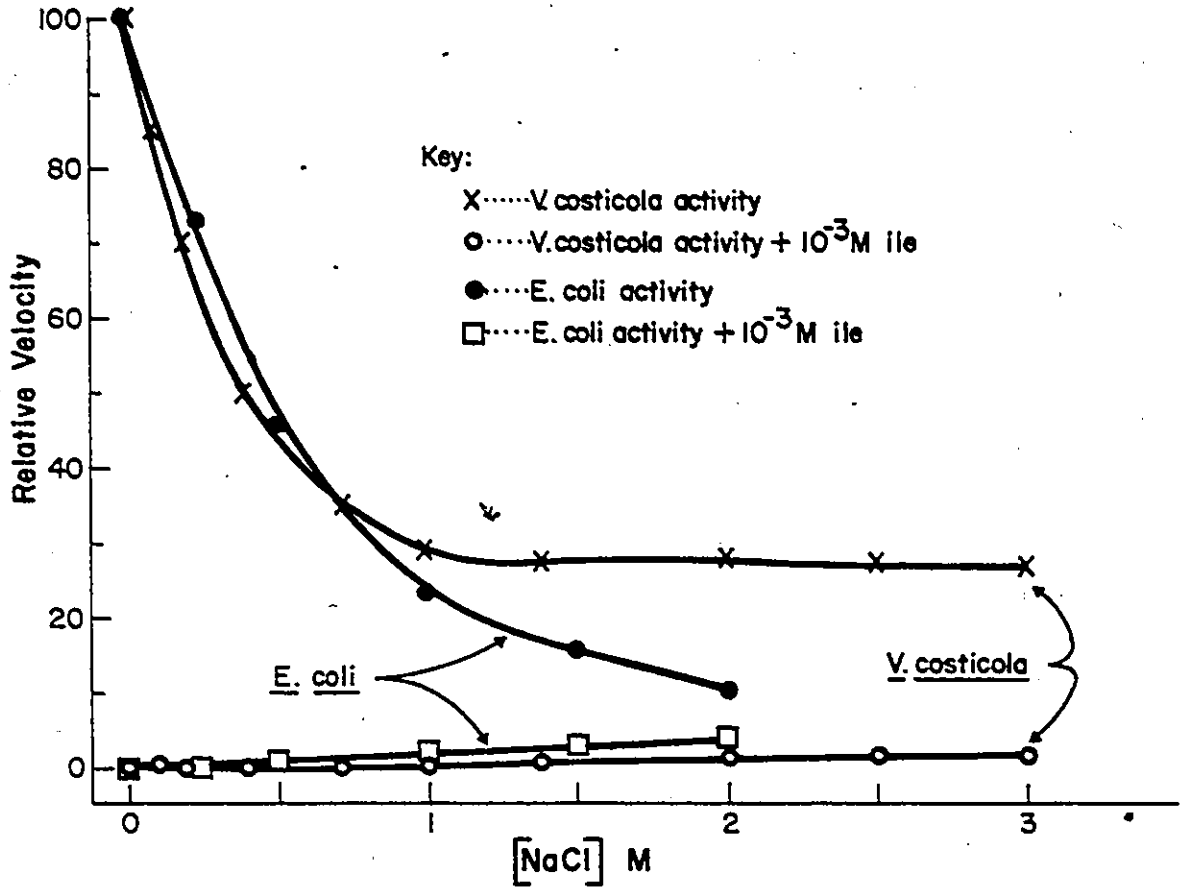
Effect of salt on feedback inhibition by isoleucine.

Isoleucine (10^{-3}M) completely inhibited the V. costicola TDase over the entire range of salt concentration tested (Fig. V-4). At lower isoleucine concentrations the effectiveness of inhibition decreased with increased salt concentrations. The K_i, i.e. the concentration of isoleucine necessary for half-maximal inhibition, increased from 10^{-4}M in the absence of added salt to 5×10^{-4}M in the presence of 1,2 or 3M NaCl (Fig. V-5). The enzyme was about half as sensitive to feedback inhibition at moderate to high salt concentrations than in low salt concentrations. The TDase maintained decreased, albeit consistent function over the 1-3M salt range.

The E. coli TDase inhibition was more sensitive to salt than the V. costicola enzyme. In 1-2M NaCl 10^{-3}M isoleucine was less effective than at lower salt concentrations (Fig. V-4). Other experiments showed that inhbition by 2.5×10^{-4}M isoleucine was abolished by 0.5M NaCl; inhibition by 7.4×10^{-4}M isoleucine was abolished by 1M NaCl; and inhibition by 2×10^{-3}M isoleucine was abolished by 3M NaCl; (Tab. V-1). With V. costicola TDase the inhibition by 2.5×10^{-4}M isoleucine was slight but still evident at 1-3M NaCl, the inhibition by 7.4×10^{-4}M isoleucine was about 70% at 1-3M NaCl, and 10^{-3}M isoleucine completely inhibited.

Fig. V-4

Effect of NaCl on activity and 10^{-3}M isoleucine inhibition of threonine deaminase from V. costicola and Escherichia coli B. Specific activities of the crude enzyme extracts at 0M NaCl were 57 and 121 mμmoles αKB/min/mg prot. respectively. E. coli enzyme extract prepared as outlined in Tab. V-1. V. costicola extract prepared as outlined in "methods" and both extracts added to identical reaction mixtures which contained 0.01M threonine (less than the K_m concentration for V. costicola and near the value for E. coli, see Tab. V-11), 10 μg/ml pyridoxal phosphate and tris buffer pH 7.8 ± 0.1 with the specified NaCl and isoleucine concentrations.


Relative Velocity
100
80
60
40
20
0
0
1
2
3
[NaCl] M
Key:
X·····V. costicola activity
O·····V. costicola activity + 10⁻³M Ile
●·····E. coli activity
□·····E. coli activity + 10⁻³M Ile
E. coli
V. costicola

Activity and feedback inhibition of TDase extracted from cells at different phases of growth.

It was important to know, both for repression studies and for an understanding of the physiology of V. costicola, if cells harvested and extracted at different points during growth yielded TDases with properties different from those already described in cells harvested during exponential phase. Tab. V-2 shows that the specific activity and the feedback inhibition of the TDase was roughly constant during most phases of growth. Any deviations occurred during the pre-log or stationary phases.

Effects of salt on kinetics of V. costicola threonine deaminase.

The addition of salt to TDase reaction mixtures resulted in inhibition of activity and a decreased sensitivity to isoleucine inhibition (Figs. V-4, V-5). These experiments were carried out at a threonine concentration of 0.01M. To better describe the effects of salt, further experiments were carried out at various concentrations of threonine (Fig. V-6 curve A, Fig. V-7 curve A). The data from the curves was rearranged to obtain a double reciprocal (Lineweaver-Burk type) plot (Fig. V-8). The pattern obtained was typical of a competitive inhibition. Because salt bears no structural or chemical relation to the substrate, threonine, NaCl is described as a "pseudo-competitive" inhibitor of enzyme activity.

The maximum velocity (V_{max}) estimated from Fig. V-8 was not affected by salt. The K_m, which can be interpreted as an estimate of affinity of the enzyme for the substrate, was increased 3 fold by the addition of 2.0M NaCl. Whatever the nature of the physical-chemical action of salt on the enzyme, salt

Fig. V-5

Effect of isoleucine and NaCl concentration on feedback inhibition of V. costicola threonine deaminase activity. The specific activities of the crude extracts at 0.2, 1, 2, and 3M NaCl were 60,24,13 and 10 mμmoles αKB/min/mg prot. respectively. The threonine (substrate) concentration in the pH 7.8 ± 0.1 reaction mixtures was 0.01M.

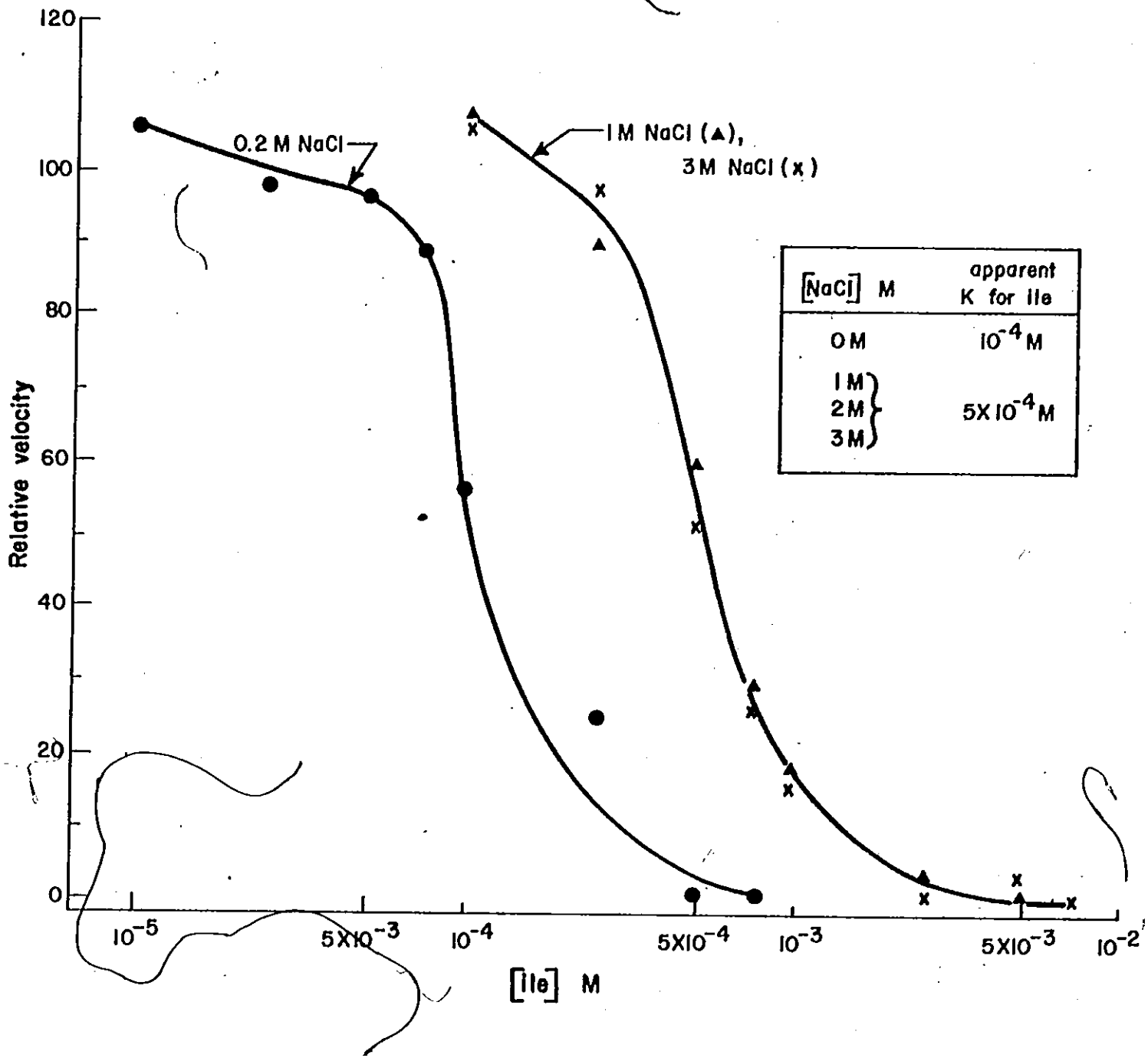

120
100
80
60
40
20
0
Relative velocity
0.2 M NaCl
1 M NaCl (▲),
3 M NaCl (x)
[NaCl] M
apparent K for Ile
0 M
10⁻⁴ M
1 M
2 M
3 M
5X10⁻⁴ M
10⁻⁵
5X10⁻³
10⁻⁴
5X10⁻⁴
10⁻³
5X10⁻³
10⁻²
[Ile] M

Table V-1

Effect of NaCl on isoleucine inhibition of E. coli threonine deaminase

NaCl M	spec. act.	% inhibition $2.5X10^{-4}M$ ile	% inhibition $7.4X10^{-4}M$ ile	% inhibition** $10^{-3}M$ ile	% inhibition** $2X10^{-3}M$ ile
0	126*	35	99	100	100
0.25	115	37	85	-	-
0.5	77	0	37	-	-
1	67	0	0	10	57
2	43	0	0	6	17
3	31	0	0	0	0

*mμmole αKB/min/mg prot. produced by an E. coli crude extract from cells grown in a minimal medium to log phase (ingredients, g/l: K_2HPO_4 7.0, KH_2PO_4 3.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 0.1, glucose 1.0, pH 7.0). Cells washed in 0.5M K_2HPO_4 pH 8.0 and extracted in 10^{-3}M dithiothreitol, 100μg/ml pyridoxal phosphate and 0.1M K_2HPO_4 pH 8. Reaction mixtures contained 0.01M threonine, 20 μg/ml pyriodoxal phosphate, 0.1M tris-PO_4 buffer pH8 as well as NaCl and isoleucine as specified.

**separate experiment with similar enzyme specific activities.

Table V-2

Effects of growth phase on V. costicola TDase activity and feedback inhibition

growth phase	(660nm abs.)	0.2M reaction sp. act.	0.2M reaction 10^{-4}M ile inhibition	2.0M reaction sp. act.	2.0M reaction $5X10^{-4}$M ile inhib.
pre - log	0.075	68*	29%	19	81%
early log	0.15	49	28%	19	48%
mid log	0.25	46	26%	15	41%
late log	0.65	57	18%	22	42%
linear	0.75	55	33%	21	42%
stationary	1.0	47	30%	16	62%

*mμmoles αKB/min/mg protein, cells grown 1M NaCl SGP medium to indicated growth phase.

Fig. V-6

Effects of threonine concentration on threonine deaminase activity (A) at 0.2M NaCl. 8 x 10^{-4}M isoleucine (D) inhibition was virtually complete at all threonine concentrations tested but could be partially alleviated by 8 X 10^{-4}M valine (C) or 5 X 10^{-3}M valine (B). Standard procedures were used in these experiments, the reaction mixture pH was 7.8 ± 0.10. Relative velocity X 3.33 = specific activity in μmoles αKB/min/mg prot.

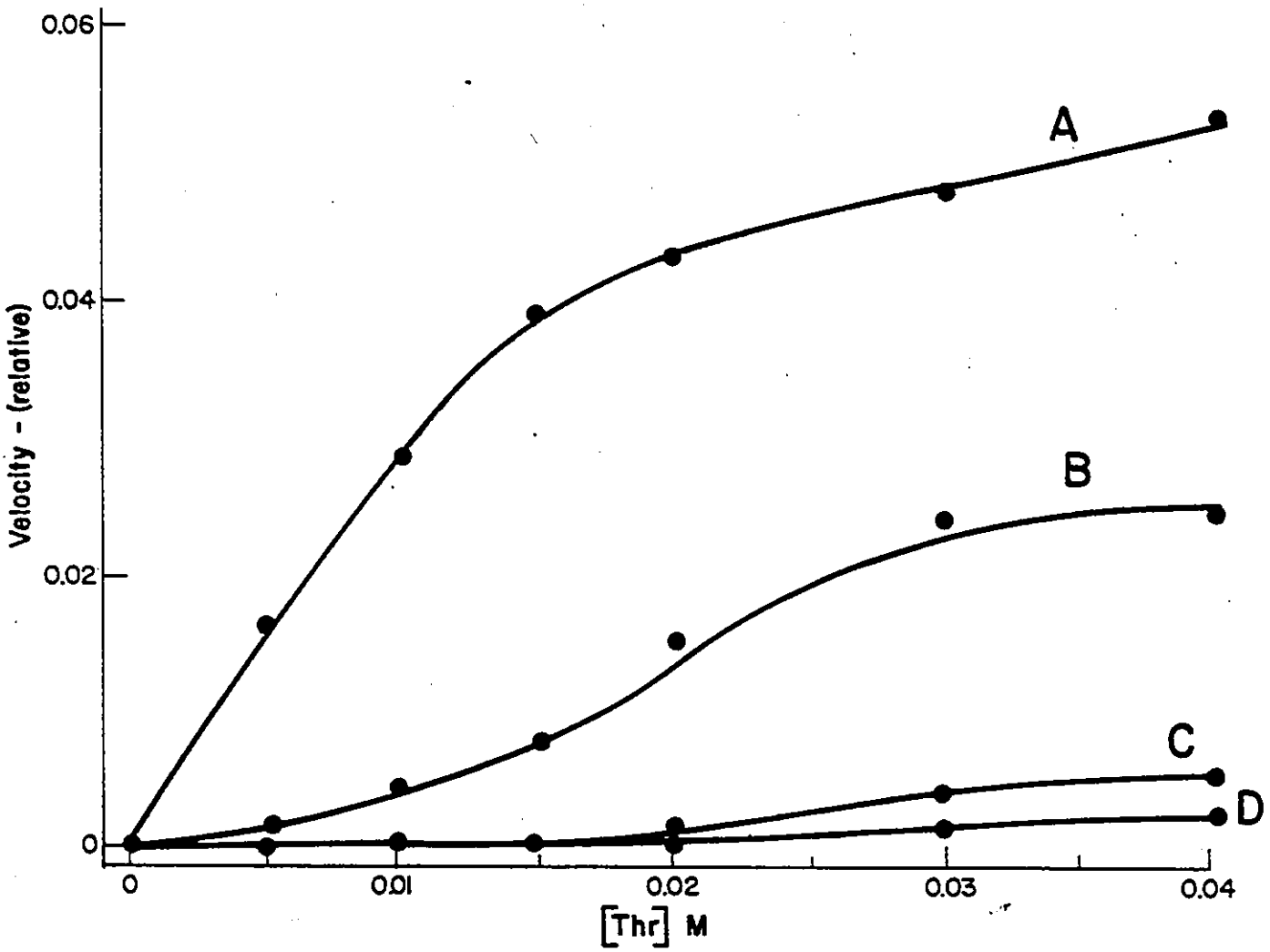

0.06
0.04
0.02
0
Velocity - (relative)
A
B
C
D
0
0.01
0.02
0.03
0.04
[Thr] M

Fig. V-7

Effects of threonine concentration on threonine deaminase activity (A) at 2.0M NaCl. 8×10^{-4}M isoleucine (D) inhibition could be partially alleviated by 8×10^{-4} valine (C) or 8×10^{-3}M valine (B). Standard procedures were used in these experiments, the reaction mixture pH was 8.0 ± 0.10. Relative velocity X 3.33 = specific activity in μmoles αKB/min/mg prot.

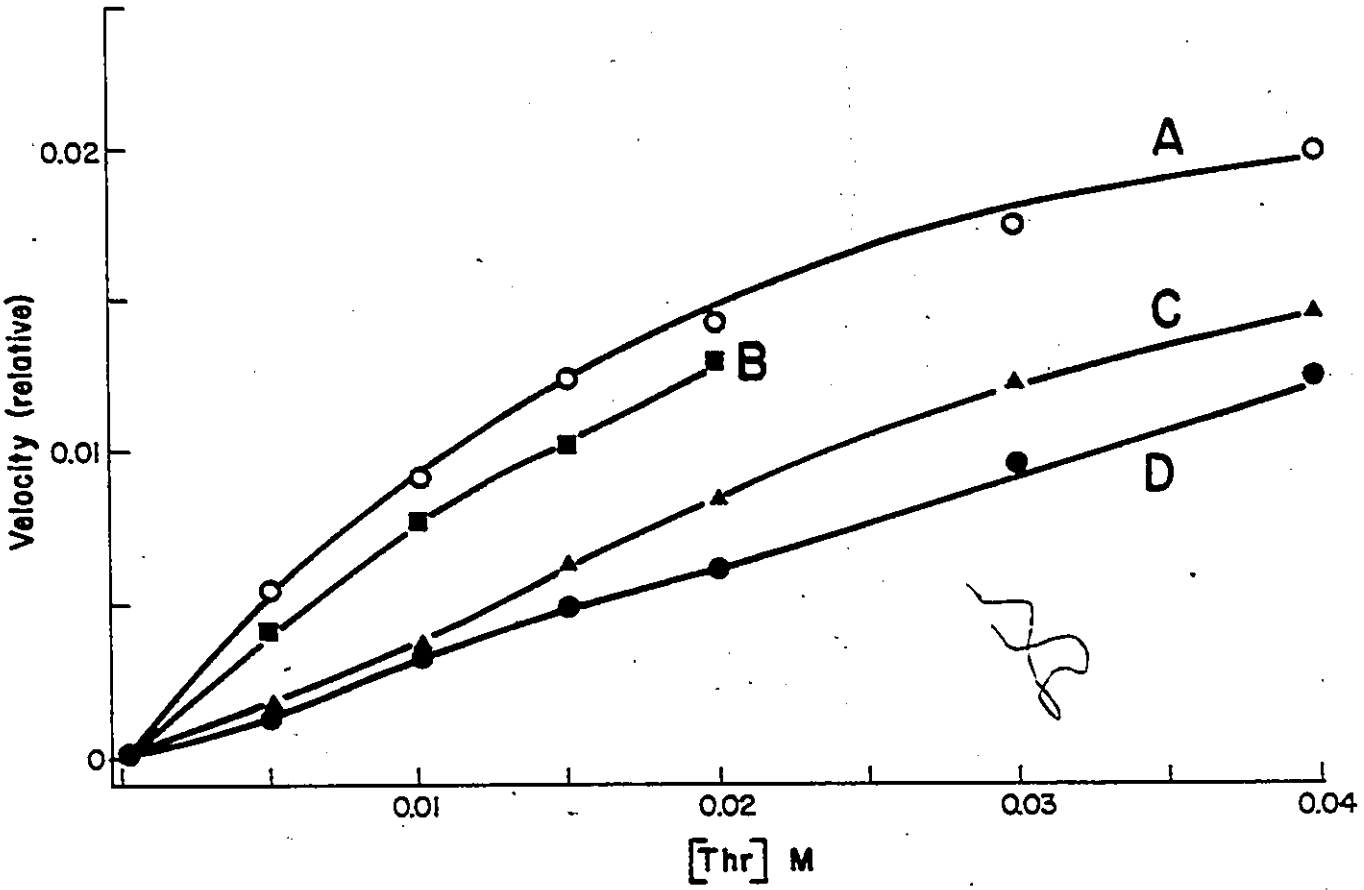
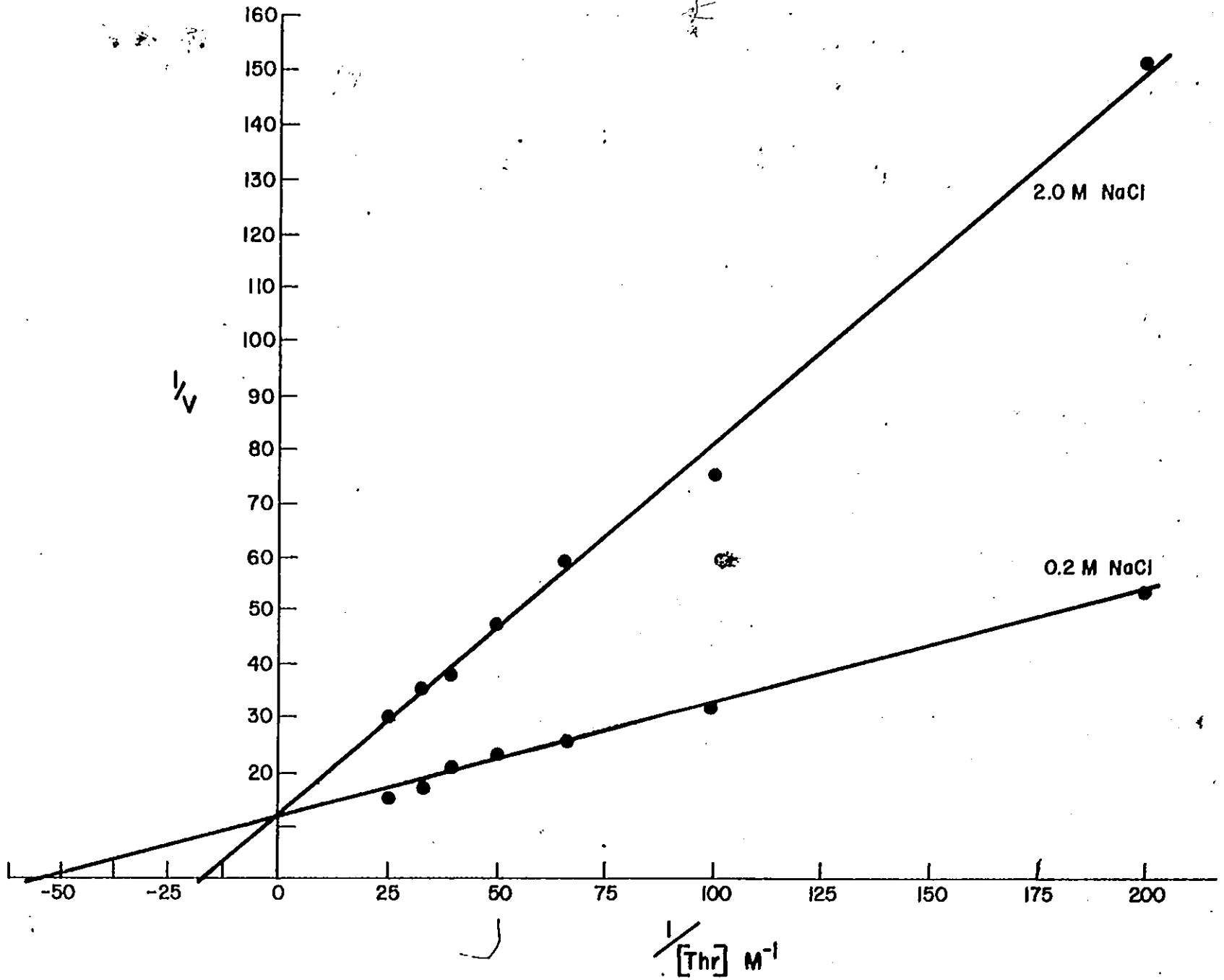

Velocity (relative)
0.02
0.01
0
A
B
C
D
0.01
0.02
0.03
0.04
[Thr] M

Fig. V-8

Double reciprocal plots of threonine saturation curves at 0.2M and 2.0M NaCl. Data from Fig. V-6 curve A and Fig. V-7 curve A. K_m (0.2M NaCl) = 0.017M threonine, K_m (2.0M NaCl) = 0.059M threonine, V_{max} = 0.29 µmoles αKB/min/mg prot.


160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
1/v
2.0 M NaCl
0.2 M NaCl
-50
-25
0
25
50
75
100
125
150
175
200
1/[Thr] M⁻¹

competitively interferes with the threonine binding processes.

Effects of effectors, isoleucine and valine, on the threonine saturation curves.

Isoleucine at both low and high salt concentrations interacted with TDase and appeared to reduce the effectiveness of threonine binding (Figs. V-6, V-7). However, the double reciprocal plots (Figs. V-9, V-10, data from Fig. V-6 and V-7) show that V_{max} was not altered by feedback effectors. The patterns of inhibition, i.e. the sigmoidal curves in the presence of effectors, are characteristic of "K" type regulatory enzymes (Monod et al. 1965).

Valine is known to allieviate inhibition of TDase by isoleucine. One of the physiological implications of such a mechanism for relief of inhibition is that an excess of valine will cause increased synthesis of isoleucine because of decreased sensitivity of TDase to isoleucine. In the V. costicola TDase, valine partially overcomes isoleucine inhibition at low as well as high salt concentrations (Figs. V-6, V-7). At 2.0M NaCl TDase is less sensitive to both isoleucine inhibition and valine alleviation than is the enzyme in low salt concentrations.

Empirical Hill coefficients associated with salt and feedback effector effects of enzyme activity.

Sigmoidal substrate saturation curves in regulatory enzymes can mean that the enzyme interacts with more than one molecule of substrate. The extent of interactions can be estimated using the empirical Hill plot (Atkinson et al. 1965). The slope

of the plot can be interpreteted as equivalent to or greater than the number of interacting substrate-binding sites per enzyme molecule. A Hill coefficient of value n=1 usually implies Michaelis Menten type kinetic behavior; if n > 1 this can be indicative of multi-substrate "allosteric" effects and cooperative interaction between substrate or effector sites on an enzyme molecule.

Salt has been shown to be a pseudo-competitive inhibitor of TDase activity (Fig. V-8). Empirical Hill plots of the threonine saturation curves in the absence of effectors (Figs. V-6A, V-7A) at 0.2M NaCl and at 2.0M NaCl showed that salt did not cause any cooperative effects (Fig. V-11A, B, Tab. V-3A). The presence of 2M NaCl, however, did decrease threonine cooperative interactions: with the same feedback effector concentrations, more than one molecule of threonine interacted with the enzyme at 0.2M NaCl (Fig. V-11 B&C), but 2.0M NaCl reduced the n value to approximately 1 (Fig. V-12 B&C, Tab. V-3 B,C). On the other hand, isoleucine homotropic cooperativity was not affected by salt; Hill plots of the isoleucine effects on activity (Fig. V-5) showed that two molecules of isoleucine were involved with inhibition at both low and high salt concentrations (Fig. V-13).

An interesting result was obtained when NaCl was treated as an effector of enzyme activity and plotted to obtain a Hill coeeficient (Fig. V-14). Up to 1M NaCl, salt acted as a simple first order inhibitor (n=1) of enzyme activity, which was also shown by double reciprical plot data (Fig. V-5). However, an inflection occurred in the Hill plot at exactly 1M NaCl; at higher NaCl concentrations the n became zero. Possibly the enzyme

Fig. V-9

Double reciprocal plots of effects of isoleucine and valine on threonine saturation curve, 0.2M NaCl. Data from respective curves of Fig. V-6. The plots hypothetically converge at the $1/V_{max}$ ordinate value. Dashed line is plot of threonine saturation curve in absence of feedback effectors, transposed from Fig. V-8.

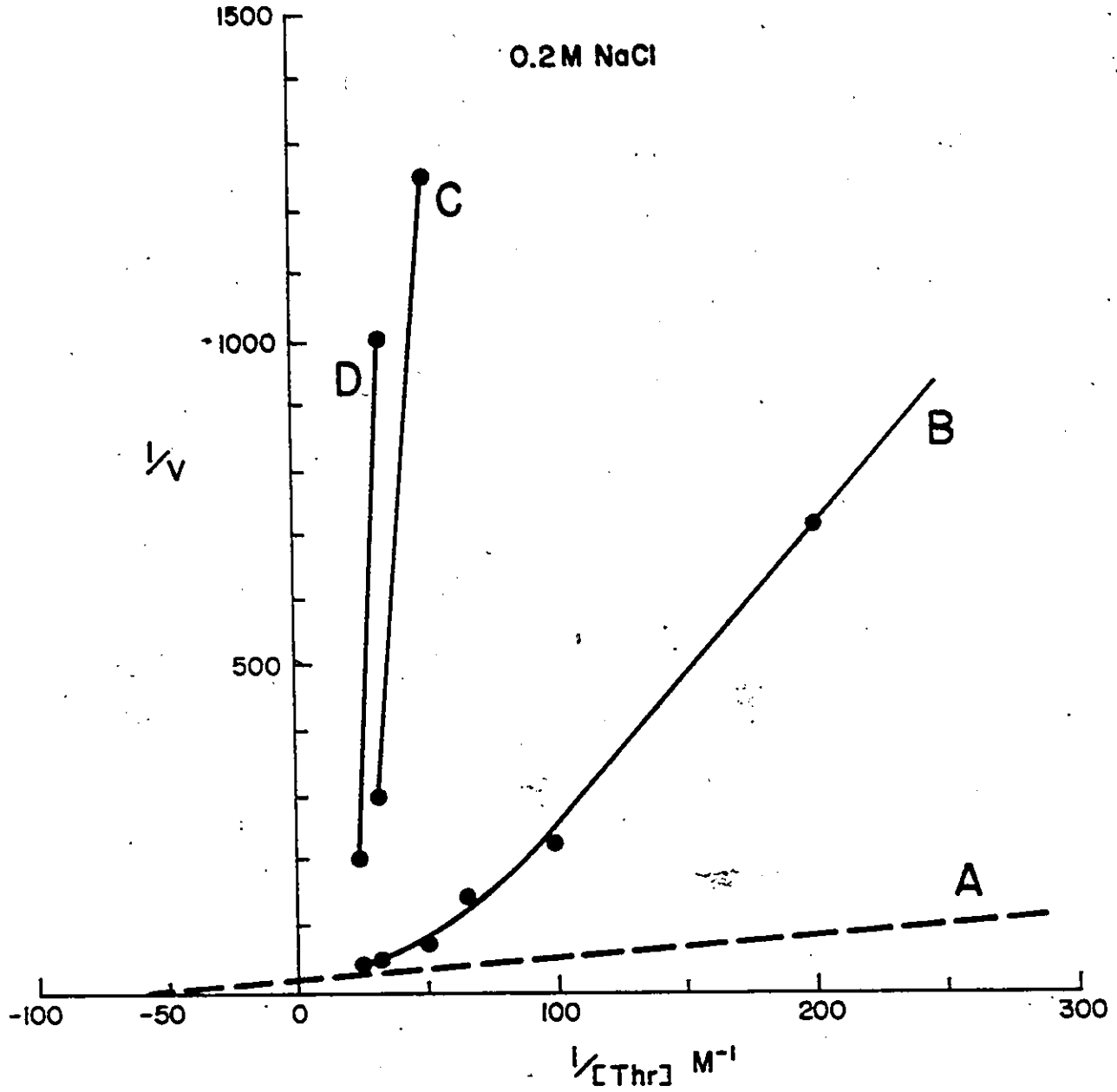

0.2 M NaCl
1500
1000
500
1/V
A
B
C
D
-100
-50
0
100
200
300
1/[Thr] M⁻¹

Fig. V-10

Double reciprocal plots of effects of isoleucine and valine on threonine saturation curve, 2.0M NaCl. Data from respective curves of Fig. V-7. The plots hypothetically converge at the $1/V_{max}$ ordinate value. Dashed line is plot of threonine saturation curve in absence of feedback effectors transposed from Fig. V-8.

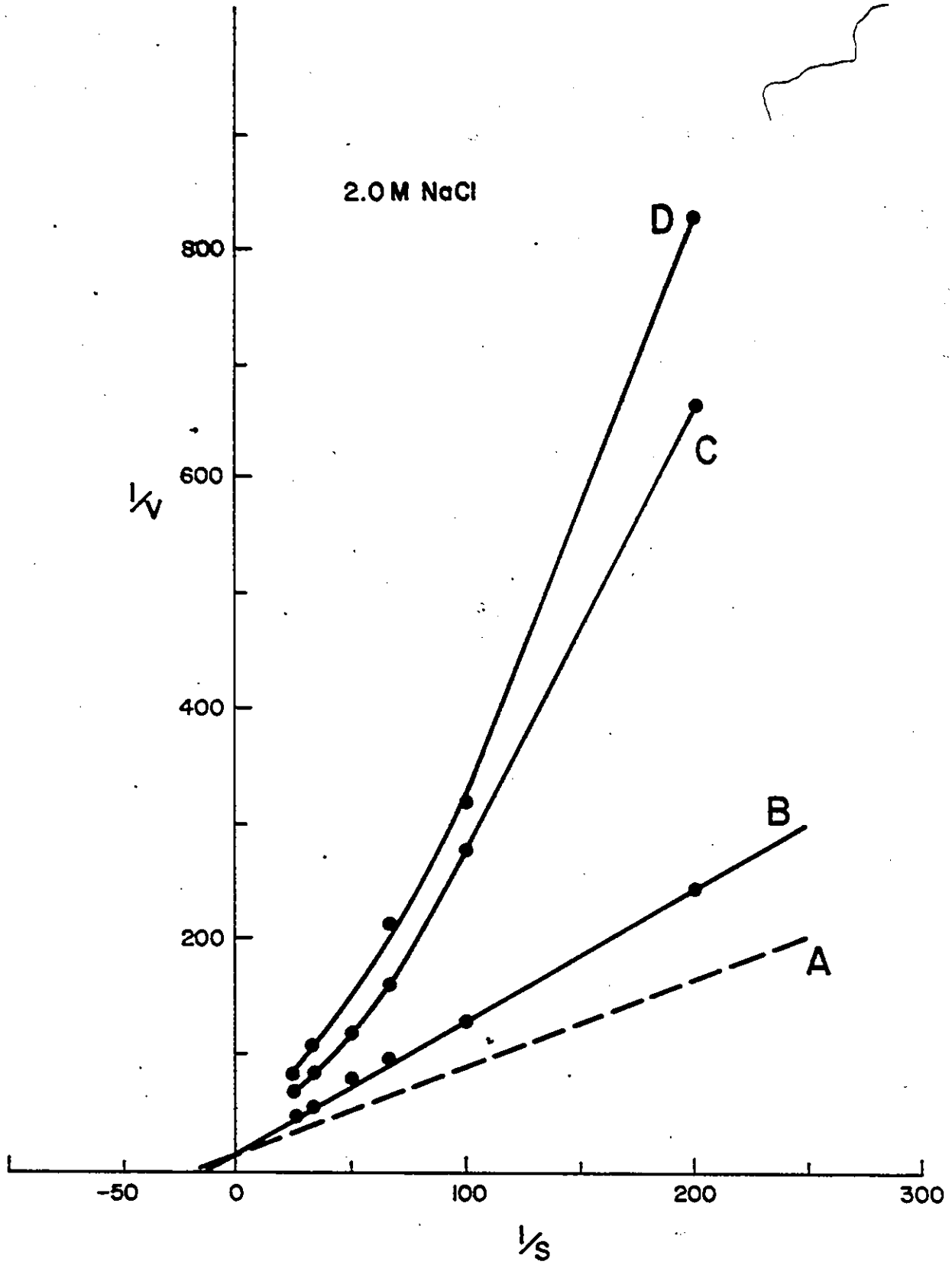
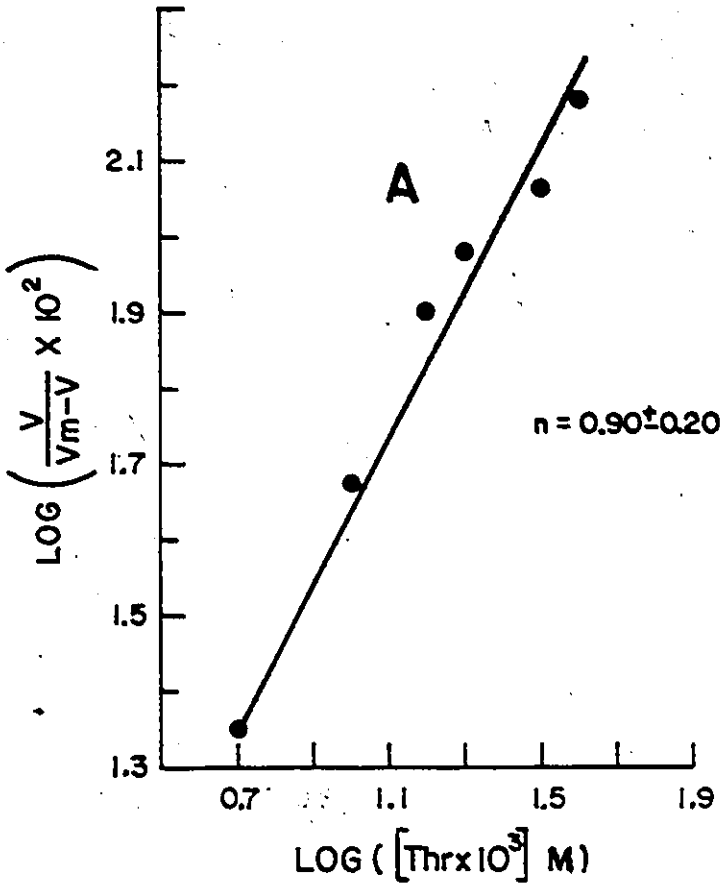

2.0 M NaCl
D
C
B
A
800
600
400
200
1/v
-50
0
100
200
300
1/s

Fig. V-11

Empirical Hill plots of data from respective curves of fig. V-6 at 0.2M NaCl. Reaction mixtures: A contained no additions, B contained 8×10^{-4}M isoleucine + 5×10^{-3}M valine and C contained 8×10^{-4}M isoleucine + 8×10^{-4}M valine. Tab. V-2 lists the reaction mixture conditions and associated Hill coefficients.


A
0.2M NaCl
n = 0.90±0.20
LOG (V/Vm-V X 10²)
LOG ([Thr x 10³] M)
2.1
1.9
1.7
1.5
1.3
0.7
1.1
1.5
1.9

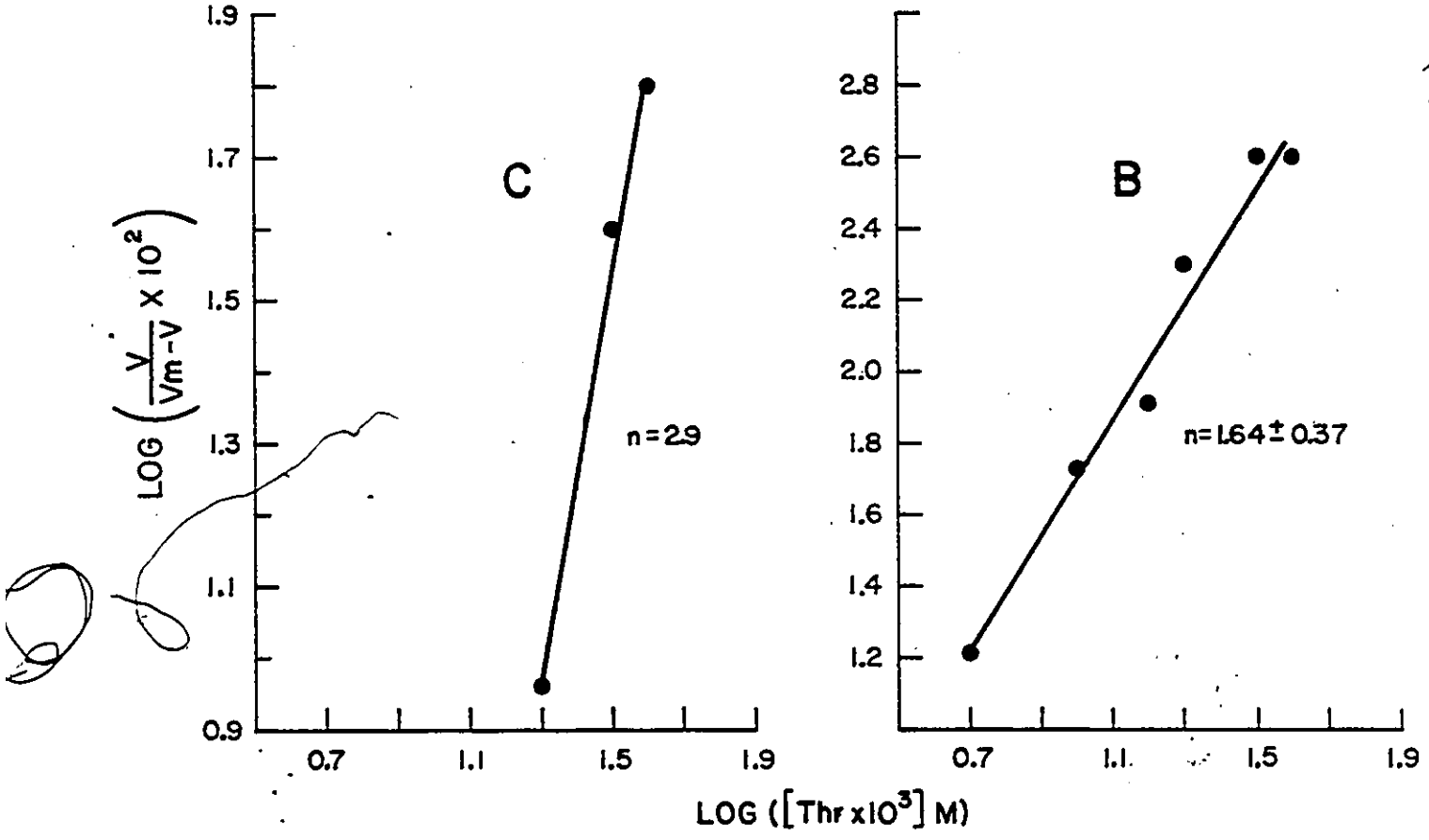

C
n = 2.9
B
n = 1.64 ± 0.37
LOG (V/Vm-V X 10²)
LOG ([Thr x 10³] M)
1.9
1.7
1.5
1.3
1.1
0.9
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
0.7
1.1
1.5
1.9

Fig. V-12

Empirical Hill plots of data from respective curves of Fig. V-7 at 2.0M NaCl. Reaction mixtures : A contained no additions, B contained 8 X 10^{-4}M isoleucine + 8 X 10^{-3}M valine, C contained 8 X 10^{-4}M isoleucine + 8 X 10^{-4}M valine and D contained 8 X 10^{-4}M isoleucine. Tab. V-2 lists the reaction mixture conditions and associated Hill coefficients.

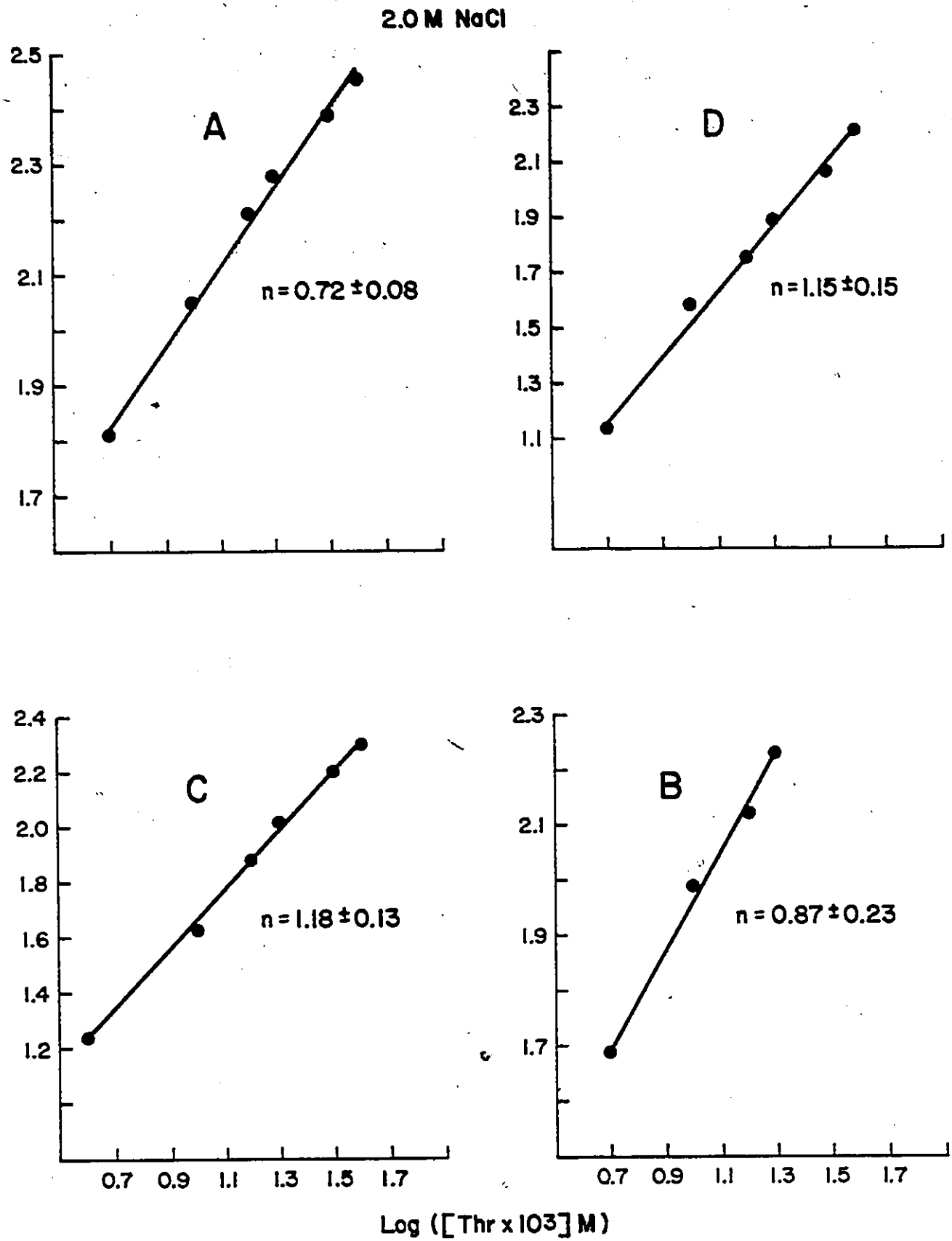

2.0 M NaCl
A
n = 0.72 ± 0.08
D
n = 1.15 ± 0.15
C
n = 1.18 ± 0.13
B
n = 0.87 ± 0.23
Log ([Thr x 10³] M)

Table V-3

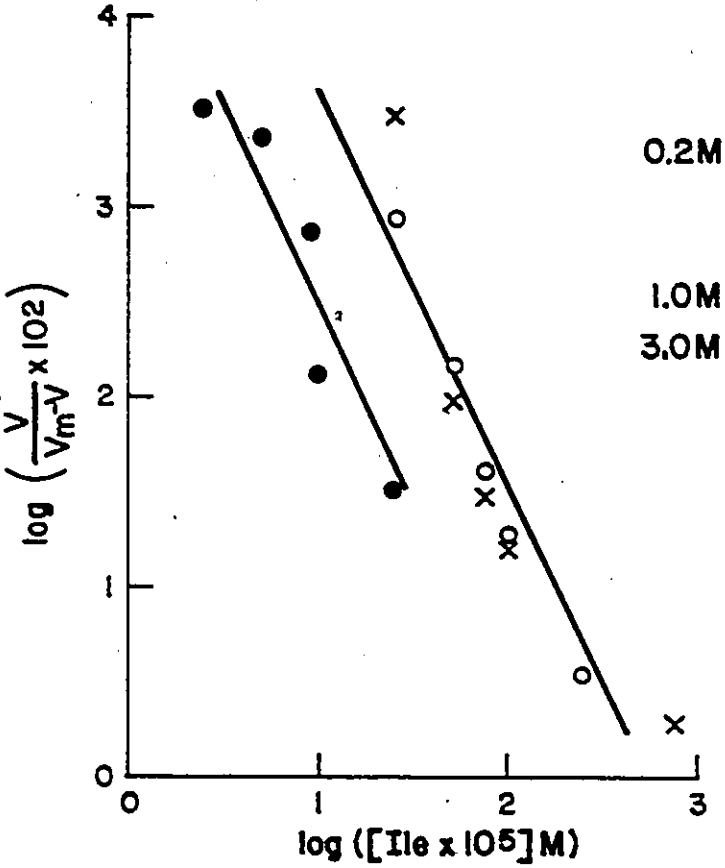
Effects of salt concentration and feedback effectors on the empirical Hill coefficient.

Figure Key	addition to reaction mixture	0.2M NaCl n	2.0M NaCl n
A	none	0.94 ± 0.19[a] 0.90 ± 0.20	1.04 ± 0.13 0.72 ± 0.08
D	$8X10^{-4}M$ isoleucine	---[b]	1.15 ± 0.15
C	$8X10^{-4}M$ isoleucine+ $8X10^{-4}M$ valine	2.9	1.18 ± 0.13
B	$8X10^{-4}M$ isoleucine+ $8X10^{-3}M$ valine	1.64 ± 0.37[c]	0.87 ± 0.23

a. Empirical Hill coefficient,n, with 95% confidence interval limits.
b. Complete inhibition of activity, n > 1.
c. The concentration of valine in the 0.2M NaCl experiment was $5X10^{-3}M$.

Fig. V-13

Empirical Hill plots and coefficients for the isoleucine concentration effect on feedback inhibition presented in Fig. V-5.

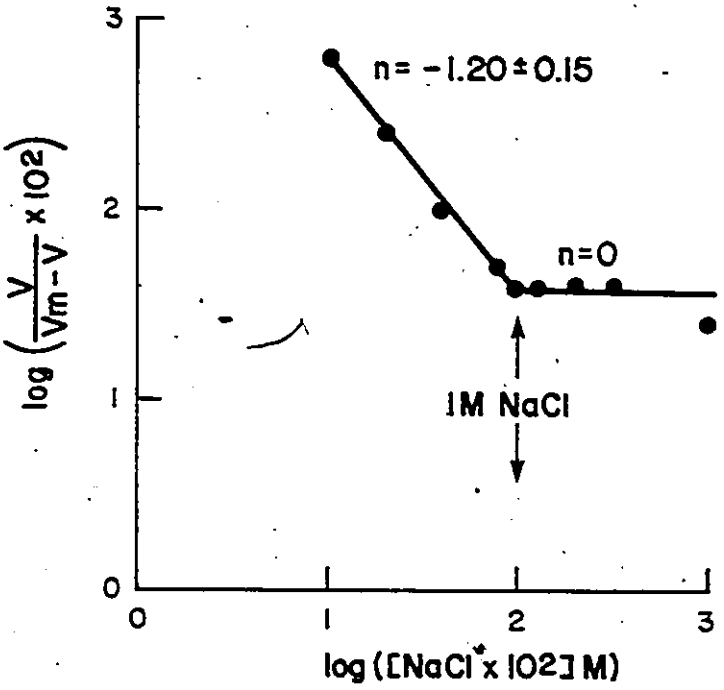


0.2M NaCl (●) n=-2.2±1.3

1.0M NaCl (○)
3.0M NaCl (×) } n=-2.1±0.7

Fig. V-14

Empirical Hill plot of data from an experiment on the effect of NaCl concentration on enzyme activity (Fig. V-4). In this analysis salt was assumed to interact with the enzyme in some manner analogous to a substrate or inhibitor.


n= -1.20 ± 0.15
n=0
1M NaCl
log (V/(Vm-V) x 102)
log ([NaCl] x 102 M)
0
1
2
3

changed in conformation at 1M NaCl and salt no longer had an interaction with the enzyme. It was also possible that a site for Na^+ was filled by 1M NaCl and added NaCl no longer had any effect. The Hill plot is not suitable for application at ligand concentrations high enough to cause site saturation (Atkinson et al. 1965). At any rate, the Hill plot emphasizes the significance of the 1M NaCl inflection point. One M NaCl is the concentration above which only small effects on the activity or feedback sensitivity were caused by addition of more NaCl (Figs. V-4, V-5). It is possible that 1M NaCl is also the concentration of NaCl required for maximum stability (see below).

Effect of pH on threonine deaminase activity and feedback inhibition.

Fig. V-15 shows the effect of pH on the activity of TDase. Optimal activity occurred at about pH 9.0 at both 0.2M and 2.0M NaCl. A pH 8-9 optimum is typical of microbial TDases. In contrast to the activity, the feedback inhibition by isoleucine increased as the pH was lowered (Tab. V-4). At pH 7.5 only 6-21% of the maxmimal activity remained but concentrations of isoleucine which hardly inhibited at pH 9, inhibited 75-87%. Most experiments were run at pH 7.8-8.0, to achieve a compromise between activity and feedback inhibition.

Effects of various treatments and agents on the retention of TDase activity and sensitivity to inhibition.

Since the V. costicola TDase had maximal activity in the absence of salts, attempts were made to extract cells in salt-free media or to resuspend ammonium sulfate fractions in buffers

Fig. V-15

pH response of partially purified threonine deaminase. Extract and reaction mixture conditions described in Tab. V-4.

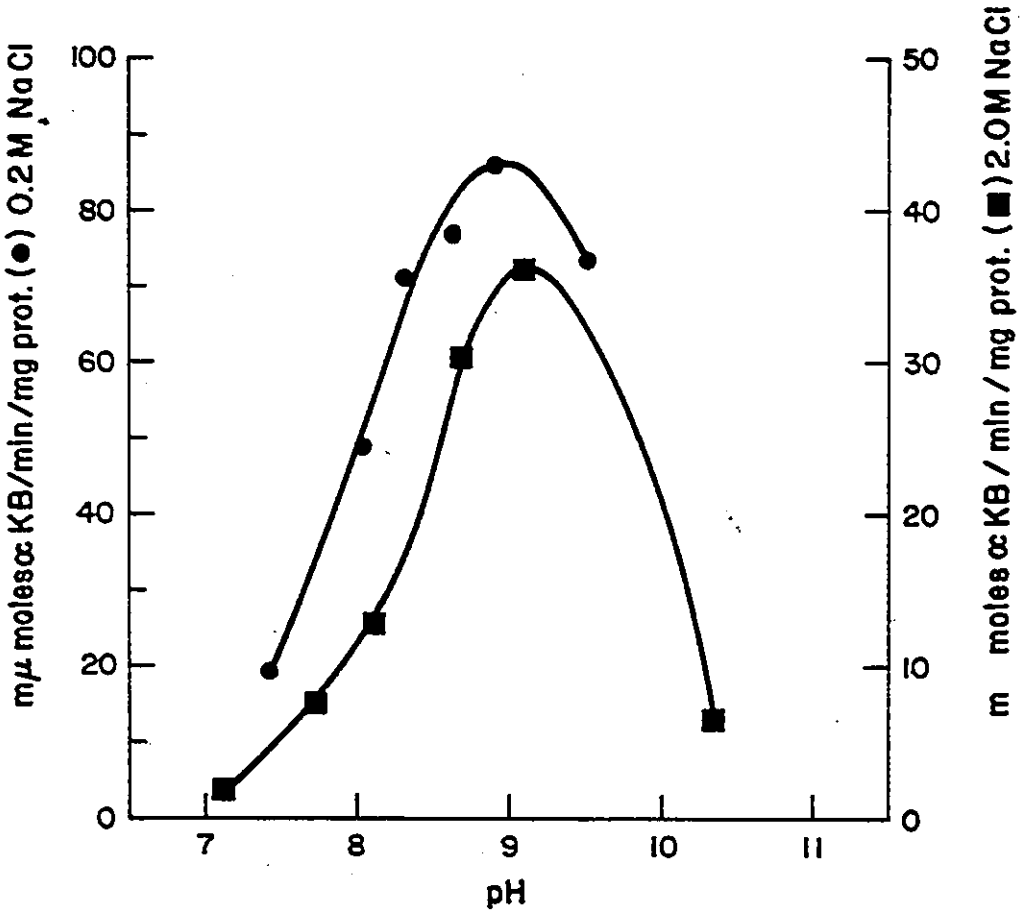

mμ moles∝ KB/min/mg prot. (●) 0.2 M NaCl
m moles ∝ KB / min / mg prot. (■) 2.0M NaCl
pH
0
20
40
60
80
100
10
30
50
7
8
9
10
11

Table V-4

V. costicola TDase: Effect of pH on activity and feedback inhibition.

NaCl	pH	Relative Velocity	10^{-4}M ile inhibition	$4X10^{-4}$M ile inhibition
0.2M	9.1(max)	100[a]	<10%	
	8.2	73	23%	
	7.8(normal)[b]	44	45%	
	7.5	21	87%	
2.0M	9.1(max)	40		<10%
	8.2	16		25%
	7.8(normal)	10		32%
	7.5	6		75%

a. Approximate specific acitivity mμmoles KB/min/mg prot. ammonium sulfate fractioned enzyme dialysed overnight at 4C against the extraction buffer: 10^{-3}M dithiothreitol, 10^{-3}M Mg-EDTA, 0.5M NaCl, 0.1M tris, 0.5M KH_2PO_4 pH7.8. Reaction mixtures contained 0.01M threonine, 10μg/ml pyridoxal phosphate, NaCl and buffer-0.1M KH_2PO_4, 0.1M tris, 0.1M glycine.

b. pH 7.8 was that used for most of the experiments.

containing little or no salt, e.g. 0.1M tris pH 7.8. In all cases where the enzyme was removed from salt solutions the activity disappeared within several hours. In a dialysis experiment a crude extract (relative act. 100, isoleucine feedback inhibition 100%) was divided into two equal portions and dialysed against 0.1M tris pH 7.8 or against 1M NaCl, 0.1M tris pH 7.8. The extract which was dialysed against tris lost all activity over 4 hr. at 4C, while the extract dialysed against tris-NaCl retained all activity and maintained good feedback inhibition (67.5%). Once the activity had been lost during dialysis it was not possible to recover it by dialysing against 1M NaCl, 0.1M tris, pH 7.8.

To determine what substances might stabilize the TDase activity in the virtual absence of salts, an experiment which resulted in the data of Tab. V-5 was carried out. The only unequivocal stabilizers at both 25C and 0C were 1M NaCl, 1M KCl and allothreonine, the other isomer of threonine with respect to the second assymetric carbon (C_3). Allothreonine was used by P. T. Magee to help stabilize yeast TDase (personal communication). A more stringent experiment attempted to determine if allothreonine stabilized the enzyme in the absence of salts. A 4 hr. 4C dialysis was run using crude extract which had been sieved through G-25 Sephadex to remove small molecules. The extract dialysed against 1M NaCl, 0.1M tris pH 7.8 retained most of its activity and feedback inhibition. The extract dialysed against 2.5×10^{-3}M threonine, 0.1M tris, pH 7.8 lost all of its activity. Substitution of allothreonine for threonine caused the retention of 10% of the original activity and slight

Table V-5

Stability of V. costicola TDase at 25 and OC, with salts or other substances present.

compound	zero time	25C,135min.	OC, 12hr.	OC, 60hr.
NONE	24 (40%)*	15 (55%)	9 (42%)	0
1M NaCl	19 (31%)	27 (37%)	21 (42%)	10 (45%)
1M KCl	38 (69%)	45 (72%)	46 (70%)	31 (75%)
10^{-3}M DTT**	23 (49%)	29 (55%)	15 (49%)	2
10^{-3}M EDTA	18 (40%)	16 (40%)	6 (47%)	0
10^{-3}M $MgCl_2$	21 (48%)	16 (42%)	9 (40%)	1
$5X10^{-5}$M isoleucine	19 (42%)	1 (71%)	5 (40%)	1 (100%)
10^{-3}M allothreonine	9 (93%)	9 (91%)	11 (79%)	5 (76%)
10µg/ml pyridoxal phosphate	22 (39%)	17 (45%)	12 (17%)	0

*Relative activity retained after inactivation period; ( )% activity in presence of 5 X 10^{-4}M ile. Protein concentration 12.5 mg/ml. Enzyme extracted from 1M NaCl SGP grown cells, centrifuged 1 hr. 50,000 X g, supernatant put through a Sephadex G-25 column, concentrated by ultrafiltration then distributed into the inactivation mixtures at zero time. Aliquots removed from inactivation mixtures at indicated times and assayed in 1M NaCl reaction mixtures. 5 X 10^{-4}M isoleucine was used to check feedback inhibition, relative activity x 2 = specific activity.

** dithiothreitol

Table V-6

Stability of crude and partially purified *V. costicola* TDase at 1M salt.

enzyme	inactivation period 0 time	90 min	150 min
crude extract (12.5 mg prot./ml)			
specific activity remaining :	15*	17	11
$7X10^{-4}$M ile feedback inhibition :	100%	100%	100%
$(NH_4)_2 SO_4$ fraction (7.0mg prot./ml)			
specific activity remaining :	42	27	26
$7X10^{-4}$M ile feedback inhibition :	100%	81%	50%

*mμmoles KB/min/mg prot., 1M NaCl activity remaining after inactivation period. Inactivation mixtures contained 0.5M NaCl, 0.5M KCl, dithiothreitol, Mg-EDTA and tris pH 7.8.

feedback inhibition. Clearly, the salts were the only effective single stabilizers. But even salts did not completely stabilize partially purified TDase at 37C (Tab. V-6).

It is unusual that none of the TDase ligands, isoleucine, pyridoxal phosphate (Tab. V-5) and threonine, stabilized the enzyme. Isoleucine caused an increased rate of inactivation at 25C compared to the control (Tab. V-5) yet acted to stabilize yeast TDase (Cennamo et al. 1964, McDonald & Kaplan 1973). Interactions and concerted actions of substances on the TDase stability are undoubtedly important; for instance, in low salt reaction mixtures the TDase was stable (i.e. showed activity which was linear with time) for at least 1 hr. These effects were not investigated.

A preliminary molecular weight estimate for the V. costicola TDase.

The TDase molecular weight was estimated using a Bio-Gel 1.5m column as 80,000-94,000 Daltons (Fig. IV-16, p. 148). This molecular weight estimate could not be confirmed by gel chromatography at low salt concentrations because of the instability of the enzyme. The assumptions about marker proteins and gel effects at 1-2M NaCl concentrations discussed in the context of the ATCase molecular weight estimate (Ch. IV p.145-147) also apply to the TDase estimate. If the estimate is accurate the V. costicola enzyme is one-half the size of most microbial TDase which are tetrameric with about 200,000 molecular weight. Moreover, even though some activity was lost in the column, the TDase effluent which came out at K_{av}=0.11 was fully sensitive to inhibition by isoleucine: 5 x 10^{-4}M isoleucine at 1M NaCl

pH 7.8 inhibited the chromatographed enzyme 75%. TDases which had the same molecular weight as the Vibrio enzyme were either inactive apodimeres (Calhoun et al. 1973) or very low activity, feedback insensitive dimers (Grimminger & Feldner 1974) from E. coli, or in the case of the Thermus TDase, were completely insensitive to isoleucine feedback inhibition (Higa & Ramaley 1973).

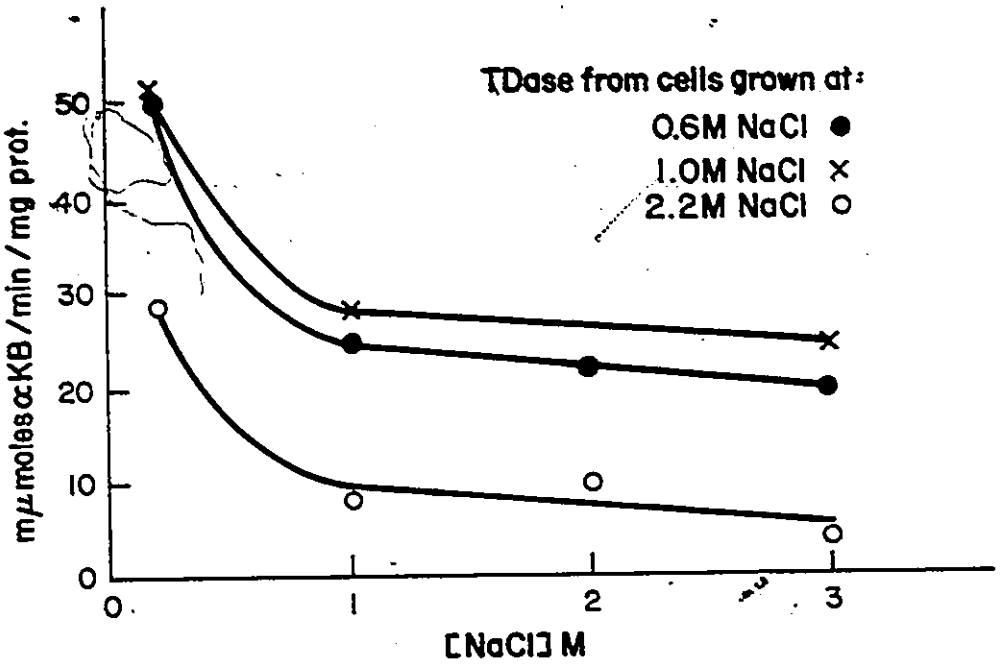
Results: Experiments on the synthesis and repression of V costicola biosynthetic threonine deaminase.

Effects of salt on TDase activity extracted from cells grown at different salt concentrations.

Individual moderately halophilic bacteria are often able to grow over a wide range of salt concentrations. V. costicola in complex media, for instance, can grow well over an eight-fold range of salt concentrations. Cells must maintain activities under different environmental conditions; one possible explanation for such versatility is that cells make different enzymes, i.e. isozymes, under different conditions. For instance, V. costicola might make a salt-dependent TDase at high medium salt concentrations, and a non-halophilic enzyme at low medium salt concentrations. Fig. V-16 shows that, although the specific activities varied, TDases extracted from cells grown at 0.6, 1.0 and 2.2M salt concentrations had identical salt response curves. Moreover their feedback inhibition response to isoleucine was typical (see Fig. V-16 caption).

Fig. V-16

Effect of salt on TDase activity extracted from *V. costicola* cells grown at various salt concentrations. Cells from cultures were harvested and resuspended in standard 1M salt extraction buffer, then their activity (and feedback inhibition, not shown) tested at various reaction mixture salt concentrations. Feedback inhibition by 2×10^{-4}M isoleucine in all 0.2M NaCl reaction mixtures ranged from 41-54%; inhibition by 7×10^{-4}M isoleucine in all 1-3M NaCl reaction mixtures ranged from 26-48%.


TDase from cells grown at:
0.6M NaCl
1.0M NaCl
2.2M NaCl
mμmoles αKB/min/mg prot.
50
40
30
20
10
0
0
1
2
3
[NaCl] M

TDase activity from V. costicola grown in media containing amino acids.

Extracts prepared from V. costicola cultures grown in complex amino acid media, 1% proteose peptone and 1% tryptone or 1% Difco casamino acids + 0.005% $MgSO_4$, had no detectable TDase activity. This result suggested that amino acids in the medium caused a repression of the synthesis of the enzyme. TDase repression phenomenon are well known in various non-halophilic microorganisms. I attempted to determine the medium conditions and components associated with the TDase repression in the moderate halopile.

When added to SGP medium, large amounts of casamino acids were required to bring about complete repression of TDase activity (Tab. V-7). The degree of repression obtained was not significantly affected by the medium salt concentration.

With one exception, the branched chain amino acids when added singly or in combinations to 1M NaCl SGP medium, even at high concentrations (0.1M), did not cause a significant repression of TDase activity (Tab. V-8). Tenth-molar valine reduced the activity by about 50%. Whenever isoleucine was added alone the highest specific activities occurred. The extracted TDase retained feedback sensitivity to isoleucine.

Repression was obtained when 18 amino acids were added to the 1M NaCl SGP medium at the concentrations indicated in Tab. V-8. To determine if some amino acids might be more important than others in causing repression, experiments were run using arbitrary combinations of amino acids in media (Tab. V-8). The "aspartic acid family" (group B) could repress the TDase.

Table V-7

Effects of addition of casamino acids to salts-glucose medium on the specific activity of extracted TDase

exp.	medium	casamino acid addition	1M NaCl specific activity	$2X10^{-4}$M isoleucine feedback inhib.
1.	1M NaCl SGP	--	47*	43%
	"	0.05%	25	28%
	"	0.5 %	0	0
2.	0.6M NaCl SGP	--	12	NT*
	"	0.5%	8.3	NT
	1.0M NaCl SGP	--	6.4	NT
	"	0.5%	2.5	NT
	2.2M NaCl SGP	--	4.1	NT
	"	0.5%	2.7	NT

* mμmoles αKB/min/mg prot. NT=not tested.

Table V-8

Effects of various amino acid additions to salts-glucose medium on the specific activity of extracted TDase

SGP medium NaCl conc.	addition		1M NaCl specific act.	2.5×10^{-4}M isoleucine feedback inhib.
1M	--		20*	24%
1M	10^{-2}M ile, val, leu		18	22%
"	10^{-1}M ile, val, leu		26	42%
1M	10^{-2}M ile		34	37%
"	10^{-2}M ile, val		25	56%
1M	10^{-1}M leu		15	53%
"	10^{-1}M val		9	56%
0.6M	--		23	34%
"	10^{-2}M ile		26	48%
1.6M	--		17	50%
"	10^{-2}M ile		32	53%
1M	amino acid groups*	A,B,C,D	3	100%
"	"	A,B	6	41%
"	"	B	7	59%
1M SGP without glucose	amino acid groups	A,B,C,D	17	68%

*mμmoles αKB/min/mg prot. Compilation of several experiments, some figures are means. Amino acid groups (final concentration of L form in the medium g/l):
A glutamic (1), arginine (0.1), proline (0.1), histidine (0.1).
B aspartic (1), threonine (0.2), glycine (0.1), serine (0.1), lysine (0.1), alanine (0.1), isoleucine (0.1), leucine (0.1), valine (0.1).
C cysteine (0.1), methionine (0.1).
D phenylalanine (0.1), tyrosine (0.1), tryptophan (0.1).

However, when glucose was removed from the medium the repression effects disappeared.

Effect of isoleucine, valine and leucine additions to 1M NaCl SGP on the growth of V. costicola.

Although the branched-chain amino acids did not cause TDase repression, they did affect the growth of V. costicola. From the growth curves of Fig. V-17 and from another similar experiment the following effects (in relation to growth in SGP medium with no additions) were deduced:

amino acid addition	effect on growth rate	effect on yield (max. growth)
isoleucine	decreased	decreased
valine	none	decreased
isoleucine + valine or leucine alone	increased	increased
ile + val + leu	decreased	decreased.

Flannery and Kennedy (1962) also noted that isoleucine or valine added separately depressed V. costicola growth but when added together were stimulatory.

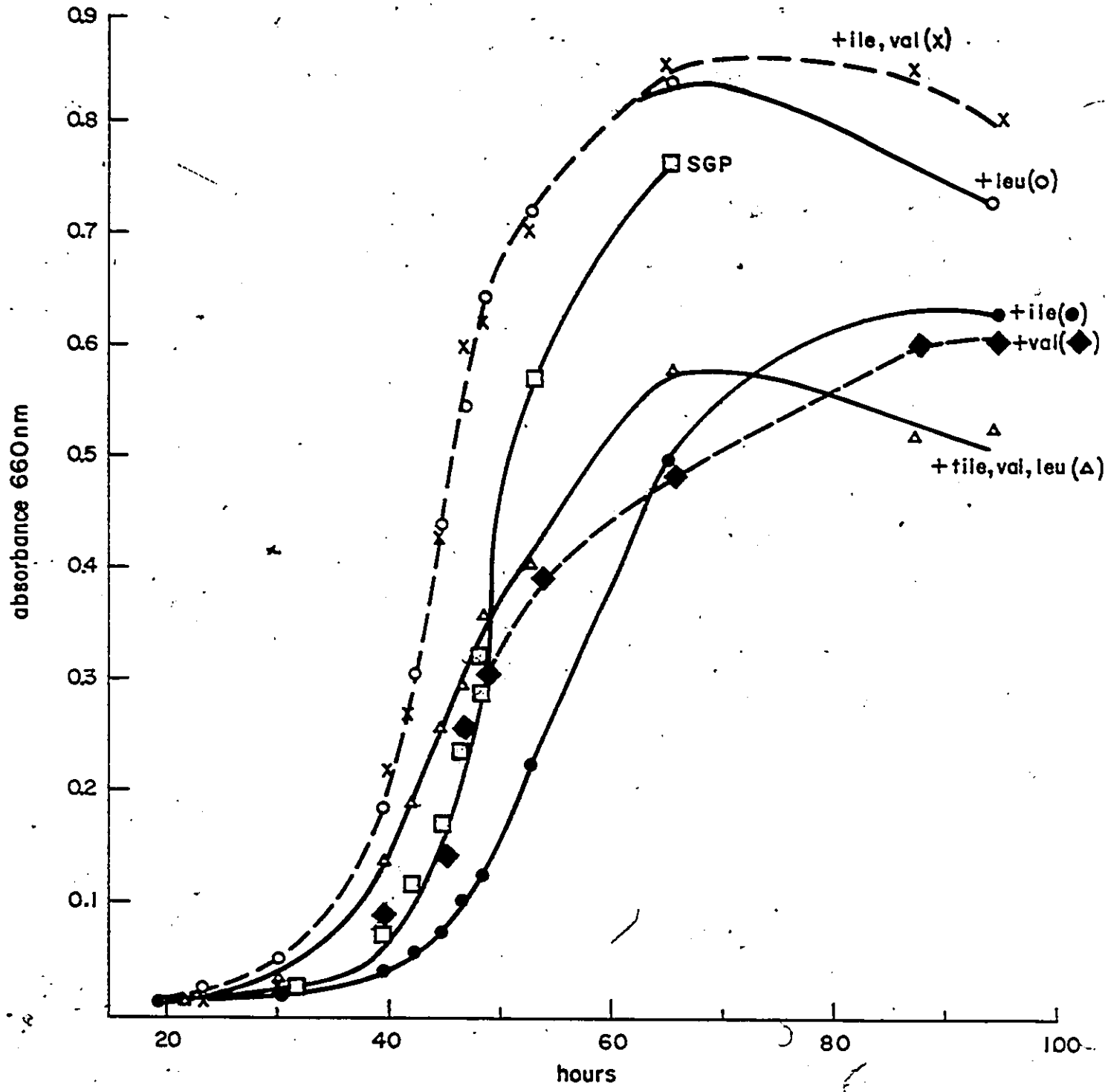
Discussion

The salt requirements of enzymes from moderately halophilic bacteria.

It has been well documented that most of the enzyme activities from extremely halophilic bacteria require substantial concentrations of salts for activity and stability (Ingram 1957, Brown 1964, Larsen 1967, Kushner 1968, Lanyi 1974). Such salt

Fig. V-17

V. costicola growth curves in the presence of various branched-chain amino acids. All amino acids added to 1M NaCl SGP medium to achieve a final concentration of 0.1 g/l.


absorbance 660nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
hours
+ile, val (x)
+leu(o)
SGP
+ile(●)
+val(◆)
+tile, val, leu (△)

requirements have been attributed to several interrelated enzyme phenomena. Participation of ions in the chemical reactions mediated by enzymes (Suelter 1970), electrostatic shielding of charged groups within proteins, stabilization of hydrophobic interactions by moderate to high salt concentrations, and effects of salts on local protein residues (Lanyi 1974) probably are important in enzyme salt dependence. The importance of any particular salt effect depends upon the individual enzyme's characteristics. Lanyi (1974) further pointed out: "The requirement of unusually high concentrations of NaCl or KCl for maintaining the optimally folded structure of halophilic proteins raises the possibility that these macromolecules are put together according to different rules than are nonhalophilic systems. This is probably not the case. It is necessary merely to assume that some of the interactions, which are ordinarily involved in stabilizing protein structure, are weaker in halophilic systems, whereas others are stronger because of the pressure of salt."

Not all the enzymes from moderately halophilic bacteria are salt dependent (and as well, not all the enzymes from non-halophilic bacteria are inhibited by salts). Tab. V-9 shows that about half of the enzyme activities studied in each organism did not require and, moreover, were inhibited by salt; as was, for example the V. costicola TDase. The rest of the enzyme activities were more in character with what might be expected from enzyme activities of cells with moderate intracellular salt concentrations (Ch. III), e.g. the V. costicola ATCase.

It has already been mentioned that the distribution of

Table V-9

Salt requirements of enzymes from moderately halophilic bacteria.

Enzyme	organism	NaCl or KCl requirement for: maximal activity	stability	% max. act. at salt conc. required for: stability	% max. act. at 1-2M NaCl or KCl	reference
threonine deaminase	V. costicola	<0.1	1 (est.)	20-40	20-40	this dissertation
isocitrate dehydrogenase	"	<0.1	0.5-1	20-60	5-20	Wydro 1974
pyruvate kinase	"	<0.1	<0.1	-	-	E. de Medicis, commun.
glycerol dehydrogenase	"	0.5-1	-	-	80-100	Baxter & Gibbons 1954
aspartate transcarbamylase	"	1.8	1 (est.)	40.100	40-100	this dissertation
succinic dehydrogenase	P. (M.) denitrificans	<0.1	-	-	30-45	Baxter & Gibbons 1956
malic dehydrogenase	"	<0.1	-	-	20-30	"
isocitric dehydrogenase	"	0.2	-	-	10-20	"
glutamic dehydrogenase	"	0.3	-	-	30-80	"
nitritase (reductase)	"	0.5	-	-	40-80	Robinson 1962
α-ketoglutaric dehydrogenase	"	0.7	-	-	20-90	Baxter & Gibbons 1956
lactate dehydrogenase	"	0.75	-	-	50-95	Robinson 1952
lactate dehydrogenase	"	1.5-2	-	-	90-100	Baxter & Gibbons 1956
cytochrome oxidase	"	2-3	-	-	70-100	"
phenylalanine transaminase	Pseudomonas #101	<0.1	-	-	30-50	Shiio et al. 1956
aspartic transaminase	"	<0.1	-	-	60-80	"
formic dehydrogenase	"	0-0.2	-	-	≤ 60	Yamada & Asano 1954a
aldose dehydrogenase	"	1.5-3	-	-	70-100	Maeno 1965
alkaline phosphatase	"	2	1-4	100	100	Yamada et al 1954
glucose oxidation system	"	2	-	-	50-100	Yamada & Asano 1954a
glucose dehydrogenase	"	2-3	-	-	50-100	"
hydroxylamine reductase	Micrococcus #203	<0.1	-	-	-	Kono & Taniguchi 1960
extracellular protease	Bacillus #21 1	0.5-0.75	-	-	50 (est.)	Onishi 1974b
cytochrome oxidase	Micrococcus #203	1-2	-	-	100	Yamada & Asano 1954b
extracellular amylase	Micrococcus halobius	1.4-2	2 or 50mM $CaCl_2$	100 or 0	80-100	Onishi 1972
extracellular nuclease	Micrococcus varians	2-3	0.25 or 10mM Mg	90-100 or 20-40	70-85	Kamekura & Onishi 1974a

cellular ions might be important in determining whether a particular process or enzymatic reaction may have evolved a salt requirement (Ch. III). That much of the salts are not free in the cytoplasm is strongly suggested by a virtually complete inhibition of V. costicola in vitro protein synthesis by 0.5M NaCl or KCl (R. Wydro, T. Hiramatsu & D. J. Kushner, unpublished data). Moreover, measurements of ions in permeabilized V. costicola (Ch. III, p. 97-99) and in Pseudomonas #101 membranes (Masui & Wada 1973) showed that a large amount of ions can be bound. If a large proportion of the major intracellular cations were associated with the cell envelope of moderate halophiles, then envelope or membrane associated enzymes ought to be salt-dependent or at least markedly tolerant, while cytoplasmic enzymes need not be salt tolerant. Indeed, this trend is partially bourne out in the data of Tab. V-9: sone of the most salt dependent enzymes, lactate dehydrogenase, cytochrome oxidase and alkaline phosphatase are putatively membrane associated.

On the other hand, some unknown factors could be affecting the salt response of enzymes or protein synthesis in vivo which could be absent in vitro. Betaine or a derivative has been implicated in imparting salt resistance to succinate oxidation and respiration in moderately halophilic bacterium Ba_1 (Rafaeli-Eshkol & Avi-Dor 1968). In low salt-grown cells or high salt-grown washed cells betaine was able to stimulate respiratory activity in the presence of 2M NaCl to the levels found in high salt-grown cells. In another case, some unidentified protein factors increased the salt optimum for activity of purified

aldose dehydrogenase of the moderately halophilic Pseudomonas #101 from 0.5M to 2-3M NaCl (Maeno 1965) (but this effect may have been due to an instability of dilute enzyme at high salt concentrations).

If the salt inhibition effects on enzyme activities illustrated in Tab. V-9 were not due to the absence of appropriate stabilizing ions or factors, and if the intracellular salts are free in the cytoplasm then it is possible that many enzymes from moderately halophilic bacteria are genuinely inhibited by the salt concentrations extant in the cells. It has been suggested that cellular enzymes may not operate under optimum conditions inside the cells (Larsen 1962), and that full enzyme activity of each enzyme molecule is not demanded by normal metabolism (Lamanna et al. 1973). In salt inhibited enzymes, considerable residual activity often remained at 1-2M NaCl, close to the intracellular salt concentrations, or in the case of a few measurements, at the salt concentrations required for stability (Tab. V-9). Although some enzymes from moderately halophilic bacteria are inhibited by salts, they are not completely disfunctional at physiological intracellular salt concentrations. The residual activity and feedback inhibition of the V. costicola TDase at 1-3M salt for instance, may render the enzyme fully competent in vivo.

The potential importance of divalent cations and various anions should also not be underestimated in moderately halophilic enzyme salt response phenomena. Although Na^+ and K^+ are almost certainly the major intracellular cations of moderately halophilic bacteria, some enzymes may be completely dependent upon

particular anions or divalent cations for activity and/or stability. In the extracellular amylase secreted by Micrococcus halobius, 50mM $CaCl_2$ could stabilize as effectively as 2.0M NaCl, but the $CaCl_2$ could not support activity alone (Onishi 1972). Likewise, the extracellular nuclease from Micrococcus varians could be stabilized by 10mM Mg^{+2} but required 2-3M NaCl or KCl for maximal activity (Kamekura & Onishi 1974a). In a series of papers on several enzymes from the moderately halophilic marine V. alginolyticus, the importance of the roles of magnesium and chloride ions was illustrated. Pyrophosphatase was inactivated by an in vitro incubation with the measured cellular Na^+, K^+ and Cl^- concentrations. But in the presence of Mg^{+2} the inactivation effects were prevented (Unemoto et al. 1973). Unlike the enzyme from E. coli, the V. alginolyticus alkaline phosphatase was stimulated by anions, Cl^- giving the optimum V_{max}/K_m value (Hayashi et al. 1973). Chloride anion also modified the reactions of the V. alginolyticus cyclic phosphodiesterase; while inhibiting the cyclic phosphodiesterase activity it acted as an activator for two other activities associated with the enzyme, 3'-ribonucleotide and di-p-nitrophenyl phosphate hydrolysis (Unemote & Hayashi 1969). The cell envelope 5'-nucleotidase required both Mg^{+2} and an appropriate anion, Cl^- being the most effective, for maximal activity while exhibiting no requirement for monovalent cations (Hayashi et al. 1970). There is no information on such effects on enzymes from other moderate halophiles.

Kinetic parameters of halophilic enzymes: Is there evidence for consistent unusual values or phenomena?

Considering that an individual enzyme's salt requirements may be the result of a unique combination of physio-chemcial interactions (Lanyi 1974), and that kinetic parameters such as the Michaelis constant may evolve over long periods in response to cellular physiological conditions (Hochachka & Somero 1973, Crowley 1975), there seems to be no a priori reason to assume that the kinetics of halophilic enzymes should exhibit consistently unusual patterns. The halophiles appear to share the basic life processes with the rest of the microbial world and, although their structures may differ, their metabolism exhibits generally "normal" patterns (see Ch. 1). Thus, within the thermodynamic and chemical constraints of metabolic processes, their enzymes should exhibit general similarities to non-halophilic bacteria with accompanying natural statistical variation in kinetic parameters. On the other hand, the striking kinetic differences in halophilic enzymes, such as the high K_m values of V. costicola ATCase, might point toward real physiological differences associated with halophilism.

Data on the K_m values of ATCase from various sources Tab. V-10 show that both halophilic enzymes had the highest K_m values. The only other data where similar comparisons can be made involve NADP dependent isocitrate dehydrogenase (IDase), biosynthetic threonine (TDase) and alkaline phosphatase (APase) studies. The Halobacterium salinarium IDase at the optimum 1M salt concentrations had K_m values for isocitrate and NADP of 0.5-1.3 x 10^{-4}M and 1.2-1.3 x 10^{-4}M respectively (Aitken et al.

Table V-10

K_m comparisons, ATCase from various sources.

	M Km			
	ASP.	CP.	M NaCl	reference
H. cutirubrum	0.01	0.002	(3.5)	Norberg et al. 1973
V. costicola	0.2	0.01	(1.8)	this dissertation
S. fecaelis	0.02	0.0002	(0)	Prescott and Jones 1970
E. coli	0.02	0.0002	(0)	Bethell et al. 1968
S. cerevisiae	0.003	0.004	(0)	Kaplan et al. 1967
Cod ovary	0.002	0.0004	(0)	King and Baker 1975
Mouse spleen	0.008	0.002	(0)	Hoogenraad et al. 1971

Table V-11

Threonine K_m values of biosynthetic threonine deaminase from various organisms.

Organism	threonine K_m (mM)	salt concentration	reference
Vibrio costicola	17	0.2M NaCl (near optimum)	this dissertation
	60	2M NaCl	"
E. coli	4.8-6	0.5M buffer	Harding 1969
Salmonella typhimurium	1-4	0	Maeba & Sanwal 1966
Azotobacter chroococcum	56	0	Gupta 1971
Rhodospirillum rubrum	6.8	0	Feldberg & Datta 1971
Bacillus stearothermophilis	3.5	0 (55C)	Thomas & Kuramitsu 1971
	8.3	0.1M NaCl (55C)	"
Thermus X-1	14	0 (70C)	Higa & Ramaley 1973
Saccharomyces cerevisiae	10	0	Robichon-S. & Magee 1968
Schizosaccharomyces pombe	20	0	McDonald & Kaplan 1973

1970). The values for the V. costicola enzyme at the low salt optimum (<0.1M) were 6 x 10^{-5}M and 1 x 10^{-4}M respectively (Wydro 1974). All the above values were similar to those reported for other non-halophilic bacterial NADP specific enzymes (Wydro 1974). The V. costicola TDase had a threonine K_m value near the optimum low salt concentration only slightly higher than several other bacterial enzymes and well within the range of values presented in Tab. V-11. The K_m value at 2.0M NaCl reflects salt inhibition effects. Egami (1955) stated that the K_m value determined for the alkaline phosphatase of moderately halophilic Pseudomonas #101, 6.5 x 10^{-3}M, was of the same order of magnitude as the enzyme from animal sources. The K_m of the moderately halophilic marine V. alginolyticus enzyme was 0.2-0.4 x 10^{-3}M at pH 10.3 but about 200 x less at pH 7.5 (Hayashi et al. 1973). The K_m values of the halophilic enzymes seem to be similar to those of the non-halophile; only the halophilic ATCase K_m values are high. The high aspartate K_m values would be particularly interesting if high intracellular concentrations of acidic amino acids were important in halophilic osmotic regulation (see Ch. III discussion p. 109, and Measures 1975).

Characteristics and feedback regulation properties of TDase from various microorganisms compared with V. costicola TDase.

V. costicola TDase activity feedback inhibition was reduced by M NaCl or higher, yet NaCl or KCl were required for enzyme stability. As with the Vibrio enzyme, Salmonella TDase (Maeba & Sanwal 1966, Burns & Zarlengo 1968) homotropic

interactions induced by isoleucine were removed or reduced in presence of valine or salts. In contrast to the Escherichia coli and V. costicola enzymes, the TDases from Bacillus stearothermophilis (Thomas & Kuramitsu 1971), Salmonella typhimurium (Burns & Zarlengo 1968), several marine planktonic algae (Antia et al. 1972) and yeast (Holzer et al. 1964) were activated by monovalent cations.

The influence of salts, specifically or relating to ionic strength in stabilizing or protecting TDase from inactivation by dilution has been noted by many workers. An interesting example is the requirement of 1-2M NaCl or KCl to stabilize the hydrophobic Rhodospirillum rubrum enzyme (Feldberg & Datta 1971b). V. costicola TDase required either NaCl or KCl for stability; except for $MgCl_2$, other salts were not tested. E. coli TDase required either threonine or high concentrations of phosphate buffer to prevent inactivation of dilute enzyme preparations (Harding 1969). Experiments using stabilized E. coli TDase resulted in an alternate interpretation of the effect of valine; instead of relieving isoleucine inhibition it acted as an inhibitor in the same way as isoleucine (Harding et al. 1970). This could mean that there is no separate site on the enzyme for valine binding; valine may bind at the isoleucine site.

The V. costicola TDase had, at least at low salt concentrations, a definite hyperbolic to sigmoidal shift in the threonine saturation curve when isoleucine was added, accompanied by an increase in the threonine Hill coefficient from 1 to 2 or 3 (Tab. V-3). An increase in valine concentration decreased

the Hill coefficient. Cooperative effects were evident with threonine and presumably with isoleucine (n=2, Fig. V-13). The Hill coefficient for isoleucine was not affected by 2.0M NaCl. The possibility remains that threonine cooperative effects would be observed at high salt concentrations if higher feedback effector concentrations were tried. The V. costicola TDase results are consistent with the "K" model of allosteric enzymes (Monod et al. 1965). The kinetic behavior and many of the characteristics of the V. costicola enzyme indicate that if behaves like other well-known microbial TDase, except that it is more salt-tolerant.

Many meaningful comparisons between the V. costicola TDase and other TDases require a purified enzyme. It was not possible so far to purify the enzyme, but several recently developed techniques might be suited for use with halophilic enzymes which, for the most part, have proven difficult to purify. The instability of E. coli TDase has hampered its purification, but Grimminger et al. (1973) have reported that affinity chromatography has allowed purification. Yon (1974) has purified ATCase from wheat germ by chromatography under conditions where hydrophobic interactions could take place between the protein and the Sepharose column gel, a technique called "hydrophobic chromatography". Finally, hydrophobic chromatography in the presence of high salt concentrations has been suggested for use in purifying unstable enzymes requiring high ionic strength (Bigelis & Umbarger 1975). Ammonium sulfate and glycerol stabilized yeast α-isopropylmalate isomerase and allowed the protein to be differentially retained on a sepharose affinity column. The combination of affinity

Tab. V-12

Regulatory enzymes from halophilic bacteria

Enzyme	source	effectors	allosteric regulatory class	M NaCl or KCl required for: activity	regulation	ref.
biosynthetic threonine deaminase	V. costicola	isoleucine, valine	K*	<0.1	<0.1	this dissertation
NADP-isocitrate dehydrogenase	V. costicola	α-ketoglutanate glyoxylate, oxalacitate	competitive	<0.1	<0.1	Wydro 1974
pyruvate kinase	V. costicola	AMP	K?	<0.1	<0.1	E. deMedicis, commun.
aspartate transcarbamylase	H. cutirubrum	CTP	V	3-4	3-4	Liebl et al., 1969, Norberg et al., 1973.
catabolic threonine deaminase	H. cutirubrum	ADP	K	3-4	3-4	Lieberman & Lanyi 1972.
NADP-isocitrate dehydrogenase	H. cutirubrum	oxalacetate, glyoxylate	competitive	1	0.5-1	Hubbard & Miller 1970, Aitken et al., 1970.
NADP-malic dehydrogenase	H. cutirubrum	acetyl CoA	K	3	3	Vidal & Cazzulo 1972.
citrate synthase	H. cutirubrum	NADH, ATP	competitive	1-3	1-3	Cazzulo 1973, Higa & Cazzulo 1975.

* K or V classes of Monod et al., 1965. Competitive implies that only Michaelis-Menten kinetics were observed.

chromatography with hydrophobic or high salt interactions promises to allow purifications which were previously impossible.

Some enzymes from extremely halophilic bacteria can be inactivated by dialysis against dilute solutions, then reactivated by dialysis against high salt concentrations. This permitted purification of Halobacterium salt dependent malic and isocitrate dehydrogenases using conventional methods at low salt concentrations with inactive enzymes. In the final steps of the purification the purified enzymes were reactivated (Holmes & Halvorson 1965, Hubbard & Miller 1969). V. costicola TDase was irreversibly inactivated by dialysis against dilute solutions.

Feedback regulation in halophilic enzymes.

It is now clear that the regulation of enzymatic activity by allosteric effectors is a general phenomenon extending to the halophilic bacteria (Tab. V-12). The regulatory enzymes from V. costicola showed no requirement for salts, in marked contrast to the enzymes from Halobacterium. However, the V. costicola TDase had regulatory function even at 3M NaCl. Regulatory enzymes from halophilic bacteria may provide sensitive systems to help define requirements and limits for metabolic function in halophilic bacteria.

Feedback regulation of biosynthetic enzymes, metabolite pools and osmotic regulation.

Measures (1975) has suggested that free amino acid pools, mainly glutamate and proline, are involved in the maintenance

of osmotic equilibrium in non-halophilic bacteria. However, large pools of amino acids could affect repression and feedback mechanisms. Stebbing (1974) has pointed out that feedback controls of biosynthetic pathways would tend to change pool concentrations in the wrong direction. If the medium salinity were to increase, water would flow out of the cells, increasing the effective concentrations of the end products. Such increases would cause increased feedback inhibition and decreased synthesis of end products, whereas the required response would be to increase the pool size and attain a new osmotic equilibrium. The implications are that (1) to be effective osmotic regulators, amino acids such as glutamate or proline would have to be ineffective feedback inhibitors, and moreover, (2) should be synthesized by an enzyme sensitive to some aspect of osmotic regulation. Enzymes involved in glutamate metabolism such as glutamate dehydrogenase (Measures 1975) or aspartic-glutamic transaminase (Owen & Hochachka 1974) could be candidates for such regulation. The significance of amino acid pools in halophiles has yet to be shown.

Conclusions from experiments on V. costicola TDase synthesis and repression.

There are two threonine deaminase enzymes in E. coli. The isoleucine sensitive biosynthetic enzyme is synthesized when minimal salts-glucose media are utilized for growth (Umbarger & Brown 1957). Repression of TDase activity occurs in enterobacteria (Freundlich et al. 1962) and in bakers' yeast (Robichon-Szulmajster & Magee 1968, Bussey & Umbarger 1969) if all three branched-chain amino acids are present in excess

in the medium ("multivalent" repression). In V. costicola the biosynthetic enzyme was produced in minimal medium, could be completely repressed in complex amino acid media, but was not multivalently repressed by the branched-chain amino acids together. Pseudomonas aeruginosa (Marinus & Loutit 1969) and Schizosaccaromyces pombe (McDonald 1971) TDases also did not exhibit repression in presence of the three branched chain amino acids.

So far, any explanation of the results of the repression experiments must necessarily be speculative. Clearly, effective repression regulation does exist in V. costicola but the amino acid requirements for the effect imply that other amino acid biosynthetic pathways are involved. Possibly the branched-chain amino acid pathway enzymes could not be repressed unless the entire synthetic machinery for the aspartic acid "family", beginning with aspartokinase, was also repressed.

The addition of either isoleucine or valine to growth media caused two effects. Adding either amino acid alone caused growth inhibition (Fig. V-17), yet isoleucine alone caused an increase in TDase specific activity while valine alone caused a specific activity decrease (Tab. V-8). The valine effects were probably caused by the repression of TDase and consequent growth inhibition. When isoleucine was added with valine both the growth and repression effects were removed. The cause of isoleucine "derepression" remains unknown.

The V. costicola repression effects discussed above appear to be complicated by glucose catabolite repression. Removing glucose from the medium which had the group A,B,C and D amino

acid additions increased the synthesis of V. costicola TDase. Whitlow and Polglase (1974) have shown that the valine inhibition of growth of E. coli K-12 was overcome if cyclic AMP was included in the medium or if the carbon source was acetate instead of glucose. In V. costicola removing glucose from the medium should relieve the valine repression of TDase. The valine effect on growth should, therefore, disappear, a testable prediction.

Do moderately halophilic bacteria produce different enzymes as a response to environmental salt concentration changes?

The data on V. costicola TDase supports other existing evidence that halophiles do not radically change their metabolism and enzyme content in response to changes in medium salt concentrations. V. costicola glycerol dehydrogenase extracted from cells grown in either 4% or 15% NaCl media showed identical NaCl and KCl salt response patterns (Baxter & Gibbons 1954). In several studies of extracellular enzymes produced by moderately halophilic bacteria, only the amounts of enzymes produced, and not the properties of the enzymes, were affected by medium salt concentrations (Onishi 1972a, b, Kamekura & Onishi 1974 a, b). Preliminary studies on V. costicola ribosomes indicated that their sedimentation patterns were not greatly affected by growth medium salt content if the extraction buffer contained no more than 0.75M salts (Wydro et al. 1975). In contrast to the relatively small effects of salts, temperature changes did cause marked enzymatic changes in thermophilic Bacillus stearothermophilus, with accompanying metabolic shifts (Jung et al. 1974).

Chapter VI

Conclusions: Toward a better understanding of the moderately halophilic mode of existence.

Unlike the extremely halophilic bacteria whose relationship to the rest of the microbial world seems distant, the moderately halophilic bacteria are related to marine and non-halophilic bacteria. V. costicola for instance is closely related to several marine Vibrios (Ch. II, p. 30). Using the electrophoretic mobility of the bulk ribosomal proteins as a taxonomic trait, Falkenberg et al. (1976) and Wydro (unpublished data) have shown that the proteins from V. costicola ribosomes are only slightly more acidic than those from E. coli ribosomes. However, the Halobacterium ribosomal proteins were markedly more acidic than the others. Interesting taxonomic comparisons using other halophiles could be made using this technique: of special interest might be to compare the ribosomal proteins from Halobacterium volcanii, which has only a moderate 1.7-2.5M salt requirement (Mullakhanbhai & Larsen 1975), with extremely halophilic Halobacterium strains. Taxonomic comparisons with halophilic blue-green bacteria (e.g. Brock 1976) could also prove useful in better defining the relationships between halophiles and non-halophiles.

The physiological requirement of moderate halophiles for 0.5-2.5M NaCl for best growth is poorly understood. Presumably halophilic bacteria have evolved cellular structures and components which are stabilized and activated by the saline conditions of their milieu. The extremely halophilic bacteria offer a striking example; their cell envelopes, enzymes and ribosomes

are dependent upon high salt concentrations. However, the salt requirements of V. costicola structures and components are less definite.

Certain activities from V. costicola such as in vitro protein synthesis (R. Wydro, T. Hiramatsu & D. J. Kushner, unpublished) and several enzyme activities (Tab. V-9, p. 192) including the threonine deaminase activity, are inhibited by the salt concentrations extant in the cells (i.e. 0.7M K^+ plus enough Na^+ to bring the internal salt concentration to approximately the same level as the medium). It is possible that some of these activities were unstable when extracted, or that enough residual activity remained in vivo at high salt concentrations to meet physiological requirements.

It is also possible that most of the salts in the cell are not free, and separated from the cytoplasmic proteins and ribosomes. Isolated membranes (Masui & Wada 1973) and permeabilized V. costicola (Ch. III, p. 97) bound large amounts of ions. If the distribution were such that the bulk of salts were associated with the cell envelope, then membrane-associated enzymes should show salt dependencies. To some extent this is confirmed by the data in Tab. V-9, cytochrome oxidase from two bacteria being the best example. V. costicola salt-dependent ATCase should also be located in an area where salts are present. Future research must reconcile the apparent conflicts between salt-dependencies of some processes and salt-inhibitions of others in moderately halophilic bacteria.

Regulation of the activity and enzyme synthesis have been shown to operate in V. costicola. The biosynthetic threonine

deaminase was sensitive to feedback inhibition by isoleucine. It maintained feedback inhibition at high salt concentrations, in contrast to the E. coli TDase, where such salt concentrations abolished feedback inhibition. The TDase could also be repressed, but more than the three branched-chain amino acids in the medium were required (Tab. V-8, p. 188). In the extremely halophilic bacteria, feedback inhibition has been reported (e.g. Liebl et al. 1969) but there are no reports on repression phenomena. Whether repression operates in the extreme halophiles is another area worth considerable future effect, especially since unusual systems may exist.

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