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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Vibrio costicola, a moderately halophilic bacterium, has the ability to grow over a wide range of NaCl concentrations. Studies were undertaken to investigate the possibility that proteins functioning in such environments might have an unusual pattern of turnover.

The turnover rate (about 5 %/hr.) of pulse-labelled proteins, at all salt concentrations supporting growth, was substantially higher than rates reported for other organisms. The rate was especially elevated in medium with NaCl concentrations less than 1.0 M. Turnover rates of pulse-labelled proteins seems to be determined by the salt concentration in which the cells are subsequently incubated rather than which they are labelled. There was no correlation, either positive or negative, between the growth rate and the rate of protein breakdown.

Tetracycline or chloramphenicol addition, while both inhibiting protein synthesis, resulted in inhibition only by the former antibiotic, with chloramphenicol having no effects on protein breakdown.

Impairments of energy production by addition of various inhibitors was studied. Turnover was inhibited by cyanide but not azide, dinitrophenol or iodoacetate. Succinate also led to a severe reduction in rates of protein breakdown. The possible reasons for the varied effects of these agents is discussed.

A cell-free system was successful in obtaining a rate of breakdown 45 % of that found in whole intact cells. Tetracycline addition resulted in an inhibition whereas cyanide addition precipitated a stimulation of protein degradation in this system.

Guanosine tetraphosphate (ppGpp), a nucleotide previously implicated in protein turnover, stimulated this process in V. costicola. The possible action of tetracycline and cyanide on cellular levels of ppGpp is discussed.

Increases in protein breakdown occurred with a 10°C decline in incubation temperature. Lower NaCl concentrations concomitant with lower temperatures caused a substantial increase in the rate of turnover.

Polyamines, nitrogenous polycationic bases, were found in high cell-associated concentrations in this bacterium. Putrescine (a polyamine) addition brought about lowered breakdown rates especially in medium containing lower NaCl concentrations. The possible role of polyamines for helping to maintain "normal" protein conformation is discussed.

Separation of proteins with respect to either their individual molecular weights or net charge was carried out. Some evidence is presented for an elevated rate of turnover for proteins possessing a higher net negative charge.

Protein turnover, in V. costicola, is probably governed by an interplay between miscoding caused by too high, and

conformational changes, caused by too low, salt concentrations.

INTRODUCTION

INTRODUCTION

Extreme Environments

Organisms which grow in extreme environments have provided an interesting field of research in recent years.

Extreme environments are generally thought of as having one or more factors outside the range in which most, but not all, living organisms can carry out their life processes and grow. Such factors, to only name a few, as oxidation-reduction potential (Eh), hydrostatic pressure, temperature and salinity may cause, by themselves or in combination, an extreme environment. These and other contributing factors can cause an inhibition of the life processes and/or structural damage.

Thermoduric organisms, including eukaryotic algae, such as Cyanidium caldarum, or Bacillus stearothermophilus, a bacterium, can grow optimally at temperatures of 56-60°C and 70-75°C respectively (Brock, 1967). Mesophilic organisms such as the bacterium Escherichia coli or many freshwater or marine algae would be virtually excluded from growing at such high temperatures.

Salinity of the environment also severely limits the number of taxonomic groups able to grow. In the Dead Sea, with the concentration of salts at the saturation point, only eight species of bacteria and about two species of blue-green

algae can be isolated (Kushner, 1978).

The question that naturally arises from the durability to, and even requirement for, a more extreme environment by certain microorganisms, as compared to their counterparts existing at less extreme environments is - what attributes at the sub-cellular or molecular level allow for this unusual existence?

The pages that follow will hopefully add to help answer this perplexing question and in so doing, as occurs in science, open other perplexing questions.

Taxonomic Distribution of the Halophilic Organisms

Bacteria of various kinds are capable of growth at salt concentrations ranging from almost zero to that of saturated brine. It is this wide range that enables bacteria to be grouped according to their salt requirements. Usually these bacteria are classified, according to their ability to grow in increasing salt, as terrestrial or freshwater, marine, moderately halophilic, or extremely halophilic. This may be visualized by plotting a distribution curve of the Earth's total bacterial population against salt concentration. This plot would reveal an asymmetrical broad peak, or possibly two peaks, in the freshwater-seawater region (ca. 0 - 0.68 ionic strength) (Brown, 1964a). The extreme halophiles would be found at the upper end of this plot. Such exotic locations

as salt evaporation ponds, for obtaining crude solar salt, the Dead Sea, and so on would play host for these halophilic organisms. Moderate halophiles, while generally not found in nature growing under such extreme saline conditions, do require relatively high salt concentrations for growth. These environments include locations, to name only two, such as bacon curing brines or freshwater-seawater regions.

The halophilic bacteria, being among the most "salt-tolerant" of organisms, are capable of exploiting these environments which are inimical to most living forms. Extremely halophilic bacteria of the genera Halobacterium and Halococcus can grow in saturated NaCl (ca. 32 % or 5.2 M) with the lower limits of growth at 12 - 15 % NaCl (Kushner, 1978). Moderate halophiles, which were first described by Baxter and Gibbons (1954), have lower, but still, definite salt requirements of 0.5 - 4.0 M NaCl (ca. 3 - 20 %). Moderate halophiles, therefore, can grow in salt concentrations from those in which non-halophiles can grow, up to the saline environment of extreme halophiles.

A Moderately Halophilic Bacterium - Vibrio costicola

The moderate halophiles (Gr. salt-lover) include many different bacterial species, both Gram positive and Gram negative (Brown, 1976; Kushner, 1978).

Vibrio costicola, a moderately halophilic bacterium, is a Gram negative, aerobic, curved rod (0.5 X 2-4 u). A similar organism was originally isolated in pork ribs marinated in salt brine (Smith, 1938). It was then named Vibrio costicolus because of its comma shape and the latin words "costa" meaning rib and "cola" meaning dweller. Robinson (1950) isolated this organism from bacon curing brines, in Canada, some years after the previous culture was lost. The name was subsequently changed, in the 8th edition of Bergey's Manual of Determinative Bacteriology, to Vibrio costicola.

Recently this bacterium has received much attention due to the wide range of salt concentrations supporting its growth. As previously stated, this range overlaps the upper and lower NaCl concentration growth range of mesophilic and extremely halophilic microorganisms. respectively. Thus V. costicola has proved to be valuable in studies of the physiological and molecular aspects of halophilic bacterial life.

Some Characteristics of Moderately Halophilic Bacteria

V. costicola can grow in minimal medium with 0.5 M - 3.0 M NaCl (Forsyth and Kushner, 1970; Shindler, 1976) with best growth in the range 1.0 M - 2.5 M NaCl. This vibroid-shaped organism's requirement for such a saline environment, as found in other moderate halophiles (Brown, 1964a), is a stable genetic characteristic (Forsyth and Kushner, 1970).

Many halophilic proteins, including those of V. costicola (Wydro, 1977), have a different composition of amino acids than their non-halophilic counterparts (Lanyi, 1974). In such proteins is found a reduced number of hydrophobic residues in favour of increased apolar residues. Additionally, a general excess of acidic residues has been observed (Wydro, 1977).

Various studies have attempted to determine the "intracellular" salt concentrations of V. costicola (Christian and Waltho, 1961; Shindler et al., 1977). These types of studies were important, since a knowledge of how intracellular ionic content varies with that of the external milieu is essential in comparing how growth at different salt concentrations affects cellular components, including the conformational states of proteins.

Non-halophiles have, in general, a slightly elevated K^+ level internally, with their Na^+ level being quite low relative to the external milieu. In contrast to this, moderately halophilic bacterial accumulation of K^+ is very pronounced with substantial quantities of Na^+ being found also. The intracellular ion concentration of V. costicola, grown at its optimum salt concentration (ca. 1.0 M NaCl) is at least as high as that of the environment (Table 1). However, as V. costicola is grown at progressively higher salt concentrations approaching that at which extreme halophiles can grow (ca. 18 % NaCl), the intracellular ion concentration is lower than

that of the external milieu (Shindler et al., 1977).

The question that naturally evolves from such findings in moderate halopiles is - in what form is K^+ and Na^+ found intracellularly--free or sequestered (bound)? At present, no firm answers to this question can be drawn but there is growing evidence for sequestering of ions.

Masui and Wada (1973) found that the isolated cell envelope of a moderately halophilic Pseudomonad No. 101 contained large quantities of fixed Na^+ and K^+. This finding seemed to contradict the concept that the ions were free in the cytoplasm. Unfortunately, from this type of study, it is impossible to apply the amount of ions bound by broken cell preparations to those found in a living cell. Also the binding properties of envelopes would probably be changed upon cellular disruption and removal from the environment of the living cell (Kushner, 1978).

From recent studies, as shown in Table 1, the internal salt concentrations of V. costicola seems to be as high as those external to the cell at most salt concentrations of the medium. The actual localization of the bulk of the ions either in the cytoplasm or other cellular structures, such as the cell envelope, is unknown since most of the experimental methods involve disruptive techniques. In NaCl concentrations up to 1.6 M, the sum of internal Na^+ and K^+ ions is greater than that of the external ions. Only in the 2.0 M NaCl medium is

TABLE 1[a]

Effect of medium NaCl concentration on cell-associated cations in *Vibrio costicola*

Medium NaCl conc. (M)[b]	Cell-associated cations (molal concentration)				
	Na^+	K^+	K^+/Na^+	$Na^+ + K^+$	Mg^{++}
0.6	0.51	0.52	1.04	1.03	0.051
1.0	0.58	0.66	1.13	1.24	0.056
1.6	1.09	0.59	0.54	1.68	0.038
2.0	0.90	0.57	0.63	1.47	0.042

a From Shindler et al, 1977.

b Cells grown in SGS medium

the total $Na^+ + K^+$ concentration found to be slightly lower than the external NaCl concentration. The internal ions increase, although not proportionally, with increases in the external NaCl concentration. The K^+/Na^+ ratio decreases, as shown, with increasing concentrations of NaCl. Actively metabolizing V. costicola maintains, as shown in Table 1, a 75-100 fold concentration gradient of K^+ with respect to the medium. The NaCl concentration of the medium did not alter the cellular K^+ level, however, it did have a large effect on the cellular Na^+ concentration. At external concentrations above 1.0 M NaCl more than half of the cellular monovalent cations were Na^+.

Other studies (Masui and Wada, 1973) have found evidence contrary to that discussed for V. costicola. The moderately halophilic bacterium "Pseudomonad 101" growing in the range 1.0 - 3.0 M NaCl, has intracellular Na^+, K^+ and Cl^- concentrations which are almost independent of the NaCl concentrations of the medium. Additionally, in a Gram negative, unidentified, extremely halophilic bacterium (Matheson et al., 1976) the internal ionic levels were also found to be much lower than the external milieu. To counterbalance this ionic imbalance it has been shown that many extremely halophilic organisms (Brown, 1964b, 1976) can maintain lower internal ionic concentrations than the medium supporting growth by having higher cellular levels of "compatible solutes," such as

glycerol. "Compatible solutes" with their ability to allow for cellular adjustment to the different concentrations of salt is due to the lowering of intracellular water activity. In a very recent study Unemoto and Hayashi (1979) have shown that the amino acids proline and glutamic acid acted as compatible solutes in a slightly halophilic bacterium (growth in 0.2 - 1.5 M NaCl). Their concentrations were found to increase concomitant with an increased external NaCl concentration. These substances, or others, acting as compatible solutes in many extremely and slightly halophilic organisms, will probably be found in moderately halophilic bacteria although no measurements have been made as yet.

A better approach to elucidate this question of cellular ionic levels (Wydro et al., 1977) was to explore the effects of salt changes on physiological functions. Wydro et al. (1977) concentrated on in vitro protein synthesis as their physiological index of intracellular ionic conditions. This represented an excellent approach as it encompassed the stability of ribosomes, amino acid activating enzyme activity and incorporation of proper amino acids as directed by a known messenger at different salt concentrations. Their study suggested the sequestration of ions, since in vitro protein synthesis in V. costicola only occurred in 0.1 - 0.2 M NaCl containing medium. In addition, higher salt concentrations induced erroneous protein production in poly-U-directed

phenylalanine incorporation experiments (Wydro, 1977). It was suggested by Wydro et al. (1977), that since growing V. costicola must make proteins there must exist regions secluded from high salt concentrations where protein synthesis takes place. Thus, other cellular components, such as the cell envelope, may bind excess ions leaving other regions in relatively low ionic conditions. Additional evidence for the concept of solute protected areas was suggested with the mesophilic bacterium E. coli (Wydro et al., 1975). This organism, while being capable of growth in 0.5 M NaCl, suffers ribosomal aggregation at this concentration in cell-free extracts.

Several recent research documents concerned with V. costicola are available which offer extensive information on other interesting facets of this bacterium and other moderately halophilic bacteria. Shindler (1976) and Rossignol (1978) have studied, in detail, the effects of salt on the stability and activity of specific halophilic enzymes. The effect of salt on an in vitro protein synthesizing system prepared from V. costicola was studied by Wydro (1977). In addition, the latter study gives an analysis and comparison of the structural make-up of ribosomes from V. costicola and other bacteria.

Solutes, Stability and Halophilic Proteins

The success in which halophiles have adapted to life

under conditions of extreme salinity is seen by the fact that they have basically the same metabolic apparatus, functionally speaking, as non-halophilic bacteria. Pathways of protein synthesis, ATP generation and gene replication (Hochacka, 1973) are some examples of similar cellular biochemical reactions. This provides probable evidence that for such parallelling functional similarities to exist, enormous underlying biochemical differences must distinguish halophiles from non-halophiles.

In a bacterial cell, comprised of lipid, protein and other types of biological material, conformational changes, if they occur, can arise with changes in a_w [*1] or solute concentration. These molecular conformational changes are most likely to proceed, as will be discussed later, initially, in the protein component in response to changing ionic influences (Brown, 1964a).

Various studies with moderate halophiles have shown a difference in salt requirement for "cell-membrane associated" enzymes versus the so-called "soluble" enzymes (as indicated in brackets below) for maximum activity and stability. In

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*1 a_w (water activity) is defined by the equation:

$$a_w = \frac{P}{P_0} = \frac{n_2}{n_1 - n_2}$$

where p = vapour pressure of the solution and P_0 that of the solvent, pure water; n_1 and n_2 are the number of moles of solvent and solute respectively.

moderate halophiles it has been demonstrated that "cell-membrane associated" enzymes such as cytochrome oxidase (2 - 3 M NaCl), lactate dehydrogenase (1.5 - 2 M NaCl) (Baxter and Gibbons, 1956), and alkaline phosphatase (2 M NaCl) (Yamada et al., 1954; Brown, 1964a) require much higher salt concentrations for stability and activity than the "soluble" enzymes isocitrate dehydrogenase (0.2 - 0.7 M NaCl) (Wydro, 1974), pyruvate kinase (0.6 - 1.0 M NaCl) (De Médicis and Rossignol, 1977; Rossignol, 1978).

Theories on Halophilic Salt Requirements

(A) Baxter, Gibbons and Brown Hypothesis

A hypothesis for the requirement, by halophilic membranes and many proteins, for higher salt concentrations was put forward by these researchers individually (Baxter and Gibbons, 1954; Brown, 1976).

Simply stated, their hypothesis entails the neutralization, by Na^+ of the structural protein surface charge. In the absence of salt the repulsion of the charges leads to disruption of protein structures and concomitant loss of activity.

Generally, procaryotes and eucaryotes have ribosomes whose proteins are strongly basic. However, extreme halophiles, and to a lesser extent, moderate halophiles, show quite high amounts of acidic ribosomal amino acid residues

(Bayley, 1966; Wydro, 1977). These differences in amino acid composition, being most pronounced in extreme halophiles, relative to non-halophiles, has also been noted in other protein structures such as the cell wall (Reistad, 1970) and the cell membrane (Kushner et al., 1964). Brown (1964b) speculated that the requirement for higher salt concentrations was for the cancelling of the acidic, or negative, groups in order for the protein to assume its native conformation. He also (1964b) demonstrated that succinylation of ϵ-amino groups of lysyl residues, leading to an increase in acidity, of cell membranes of a non-halophilic bacterium conferred halophilic properties as seen by a need for elevated salt levels for stability.

Thus, salts seem to be required, in part, to act in a counter-ion shielding function to neutralize electrostatic repulsion.

(B) Lanyi and Stevenson Hypothesis

Lanyi and Stevenson (1970) proposed that the need for higher salt concentrations was not solely attributable to a counter-ion shielding function. It was estimated that the electrostatic effects could be neutralized at between 0.2 - 0.5 M NaCl, even in extreme halophiles. This is also noted with DNA (Inman and Jordan, 1960), bovine serum albumin (Edsall et al., 1950) and other highly charged macromolecules

which are also seen to behave as neutral species at only 0.1 - 0.2 M NaCl.

Secondly, counter-ion shielding cannot account for the differences observed in effectiveness of both anions and cations towards various halophilic enzymes (Hochstein and Dalton, 1968; Lanyi and Stevenson, 1970).

Thirdly, Wada (1960) found that counter-ion shielding did not stabilize a poly-L-glutamic acid helix. In fact, lowering the pK of the acid group in concentrated salt solutions leads to disruption of such a structure (Ciferre et al., 1968).

Therefore, Lanyi and Stevenson (1970) proposed, that while counter-ion shielding or electrostatic interaction was important at low salt concentrations, another ionic function occurred at higher salt concentrations. The particular need for higher levels of salts was suggested for maintenance of enzyme activity and stability by hydrophobic bond interaction. This kind of bond derives stability from the increase of entropy in the surrounding water upon formation. NaCl and other salts that decrease the solubility of organic compounds in water tend to increase the stability of hydrophobic bonds (Lehninger, 1975).

The changes in water structure around apolar residues plays a role in the formation of hydrophobic bonds. At equilibrium, when the random chain is fully folded, the increase in the entropy of the surrounding water molecules is

greater than the decrease in the entropy of the now correctly coiled polypeptide chain. Hydrophobic bonds, in addition, are seen to be more stable at higher temperatures due to the endothermicity of their formation.

NaCl and KCl with their "salting-out" or lyotropic nature tend to increase this form of bond (Lehninger, 1975; Rossignol, 1978). In addition, polycationic molecules such as polyamines, spermidine for example, and divalent cations, such as Mg^{++}, have been shown, by themselves, to maintain partial enzyme stability and activity in some halophilic proteins (Lanyi and Stevenson, 1970). It is thought that at low salt concentrations, and in the presence of these polyvalent cations, various halophilic proteins may be in partially folded conformations with increased stability being achieved through counter-ion shielding. However, the salt concentration would have to be increased further to achieve the proteins native conformation through hydrophobic bonding with a lowering of its solubility in the solvent (Tanford, 1973).

Many halophilic proteins, as discussed, tend to be more acidic in nature but also contain a reduced number of hydrophobic residues (Lanyi, 1974; Wydro, 1977). This being the case, higher salt concentrations would be required to reduce unfavourable steric conditions and counterbalance the reduced number of hydrophobic bonds. The saline environment of many halophilic proteins, in fact, would require that the organism

would have a lower percentage of hydrophobic bonds in favour of increased apolar residues and an excess of charged acidic (negative) residues. These proteins would require high salt concentrations to direct hydrophobic bonding concomitant with the neutralization of excess ions in solution by apolar and acidic residues respectively. Therefore, in an "ion deficit" situation, where there is a reduction in ionic strength of the external milieu of a halophilic organism, it would be expected that many salt-dependent halophilic proteins would cease to maintain their native conformations.

Protein Turnover

While our knowledge of protein synthesis is well developed, that of protein degradation and turnover is still quite rudimentary. Much time and effort have been devoted, in the past and present, on the regulation of protein biosynthesis whereas investigations on factors involved in removal of cellular proteins have been sporadic. Recently, however, studies on protein turnover have attracted renewed interest due to their implications in many aspects of cellular development and survival.

Protein turnover, as experimentally determined, has been defined (Neurath, 1964) as the time required to remove an amount of protein under investigation. The rate of disappearance of radioactivity, for example, a readioactive amino acid such as ^{14}C-leucine, from a prelabelled protein to which

a non-radioactive form of the same amino acid is subsequently added gives a rate of protein turnover. The protein, as it is being hydrolysed to smaller molecular weight peptide units and amino acid residues, releases the radioactive residues. The amount of radioactivity released (dpm) during a certain time period can then be counted. The calculated rate, it must be remembered, gives an average for proteins experiencing rapid breakdown and those with lower breakdown rates (Goldberg, 1972; Siekevitz, 1972). As will be discussed, the theories on selectivity and control of protein turnover are numerous which demonstrates the need for further studies to elucidate this process.

Theories on Control of Protein Turnover

Why do protein molecules get degraded? Unfortunately, only a universal negative, circumstantial, answer exists, that because all instances of degradation of specific proteins seem to follow first-order kinetics, then the proteins are degraded in a more or less random fashion. In other words, they are not degraded because they "age" and therefore become more susceptible to a proteolyic mechanism.

Protein turnover involves intracellular proteolytic enzymes, together with peptidases, catalyzing the hydrolysis of proteins to amino acids. However, at this time, the factors involved which make a protein more susceptible to proteolysis

are obscure. As a result, it is not possible to make any final statements as to which properties and conditions are decisive for the increased proteolytic susceptibility of cell proteins. Various theories have been proposed and will be dealt with individually.

(A) Deamidation Reactions

Strehler (1977) has theorized that the enhanced hydrolysis of peptide bonds can arise from the fact that two of the amino acids present in proteins are asparagine and glutamine which possess amide groups on the termini of their "R" groups. This is a potential source of structural instability since amides, like peptide bonds, are subject to hydrolysis.

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH_2 + H_2O \dashrightarrow R\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + NH_3$$

The amidinated amino acids lead to a protein with more acidic properties due to the removal of the amide ammmonia, leaving a free carboxyl group. It was speculated that a deamidation reaction process may play a role determining the turnover of a protein. Of course, the control of turnover by deamidation would depend upon the formation of more acid charged proteins being derived from more neutral ones resulting in selective targets for the endogenous cellular proteases. Thus a certain half-life could be conferred on a particular protein by the occurrence of one or more deamidation reactions.

For example, a single deamidation event could give rise to a first-order exponential disappearance of a protein. However, if two or more deamidations per molecule were prerequisite for degradation, the molecule could have an increased half-life before breakdown. Therefore, a direct relationship between breakdown and the number of deamidation events occurring with a protein may be decisive for determining its respective half-life.

Robinson et al. (1970) have also ventured to correlate the turnover times with the percentage of glutaminyl and asparaginyl residues in protein. It was found that larger percentages of these residues lead to shorter half-lives. A deamidation reaction of these residues was thought to lead to opening of the protein structure thus making it more susceptible to proteolysis.

(B) Hydrophobic Interactions

Tanford (1973) proposed a hypothetical mechanism for the enhanced proteolysis of a protein. Different degrees of exposure of superficial hydrophobic areas of substrate proteins could cause different affinities for proteolytic enzymes. It was thought that the primary reaction of intracellular protein catabolism, for many proteins, was the change from a more stable conformation (more buried apolar residues) to a less stable conformation (more superficial hydrophobic areas).

Most water soluble proteins have approximately 25 to 30 % hydrophobic amino acid side chains, 45 to 50 % are typically ionic or contain uncharged hydrophilic side chains. The remaining residues have relatively little preference for being in or out of the aqueous environment (Tanford, 1973). In the native conformation of such a protein a substantial fraction of the hydrophobic side chains are typically buried in the interior of the molecule. The free energy gained, by the surrounding water molecules, is a major factor in the stability of the native conformation relative to a more flexible conformation in which the hydrophobic side chains would be exposed to the solvent. In nature, the native conformation is, in fact, in dynamic equilibrium with other possible conformations with reversible, and occasionally irreversible, transitions taking place where the solvent medium is altered.

In one study (Yutani et al., 1977) the effect of a single amino acid substitution (missense mutation) on stability of conformation of a protein was studied. It was demonstrated that a single amino acid substitution could alter the susceptibility to proteolysis of a protein molecule without a gross change in its conformation. They suggested that the stability of a protein may be greatly increased by an increase in hydrophobicity by substitution of a few suitable amino acid residues. The dual concepts of hydrophobic interactions and miscoding may be related to a protein's stability.

(C) Thermodynamic Control

A positive correlation has been found between the *in vitro* thermal stability and *in vivo* turnover rates of proteins (McLendon and Rudony, 1978). This particular study of the thermodynamic model for turnover of proteins suggested that intracellular degradation is controlled by intramolecular conformational equilibrium.

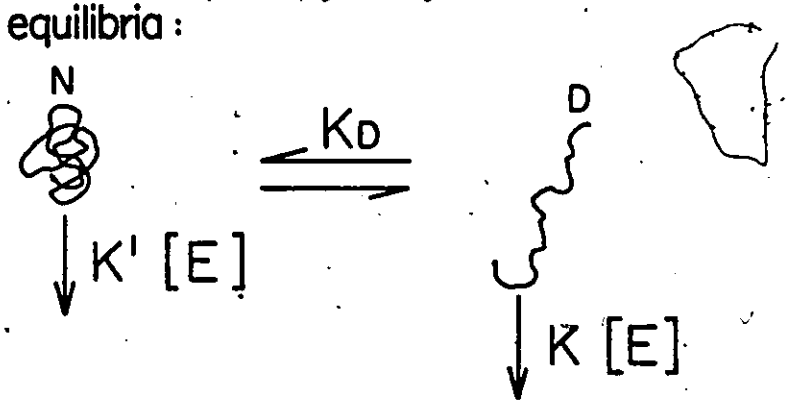
It must be remembered that the "native" protein conformation, as determined for example by X-ray crystallography, is in dynamic equilibrium with a number of other structures which may be partially or completely unfolded. These interconversions occur quite rapidly since the actual energy difference between the unfolded and folded states is quite low (5 - 15 Kcal/mole) for proteins (Tanford, 1973).

McLendon and Rudony have questioned whether the many molecular aspects of protein degradation depend simply upon the dynamic equilibrium among the different conformational states. If this is the case, conformer equilibrium between folded and unfolded states may be more important than the "native" conformation in determining a proteolytic event. Since an unfolded protein is a potentially very reactive substrate for proteolytic enzymes the equilibrium concentration of unfolded forms, determined by the unfolding equilibrium constant K_u (Figure 1), might govern the overall rate of degradation -d protein/dt.

Figure 1

Control of Proteolysis by Conformer Equilibria

Control of proteolysis by conformer equilibria :



$K >> K'$

peptide products

N native protein
D denatured protein

This proposed model of thermodynamic modulation of protein degradation is referred to as the "thermodynamic control" or also as the "thermodynamic hypothesis" of protein turnover.

(D) Influence of Molecular Weight

In the opinion of Kalish et al. (1979) and others (Schimke et al., 1970; Dice and Goldberg, 1975) one important property for the selective catabolism of short-lived proteins is their high molecular weight. This would probably imply more susceptible bonds, less stable native conformations, more different conformations and more chance of errors in amino acid sequences. It must be noted that several examples are known where small proteins appear to turn over rapidly (Kemshead and Hipkiss, 1976); but no exceptions have been reported where large proteins are stable (Dice and Goldberg, 1975; Goldberg and St. John, 1976).

(E) Influence of Protein Isoelectric Point

Dice and Goldberg (1975) found another feature of protein structure which correlated with an enhanced turnover rate.

Proteins with acidic isoelectric points were found to be degraded more rapidly than those having neutral or basic isoelectric points (IP). For example, basic bacterial ribo-

somal, basic IP, or neutral charged proteins were degraded more slowly than various acidic (negative) soluble proteins (Goldberg and Dice, 1974). The authors stressed, however, that protein size and charge seem to be independent factors influencing intracellular half-lives.

The manner in which protein charge influences protein proteolytic susceptibility is still uncertain. On one hand, the effects of charge may reflect the selectivity of the degradative enzymes. It could be visualized that the protease(s), in question, could be cationic molecules or could be specific for acidic residues within the substrate polypeptide. On the other hand, these findings may indicate that acidic polypeptides share certain conformational features that promote their hydrolysis.

One could also imagine that the native conformations of acidic polypeptides are inherently less stable. Therefore, these proteins could be more susceptible to spontaneous denaturation (Brown, 1964b), which may be the rate-limiting step in protein turnover. Finally, it could be envisioned that acidic proteins may preferentially group in regions of the cell where the degradative enzyme(s) are localized.

(F) Abnormality of Protein Structure

Pine (1972), using E. coli, found that intracellular degradation may be confined to a limited class of proteins dur-

ing growth, particularly those with abnormal structures. This has been subsequently tested by observing the intracellular proteolytic response, either to environmental changes (Kirschbaum et al., 1975; Vinopol, 1975), or artificial changes induced in the cellular protein. For example, the incorporation of amino acid analogues (Kemshead and Hipkiss, 1976; Wheatley et al., 1978), heating, cooling, or monitoring the effects of reduced fidelity of proteins synthesis (Pinkett and Brownstein, 1974). Increased proteolysis, as a consequence of errors induced in translation, has been documented in bacteria incubated with streptomycin (Hipkiss and Kogut, 1973; Pinkett and Brownstein, 1974). It was suggested from these studies that, for a number of cellular proteins, turnover has an important regulatory and survival role which is controlled by the substrates conformational state.

In nature, abnormal proteins can result from mutational, biosynthetic or post-translational events. Unfortunately, the frequency of these occurrences is very difficult, or even impossible to determine (Goldberg and St. John, 1976).

The strongest evidence that "normality" of protein conformation can influence intracellular half-lives comes from the study of proteins with abnormal tertiary structures. Both bacterial and animal cells have been found to degrade such polypeptides especially rapidly (Platt et al., 1970). Work with E. coli B-galactosidase nonsense fragments (Lin and

Ionizing radiation, for example, which can induce free radical formation can bring about cleavage of protein disulphide bonds. This may in turn cause abnormality of conformation (Pollard, 1969).

Zabin, 1972) or with puromycin-induced, prematurely released, polypeptides from ribosomes (Goldberg and Dice, 1974) has strengthened this view. Specificially, these abnormal protein components are quickly catabolized leaving the pre-existent "normal" proteins unscathed.

Kemshead and Hipkiss (1976), using E. coli, demonstrated the in vivo and in vitro degradation of certain abnormal proteins of shortened chain length. Puromycin-peptides from puromycin treated cells and peptides from cyanogen bromide-treated purified alkaline phosphatase were found to be degradable in vitro. It was noted that these shortened peptides could form oligomers. If the oligomer formed was greater than 30,000 MW its in vitro degradation was significantly inhibited. Oligomers of less than 30,000 MW, however, were found to be rapidly degraded in the cell-free extracts. It was suggested that E. coli cell-free extracts may contain a protease-peptidase system active only against peptide complexes less than 30,000 MW. It was also suggested that the degradation of abnormal proteins or oligomers of molecular weight greater than this may require one step additional for degradation. This could be an aggregation of abnormal polypeptides. This phenomenon has been visualized in electron micrograph studies (Schachtel, 1968) in E. coli cells with induced protein abnormalities. The aggregation process may possibility elicit a recognition from the responsible protease(s).

Wheatley et al. (1978) suggested that a common degrad-

ation system is probably present in all cells, which operates in an indiscriminate manner. When proteins are released from the ribosomes they are susceptible, be they normal or abnormal. It then simply becomes a question of determining the relative merits of abnormal and normal proteins as substrates for proteolysis. The stabilization of polypeptides comes when they enter into associations by folding on themselves, linkage with other subunits, co-factors or substrates, ionic interactions, and/or when they enter cellular structure. As a result, the inability of some ribosomal products to find protection by any of these latter associations, due to altered conformation, leaves a higher percentage of them at risk to proteolysis for longer.

(G) Role of Guanosine Tetraphosphate (ppGpp)

The levels of the nucleotide guanosine 5'-diphosphate-3'-diphosphate (ppGpp) formerly known as Magic Spot II, correlates inversely with the rate of stable RNA synthesis and directly with rates of protein turnover (Edlin and Donini, 1971; Cashel, 1975; Goldberg and St. John, 1976; Martegani and Alberghina, 1979) in various microorganisms studied. Amino acid starvation leads to the restriction of protein synthesis concomitant with a ribosomal idling reaction (Cashel, 1975). Under this circumstance the cellular levels of ppGpp rise quickly (Block and Haseltine, 1974) many-fold. The levels

of this nucleotide are also seen to increase upon carbon/energy source depletion (Pao and Gallant, 1978) and ionic changes (Block and Haseltine, 1974).

It has been suggested that this nucleotide may function as an allosteric modifier of proteins or may simply be hydrolyzed. The eventual energy gained from the release of this nucleotides phosphate groups may enhance conditions for proteolysis (Taguchi, 1978).

The fundamental problem in evaluating the importance played by each control mechanism is to determine which ones have truly important influences upon the turnover rates and whether the observed correlations are in fact causal relationships.

Energy Requirement for Protein Turnover

In bacteria, such as E. coli, intracellular protein degradation can be inhibited by agents which disrupt the cell's energy generating mechanisms (Mandelstam, 1960; Kowit and Goldberg, 1977; Olden and Goldberg, 1978). Such findings suggest an energy-dependent step in protein catabolism. However, thermodynamically, the hydrolysis of peptide bonds should be an energy-releasing process (Strehler, 1977). In addition, no known proteolytic enzyme requires metabolic energy (Olden and Goldberg, 1978).

The effects of various metabolic inhibitors on protein

turnover, has generally been attributed to a reduction in the steady-state level of ATP (Schechter et al., 1973). However, the ATP levels in E. coli have been reduced to very low levels (up to 75-85 % reduction) without affecting proteolysis (Olden and Goldberg, 1978). This may imply that phosphate bond energy is not the actual source of energy for this process. The actual energy source may be respiration or the so-called "energy-rich membrane state" (ERMS). Such membrane-associated functions as proline or lactose transport (Harold, 1972) are driven by this energy source. This involvement of the energized membrane in protein turnover may also account for the repeated failures of many investigators to successfully demonstrate protein breakdown in cell-free preparations (Powell et al., 1973; Schimke and Katunuma, 1975).

Experiments to date do not distinguish between a requirement for ATP itself or for other high energy compounds, whose levels may depend on the ATP concentration. Previous experiments, while demonstrating a requirement for high energy compounds, such as phosphates, still do not define a direct role for such compounds in the hydrolysis of peptide bonds.

Alternatively, this metabolic energy may be required to activate the responsible proteases(s) or to actually alter the conformation of the protein substrates, thereby rendering them more susceptible to proteolysis. This model remains

to be proven since degradation of abnormal proteins also has an energy component involved (Schimke, 1973). Schechter et al. (1973) suggested the need for ATP to drive protein synthesis for proteolytic enzyme production or other related protein fractions. However, others (Mandelstam, 1960; Goldberg, 1972; Goldberg and St. John, 1976) have shown that effective degradation of abnormal proteins can occur in the absence of protein synthesis.

Compartmentalization of Bacterial Protein Turnover?

The role of different subcellular fractions in the process of protein turnover has yet to be fully elucidated. A potentially important mechanism for protease action could involve the compartmentalization of these enzymes in order to prevent continual interaction with all cellular proteins. This concept is somewhat analogous to lysozyme vesicle function.

Yeast have been shown to have differential subcellular localization of proteolytic activity (Matern et al., 1974). The phenomenon appears applicable to some bacterial cells also but often with conflicting localizations (Goldberg and St. John, 1976).

In an early study (Pine, 1965) it was demonstrated that in E. coli approximately 25 % greater turnover of proteins occurred in the membrane fraction than any other. A more recent study (Miller, 1975) has demonstrated that the pro-

teases responsible for degradation appear to be cyoplasmic. Contrary to this (Goldberg et al., 1975; Chaloupka et al., 1977; Vachova-Philoppova and Chaloupka, 1978) it has been shown the greatest turnover of proteins, or at least their large fragments, appears to proceed in the periplasm.

The question of localization, as seen, has proven to be evasive, as most of the techniques and approaches tend to be rather disruptive and inexact, leading often to conflicting results.

Cellular Need for Turnover of Proteins

The many possible control mechanisms and other parameters of protein turnover have been explored to some extent. The resulting information gained has revealed intriguing insight into this process. Consequently, the question arises - why have microorganisms developed such seemingly elaborate, and in some cases, precisely regulated pathways of a protein's turnover?

One of the primary reasons which can readily be given suggests that proteases selectively degrade defective proteins, however biologically defined, that would otherwise interfere with normal cell function (Adams et al., 1972; Capeceli et al., 1974; Goldberg and St. John, 1976; Simon et al., 1978; Martegani and Alberghina, 1979). The elimination of

these abnormal and potentially harmful polypeptides resulting from mutations, biosynthetic errors and/or spontaneous or induced denaturation must have important consequences.

Secondly, the process of turnover serves an important physiological response under periods of poor nutrient availability. The overall release of amino acids from cellular proteins has been documented (Goldberg and St. John, 1976) to be closely regulated during "hard times."

Thirdly, the rate limiting enzymes regulating the flux of substrates through key metabolic pathways seem to be turned over much quicker than other cellular enzymes (Dice and Goldberg, 1975). This suggests an important role for this process in regulating the cell's biochemical machinery.

The elucidation of the exact role played by protein turnover and its regulating factors is at a rudimentary stage. The missing pieces to enhance our understanding of this process will only be derived from unique approaches and model systems.

Environmental Effects on Protein Turnover

Bacterial cultures, in general, in exponential growth have low rates of protein turnover indicating that the proteins, on average, made during this period are stable, although some specific bacterial proteins, such as L-serine deaminase, can show a rapid degradative rate during exponential growth (Beeraj *et al.*, 1978). For *E. coli* rates of

0.5 to 2.0 % of cellular protein turnover per hour have been observed (Nath and Koch, 1970; Pine, 1972; Goldberg and St. John, 1976).

Many of these studies have focused on mesophilic types of bacteria, such as E. coli, and to a much lesser extent on organisms growing in more "extreme" environments. Several past studies of organisms existing in extreme types of environment have concentrated on the thermophilic bacterium Bacillus stearothermophilus (Allen, 1953; Bubela and Holdsworth, 1966). B. stearothermophilis grows best in the temperature range between 55 - 65°C. This ability to grow at higher temperatures has had various explanations. Two of the main ones are: (1) that the proteins are probably heat stable (Epstein and Grossowicz, 1969; Pfueller and Elliot, 1969; Amelunxen and Murdoch, 1978). In fact certain exoproteins of this thermophile are heat stable such as the α -amylase and the purified flagellar proteins. The alternative suggestion (2) by Bubella and Holdsworth (1966) proposes that the survival of the thermophile at higher temperatures might be its ability to replace the heat damaged material at such a rate that overall metabolism is not affected. It was this latter hypothesis that was tested by them. At 40°C B. stearothermophilus had a much greater protein turnover rate (5 - 7 % per hour) than E. coli grown at the same temperature. This rate was found to increase as the temperature increased. With these results,

the comparative values for thermophiles and mesophiles, with respect to their turnover rates, may provide some evidence that the ability to grow under more extreme conditions may be associated with a higher intracellular proteolytic rate and rebuilding capacity.

It was also suggested by the latter researchers that the proteolytic enzyme(s) involved could show increased activity concomitant with temperature increases in thermophiles.

When halophilic membranes are subjected to an "ion deficit", the membrane proteins may be assumed to suffer conformational changes (Brown, 1976) before or during their dissolution but at present only limited evidence attests to this idea. It has been observed (Brown, 1961) that the isolated cell envelopes of a marine Pseudomonad is subject to increased proteolysis under conditions of an "ion deficit". This enhanced degradation is thought to be related to conformational changes of the membrane proteins. Brown (1960), in an earlier study, found a definite inhibition of a cell wall lytic enzyme by the polyamine, spermine, and the divalent cations Mg^{++} and Ca^{++}. This provided some evidence of a predominately electrostatic effect and led to the suggestion that conformational changes were initiated by exposure of negative charges of the residues comprising the protein in marine bacteria; carboxyl groups especially. Although this kind of evidence

is not conclusive, it seems that the potential for structural change could be substantial. In either marine organisms or halophiles, the membranes can be exposed to a very large "ion deficit," large enough to allow for significant changes in hydrophobic bond strengths (Tanford, 1973). This increase in abnormality of structure would surely set the stage for enhanced protein degradation.

Kamekura and Onishi (1973) isolated a moderately halophilic Bacillus strain which was capable growth in a defined medium containing 0.5 - 4.0 M NaCl, with optimum growth at 1.0 - 2.0 M NaCl. It was found that a 1.0 M NaCl-containing medium was optimal for production of one particular extracellular protease they studied. The activity of this enzyme produced, after partial purification, was assayed in a reaction mixture at different NaCl concentrations. Maximal activity was noted at 0.5 M NaCl with almost no activity being observed at 3 M NaCl. This type of control on activity of intracellular proteases may be of importance, although no reports of this are known.

The combined studies, relating changes in solute concentration and proteolytic activity, may demonstrate an interplay between protein conformational changes paralleled by an increase in activity of the responsible protease(s).

Work with organisms capable of growth under unusual

ranges and conditions of environment, as seen, has provided some informative clues as to the probable causative factors associated with enhancement of protein turnover and survival in bacteria. New approaches and continued research will hopefully solve some of the elusive questions that remain with respect to protein turnover.

GOALS OF THIS RESEARCH

The laboratory of Dr. D. J. Kushner has, for years, been concerned with the study of halophilic organisms. The unusual attributes of the growth and survival of these organisms has allowed the opening of many lines of research; protein turnover being a previously unexplored area.

We have been especially interested in the stability of proteins of halophilic bacteria, since such proteins may come in contact with a wide range of salt concentrations. In addition, the proteins of V. costicola, in general, are more acidic and less hydrophobic than those of the mesophile E. coli (Wydro, 1977). From previous studies, including those of this laboratory (Kushner, 1978), it appears that most of the enzymes of extreme halophiles and some of those of moderate halophiles are adapted to function in the presence of high salt concentrations.

The moderately halophilic bacterium V. costicola was chosen, as much previous work on various facets of its physiology and molecular biology have been done. This organism has to survive in environments which probably alter the conformation of some cellular proteins. This latter possibility along with information obtained from studies of other organisms existing in less mesophilic conditions, such as thermophilic

organisms, has provided the impetus for this study.

The studies presented in this thesis aimed first at ascertaining any differences in the turnover of the proteins of this moderately halophilic bacterium relative to non-halophilic bacteria, such as E. coli.

Some experimental approaches were designed to elucidate the relevance of environmental conditions on the process of protein turnover in V. costicola cells under different conditions of exponential growth.

Polyamines, well known for their structure-stabilizing effects on many cellular components, including proteins, were thought to be of possible relevance to protein turnover. Consequently, a study of polyamines with respect to their effects on protein breakdown was done in order to reveal any relationship between these molecules and turnover in V. costicola.

An initial attempt to partially characterize the proteins of this bacterium was done in order to look for any obvious trends relating these characteristics and turnover rates as reported in various published literature for other organisms.

It is hoped, finally, that the present study will offer information helpful to elucidate the relevance of the process of turnover on the existence of organisms under conditions of unusual environment.

MATERIALS AND METHODS

MATERIALS AND METHODS

Culture Maintenance and Growth Media

Vibrio costicola NRC 37001 was maintained on agar slants, at 4°C, with subculturing onto fresh agar slants at 8-12 week intervals or less. The medium in the agar slants consisted of 0.5 % Proteose Peptone (Difco), 0.5 % Tryptone (Difco), 1.5 % Bacto-agar (Difco), and 1.0 M NaCl. Before autoclaving at 20 lbs/ft^2 , the pH was adjusted to 7.5 (Forsyth and Kushner, 1970).

The mineral salts-glucose-sodium phosphate (SGS) medium (Forsyth and Kushner, 1970) was prepared as follows: (amounts expressed in g/L) Na_2HPO_4, 10.86; KH_2PO_4, 1.12; $MgSO_4.7H_2O$, 0.10; $(NH_4)SO_4$, 2.0; NH_4Cl, 2.0; NaCl, variable. The pH was adjusted to 8.4, with 10 N NaOH prior to sterilization for 20 minutes at 20 lbs/ft^2 (final pH 7.3). Glucose was added just before use (final concentration 1 % v/v).

Escherichia coli MRE 300 was maintained and transferred as with *V. costicola* but the NaCl was omitted in the agar slant preparation.

Ionic Content of SGS Minimal Medium (1.0 M NaCl)

Ion	Molarity
Na^+	1.14
K^+	0.008
Mg^{++}	0.00041

Ion	Molarity
NH_4^+	0.068
Cl^-	1.04
PO_4^{-3}	0.08
SO_4^{-2}	0.016

Culturing

A small amount of SGS - glucose medium (NaCl as indicated) was put into an agar slant tube of V. costicola or E. coli. The colonies were scraped with an inoculating loop into solution. Subsequently the bacteria-containing solution was poured into 50 mls of the same NaCl-containing medium. Growth, at 30°C was allowed over a two day period. A quantity (0.5 ml) of the latter culture was subcultured into 50 ml of a fresh glucose minimal medium (SGS). After about 24 hours growth a volume of this culture was utilized as inoculum for experiments.

The preculture was checked for purity using a Wild M-20 phase-contrast microscope.

Cells, for experimental study, were grown at 30°C on a rotary shaker water bath in Erlenmeyer flasks (usually 500 ml) containing 1/5 volume of growth medium.

Bacteria growth was followed by changes in turbidity as measured at 600 nm on a Beckman DB spectrophotometer. Studies were generally carried out with cells in exponential growth

(OD_{600} 0.3 - 0.8) unless otherwise indicated.

Radioisotopic Labelling of Proteins

Cellular proteins were labelled with a radioactive precursor. In pulse-labelled cells L-4-5-^{14}C-leucine (Amersham Corp., final concentration 0.925 KBq[*1]/ml; specific activity 13 GBq/Mole) was added to exponentially growing cells for a period of 5 minutes. Studies involving long-labelled cells had an incubation with the isotope for 150 minutes (unless otherwise specified). After the labelling period, in both cases unless otherwise indicated, the cultures were centrifuged very briefly at 10,000 rpm (12,000 X g) with the centrifuge being brought to this speed and immediately shut off. The resultant supernatant was discarded. Washing of cells was carrried out with a small amount (usually 25 mls) of ^{12}C-leucine (200 ug/ml) containing minimal medium with appropriate NaCl concentration. Centrifugation and washing of the cells was repeated. Again the resultant supernatant was discarded. Resuspension of pellets, after vortexing, in a small volume of medium (+ 200 ug/ml ^{12}C-leucine), with alterations where indicated, was done to allow continuation of growth at 30°C with the same cellular concentration, OD_{600} as measured at 600 nm, of the original culture.

[*1] 1 microCurie = 37 KBq (SI units)
1 Gb = 27.03 mCi

Determination of Protein Turnover

This was determined by the conversion of acid-insoluble radioactive material into acid-soluble radioactive material (Goldberg and Dice, 1974; Chaloupka et al., 1977). At intervals, usually 0, 5, 15, 30, 60, 90, 120 and 150 minutes after "chasing" with the ^{12}C-leucine-containing medium, one ml samples were taken and mixed with one ml of 10 % TCA (Trichloroacetic acid) to precipitate proteins. After 18 hours at room temperature the precipitate was removed by filtration through a Millipore filter (porosity = 0.22 u). One ml of this filtrate was added along with 3.0 ml of Scintiverse scintillation cocktail (Fisher Scientific Co.) into a Mini-vial. "Total" radioactivity was determined by putting 0.5 ml of the culture and 0.5 ml distilled water directly into a Mini-vial along with 3.0 ml scintillation cocktail. Samples were counted in a Beckman liquid scintillation spectrometer (LS-233) with appropriate window settings for maximum ^{14}C- or ^{3}H-isotope counting. Resultant counts were corrected for quench effects by the "channels ratio method" (Wang and Willis, 1975). This allowed for correction of differences in efficiency of counting with different component changes in the medium.

The percentage protein turnover at time "t", after chasing, was determined according to the formula:

$$\frac{\text{TCA soluble dpm at time "t" - TCA soluble dpm at 0 time}}{\text{"Total" dpm in 0.5 ml culture - TCA soluble dpm at 0 time}}$$

The results, in this study, are expressed in terms of percent of total label rendered soluble in TCA, which indicates the percentage of protein degraded to forms of lower molecular weight.

Determination of Cell-free Protein Breakdown in V. costicola

Growing cells of V. costicola (500 ml of 1.0 M NaCl medium) were either long-labelled (150 min.) or pulse-labelled (5 min.) with ^{14}C-leucine. They were harvested and washed as usual then broken by sonication (as described for DEAE Sephadex Chromatography) and subsequently suspended in 4.0 mls of "cell-associated" medium. The medium, in addition, contained either tetracycline (200 ug/ml), cyanide (10 mM) or no inhibitor. The lysed cells were centrifuged briefly at 12,000 X g to remove whole cells. The remaining supernatant was centrifuged at 30,000 X g then incubated, after careful pipette removal from the resulting pellet, in small test tubes at 30°C.

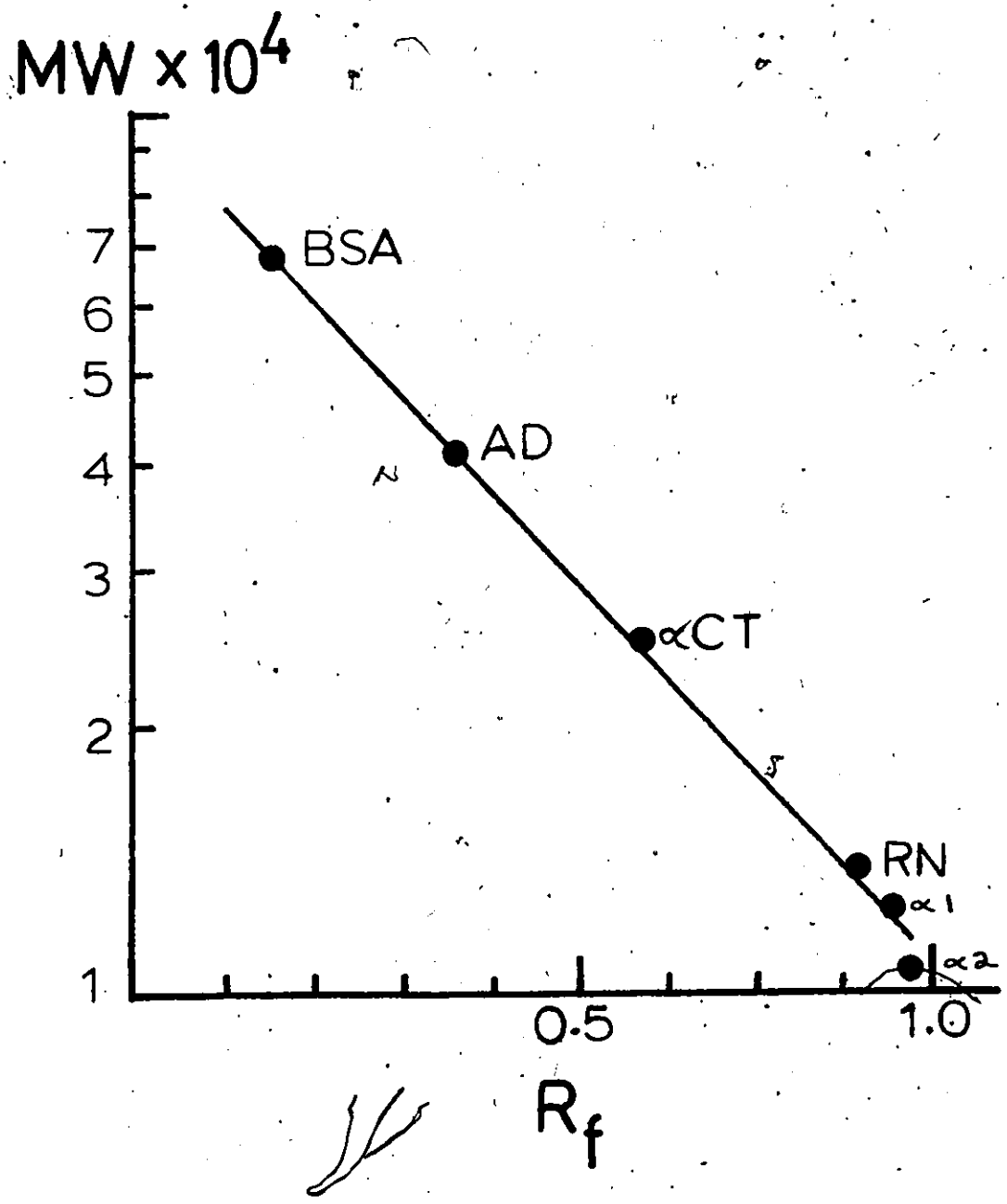
Samples (0.1 ml) were taken at 30, 60, 90, 120 and 150 minutes with 0.9 ml of distilled water being added. Standard filtration with dilution corrections were done to determine the rate of protein breakdown. 3.0 ml of Scintiverse scintillation cocktail was added directly to 1.0 ml of the cell-free suspension for determination of "Total" radioactivity.

A portion (100 ml) of the same culture was assayed for the rate of whole cell protein breakdown as previously described.

Figure 2

SDS polyacrylamide gel electrophoresis - protein molecular weight standard curve

BSA	Bovine serum albumin	(68,000 MW)
AD	Alcohol dehydrogenase	(41,000 MW)
∝ CT	chymotrypsin	(25,000 MW)
∝2, ∝1	chymotrypsin with 1 % mercaptoethanol	(2 chains of 11,000 and 13,000 MW)
RN	Ribonuclease	(13,700 MW)


MW x 10^4
7
6
5
4
3
2
1
BSA
AD
∝CT
RN
∝1
∝2
0.5
1.0
Rf

Sodium Dodecyl Sulphate Acrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (1969).

Supernatants (30,000 X and 100,000 X g) containing double-labelled (150 min. L-U-^{3}H-leucine then 5 min. ^{14}C-leucine incubation) proteins were prepared by lysis from 50 ml aliquots isolated from a 1.0 M NaCl culture at 0 and 120 minutes during the "chase" period. The lysed (as described for DEAE Sephadex chromatography) and fractionated supernatants were put into 0.01 M sodium phosphate buffer, pH 7.0, with 1 % SDS for 2 hours at 37^{o}C (1 % mercaptoethanol added where indicated). The SDS dissolves the proteins and gives them a uniform negative charge such that they migrate according to their respective molecular weight.

Approximately 50 - 100 ug of the cellular protein samples were applied per tube. Electrophoresis was performed for 5 hours at a constant current of 8 mA per gel (at 30 V) with the positive electrode in the lower chamber. Bromphenol blue (1 %) was added with the proteins. The bromphenol blue is a marker which migrates slightly faster than proteins of 10,000 MW.

After electrophoresis the tubes were put in 50 % methanol. Next day they were put into a Coomassie briliant blue solution (1.25 g of CBB in 454 ml of 50 % methanol and 46 ml of glacial acetic acid) for 2 hours. They were then subsequently washed with 7.5 % acetic acid.

The marker proteins used were bovine serum albumin (MW 68,00), alcohol dehydrogenase (MW 41,000), α -chymotrypsin

(MW 25,000, 2 chains of MW 11,000 and 13,000 in mercaptoethanol) and ribonuclease (MW 13,700) (Figure 2).

Slicing and counting the proteins in the gels was done according to the methods of Palmiter et al., (1971). Gels containing different bands of protein were partially frozen on dry ice. They were then cut into discs 5 mm thick. The proteins in the gels were dissolved by incubation with 40 ul of distilled water and 0.5 ml of NCS (solubilizer) in tightly capped scintillation vials at 37°C for 12-18 hours. Scintillation cocktail was added to 4.0 ml (total volume) to allow for counting the ratio of ^{3}H to ^{14}C.

Diethylaminoethyl (DEAE) Sephadex Chromatography

A 500 ml (1.0 M NaCl SGS-grown) mid-log culture of V. costicola was initially long-labelled for 180 minutes with ^{3}H-leucine then pulse-labelled with ^{14}C-leucine for a further 5 minutes. Standard washing of the cells was carried out as described before with half of the culture. The cells were then resuspended in 5 ml of "cell-associated ion" medium (Shindler et al., 1977) which contained 0.6 M KCl, 0.6 M NaCl, 10.0 mM Tris-HCl (pH 7.6), 40.0 mM $MgCl_2$. This suspension was then lysed, in an ice bath, with a Branson Sonifier. Alternating periods of sound, using a micro-tip, at a setting of 7.5 for 15 seconds with rest periods of 60 seconds between was found to effectively disrupt the bacterial cells. This sequence of sonification was repeated 5 times. The resulting

viscous suspension, after centrifugation at 30,000 X·g (30'), was centrifuged at 100,000 times gravity for 120 minutes. This sequence of steps for lysis and fractionation was repeated for the remaining growing culture after 120 minutes further incubation.

A-50 DEAE Sephadex was swelled for 5 hours in 0.1 M Tris-HCl (pH 8.3) in a boiling water bath. The swollen ion exchanger (capacity of 3.5 meq/g resin) was mixed with an excess of the starting buffer Tris-HCl and carefully poured into the column (60 cm X 0.9 cm) using a column eluant reservoir. The gel was stabilized for 5 - 10 minutes. After this the column was filled with starting buffer and connected to a buffer reservoir. An operating pressure of about 1 cm H_2O/cm height of gel bed was set by adjusting the column outlet. Two bed volumes of the starting buffer were run through the ion exchanger bed to allow for attainment of bed equilibrium and stabilization. All operations were carried out at 4°C.

One ml of the previously prepared lysed cell suspension with one ml of 10 % glucose was layered onto the column using a 5 ml syringe attached in place of the eluant reservoir.

The sample was initially eluted with a 0.1 M Tris-HCl buffer at pH 8.3. A step-wise gradient elution of progressively lower pH levels was done with volumes of 200 ml of buffer. As a change of pH towards the isoelectric point of protein is achieved it is rendered neutral and therefore

cause it to be desorbed and eluted from the ion exchanger.

Each fraction of 4 ml each was analyzed for the ratio of ^{3}H to ^{14}C and the respective pH.

Molecular Weight Exclusion Chromatography

Sephadex G-100 (Medium grade; MW range 4,000 - 150,000) was swelled in a boiling water bath for 5 hours, and equilibriated in 0.1 M Tris-HCl (pH 7.5). Two bed volumes of the Tris buffer was initially run through the column (100 cm X 1.9 cm). Cell lysate preparation and addition to the column were the same as that described for DEAE Sephadex chromatography. Elution of the proteins was with Tris-HCl buffer with 4 ml fractions being collected on an LKB fraction collector at 4°C. Each fraction was analysed at 280 nm for the determination of approximate protein level.

Molecular weight versus fraction collected was standardized with 2 mg each of the marker proteins bovine serum albumin (MW 68,000), alcohol dehydrogenase (MW 39, 805), and lysozyme (MW 14,316) mixed in 4 ml of the Tris-HCl eluting buffer (Figure 3).

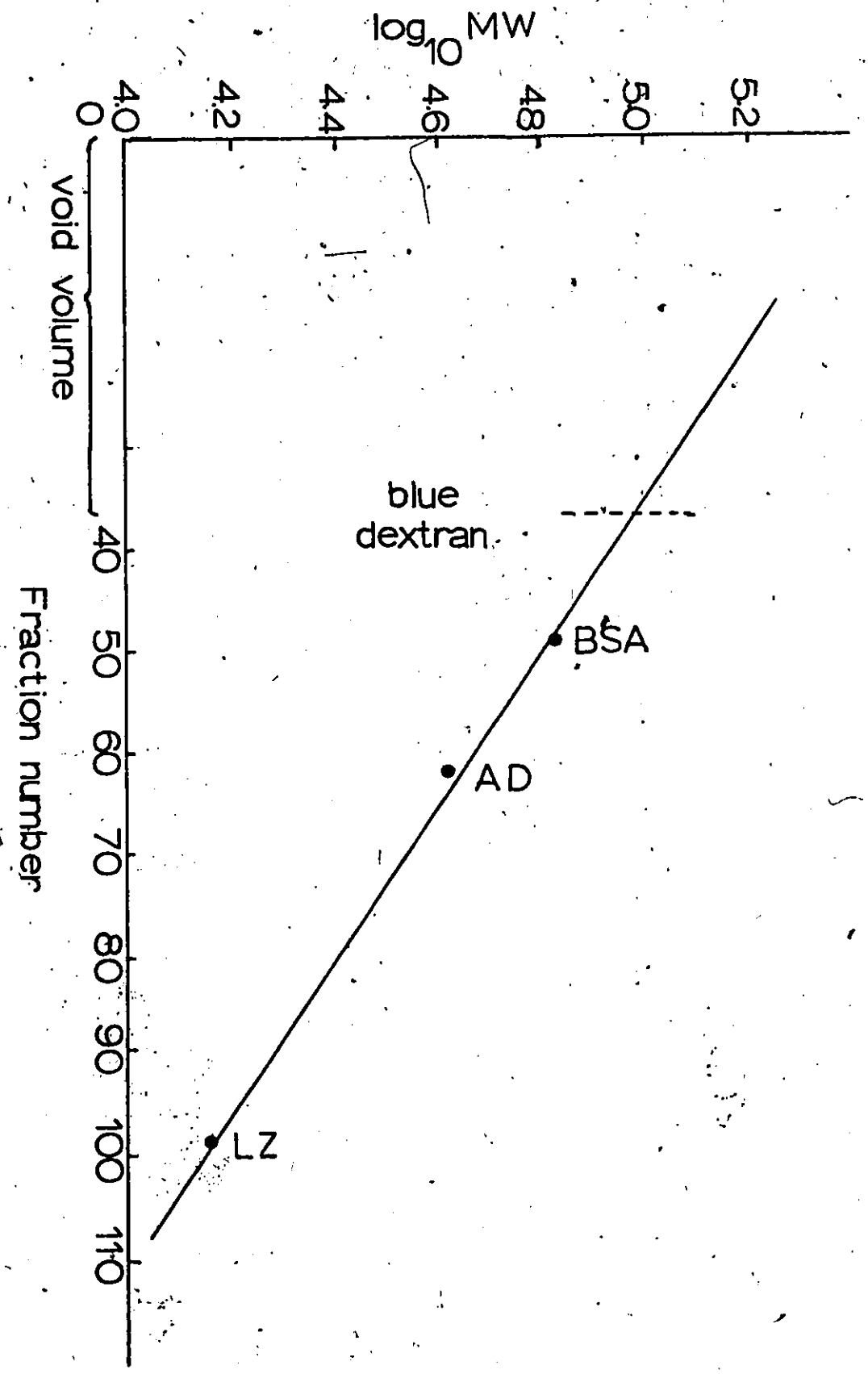
Protein Determination

Protein concentration of the samples utilized in the individual studies employed the method of Lowry et al. (1951) using bovine serum albumin for protein concentration standardization.

Figure 3

Sephadex G-100 protein molecular weight standard curve

BSA	Bovine serum albumin	(68,000 MW)
AD	Alcohol dehydrogenase	(39,805 MW)
LZ	Lysozyme	(14,316 MW)


log10 MW
4.0
4.2
4.4
4.6
4.8
5.0
5.2
0
void volume
40
50
60
70
80
90
100
110
Fraction number
blue dextran
BSA
AD
LZ

Determination of Cellular Polyamine Concentrations

The method of Dion and Herbst (1970) was used for the measurement of cellular polyamine levels.

A 1.5 L culture, with either 1.0 M NaCl or 3.0 M NaCl, was pelleted at 12,000 X g for 10 minutes. The pellet was then resuspended and washed in the same NaCl-containing medium (20 ml) using a cannula-fitted syringe. Ten ml of this suspension was centrifuged as before and 0.4 ml/15 mg wet cell weight of 0.2 N perchloric acid (PCA) was added to the pellet. This was homogenized with a Dounce hand homogenizer. Centrifugation was repeated as before, leaving a supernatant which was decanted off carefully for analysis of polyamine content. Polyamines were labelled as follows: 0.2 ml of the PCA extract was transferred to a conical centrifuge tube and dansylated by the addition of 0.4 ml dansyl chloride (30 mg/ml acetone) and 50 mg $Na_2CO_3 \cdot 10\ H_2O$. Dansylation was allowed to proceed for 16 hours in the dark at room temperature. Excess dansyl chloride was then converted to dansyl proline by reaction with 0.1 ml proline (100 mg/ml) for 30 minutes. The dansylated derivatives were extracted by the addition of 0.5 ml benzene, followed by vigorous shaking.

Microliter aliquots of the benzene extracts were applied to activated (1 hour at 110°C) silica gel G thin layer chromatography (TLC) plates, 250 u.

The separation of the dansyl polyamines was effected by development in ethylacetate/cyclohexane (2:3, v/v), followed immediately by spraying the developed TLC plate with triethanolamine/isopropanol (1:4, v/v) to enhance and stabilize (Figure 4) fluorescence. The coated plates were then dried *in vacuo* at room temperature for 16 hours in the dark. After equilibrium at atmospheric pressure for 1 hour, the TLC plate was ready for fluorometric scanning.

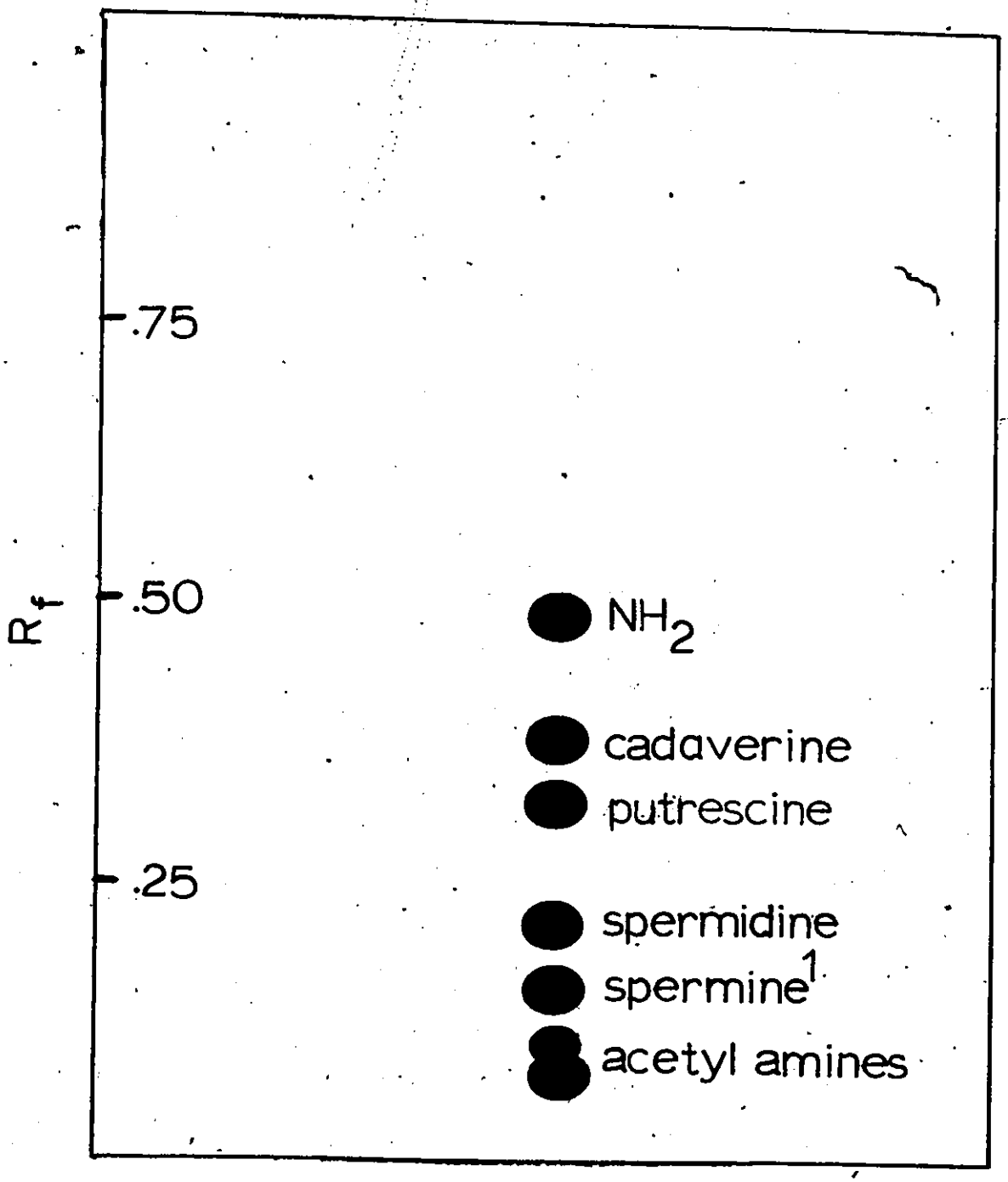
Direct quantitative analysis of fluorescent intensities were obtained with a Turner Model 111 fluorometer (G. K. Turner Assoc.) euipped with TLC scanner and recorder. The light source employed was a long-wave source (Turner Catalog #110-850). Activation at 365 mu was effected with a Turner Primary filter 7-37. Turner filter #2A-12 served as the secondary filter, allowing transmission of wavelengths longer than 512 mu (Figure 4).

Figure 4

Polyamine Silica gel G thin layer chromatography R_f values

Separation of polyamines on silica gel G thin layer chromatographic plate.

Note: no spermine was found in Vibrio costicola



1 not found in V. costicola

RESULTS

RESULTS

Protein Turnover

An initial assay of the rate of turnover of both long- and pulse-labelled proteins in V. costicola was done to obtain some indication of any unusual values relative to those of previously published data regarding other organisms.

Figure 5 presenting an individual experiment shows the time course of turnover of pulse- and long-labelled proteins in V. costicola growing exponentially in 1.0 M NaCl medium.

Several experiments with V. costicola growing in 0.5, 0.7, 1.0, and 1.5 M NaCl are summarized in Table 2. It was noted that the initial turnover rate of pulse-labelled 1.0 M NaCl-growing cells (about 5 %/hr.) was substantially higher than the rates reported for other organisms (Goldberg and St. John, 1976). For long-labelled cells the rate was much lower, about 2.5 % in a 2.5 hour period.

A determination of the effects of cultural conditions on isotope incorporation was carried out by measuring the actual amount of ^{14}C-leucine incorporated per mg protein. In V. costicola the amount of radioactive leucine incorporated corresponded to 16.3 $\pm$ 5.8 nmol/mg protein for pulse-labelled cells and 46.3 $\pm$ 7.0 nmol/mg protein in long-labelled cells (values obtained from 3 experiments utilizing 1.0 M NaCl cells).

Incorporation of this radioactive amino acid was found to be similar in 0.5 M and 1.5 M NaCl growing cells. Incorporations of 45 nmol/mg protein and 12 nmol/mg protein was found in 0.5 M NaCl long- and pulse-labelled cells, respectively. 1.5 M NaCl growing V. costicola exhibited an incorporation of 20 nmol/mg protein in pulse-labelled cells.

Table 2 shows a similar rate of degradation for cells growing in 1.5 M NaCl compared to 1.0 M NaCl cells. However, significant differences are found for cells growing in salt concentrations less than 1.0 M. Elevated levels of protein turnover, in pulse-labelled cells, became evident in 0.5 M and 0.7 M NaCl growing cells of V. costicola (Table 2). In all of these experiments, the rate of turnover of long-labelled proteins remained low (usually no more than 2.9 % over a 2 hour period) and did not change with NaCl concentration changes in the medium.

In long-labelled V. costicola any rapidly turning-over fraction must be degraded and resynthesized in the presence of ^{14}C-leucine. In the protein turnover experiments, in this study, this fraction would only represent a small part of the total calculated rate.

The turnover rates of E. coli were also studied in order to determine some comparative values between V. costicola and a non-halophilic bacterium. The turnover experiments are summarized in Table 3. E. coli growing at 30°C in minimal

Figure 5

Protein breakdown in pulse-labelled and long-labelled *Vibrio costicola* growing in 1.0 M NaCl SGS medium

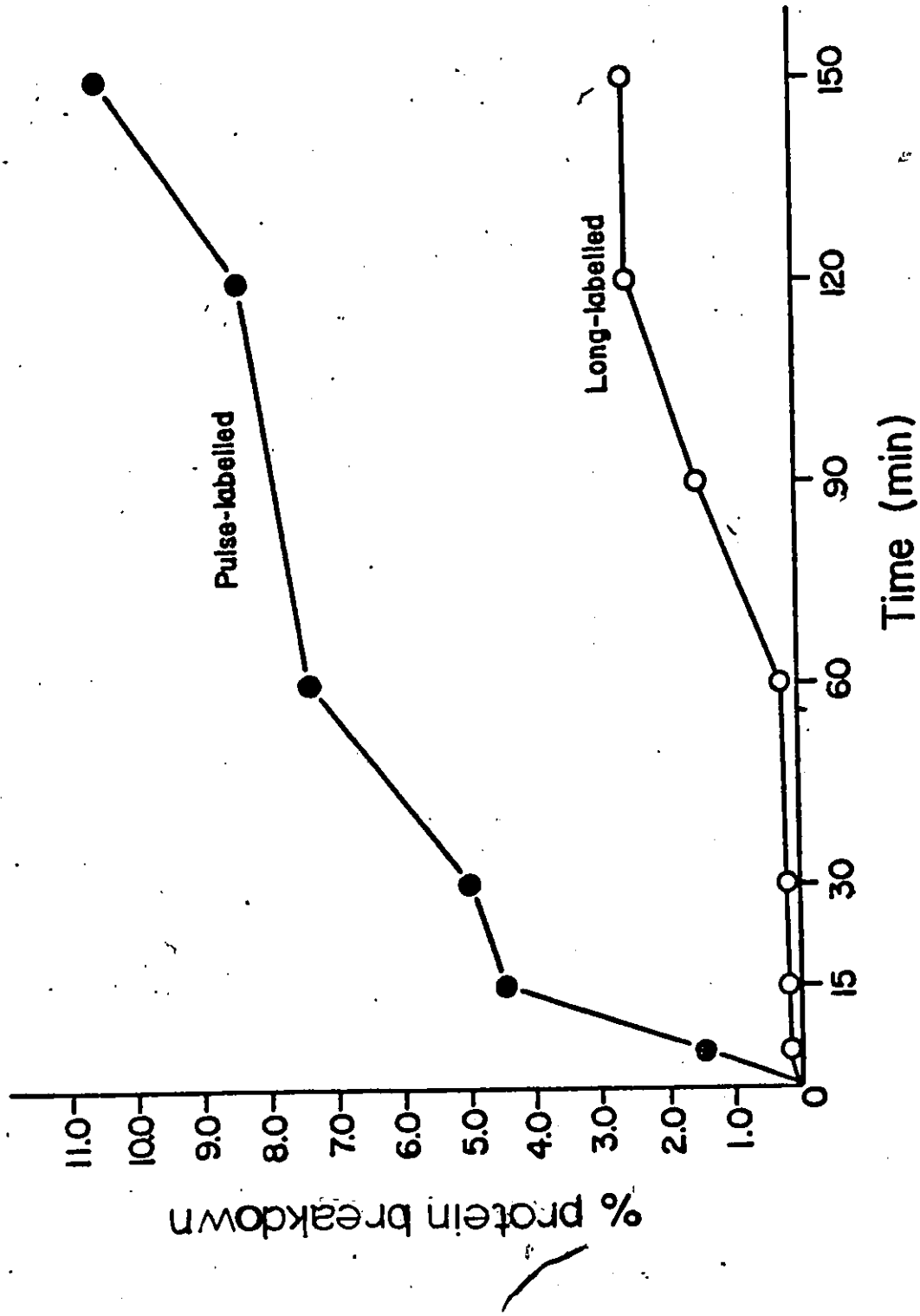

% protein breakdown
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15
30
60
90
120
150
Time (min)
Pulse-labelled
Long-labelled

TABLE 2

Turnover of proteins in V. costicola grown in different NaCl concentrations

NaCl conc. (M)	% Protein turnover at time: Pulse-labelled protein 30'	60'	90'	120'	Long-labelled protein 120'
0.5 (3)[a]	6.9 ± 1.1[b]	8.2 ± 1.5	9.9 ± 1.9	11.1 ± 0.6	2.4 ± 0.6
0.7 (1)	6.6	8.5	11.0	11.6	-
1.0 (8)	3.2 ± 1.6	4.7 ± 2.0	6.3 ± 2.3	7.1 ± 1.9	2.9 ± 0.5
1.5 (4)	3.6 ± 1.1	4.5 ± 1.2	5.4 ± 0.9	6.4 ± 0.5	2.9 ± 0.2
2.5 (1)	3.2	4.8	6.3	6.5	2.2

a Figures in parenthesis represent number of individual experiments.
b Figures are given for standard deviation

TABLE 3

Turnover of proteins in E. coli

Conditions[a]		% Protein breakdown at time (min):					
Pulsed in NaCl (M)	Transfer to NaCl (M)	30'	60'	90'	120'	150'	Long-labelled proteins at 150'
0.0	0.0	0.8	0.7	0.7	0.9	1.0	0.0
0.5	0.5	3.6	2.3	2.6	2.4	2.8	0.6
1.0	1.0	1.1	1.5	2.5	3.0	3.1	0.0
0.0	0.5	0.2	1.1	1.3	1.3	0.9	0.4
0.0	1.0	0.0	0.7	0.8	1.1	0.9	0.0

a Pulse-labelled proteins except where indicated.

b Transfers utilized cells subcultured three times prior to pulse-labelling and transfer.

medium without added NaCl had a very low rate of turnover for pulse-labelled proteins (1 % in 150 minutes). In one experiment in which cells, after pulse-labelling, were transferred directly into medium with either 0.5 M or 1.0 M NaCl, the breakdown rate of protein did not change although the growth rate decreased. In another experiment, cells were subcultured several times in medium containing 0.5 M or 1.0 M NaCl. As Table 3 indicates, a slightly higher rate of breakdown was observed in the higher salt concentration, although the rates were considerably lower than those found in V. costicola. In addition, there was practically no turnover of long-labelled proteins in E. coli under all cultural conditions.

Effect of Experimental Manipulations and Conditions on Protein Turnover:

Protein turnover rates, as explored by the stated method (Materials and Methods section), could be subject to variations which may be the result of the experimental handling of the cells. It was for this reason that turnover was analyzed under alternate conditions of procedure to elucidate the effect of these extraneous factors, if any.

(A) Changes in Washing of Cells:

A 1.0 M NaCl culture of V. costicola was pulse-labelled

TABLE 4

Effects of centrifugation on protein breakdown

Details[a]	% Protein breakdown at time: 15'	30'	60'	120'	Generation time (hrs)
Control - centrifugation step included	1.8	2.9	4.4	7.2	5.5
- centrifugation step excluded	4.2	6.4	5.8	14.0	5.6

a All experimental manipulations included except where indicated.

with a standard amount of ^{14}C-leucine. All other procedures of "chasing" with non-radioactive leucine and determination of protein turnover were carried out. However, no centrifugation of the "pulsed" culture was done. This was done as some fear that the centrifugation procedure (at 12,000 X g) may have affected the cellular level of isotope ("squeezing-out") or that the well-being of the cell was in jeopardy (affecting growth rates).

The level of protein turnover is shown in Table 4. The results seem to indicate that centrifugation tends to lead to lowered rates of turnover relative to cells that are manipulated under standard procedures without centrifugation. This procedure, as indicated by measured generation times, does not alter to any appreciable level the growth rate relative to centrifuged cells. The appearance of higher rates in non-centrifuged cells may be indicative of some anaerobic conditions being created leading to disruption of an energy component necessary for this phenomenon (this will be dealt with in detail later).

(B) Effectiveness of ^{12}C-leucine ("Chase") in Preventing Reincorporation of ^{14}C-leucine:

The non-radioactive leucine added in the "chase" period following labelling of protein with ^{14}C-leucine was used at a concentration of 200 ug/ml. It was crucial to all experi-

TABLE 5

Effectiveness of ^{12}C-leucine (200 ug/ml) in preventing ^{14}C-leucine reincorporation

NaCl conc. (M) and conditions[a]	% Protein breakdown at time: 0'	5'	15'	30'	60'	90'	120'
0.5	0.0	1.8	3.3	4.8	7.1	7.9	8.5
0.5 + CAP[b]	0.0	1.3	4.6	4.7	6.8	7.6	8.7
1.5	0.0	1.1	1.4	2.1	3.5	3.7	4.5
1.5 + CAP	0.0	2.2	1.5	2.2	3.6	4.0	4.5

a. All cultures contained ^{12}C-leucine at 200 ug/ml.

b. Chloramphenicol (100 ug/ml) completely arrested growth of V. costicola as measured by absorbance at O.D$_{600}$.

ments that this concentration of non-radioactive leucine should effectively compete with the ^{14}C-leucine for incorporation into protein in order to obtain valid turnover rates.

If the ^{14}C-leucine was allowed to be reincorporated during this period it would lead to lower apparent levels of protein turnover. Since it was known that chloramphenicol (100 ug/ml) effectively inhibited protein synthesis (by at least 90 %) as measured by incorporation of ^{14}C-leucine (see section on Protein Turnover and Inhibitors), while not affecting levels of turnover, this antibiotic was used to test the competition between radioactive leucine versus non-radioactive leucine into protein.

A 1.0 M NaCl culture of V. costicola was grown, then transferred, after standard labelling and washing, into either a 0.5 M or 1.5 M NaCl medium with or without chloramphenicol but both containing ^{12}C-leucine (200 ug/mg). If ^{14}C-leucine was being reincorporated during the "chase" period this would be seen by an apparent higher level of protein breakdown in the chloramphenicol-containing culture than in those without this antibiotic.

The rates of breakdown were calculated in all cultures (Table 5). The data show effective inhibition of ^{14}C-leucine reincorporation during the chase period since there were virtually identical rates of breakdown in chloramphenicol-containing and Control cultures.

(C) Growth Phase versus Rates of Protein Turnover:

In all studies in this thesis, unless otherwise indicated, the cells utilized were in the logarithmic phase of growth (OD_{600} 0.3 - 0.8). Nevertheless, generation times were found to differ, at times, from one batch of log cells to another. It was thought that the variations from one experiment to another in the rates of turnover may have been due to the respective generations times. In one experiment the effect of the phase of growth (as measured by turbidity at OD_{600}) and the actual generation times were compared with respect to the rate of protein turnover. Portions of a 1.0 M NaCl culture of V. costicola were isolated at different optical densities (OD_{600}) and assayed for their respective rates of protein degradation and growth rates (Table 6).

The results indicate that the phase of growth (either exponential or stationary) or generation times did not appear to significantly affect the levels of proteolysis. The use of log phase cells in this study ensured as little variation as possible, from these factors, to alter the rates of protein degradation.

Variation of Salt Concentration and Turnover Rates:

Various halophilic proteins (Brown, 1964a; Shindler, 1976; Rossignol, 1978) have been shown to require elevated

TABLE 6

Protein breakdown in V. costicola at different phases of growth

Generation Time	% Protein breakdown at time: 15'	30'	60'	90'	120'	150'	$O.D_{600}$
4.3	2.1	2.9	4.8	6.2	6.1	6.3	0.34
4.7	1.0	3.1	5.1	5.1	6.0	6.1	0.66
6.8	2.2	3.9	6.5	6.5	6.9	8.0	1.30
- [a]	-	2.4	6.0	6.0	6.1	6.2	1.70

a Culture in stationary phase of growth.

salt concentrations for maximum stability whereas others "prefer" less saline environments.

In a number of experiments (Table 7), cells were either pulse-labelled or long-labelled while growing at one NaCl concentration, then centrifuged and resuspended in fresh medium of a different NaCl concentration. The results demonstrate that the rate of turnover of pulse-labelled proteins is determined by the salt concentration in which the cells are incubated, rather than that in which they were labelled. Therefore, cells pulse-labelled in 1.0 M NaCl then subsequently transferred to 0.5 M NaCl, showed the higher turnover rate characteristic of the lower salt concentration. The reverse was found to occur for cells labelled in 0.5 M NaCl and then transferred to 1.0 M or 1.5 M NaCl. As noted before, in all cases, the turnover of long-labelled proteins remained low.

Growth before and after transfer was measured in several of the above experiments. No major or consistent changes were noted upon transfer to the same or different salt concentrations. Also there was no correlation, either positive or negative, between the growth rate and the rate of protein turnover.

Effect of Inhibitors on Protein Turnover:

The physiological state of the cell, such as the levels

TABLE 7

Effect of changed NaCl concentration on turnover of pulse-labelled proteins in *V. costicola*

Concentration		% protein breakdown at time:			
Pulsed in NaCl (M)	Transfer to NaCl (M)	30'	60'	90'	120'
0.5	0.5 (4)[b]	6.6 ± 1.1[c]	8.3 ± 1.0	10.2 ± 1.3	10.8 ± 1.0
0.5	1.0 (2)	4.6 ± 1.7	6.3 ± 4.1	8.5 ± 3.6	8.2 ± 2.3
0.5	1.5 (2)	4.9 ± 3.7	5.0 ± 4.7	5.8 ± 3.5	6.1 ± 4.2
1.0	0.5 (11)	5.3 ± 1.7	7.6 ± 2.1	8.7 ± 2.3	10.2 ± 2.8
1.0	1.0 (19)	3.0 ± 1.7	4.8 ± 1.9	5.9 ± 2.2	7.0 ± 2.2
1.0	1.5 (7)	2.9 ± 2.3	3.9 ± 2.1	4.6 ± 1.6	5.6 ± 2.1
1.5	0.5 (2)	4.8 ± 0.0	7.7 ± 0.14	10,0 ± 0.4	11.7 ± 0.5
1.5	1.0 (2)	2.9 ± 1.2	4.6 ± 0.9	5,8 + 1.4	6.8 ± 0.9
1.5	1.5 (4)	3.8 ± 1.1	4.5 ± 1.1	5,4 ± 0.8	6.2 ± 0.4

a In individual experiments with long-labelled cells the protein breakdown was about 2 % per hour under all conditions.

b Figures in parenthesis represent number of individual experiments.

c Figures given are for standard deviation.

of energy or protein biosynthesis, have been found to affect the level of protein turnover in many microorganisms (Goldberg and St. John, 1976). In both E. coli and mammalian cells, intracellular protein degradation can be inhibited by agents which perturb the cell's energy -- generating or protein biosynthetic mechanisms.

A comparison was made with V. costicola, with respect to other organisms (published data) of the effects of inhibitors of protein synthesis and cellular energy generation on protein turnover. The antibiotics tetracycline (100 % inhibition) and chloramphenicol (90 % inhibition), both at 200 ug/ml, were found to completely or almost completely inhibit protein synthesis respectively in V. costicola, as measured by ^{14}C-leucine incorporation into the trichloroacetic acid insoluble fraction (A. R. Hipkiss, personal communication).

In a standard experiment the cells, after "chasing" were further incubated in the presence of tetracycline (200 ug/ml). Tetracycline was very inhibitory to the rate of turnover in V. costicola (Table 8). This result was found to agree with published data (Cashel, 1975; Goldberg and St. John, 1976) due to the marked level of inhibition to the rate of turnover brought about by this antibiotic in E. coli.

In another experiment, tetracycline (200 ug/ml) was added to pulse-labelled cells growing in 1.0 M NaCl at different time points during the "chase" period. Figure 6 shows that

Figure 6

Effect of tetracycline on protein breakdown in *Vibrio costicola*

To pulse-labelled cells in medium with 1.0 M NaCl, tetracycline (200 ug/ml) was added at the times shown.

Control, no tetracycline

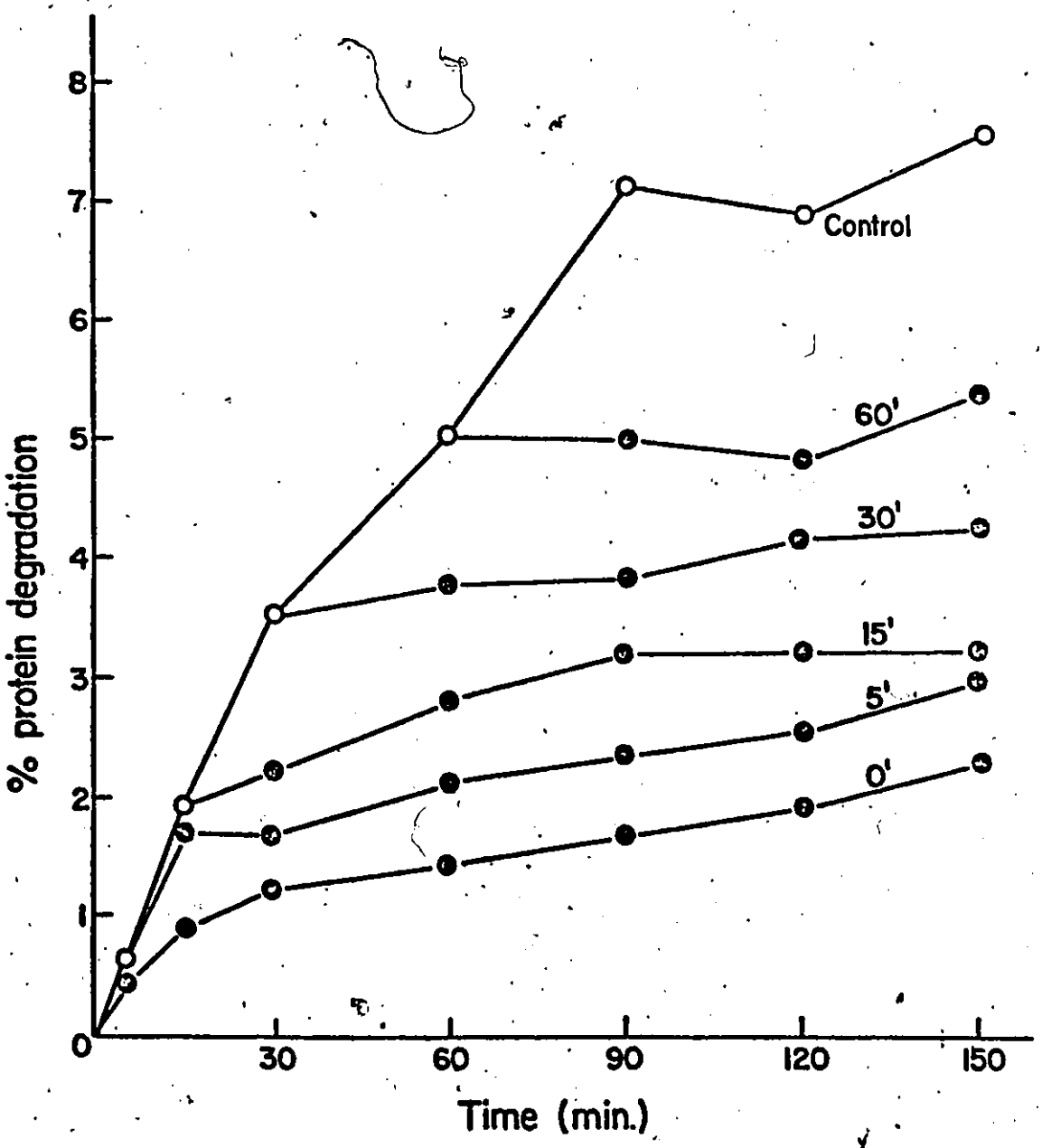

% protein degradation
Time (min.)
Control
60'
30'
15'
5'
0'
0
1
2
3
4
5
6
7
8
30
60
90
120
150

TABLE 8

Effect of metabolic inhibitors on protein breakdown in *V. costicola*

Inhibitor[a]	% Breakdown at time[b]			
	30'	% Inhibition	60'	% Inhibition
Control	3.5		6.9	
+ tetracycline (200 ug/ml)	1.2	66	1.9	72
Control	2.7		6.2	
+ DNP (0.2 mM)	2.8	0	6.2	0
+ CN^- (10 mM)	1.2	56	3.2	48
Control	2.7		5.1	
+ iodoacetate (10 mM)	-	-	5.3	0
Control	4.5		9.5	
+ azide (100 ug/ml)	5.0	0	10.5	0
+ chloramphenicol (200 ug/ml)	5.0	0	10.5	0
Control (0.5 M)	4.8		7.1	
+ chloramphenicol (200 ug/ml)	4.7	0	6.8	4
Control (0.5 M)	2.1		3.5	
+ chloramphenicol (200 ug/ml)	2.2	0	3.6	0

a All experiments done in 1.0 M NaCl medium except as indicated in last two experiments.

b Breakdown of pulse-labelled proteins.

this antibiotic acted quickly and effectively to inhibit further degradation of proteins.

Chloramphenicol, used in cells of E. coli deprived of glucose, can inhibit protein breakdown (Hipkiss, 1978; St. John et al., 1978). This agent, while effectively preventing protein synthesis, did not affect turnover in V. costicola at any salt concentration with glucose added (Table 8, Table 5).

The degradation of abnormal proteins, that can occur spontaneously or be induced (by incubation with amino acid analogs for example), in E. coli is inhibited by the addition of azide (N_3^-), cyanide (CN^-), 2-4-dinitrophenol (DNP) and other agents which inhibit ATP synthesis (Goldberg and St. John, 1976; Olden and Goldberg, 1978) whereas "normal" cellular proteins are not affected by cyanide additions, if glucose is present. Results with various inhibitors of ATP synthesis on the rate of turnover of pulse labelled proteins in V. costicola did not lead to a marked inhibitory effect except for cyanide (Table 8). It was found that cyanide lowered the rate of turnover by about 50 %. The other agents tested, including azide and iodoacetate, had virtually no significant inhibitory or stimulatory effects on this rate.

Inhibition of Streptomycin-induced Erroneous-Protein Breakdown:

In V. costicola tetracycline and cyanide are seen to

inhibit the rates of turnover. If a higher cellular concentration of abnormal proteins was responsible for this elevated level of turnover then the effects of these inhibitors upon induced abnormal proteins was thought to be of interest.

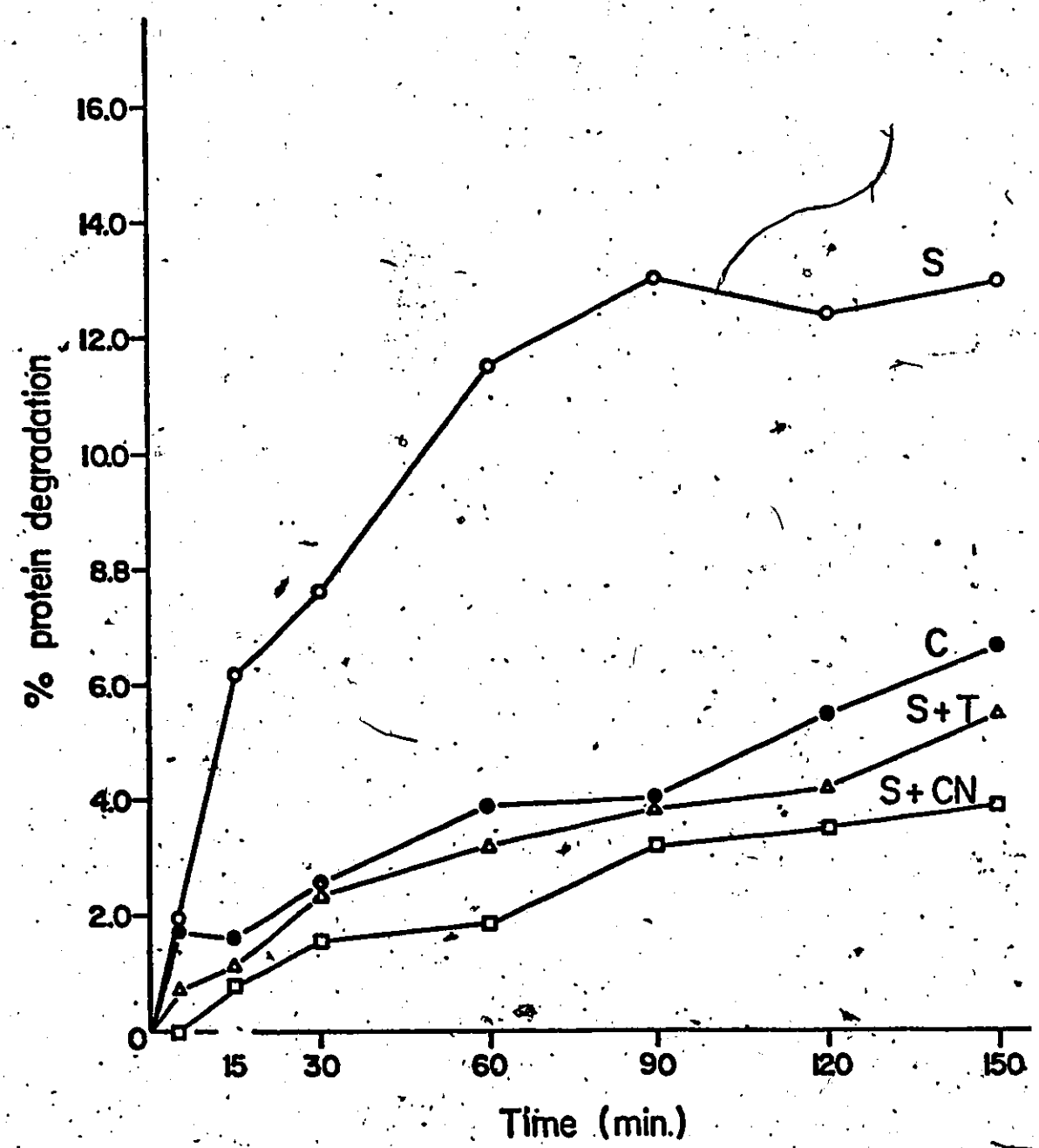
Streptomycin, an aminoglycosyl antibiotic, while preventing the binding of amino-acyl-tRNA causes miscoding and concomitant production of erroneous proteins (Davis et al., 1974). Other metabolic effects are the inhibition of protein synthesis, at high concentrations, and increased RNA synthesis. Of interesting consequence upon the bacterial cell is "anomolous" incorporation of incorrect amino acids into protein during synthesis thus leading to abnormal protein production (Gale et al., 1972). In E. coli dihydrostreptomycin causes a stimulation in proteolysis, probably due to an increase in translational errors leading to abnormal protein production (Gale et al., 1972; Hipkiss and Kogut, 1973).

A 1.0 M NaCl culture of V. costicola was pretreated for thirty minutes with dihydrostreptomycin sulphate (50 ug/ml) which is only slightly inhibitory to protein synthesis as measured by incorporation into TCA insoluble material (A.R. Hipkiss, personal communication). The cells were then pulse-labelled and standard washings then carried out. After resuspension in a small quantity of ^{12}C-leucine-containing medium (1.0 M NaCl and free of streptomycin) the cells were incubated either in medium without inhibitor or with tetracycline (200 ug/ml), or cyanide (10 mM).

Figure 7

Effect of streptomycin and other inhibitors on protein breakdown in Vibrio costicola growing in medium with 1.0 M NaCl.

C	control
S	streptomycin sulphate (50 ug/ml)
T	tetracycline (200 ug/ml)
CN	cyanide (10 mM)


16.0
14.0
12.0
10.0
8.8
6.0
4.0
2.0
0
% protein degradation
15
30
60
90
120
150
Time (min.)
S
C
S+T
S+CN

The results (Figure 7) show that pretreatment with streptomycin of V. costicola, leads to a marked stimulation of the rate of protein degradation. The two inhibitors, cyanide and tetracycline were found to inhibit this stimulated rate of degradation of the presumably abnormal proteins produced as a result of streptomycin treatment.

Change of Carbon Source and Energy Impairment - Effects on Protein Turnover:

Various studies with different carbon, or energy, sources have been done with E. coli in order to evaluate the effects of depletion of the cellular supply of energy on protein breakdown (Goldberg and St. John, 1976; Olden and Goldberg, 1978).

The glucose analogue ∝ - methylglucoside is phosphorylated then dephosphorylated by the cell, without further metabolism. This cyclic process serves to rapidly exhaust the cell's high energy phosphates. The degradation of abnormal proteins, in E. coli is only affected by this agent in the absence of glucose.

Alternate forms of carbon sources, such as succinate, have also been utilized in previous studies (Kowit and Goldberg, 1977). Succinate does not allow energy for growth through glycolysis, while not inhibiting respiration or the so-called "energy-rich-membrane state" (ERMS). E. coli

grown with succinate as the only carbon source experiences no reduction in the rate of turnover unless agents such as cyanide or anaerobic conditions are present.

V. costicola was grown in a 1.0 M NaCl medium with 1 % (v/v) glucose being present. After "pulsing" the cells with radioactive leucine they were separated by filtration on a 0.45 u (Millipore) filter and washed with medium (1.0 M NaCl) lacking glucose. The washed pellet, after being re-suspended in a small amount of medium lacking glucose, was incubated in ^{12}C-leucine-containing medium with either glucose (1 % v/v), α - methylglucoside (20 mM) or succinate (20 mM). Cyanide was added to flasks, duplicating the above conditions, to evaluate the effect of reduction of cellular respiration and simultaneous depletion of cellular energy supplies. The α - methylglucoside led to a lowering of the rate of protein degradation in V. costicola. The same results were found with E. coli (Olden and Goldberg, 1978) while using cells incubated with canavanine (an amino acid analogue of arginine producing abnormal proteins) in the absence of glucose only.

V. costicola was incubated in succinate which revealed a substantial reduction in the rate of the protein turnover.

In both cases of α - methylglucoside or succinate addition, the rate of turnover was seen to be reduced in V. costicola. Table 9 also indicates that V. costicola when incubated with succinate and cyanide demonstrated little change

TABLE 9

Cellular energy impairment - effect on protein breakdown

Conditions[a]	% Protein breakdown at time: 15'	30'	60'	90'	120'	150'	% inhibition of breakdown at 150'
+ glucose (1%)	2.1	4.6	7.5	5.5	7.5	9.8	
+glucose +cyanide (20 mM)	2.0	3.1	3.4	4.2	3.3	3.7	62
+∝MG[b] (20 mM)	0.3	1.8	3.3	3.4	3.6.	5.0	49
+ CN (20 mM) +∝MG (20 mM)	1.4	1.4	1.6	1.0	2.1	2.2	78
+ succinate (20 mM)	1.9	2.0	3.0	3.1	3.9	4.7	52
+ succinate (20 mM) + CN (20 mM)	2.8	3.6	4.1	4.1	4.1	4.1	58

a All cells were incubated in 1.0 M NaCl SGS medium.

b ∝ methylglucoside.

in the level of intracellular degradation of protein compared to succinate only-incubated cells. However, cyanide and α-methylglucoside, added together, appeared to have more of a striking inhibition upon this process. In E. coli the combined effects of α-methylglucoside and cyanide leads to an ATP reduction down to 2 - 3 % of a growing culture (Olden and Goldberg, 1978). Since these agents have the same effect in V. costicola then quite possibly the substantial lowering of cellular high energy phosphates disrupts the process of turnover of protein. The results presented for V. costicola seem to follow, for the most part, the patterns obtained with E. coli possessing high cellular levels of abnormal proteins and not those found under conditions of biosynthesis of "normal" proteins.

Modulation of Protein Turnover by Different Time Transfers and Salt Concentrations:

Normally, V. costicola, when transferred to a medium with a lower salt concentration (0.5 M NaCl), demonstrates a stimulation in the measured breakdown rate. The question of continuation of this effect upon reinstatement of higher salt concentrations was of interest.

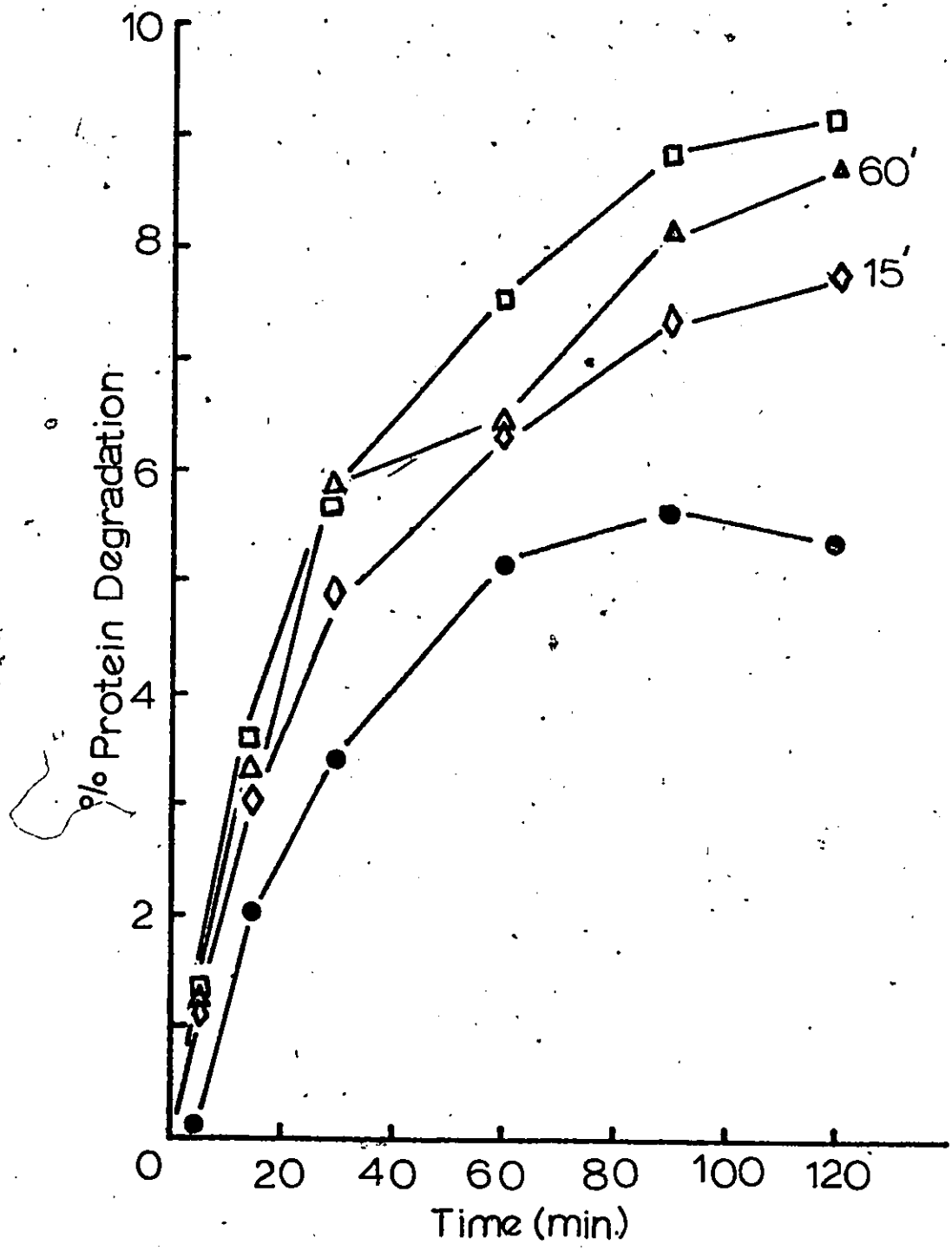
An experiment was designed to elucidate the effect on turnover of exposing pulse-labelled cells to a low NaCl concentration for different periods of time. V. costicola was pulse-labelled in a 1.0 M NaCl medium and handled according

Figure 8

Modulation of protein breakdown by different time transfers and salt concentrations

closed circles	1.0 M to 1.0 M NaCl at 0 min.*
open boxes	1.0 M to 0.5 M NaCl at 0 min.
diamonds	0.5 M to 1.0 M NaCl at 15 min.
triangles	0.5 M to 1.0 M NaCl at 60 min.

*Time refers to period following the end of the "pulse" with ^{14}C-leucine.


10
8
6
4
2
0
% Protein Degradation
20
40
60
80
100
120
Time (min.)
60'
15'

to standard procedures. These cells were subsequently incubated in 1.0 M or 0.5 M NaCl immediately following the "pulse" period. At 0, 15 or 60 minutes the 0.5 M NaCl cells had NaCl added to increase the molarity to 1.0 M NaCl final concentration.

Typical stimulation of intracellular proteolysis was evident in 1.0 M NaCl cells transferred to 0.5 M NaCl at 0 minutes following the "pulse" period (Figure 8). V. costicola seems to undergo a slight inhibition of this stimulation when changed back to 1.0 M NaCl both at 15 and 60 minutes after the initial transfer into the lower salt medium.

The rate of turnover upon reinstatement of the optimum level of NaCl (1.0 M) appears to return immediately to a slightly lowered level (although still higher than the Control 1.0 M NaCl culture). The "early changed" cells (0.5 M to 1.0 M NaCl at 15 minutes) are found to return to lower levels but are still higher than the control. The two time periods following the change at either 15 or 60 minutes show approximately the same decrease in the rate of protein breakdown.

Delayed Transfer of 1.0 M NaCl-grown V. costicola into a 0.5 M NaCl Medium:

These studies suggest that transfer of V. costicola from 1.0 M (or 1.5 M) NaCl into 0.5 M NaCl results in a stimulation in the turnover rate of pulse-labelled but not

TABLE 10

Delayed transfer from 1.0 M to 0.5 M NaCl medium

Transfer at time[b] (min)	30'	60'	90'	120'
1.0 M to 1.0 M NaCl (Control)[a]				
0	2.3	4.0	6.0	6.0
15	2.8	3.8	5.2	6.0
60	2.3	4.0	5.0	6.0
1.0 M to 0.5 M NaCl				
0	5.3	7.0	8.0	9.4
15	-	6.2	8.0	8.8
60	2.6	6.1	8.0	7.8

a Values given are for % protein breakdown at each respective time period.

b Transfer into indicated NaCl - containing medium at time following "pulse" period.

long-labelled proteins.

In order to determine the duration of increased proteolytic susceptibility of pulse-labelled proteins to a lowering of the NaCl concentration the periods of transfer to a lower salt concentration was altered. Cells, which were pulse-labelled in 1.0 M NaCl and transferred to 0.5 M or 1.0 M either immediately, at 15, or 60 minutes following removal of the isotope, were assayed for their respective rates of turnover.

It was observed (Table 10), following a delay in the transfer of 1.0 M NaCl-grown V. costicola into a medium of lower NaCl concentration, that a portion of the proteins initially present were less susceptible to the stimulated degradative activity. Transfer to 1.0 M NaCl-grown cells, at 0, 15 or 60 minutes post-pulsing, into fresh medium of the same NaCl concentration appeared to have no effect on turnover rates.

In general, the results indicate that later transfer of cells (15 or 60 minutes after pulsing) to a medium of lowered NaCl concentration (0.5 M) leads to progressively lower rates of protein breakdown for pulse-labelled proteins.

Guanosine Tetraphosphate (ppGpp) and Temperature Effects on Protein Turnover:

(A) ppGpp Effects

The cellular levels of guanosine tetraphosphate (ppGpp) has been shown to be correlated directly with the rate of protein turnover and indirectly with the rate of stable RNA synthesis in many bacteria (Edlin and Donini, 1971; Cashel, 1975; Goldberg and St. John, 1976; Martegani and Alberghina, 1979).

In E. coli the physiological concentration of ppGpp is 10 mM under conditions of carbon source starvation (Edlin and Donini, 1971) compared to only 1 - 2 mM under optimum growth conditions.

It was of interest to see how the rates of breakdown in V. costicola could be affected by extraneous addition of this nucleotide (10 mM) in 1.0 M and 0.5 M NaCl-growing, pulse-labelled cells. Table 11 clearly shows a substantial stimulation in the measured turnover rate in both 1.0 M and 0.5 M NaCl cells. It was noted that the 1.0 M cells showed a gradually higher level of degradation with time. However, the 0.5 M cells showed an immediate elevation (ca. 10 %) of the rate which continues to quickly rise to a relatively high peak value followed by a slight decline (at 165 minutes). The growth of V. costicola, as measured by the increase in turbidity (OD_{600}), was somewhat inhibited (about 25 % increase in doubling time) by the addition of this nucleotide. This may be due to a restriction the formation of stable RNA by

TABLE 11

Guanosine tetraphosphate (ppGpp), temperature - effects on protein breakdown

Experimental Details	% Protein breakdown at time: 5'	15'	30'	60'	165'	Generation Time (hrs)
1.0 M NaCl (30°C)	0.0	2.0	-	6.1	7.2	2.4
0.5 M NaCl (30°C)	4.9	7.7	8.0	7.8	9.4	3.0
1.0 M NaCl (20°C)	4.3	3.6	6.3	8.7	10.3	2.8
0.5 M NaCl (20°C)	11.1	17.9	11.6	12.4	34.0	3.3
1.0 M NaCl + ppGpp (10 mM)[a]	3.2	14.2	15.9	25.4	29.4	3.0
0.5 M NaCl + ppGpp (10 mM)	10.0	24.3	22.0	23.2	18.0	3.8

a ppGpp supplemented V. costicola cultures were both incubated at 30°C.

ppGpp (Martegani and Alberghina, 1979).

(B) Temperature Effects

Another feature which has been attributed to enhancing the rate of protein breakdown is the role of hydrophobic bonds in maintaining protein structure (Katsoyannis, 1973; Goldberg and Dice, 1974).

It has been demonstrated in V. costicola (De Médicis and Rossignol, 1977; Rossignol, 1978) that pyruvate kinase, in vitro, has a lowered enzymic activity as the temperature is lowered from 30°C to 4°C. with progressive regain of activity upon temperature reversal back to 30°C. This was postulated to be due to disruption of hydrophobic interactions in this enzyme.

V. costicola, in the present study, was assayed both at 30°C and 20°C to determine the effect of lowering the temperature (without affecting the growth rate) on the rate of protein degradation in vivo.

The lowering of the temperature to 20°C was found to slightly increase the generation times of both 1.0 M and 0.5 M NaCl-grown cells. Table 11 indicates the generation times of the 20°C grown cells was only slightly longer in both the 1.0 M and 0.5 M NaCl cells (2.8 and 3.3 hours respectively) growing at 30°C. However, the rates of protein turnover in the 1.0 M NaCl culture (20°C), although higher than Control

cells, is markedly lower than the 0.5 M NaCl cells growing at 20°C. A substantial increase in the rate of turnover of protein is seen (3- 4 fold) with V. costicola incubated in 0.5 M NaCl and 20°C. As will be discussed in detail later, the phenomenon of "cooperativity" of bond breakage (Lehninger, 1975) may be resulting in protein structural changes. Elevated rates of turnover may be the result of increased structural accessibility of the unfolding proteins to proteolytic enzymes.

The combined effect of a lowered salt concentration and a lowered temperature seems to substantially increase the rate of protein degradation relative to the 1.0 M NaCl cells at 20°C and 30°C. This may be an interplay between the hydrophobic bonds and ionic interactions of cellular proteins being disrupted and therefore leading to increased structural accessibility to proteolytic enzymes.

Polyamines and Protein Turnover:

The polyamines spermidine, spermine, putrescine and cadaverine are non-protein nitrogenous bases that are widely distributed in nature (Tabor, 1958; Dion and Herbst, 1970). The presence of high concentrations of polyamines in bacteria, such as E. coli, even when grown on minimal medium (glucose-salts) supports the idea that these substances are important.

biological compounds. Generally, E. coli, when grown on a minimal medium, does not usually develop extra biosynthetic pathways unless the products synthesized are essential for the organism (Tabor and Tabor, 1972).

The polyamines have been implicated in the stabilization of many bacterial structures including, for example, spheroplasts and ribosomes (Colbourn et al., 1961).

The main concern of the present study with respect to polyamines was their potential to lower protein turnover due to stabilization of cellular protein charge interaction, possibly brought about by their polycationic nature. This neutralization could lead to counter-ion shielding resulting in more structurally stable proteins concomitant with a lowered proteolytic susceptibility.

This facet of the study involved firstly a determination of the cellular level of polyamines at different NaCl concentrations in the medium. Secondly, the effect of polyamines on the process of intracellular protein degradation was studied.

The concentrations of polyamines in V. costicola growing in 3.0 M NaCl are essentially double those found in 1.0 M NaCl-growing cells (Table 12). It should also be noted that these concentrations for polyamines in V. costicola are many-fold higher than those found for E. coli (Igarashi, 1975). These unusually high concentrations of polyamines in V. costicola, growing on a minimal medium, were thought

TABLE 12

Summary of cell-associated polyamine concentrations in V. costicola

NaCl conc. in Medium	Spermidine[a] Concentration	Putrescine Concentration	Cadaverine Concentration
1.0 M	2.41 ± 1.24[c] (13)[b]	7.35 ± 4.35 (13)	8.84 ± 5.08 (11)
3.0 M	4.15 ± 1.18 (9)	14.00 ± 6.43 (10)	17.54 ± 6.77 (10)

a uM/gm wet weight cells.

b Figures in parenthesis represent number of individual assays.

c Numbers given are for standard deviation.

d Polyamines found in E. coli (Igarashi, 1975):
Spermidine 0.25 - 1.5 uM/gm wet wt. cells
Putrescine 1.5 - 2.5 uM/gm wet wt. cells

TABLE 13

Effect of extraneous putrescine addition on protein breakdown in *V. costicola*

Conditions	% Protein breakdown at time:				
	15'	30'	60'	165'	180'
1.0 M NaCl	2.7	2.0	3.3	4.2	6.0
+ putrescine (10 mM)	2.7	1.9	1.9	1.0	2.8
0.5 M NaCl	2.4	4.4	-	11.0	12.5
+ putrescine (10 mM)	1.5	2.5	-	6.4	7.2

to be of some significance. Therefore, the next approach involved a standard determination of protein breakdown in V. costicola, growing in 1.0 M or 0.5 M NaCl medium, with the addition of putrescine (10 mM) (Table 13).

The 1.0 M NaCl-growing cells, with putrescine added, exhibited a definite lowering of the rate of protein turnover. Putrescine caused a marked inhibition of turnover in 0.5 M NaCl. This polyamine, therefore, appears to inhibit the cellular level of protein degradation expecially in V. costicola cultured in 0.5 M NaCl. This effect is seen to occur within the early time period after incubation with putrescine in 0.5 M NaCl but only becomes evident after 60 minutes post-pulsing in 1.0 M NaCl.

Cell-free Protein Degradation in V. costicola:

Various researchers have had difficulty in obtaining in vitro systems capable of exhibiting significant levels of protein degradation (Goldberg and St. John, 1978).

Work with V. costicola (Shindler, 1976; Wydro, 1977) has resulted in the development of a successful in vitro protein synthesis system. This latter system proved useful to elucidate many questions relative to growth in environments of elevated ionic concentration. It was for this reason that development of a cell-free system for study of protein degradation was attempted.

TABLE 14

Cell-free protein degradation in V. costicola

Conditions		% Protein degradation at time: 30'	60'	90'	120'	150'
Whole intact cells [c]	PL [a]	5.9	9.0	9.8	10.9	12.1
	LL [b]	0.0	0.1	1.5	3.0	3.0
Cell-free system in cell-associated ion medium	PL	0.8	2.6	3.0	4.1	5.2
	LL	0.0	0.5	0.5	0.9	1.0
Cell-free system + tetracycline (200 ug/ml)	PL	0.6	1.9	3.0	2.1	3.1
	LL	0.0	0.0	0.0	0.1	0.0
Cell-free system + CN^- (10 mM)	PL	2.8	2.4	4.4	5.2	7.3
	LL	0.0	0.1	3.0	3.9	4.5

a Pulse-labelled proteins.

b Long-labelled proteins.

c Incubated in 1.0 M NaCl minimal medium.

In the 30,000 X g supernatants the rate of degradation of pulse-labelled cells was found to be substantially lower than that found in the whole intact cell preparation (Table 14) (ca. 45 % rate of whole intact cells). Tetracycline, when added, led to a slight inhibition of breakdown whereas cyanide slightly stimulated the rate of degradation. These effects were seen in both long-labelled and pulse-labelled cells. The effects of tetracycline in lowering breakdown were similar in the cell-free preparations and whole cells. Cyanide, however, led to a slight stimulation in the degradation of pulse-labelled proteins with a marked stimulation occurring in the degradation of long-labelled proteins. This latter effect, by cyanide, was different from its effect in whole cells.

Characterization of the Cellular Fraction Experiencing the Greatest Rate of Protein Turnover:

It has been previously demonstrated that V. costicola exhibits a difference in stability of individual proteins either to elevated or to lower concentrations of NaCl in vitro (Shindler, 1977; Rossignol, 1978) with respect to activity. In order to gain a greater insight into the molecular make-up of the proteins subject to the greatest rate of turnover various chromatographic techniques were utilized. The information obtained by these techniques allowed some charac-

terization of either the molecular weight or net charge of these proteins.

As past research has revealed, there is certain evidence for a direct correlation between turnover of individual proteins with higher molecular weight and/or acidity (more negatively charged residues) (Goldberg and Dice, 1974; Dice and Goldberg, 1975). V. costicola, as previously discussed (Wydro, 1977; Rossignol, 1978), has been shown to possess a higher net number of acidic residues in certain proteins studied to date compared to other bacteria such as E. coli. This characteristic of this moderately halophilic bacterium was thought to be a useful feature in order to demonstrate the scope of the correlation between protein charge and turnover.

(A) Molecular Weight Exclusion Chromatography:

Initial attempts to localize the cellular fraction(s) experiencing the greatest rate of turnover have been done previously with V. costicola (Hipkiss and Armstrong, unpublished results). In that particular study, long- then pulsed-labelled cells (i.e. double-labelled) were broken by sonication after a further 120 minutes incubation in an isotope-free 1.0 M NaCl medium. After subcellular fractionation the fractions were analyzed for changes in the $^{3}H/^{14}C$ ratio relative to similarly isolated and treated 0 time (post-

TABLE 15

V. costicola subcellular fraction versus change in $^{3}H/^{14}C$ ratio[a]

Subcellular fraction	$^{14}C/^{3}H$
12,000 X g (5') pellet[b]	0.79
30,000 X g (30') pellet	0.76
100,000 X g (120') pellet	0.71
100,000 X g (120') supernatant	0.94

a Hipkiss and Armstrong (unpublished results, 1978).

b Time in brackets refers to duration of centrifugation.

pulsing) cells. The double isotope technique employed was used to determine the relative turnover rates of different proteins. This method (as described in Materials and Methods) used two different isotopic forms of the same amino acid, ^{3}H-leucine and ^{14}C-leucine, to establish relative turnover rates of individual proteins. Rapid rates of turnover of individual proteins, after the long- then pulse-labelling periods, is seen as an increase in the change in the ratio of ^{3}H to ^{14}C.

In this preliminary double-label study, with V. costicola grown in 1.0 M NaCl, some evidence was found for greater turnover in the fraction sedimenting at 12,000 X g (Table 15). However, no high rate of turnover was found in material sedimenting at greater forces except for the supernatant remaining after 100,000 X g (for 120 min.). If the material that sedimented at 30,000 X g (for 30 min.) and 100,000 X g (for 120 min.) represents membranes and ribosomes, respectively, then these fractions do not contain rapidly turning-over protein.

As the 100,000 X g supernatant, which presumably contains soluble enzymes, exhibited the greatest rate of turnover it was decided to further analyze it for molecular weight of the proteins experiencing greatest breakdown. Characterization of the rapidly turning-over fraction in the 100,000 X g supernatant was carried out by passing the supernatants

Figure 9

Change in $^{3}H/^{14}C$ ratio

(during 120 min. post-pulsing period)

as determined by (Sephadex G-100) molecular

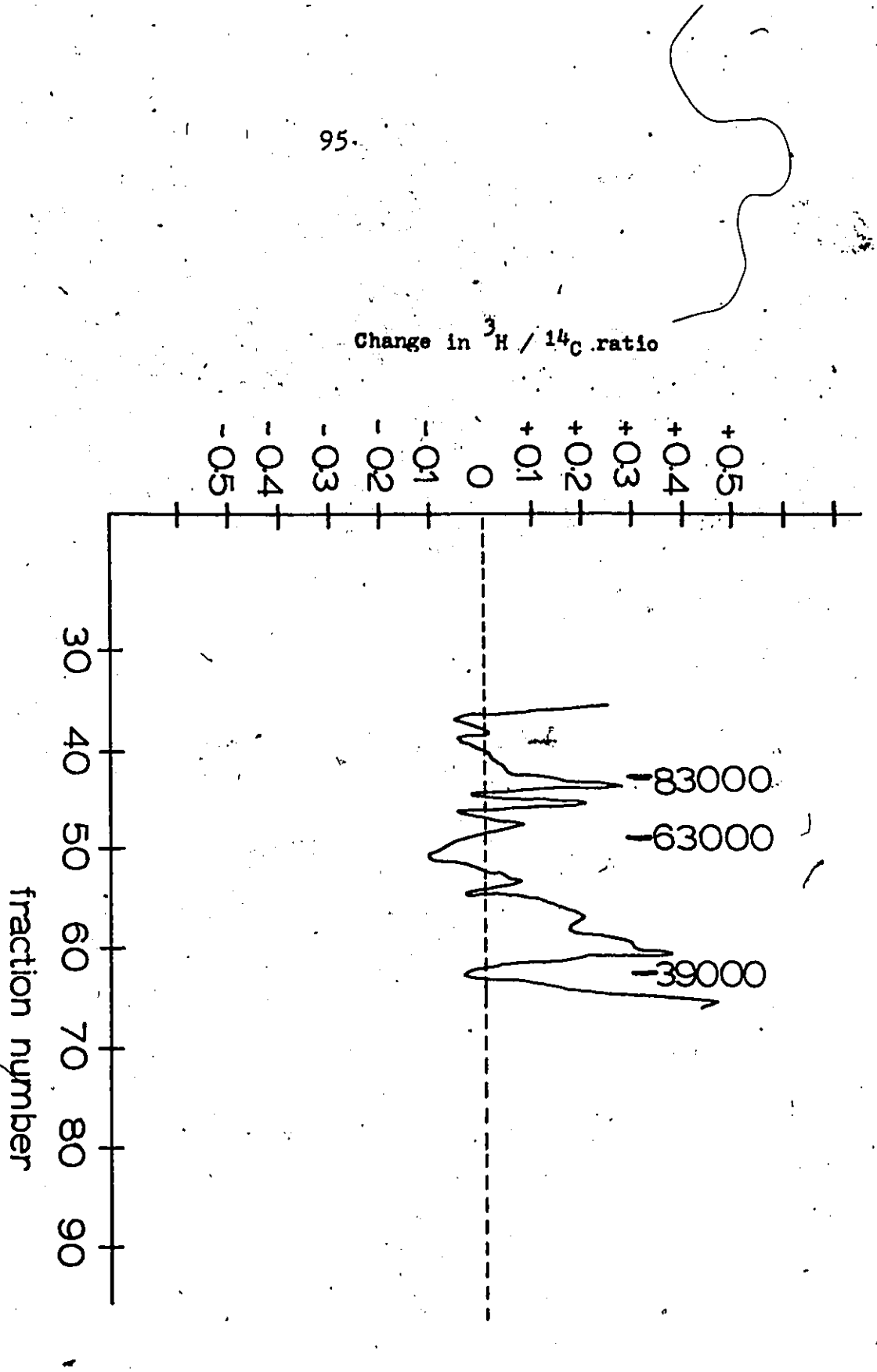
weight exclusion column chromatography

An increase in the ratio (i.e. greater than

0 base line) indicates a more rapid turnover rate

100,000 X g (after 120 min. centrifugation)

supernatant applied on column


Change in ^{3}H / ^{14}C ratio
-0.5
-0.4
-0.3
-0.2
-0.1
0
+0.1
+0.2
+0.3
+0.4
+0.5
30
40
50
60
70
80
90
fraction number
-83000
-63000
-39000

from long- and pulse-labelled cells, isolated at 0 or 120 minutes post-pulsing through a Sephadex G-100 column (samples applied as described for DEAE Sephadex chromatography) and comparing the changes in the $^{3}H/^{14}C$ ratios of each fraction. In order to establish the variability in ratio of different proteins due to experimental design, ^{14}C and ^{3}H leucine were added to V. costicola cultures simultaneously 150 minutes prior to final harvesting, washing and lysis. No systematic variation in the $^{3}H/^{14}C$ ratios were found in the protein fractions.

The results indicated (Figure 9) that the highest breakdown occurred in the proteins of molecular weight 45,000 - 60,000. Other increases in the ratio were evident in other higher MW fractions but a general trend was not obvious.

(B) Ion Exchange Chromatography (DEAE Sephadex):

The $^{3}H/^{14}C$ ratio technique was again utilized in this facet of characterization for protein charge determination.

The results (Figure 10) demonstrated that the protein fractions eluting in the acid range exhibited generally more elevated changes in the $^{3}H/^{14}C$ ratios compared to more neutral or basic proteins. It was noted, however, that these changes in the ratio were also evident in certain fractions eluting at neutral and basic pH values. The sample used (supernatant remaining after centrifugation for 120 minutes at 100,000

Figure 10

Change in $^{3}H/^{14}C$ ratio

(during 120 min. post-pulsing period)

as determined by (A-50 DEAE Sephadex)

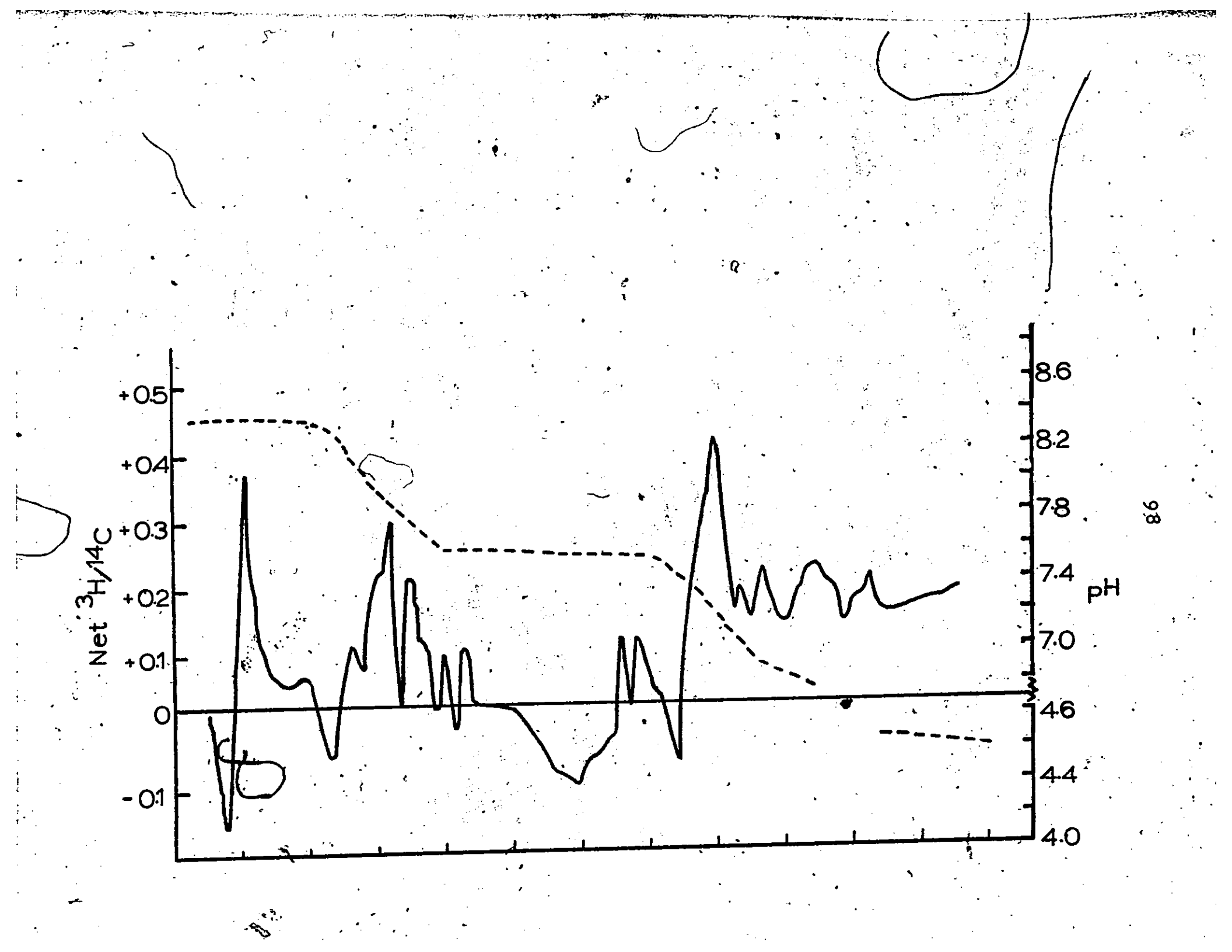
ion exchange column chromatography

Solid line — change in ratio

Dashed line — respective pH for each fraction

100,000 X g (120 min. centrifugation)

supernatant applied on column


+0.5
+0.4
+0.3
+0.2
+0.1
0
-0.1
Net $^3H/^{14}C$
8.6
8.2
7.8
7.4
7.0
4.6
4.4
4.0
pH

X g) presumably represents cytoplasmic soluble proteins although the cellular disruption by sonication may not exclude a membrane protein fraction which could have been solubilized in the process.

(C) SDS PAGE Migration and Turnover Rate:

Aliquots of a double-labelled, exponentially-growing culture of V. costicola, isolated at 0 and 120 minutes post-pulsing, were lysed (as described for DEAE-Sephadex chromatography) and the respective subcellular fractions separated by centrifugation. The subcellular fractions remaining after 30,000 X g (30 min.) and 100,000 X g (120 min.) were electrophoresed.

Table 16 A indicates that the protein fraction, MW 22,900 - 28,000, showed an increased turnover rate as indicated by a sharp change in the $^{3}H/^{14}C$ ratio. A general trend of increases in the ratios is also evident in protein fractions of greater MW, although not as pronounced as in the aforementioned protein fraction. The lower MW proteins of the 30,000 X g supernatant exhibited very low change in ratios indicating some level of stability against proteolytic attack. Unlike the latter subcellular fraction, the 100,000 X g supernatant showed a more heterogeneous pattern in turnover rates relative to the respective MW of the proteins.

TABLE 16

V. costicola subcellular fraction analysis

MW range of proteins	(A) Change in $^{3}H/^{14}C$ ratio [a]		(B) 30,000 X g sn ^{3}H long-label counts (dpm)		
	30,000 X g sn.[b]	100,000 X g sn.	0 min.[c]	120 min.	% change
37150 - 50000	0.21	0.05	360	380	+6
28000 - 37150	0.41	0.32	1340	270	-80
22900 - 28800	1.95	0.39	330	370	+12
17780 - 22900	0.14	0.36	310	350	+13
13800 - 17800	0.00	0.14	250	480	+120
10960 - 13800	0.00	0.50	370	210	-43
3160 - 10960	0.07	0.11	420	380	-10
1000 - 3160	0.18	0.21	520	400	-23

a $^{3}H/^{14}C$ ratios corrected for 0 time ratios of each respective fraction.

b sn. = supernatant.

c Time after end of "pulse" period when harvesting of cells was done.

In another experiment, utilizing a portion of the same culture of V. costicola, the cells were only labelled with ^{3}H leucine (150 minutes prior to harvesting). An aliquot of the cells was isolated immediately after this labelling period with subsequent lysis and fractionation. This was repeated with an aliquot isolated 120 minutes after the end of the long-labelling. This was done in order to give a direct indication of loss of label, from long-labelled proteins in each of the molecular weight ranges.

The results showed (Table 16 B) an 80 % loss of counts in the proteins of MW 28,800 - 37,150 and a 43 % loss of counts in the MW range 10,960 - 13,800 for the 30,000 X g supernatant.

Previous in vivo results in this present study have shown a generally lower rate of breakdown of long- versus pulse-labelled proteins. The direct approach (Table 16 B) of determination of loss of label from long-labelled proteins also shows a generally low level of loss; however, some exceptions were noted. As mentioned, a large decrease in dpm was especially prevalent in the 28,000 - 37,150 MW proteins. The data also clearly indicates that some protein fractions tend to pick up the breakdown products from other fractions. This factor, in double label studies, could have the potential to distort the change in the measured $^{3}H/^{14}C$

ratios observed. It is for this reason that only trends of increased turnover of proteins versus their respective characteristics (MW or net charge) were looked for.

In summary, the two different approaches of separating proteins, based upon their respective molecular weights, gave no strong evidence relating high molecular weight with a higher rate of turnover.

The degree of acidity, however, did show a general trend of a higher turnover for proteins eluting in the more acidic pH fractions.

DISCUSSION

DISCUSSION

Some of the "rules" describing the basis of bacterial life, which have become widely accepted, such as the stability of most cellular proteins during exponential growth, were originally derived from study of mesophilic organisms such as Escherichia coli.

Bacterial cultures, in general, in exponential growth, have low rates of protein turnover indicating that the proteins, on average, made during this period are stable, although some specific bacterial proteins can show rapid rates of turnover (Beeraj et al., 1978). Work with organisms existing in extreme environments, for example, thermophiles (Allen, 1953; Bubela and Holdsworth, 1966) has suggested that rapid breakdown and resynthesis may be a key mechanism of survival.

Previous studies of protein turnover have focused on bacteria growing in either mesophilic or extreme environments. This present study, using the bacterium V. costicola, is the first demonstration of protein turnover rates in a moderately halophilic organism. The question of the level of protein turnover and comparison between all microorganisms capable of growth in unusual environments may, in fact, be worthy of study. The accumulated data from these and future

studies may eventually help to solve the evasive questions relevant to protein turnover.

One unusual property of halophilic bacteria which makes them interesting for the study of protein turnover is the acidity of their proteins. The proteins of extreme halophiles, and to a lesser extent, many of these of moderate halophiles, are more acidic than those of non-halophilic bacteria (Wydro, 1977; Kushner, 1978). In E. coli and animal tissues acidic proteins are degraded more rapidly than neutral or basic ones (Dice and Goldberg, 1975; Turk and Marks, 1977).

It was of interest therefore to explore the process of protein turnover in the moderate halophile, Vibrio costicola, due to the nature of the environment required for growth and the unusual nature of some of its proteins as determined in other studies (Shindler, 1976; Wydro, 1977; De Médicis and Rossignol, 1977).

In the present study it was found that the overall rate of turnover in V. costicola is, in fact, substantially greater than that of E. coli (as found here and compared to published literature).

The variation in the extent of protein turnover in different experiments with V. costicola was disturbing. A possible explanation for this variation could be that the initial degradation of pulse-labelled proteins is always rapid (Pine,

1970). Therefore, any small day to day variation in the time of handling and washing the pulse-labelled cells could result in these changes in the "zero" time level of labelling and hence in the apparent turnover during extended incubation.

Even with the variability in the rates, it was consistently observed that turnover in V. costicola is always higher than in E. coli. This is apparently not a direct consequence of growth at the higher NaCl concentration. E. coli can also grow in the lower range of NaCl concentrations permitting growth of V. costicola (0.5 M and even 1.0 M NaCl) but under these conditions the protein turnover in E. coli is markedly lower than that of V. costicola. There was no consistent evidence that an increase of NaCl in the medium stimulated protein turnover in E. coli. The rates found in this study for E. coli at low salt concentrations (0.5 M NaCl or less), were found to coincide with those found by other researchers (Nath and Koch, 1970; Pine, 1972; Goldberg and St. John, 1976).

Experiments with V. costicola, grown at one NaCl concentration, then subsequently transferred to fresh medium with the same or altered NaCl concentration were undertaken to elucidate the effects of NaCl concentration on rates of breakdown. Various studies with V. costicola and other halophilic organisms (Brown, 1964a; Lanyi, 1974; Wydro, 1977; Rossignol, 1978) have demonstrated a need, by some proteins, for elevated levels of salt in the milieu for maximum stability and activity.

V. costicola when grown and transferred into 0.5 M NaCl approaching the lower salt concentration permitting growth of this organism, exhibits extremely high rates of breakdown (50 % higher than those found for V. costicola in an optimum NaCl concentration required for growth). Cells grown in or transferred to 1.5 M NaCl (either from 0.5 M or 1.0 M NaCl) were found to have the lowest rates of intracellular protein degradation.

In general, the results demonstrated that the rate of protein turnover in V. costicola is determined by the salt concentration in which the cells are subsequently incubated (during the "chase" period) rather than that in which they are labelled. Therefore, cells pulse-labelled in 1.0 M then transferred to 0.5 M NaCl experience the rate dictated by the lower salt concentration of the incubation medium. The reverse was observed for cells labelled in 0.5 M NaCl and transferred to 1.0 or 1.5 M NaCl. In all cases, the turnover of long-labelled proteins remained low. The ionic interactions, brought about by increased concentrations of NaCl, leading to concomitant stabilization of proteins may be the basis for the observations derived from the transfer experiments. Previous work by others (Brown, 1961; Goldberg et al., 1975; Schejter et al., 1978) have shown that NaCl can serve to stabilize various proteins. Perhaps the growth in or transfer to 0.5 M NaCl leads to an extensive disruption of thermodynamically stable ionic interactions in the proteins of V. costicola. The cells grown.

in 0.5 M NaCl seem to retain some elevation of turnover, relative to 1.0 M and 1.5 M NaCl-grown cells. There is a possibility that miscoding, brought about by translational errors or other factors due to the ionic concentration of the medium could be coming into play at all salt concentrations. This latter factor could be resulting in production of abnormal proteins. Although the possibility of miscoding could be used to explain the generally elevated levels of proteolysis relative to E. coli and other organisms, it cannot be used to explain the results of the transfer experiments. The immediacy of the change in turnover of the proteins upon transfer to a medium of altered salt concentration diminishes this latter possibility of miscoding. The major feature that may be affecting the rates of turnover is probably some physical requirement for the organism that is lacking, such as an adequate level of water activity.

The cell membrane, being the first major bacterial structural component to experience changes in the milieu, could suffer some protein structural abnormalities depending on the concentration of the ionic environment. Work by others (Shindler, 1976; Rossignol, 1978), as previously discussed, has demonstrated that this bacterium may have cell membrane-associated proteins with requirements for higher salt concentrations whereas soluble enzymes may require a lower ionic

milieu. Therefore, upon a shift from a high to a low ionic environment there may be disruption of structure, however, brought about, i.e. by loss of counter-ion shielding or loss of hydrophobic interactions.

Inhibitors, such as tretracycline and chloramphenicol, were used to explore the possibility that protein synthesis may be required for protein turnover. The possibility that proteolytic enzymes are synthesized as the need for the degradation of non-functional or abnormal proteins arises was investigated. Tetracycline and chloramphenicol, at the concentrations used in this study, were sufficient to completely or almost completely, (100 % and 90 % inhibition respectively) stop protein synthesis as measured by incorporation of ^{14}C radioactivity into the TCA insoluble fraction. Different results, however, were found with each respective antibiotic on the process of protein breakdown. Tetracycline was found to precipitate a marked inhibition of the process whereas chloramphenicol had virtually no inhibitory effects on breakdown. Thus, while being able to eliminate protein biosynthesis, to the extent discussed, these antibiotics exhibited vastly different properties with respect to inhibition of protein turnover. The results of tetracycline on this process in V. costicola agreed with those known for E. coli possessing high levels of abnormal proteins (Cashel, 1975; Golberg and St. John, 1976; Hipkiss, 1978). Although the results with chlor-

amphenicol differed, it has been suggested that this antibiotic does not always affect turnover in E. coli (Hipkiss, 1978). He suggested that this agent may be mainly affecting degradation of proteins with abnormal structure undergoing aggregation.

Turnover of protein, particularly those with abnormal structure, in E. coli, may be inhibited by agents which severely perturb the cell's energy-generating mechanisms (Schecter et al., 1973; Goldberg and St. John, 1976; Olden and Goldberg, 1978). Cyanide, azide (Olden and Goldberg, 1978) and iodoacetate (McKellar, 1976) while reducing the intracellular concentration of ATP by up to 80 %, do not inhibit proteolysis of normal proteins in E. coli (Olden and Goldberg, 1978) when glucose is present in the growth medium.

In E. coli it has been shown (Kowit and Goldberg, 1977) that the rapid breakdown of a single abnormal polypeptide required high energy phosphates for both an initial endoproteolytic cleavage and also for one or more subsequent proteolytic steps. Also it was demonstrated that the further degradation of small polypeptides released during turnover required this metabolic energy.

This present study investigated the energy requirement for protein breakdown in V. costicola by determining how specific impairments in energy production affected the turnover

of proteins. It was found that turnover in exponentially growing V. costicola was inhibited by cyanide but not by azide, dinitrophenol or iodoacetate. These agents inhibit the level of cellular respiration (cyanide and azide) or uncouple oxidative phosphorylation (2-4-dinitrophenol) or severely inhibited glycolysis (iodoacetate) in E. coli. The actual selectivity and inhibition levels of the different inhibitors used, however, are not clearly known in V. costicola. It has been observed, in E. coli (Goldberg and St. John, 1976; Olden and Goldberg, 1978), that inhibition of protein turnover by inhibition of energy production only acts if the intracellular level of ATP is almost completely abolished (85 - 90 % reduction). It is possible, therefore, that the agents, which did not inhibit turnover, only partially abolished the intracellular ATP level in V. costicola.

The results found in this study for cyanide concur with those found for E. coli, incubated with canavanine (an amino acid analog), with resultant high cellular levels of abnormal proteins. 2-4-dinitrophenol, an uncoupler of oxidative phosphorylation, did not affect turnover in V. costicola whereas in E. coli it does. In E. coli uncoupling agents are thought to inhibit proteolysis even in the presence of glucose, because they stimulate the hydrolysis of ATP derived from glycolysis via the (Ca^{2+} - Mg^{2+}) ATPase (Schecter et al., 1973). The role of this latter energy source and its relationship to

protein breakdown in V. costicola cannot be determined from the present type of study.

Olden and Goldberg (1978) have shown that in E. coli, in the absence of glucose with α-methylglucoside or cyanide or both of the latter two agents added, that the cellular levels of ATP are severely reduced (as low as 2 - 3 % of glucose incubated cells). These treatments are also found to markedly inhibit intracellular protein degradation in canavanine-treated E. coli. E. coli, when grown on succinate, and treated with cyanide, is subject to severe ATP depletion (over 90 % reduction) leading to a substantial reduction in the rate of turnover of abnormal proteins. E. coli grown under conditions favouring "normal" protein production, however, is not as severely affected by cyanide with respect to protein breakdown. The same experimental approaches with V. costicola revealed similar results to those observed in E. coli grown under conditions favouring production of high levels of abnormal proteins. Both cyanide and α-methylglucoside were capable of inhibiting turnover in V. costicola. Severe inhibition of protein breakdown occurred if both of the latter two agents were present. This was probably due to the simultaneous inhibition of respiration and glycolysis possibly coupled with exhausting the cellular high energy phosphate bonds. Succinate, while not allowing growth through glycolysis, does allow for respiration (therefore, maintaining an "energy-

rich membrane state") in E. coli. (Kowit and Goldberg, 1977). This substitution for glucose, in the medium supporting growth of V. costicola, led to reduction in the rate of turnover but not as severe as that found for the combined effects of eliminating respiration, by cyanide addition.

The present experiments do not reveal whether glycolysis provides the energy for the degradative process directly or whether glycolysis serves to maintain the "energy-rich membrane state" or respiration. More studies would be required to clearly define these latter two possibilities with respect to protein turnover in this bacterium. The present study may provide some evidence for the concept that ATP or some other high energy phosphate, whose levels may depend on the ATP concentration, is involved in intracellular protein breakdown in V. costicola. The experiments, however, while demonstrating some requirement for high energy phosphates, do not establish a direct role of these compounds for the hydrolysis of peptide bonds. Other alternative models could also explain this need for high energy phosphates. For example, these compounds may be required for continued protein synthesis which may be necessary for degradation. However, the hydrolysis of the proteins can take place in the presence of chloramphenicol which almost completely arrests protein synthesis in this bacterium. The metabolic energy may then alternatively be required for the activation of the responsible protease(s).

These high energy phosphates or some related factor may also be involved in the alteration of the conformation of the protein substrates thus rendering them more susceptible to hydrolysis by the cell proteases (as discussed in the Introduction).

In order to further elucidate the effects of inhibitors of either protein synthesis or energy generation, a cell-free system was attempted using the 30,000 X g supernatant fraction (presumably containing soluble enzymes and ribosomes but lacking, for the most part, cell membranes) incubated in a "reaction" medium (Materials and Methods). The attempt to produce protein degradation, in a cell-free preparation, was successful in obtaining a rate of breakdown 45 % of that found in whole intact cells of V. costicola. Tetracycline addition brought about a 40 % reduction of breakdown in the cell-free preparations (pulse-labelled cells) compared to greater than 70 % in whole intact cells. Cyanide, unlike in intact cellular preparations, brought about a slight increase in the rate of degradation of both pulse- and long-labelled cell-free preparations. The possible reasons for this cyanide-stimulated result will be discussed with the results of guanosine tetraphosphate effects. In the same cell-free preparations, the exogenous addition of ATP (A. R. Hipkiss, personal communication) did not affect the apparent rate of protein breakdown in V. costicola.

The establishment of this cell-free system is promising for use in future studies although it is still unknown whether this experimental system is exhibiting the same requirements as those of whole intact cells of V. costicola.

The effect of tetracycline and cyanide on streptomycin-induced, presumably, abnormal proteins, in V. costicola, was of interest in order to look for similar patterns of inhibition of protein breakdown relative to those seen in V. costicola growing under "normal" growth conditions. Figure 7 shows a marked increase in turnover of proteins presumably due to a higher cellular concentration of erroneous proteins. Tetracycline and cyanide both caused strong inhibition of the process of protein degradation as they did in cells without streptomycin pre-treatment (Table 8). Information gained from published results with E. coli, with high levels of abnormal proteins, and that found with V. costicola seems to suggest a naturally occurring elevated level of abnormal or structurally disrupted proteins, however biologically defined, in this bacterium at all salt concentrations.

The next facet of this study involved elucidating the effects of factors, known to be of some influence on protein turnover in other organisms, in V. costicola.

Guanosine tetraphosphate (ppGpp), as discussed previously, has been found to be directly correlated with the rate of protein turnover. Additions of this nucleotide to V. costi-

cola (10 mM - a concentration found in cells of E. coli undergoing stimulated rates of turnover (Block and Haseltine, 1974), caused a dramatic stimulation in the rate of breakdown in both 0.5 M and 1.0 M NaCl-incubated cultures. It is of interest that while both tetracycline and chloramphenicol affect protein synthesis, tetracycline also lowers the level of ppGpp by directly inhibiting the ribosomal enzyme responsible for its synthesis (Cashel, 1975; Goldberg and St. John, 1976). Since lower levels of ppGpp are associated with decreased intracellular proteolysis, it may be possible that the inhibitory effect of tetracycline is through such an action. It has been previously demonstrated (Edlin and Donini, 1971) that cyanide can cause accumulation of ppGpp in E. coli grown on a glucose medium. Possibly, a similar effect by cyanide could explain its actions, in vitro, in the cell-free preparation of V. costicola. Perhaps the inhibition of respiration by cyanide, in vivo, caused a severe inhibition of protein breakdown therefore, over-riding any stimulation due to ppGpp accumulation. The validity of cell-free preparations in assessing factors relative to protein turnover, however, still remains an open question.

Another factor which seems to be of importance in determining the rate of turnover of proteins, in growing cells, is the incubation temperature.

Thermal "melting curves" of proteins have been shown to

exhibit steep transitions from the folded to unfolded form over very short temperature intervals (less than 10°C) (Lehninger, 1975). This indicates that unfolding or denaturation is a cooperative process, in which the rupture of the first few weak bonds, hydrophobic interactions being one example, greatly increases the probability that the remainder will rupture.

Lowering the incubation temperature of V. costicola by 10°C does not significantly increase the respective generation times of 0.5 M or 1.0 M NaCl-grown cultures, but it does significantly affect rates of breakdown. At both salt concentrations there is an increase in the rate, especially in 0.5 M NaCl.

Many halophilic enzymes possess limited capability for hydrophobic bonding (Wydro, 1977; De Médicis and Rossignol, 1977; Rossignol, 1978) which may be severely affected by changes in the temperature of the environment. This factor, along with the possibility of cooperativity of bond breakage, may have some bearing on the resulting large increase in turnover of protein accompanied by the lowering of the incubation temperature. It was observed that the 1.0 M NaCl cells do not experience as great a stimulation in the rate of proteolysis as that found in 0.5 M NaCl. This may indicate that the combined effect of a lowered salt concentration and a concomitant lowered temperature is upsetting a delicate

balance of the ionic interactions and hydrophobic bonding required by some halophilic proteins. This could result in an increased structural abnormality and accessibility by proteolytic enzyme(s).

The influence of other agents, such as polyamines, was of interest due to the very high cell-associated concentrations of the three polyamines, spermidine, putrescine, and cadaverine in V. costicola relative to the concentration known for E. coli and other organisms (Igarashi, 1975). Their potential as stabilizers of various bacterial and mammalian subcellular structures was thought to be of some significance to protein turnover. In the present study the cellular levels of all polyamines, in 1.0 M NaCl, were found to be many-fold higher than those found in E. coli. It was of interest that the concentrations found in 3.0 M NaCl were essentially double those found in lower salt concentrations.

The inhibitory effects of putrescine, on the rate of intracellular protein degradation was noted, especially in the first 30 minutes of the 0.5 M NaCl-grown cells (43 % inhibition) relative to the 1.0 M NaCl-grown cells (5 % inhibition). These results may indicate a potential of polyamines as stabilizers of cellular protein charge interaction due to their polycationic nature, leading to counter-ion shielding concomitant with conformation stabilization. This stabilization could result in a lowered access to proteolytic enzymes which would

subsequently lead to a lower turnover rate. Possibly, their synthesis in higher concentrations, in this bacterium, is for this postulated reason.

Figure 8 shows that this bacterium, originally grown in 1.0 M NaCl, when changed from 0.5 M to 1.0 M NaCl exhibits similar patterns of turnover at different "change" time points. Upon reversal back to the higher NaCl-containing medium the cells experience a reduction in their respective rates of degradation. However, this rate is still found to be elevated, relative to the Control cells, in the "delayed change cells" at 15 or 60 minutes. The initial contact with the lowered salt may leave some proteins more susceptible to proteolysis, possibly due to conformational changes, even when changed back into a more optimum NaCl concentration for growth.

When V. costicola was transferred at different time points from a medium of higher NaCl concentration (1.0 M) to one of a lower concentration (0.5 M) interesting results were obtained. It was observed that following a delay in the transfer of the higher-salt cells to a medium with lower salts that a portion of the proteolytically susceptible proteins initially present were in some way made less susceptible to proteolysis. The decreased susceptibility of proteins to proteolysis upon "delayed" transfer into a lower salt concentration (0.5 M NaCl) may be due to allowance of time for assuming a more protected conformation. Longer pre-incubation in a more

optimal (1.0 M NaCl) salt concentration may allow for ions or other compounds, such as polyamines, to encourage maximum bonding, as previously discussed, concomitant with lowered accessibility to proteolytic enzyme attack and/or recognition of abnormality of structure.

The final goal of this study was to attempt a preliminary characterization of the proteins being turned-over at the greatest rate.

The generally elevated level of acidic residues found in many proteins of V. costicola, studied to date (Shindler, 1976; Rossignol, 1978) may have an important bearing on the high rates of protein breakdown in this bacterium. The different chromatographic separations of proteins based either upon their respective molecular weights or net charge has revealed a potential connection between an incidence of increased turnover and possession of a higher net negative charge for proteins in V. costicola.

In conclusion, although it has been shown that net charge may be a determinant of protein turnover, in V. costicola, it should be emphasized that other factors could influence the degradation rates of proteins. These factors may include the nature of the amino acid residues between the peptide bonds which are exposed, the conformation of the protein as affected by interactions with small molecules, and the extent to which proteins are in the associated or dissoci-

ated states. Further elucidation of the molecular characteristics of proteins experiencing the greatest turnover, in V. costicola, may result from different approaches such as work with isolated, individual, proteins.

OVERALL CONCLUSIONS

It is difficult to ascertain, in V. costicola and other moderate halophiles, the actual ionic concentrations in which different biological reactions take place (Kushner, 1978). Although some of the proteins of the outer layers are exposed to high NaCl concentrations of the external milieu, internal proteins may function in much lower concentrations. It has been shown that elevated salt concentrations can cause miscoding of in vitro protein synthesis in V. costicola (Wydro, 1977) and E. coli (Szer and Ochoa, 1964). Possibly, a certain proportion of ribosomes involved in protein biosynthesis is exposed to high enough ionic concentrations to cause miscoding. In this case, the high turnover rate could be a mechanism for correcting the erroneous proteins produced. Other studies, with a number of halophilic enzymes and proteins, especially of extreme halophiles, have shown a requirement for high salt concentrations to maintain a "native" configuration (Lanyi, 1971; Kushner, 1978). It seems possible, therefore, that even properly-made proteins are subject to conformational changes upon exposure to lower salt concentrations which may lead to increased hydrolysis. The highest rate of turnover of proteins occurred at 0.5 M NaCl, which approaches the lower limit of growth of V. costicola. The limit to growth at such

lower concentrations may in fact be due to the increased turnover of protein itself.

Transfer experiments between different salt concentrations indicated that the concentration of NaCl in the medium into which V. costicola is transferred determines the rate of degradation. If the higher rate of breakdown reflects errors in the synthesis of protein then such errors do not seem greater in 0.5 M NaCl than in higher salt concentrations. If they were, proteins from cells labelled at the lower salt concentration would probably still be degraded at a higher rate after transfer to a medium of elevated salt concentration.

The energy requirements for protein turnover, in V. costicola, coincide with those found for E. coli possessing high levels of induced abnormal proteins. This, along with elevated turnover rates, may indicate a higher level of abnormal protein formation under all NaCl concentrations, in V. costicola.

The correlations between either acidity or molecular weight of a protein and its susceptibility to enhanced turnover were tested. The results, however, were not the same or as clear-cut as those found for E. coli in published data (Goldberg and St. John, 1976; Turks and Mark, 1977; Kalish et al.,1979). Isolation and characterization of individual halophilic proteins, with respect to turnover rates, would probably lead to more conclusive evidence for or against these correlations.

In this moderately halophilic bacterium, it seems that a change in protein conformation brought about by a too low salt concentration, may stimulate the rate of turnover. Protein turnover, in V. costicola, is probably governed by an interplay between miscoding caused by too high, and conformational changes, caused by too low, salt concentrations.

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PROTEIN TURNOVER IN A MODERATELY HALOPHILIC BACTERIUM

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