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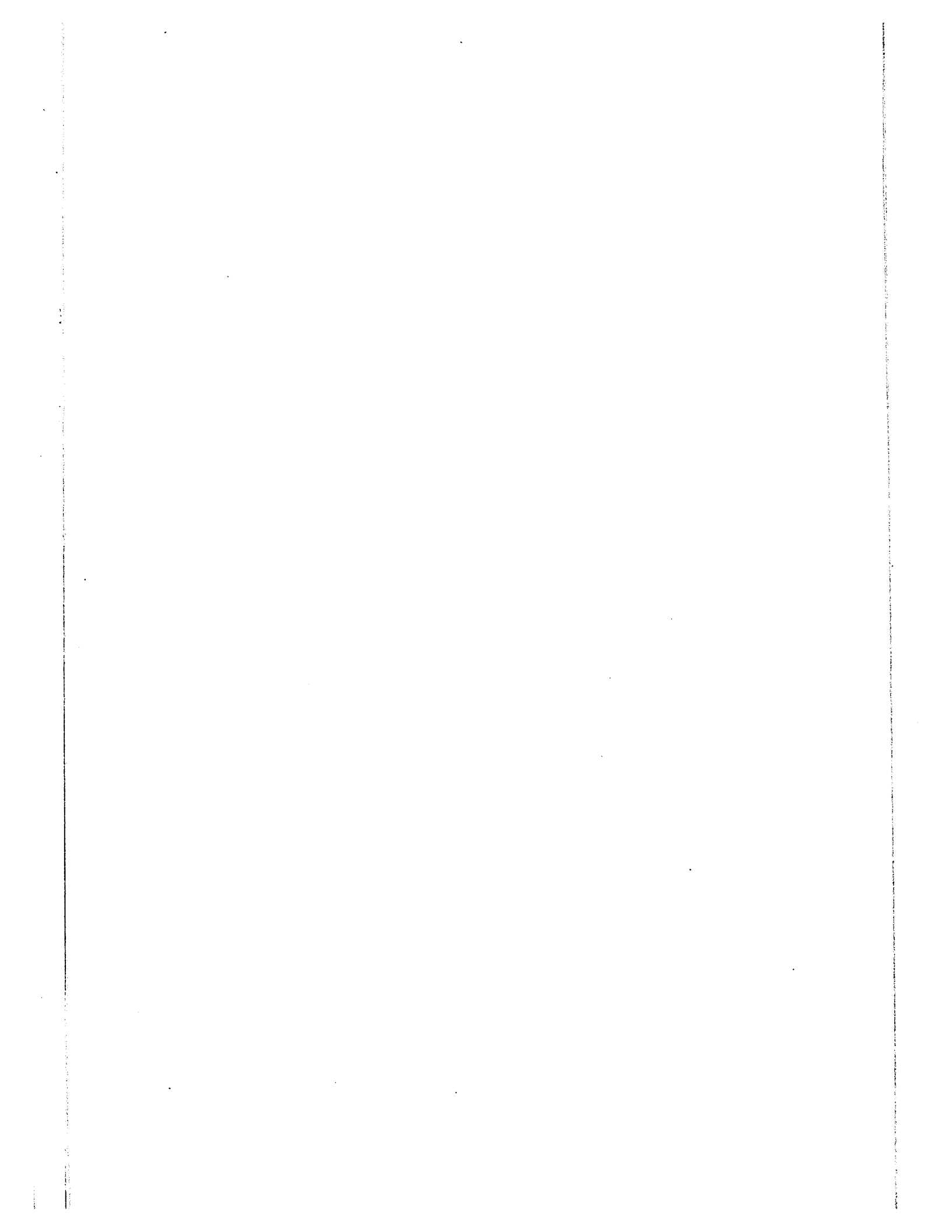
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PURIFICATION AND PROPERTIES OF HALOBACTERIUM  
CUTIRUBRUM L-ALANINE DEHYDROGENASE.

Thesis presented by  
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in partial fulfilment of the requirements  
for the degree of Master of Science.

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## I. ABSTRACT AND AIMS OF THE PROJECT

The purpose of this work was to purify and study an extreme halophile enzyme unrelated to nucleic acid metabolism in order to compare its properties with those of H. cutirubrum polynucleotide phosphorylase, DNA-dependent and RNA-dependent RNA polymerase, and alkaline phosphatase. The last four enzymes were found to be unusually small by Fitt and his collaborators and it was desirable to examine in detail a protein unrelated to nucleic acid metabolism. L-alanine dehydrogenase was chosen because its assay is straightforward and preliminary studies showed that it was highly active in crude extracts of H. cutirubrum.

The enzyme was purified approximately 100-fold by a simple procedure and found to have a molecular weight of 72,500, about one third that of the two well-studied alanine dehydrogenase from non-halophiles. It catalyses the oxidative deamination of L-alanine and L- $\alpha$ -aminobutyric acid to pyruvate and  $\alpha$ -ketobutyric acid, respectively, and the reductive amination of pyruvate and several other  $\alpha$ -keto acids.

An unusual feature is the absolute requirement of the enzyme for  $K^+$  for catalysis of the oxidative deamination reaction;  $Na^+$ ,  $Li^+$ ,  $Cs^+$ , and  $NH_4^+$  are unable to replace  $K^+$ . In contrast, in the reductive amination reaction the enzyme is fully active in the presence of high concentrations of  $K^+$ ,  $Na^+$  or  $NH_4^+$  and partially active with  $Cs^+$  or  $Li^+$  -  $NH_4^+$ , is, of course, an essential substrate in this reaction as well.

As in the case of most other halophilic enzymes, the L-alanine dehydrogenase requires a high salt concentration absolutely for stability and activity. The activity of the enzyme increases with temperature up to 70°C, but the protein itself is not thermostable.

## II. INTRODUCTION

### A. Historical Survey of Halophilic Bacteria.

Halophilic bacteria are unique organisms having a peculiar requirement for a high salt concentration. In contrast to the fact that the presence of large amounts of salt not only inhibits the growth of most familiar terrestrial microorganisms, but is also lethal to them, halophilic bacteria require at least 0.5 M salt in the surrounding medium for growth and survival. Much effort has been devoted to attempts to explain these unusual properties.

In a broad sense, marine bacteria which require between 0.005 M to 0.2 M NaCl for growth (MacLeod 1965); salt-tolerant bacteria which are able to grow in high salt concentration of 10% or more, but do not require them; and moderate and extremely halophilic bacteria, which have an absolute need for a high salt concentration for survival and growth could all be called halophilic, as the etymology of the word "halophilic" implies "salt-loving". However, the term is used more restrictively in practice (See p 5).

Since the turn of century, when several biologists recognized that the reddening of heavily salted protein products was caused by bacterial growth, the basis for the ability of

organisms to adapt to high concentrations of salt excited the interest of researchers. Early work was carried out by such scientists as Poulsen (1879), Farlow (1880), Kellerman (1915), Klebahn (1919), Harrison and Kennedy (1922), Sturges and Heideman (1924), Baas-Becking (1928), Petter (1931), Schoop (1935), Lochhead (1935), Hof (1935), Kluyver, Hof, and Boezaardt, Elarzari-Volcani (1940), etc. Baas-Becking stated that extreme halophiles are at the borderland of physiological possibilities (Larsen 1973).

Even though fairly detailed descriptions of these organisms and their relationship to salt were presented by the above mentioned scientists, studies on the biochemical basis for the salt requirement of extremely halophiles did not start until the work of Gibbons and Baxter (1953).

## B. Classification of Halophilic Bacteria.

Halophilic bacteria may be classified according to the level of their salt requirement. Thus Baxter and Gibbons (1956) divided most of the halophilic bacteria into one of two broad groups on the basis of the sodium chloride concentration ranges in which they would grow:

1) moderate halophiles which grow in concentrations of sodium chloride ranging from 1 or 2% (0.5 M) to about 20% (3.5 M);

2) extreme halophiles which will not grow unless the salt concentration is 15% (w/v) (3 M) or higher and will grow even in saturated brines with salt concentrations of about 31% (w/v) (5 M). Moderate halophiles include many different gram positive or gram negative bacterial species. Among these, Micrococcus halodentrificans and Vibrio costicola have been studied the most. Few moderate halophiles have pigments (Kushner 1968). Their classification seems to have caused less controversy than that of the extreme halophiles.

Reviews of taxonomy of halophiles by Flannery (1956), Ingram (1957), Larsen (1962, 1967, 1973), Brown (1964) and Kushner (1968), provide clear evidence that the taxonomy of extreme halophiles has been controversial, in part because the morphology of the organisms depends on the growth conditions and the handling of the bacterial cultures.

Colwell and Gibbons (1968) recently reassessed the taxonomy of extremely and moderately halophilic bacteria. They analyzed by computer (Colwell and Liston, 1961; Colwell, 1963; Qualing and Colwell, 1964) various features of 63 strains of extreme halophilic bacteria and 6 strains of moderately halophilic bacteria, which were isolated in different parts of the World from solar saltern saline lakes, salted cow and buffalo hides, salt fish and beans, sausage casings and ham-curing and bacon-curing brines. Some 185 different features were compared, including motility, staining, growth on various media, including those at different pH values and different salt concentrations, sensitivity to antibacterial agents, possession of specific enzymes, fermentative ability and other biochemical reactions. They concluded that many of those published as separate organisms in the literature among the published or classified were identical. Consequently, Gibbons proposed a major reclassification in the latest and Eighth Edition of Bergey's Manual of the Determinative Bacteriology (1974) compared with the Seventh Edition (1957).

In the Eighth Edition of Bergey's Manual, halophilic species are now all placed in the family Halobacteriaceae rather than being distributed amongst the Pseudomonadaceae or Micrococcaceae, a major simplification. The Halobacteriaceae have in turn been subdivided into two genera, Halobacterium and Halococcus. However, Gibbons recognized only two species of

Halobacteria, H. halobium and H. salinarium, in contrast to the five species previously listed. Therefore both classifications will be discussed.

In the eighth edition of Bergey's manual, Gibbons placed the genera Halobacterium and Halococcus under the same family of the Halobacteriaceae while, in the seventh edition, Breed et al. (1957), placed the genus Halobacterium under the Pseudomonadaceae and the halophilic cocci were placed in the family Micrococcus morrhuae.

Even though the halobacteria are some way related to Pseudomonas and the halophilic cocci to Micrococcus, the authors of the eighth edition argued that, based on the recognition of other characteristics such as the high ionic requirement for growth and maintenance of cell structure, cell wall composition, lipid content, major and minor DNA component and pigmentation, etc., halophilic rods and cocci should be assigned to the Halobacteriaceae, a new independent class under the taxonomical vernacular name Gram-Negative Aerobic Rods and Cocci.

However, in the Eighth Edition, Halobacterium salinarium and Halobacterium halobium were named as the only established species and Halobacterium trapanicum, Halobacterium marismortui and Amoebobacter morrhuae merely as uncertain species, incertae sedis, under the genus Halobacterium. In contrast to

the above, in the Seventh Edition, five species, H. salinarium, H. cutirubrum, H. halobium, H. marismortui and H. trapanicum were placed in the genus Halobacterium.

Even though the author of the Eighth Edition ruled that Halobacterium cutirubrum should be considered a subjective synonym of Halobacterium salinarium, agreement on this issue requires further study. Synonymity between H. cutirubrum and H. salinarium was based mainly on the fact that the proteolytic capacity of the original culture of H. cutirubrum had disappeared with ageing in the hands of reviewer and became similar to H. salinarium.

It is also interest to note that only one species, Halococcus morrhuae, was placed in the genus of Halococcus according to the Eighth Edition, while in the Seventh Edition three species, Micrococcus morrhuae, Sarcina littoralis and Sarcina morrhuae were recognized.

In this thesis, the nomenclature will be that of the Seventh Edition of Bergey's Manual, since it has been used in virtually all the published work on the biochemistry of extreme halophiles and examination of the current literature shows that Gibbon's proposals concerning the species of Halobacterium are far from being generally accepted.

C. Extremely Halophilic Bacteria.

1) General characteristics of the Halobacteriaceae.

Extreme halophiles are now placed in the family Halobacteriaceae. General features of these microorganisms, rods and cocci, are that they require high concentrations of sodium chloride (2M or over 15%) for growth and survival. Therefore, these organisms are found where NaCl and other required ions occur in adequate concentrations: in salterns some salt lakes, the Dead sea and proteinaceous material preserved with solar salts, such as fish, intestines (sausage casings) and hides.

These organisms are strict aerobes, growing on surfaces exposed to air or sun, in shallow layers of liquid medium or in well aerated liquid cultures. They grow well between 30° - 50°C, with an optimum around 40°C for the rate of growth and of about 35°C for cell production. The pH ranges for growth are between 5.5 - 8.0 with an optimum around 7.3. Growth is slow even under the best conditions, with a mean generation time of about 7 - 15 hours. Colonies on agar are small (2mm), round convex, translucent and pinkish mauve to bright red or vermilion in color. These organisms liquefy gelatin, are positive for oxidase and catalase, and produce H<sub>2</sub>S and thiosulfate from peptone and cysteine respectively. Proteins and proteoses are preferred for growth in complex media, which should be supplemented with at least 3M NaCl, plus Fe<sup>++</sup>, Mg<sup>++</sup>, and

K<sup>+</sup> (Gibbons, 1968). Most strains are sensitive to polymyxin, but not sensitive to aureomycin, chloromycetin, penicillin, and tetracycline (Bergey's Manual, 8th Edition, 1973). As will be discussed later in more detail, some strains of halophiles are capable of photophosphorylation mediated by a purple membrane containing a rhodopsin-like pigment (Henderson, 1977) and may possess vacuoles (Cohen-Bazier and Pfennig, 1969; Walsby, 1972).

## 2) Genus Halobacterium.

The genus Halobacterium consists of rod shaped microorganisms (0.6 - 1.0 by 1 - 6  $\mu$ m) that occur singly, but in deficient media they may be pleomorphic. These bacteria reproduce by binary fission and may be either nonmotile or motile (by means of a tuft of polar flagella). They are best observed by phase contrast microscopy.

They retain their rod shape in 3.5 M NaCl, but at lower NaCl concentrations the cells change shape and become spherical at 1.5 M owing to the loss of the cell wall. Below 1.5 M NaCl, the cell suspensions become viscous as a result of partial lysis and only few cells, if any, can be detected in 0.5 M NaCl. Maintenance of the cell structure is enhanced by MgCl<sub>2</sub> or CaCl<sub>2</sub> together with NaCl.

Brown and Gibbons (1955) described the requirement of a high concentration of Mg<sup>++</sup> ions for good growth. Their observation of transformation of the rod into the coccoid form in the absence of Mg<sup>++</sup> ion was further supported by Kushner (1964)

and others (Soo-Hoo and Brown, 1967) who found that lysis of the cells could be effectively prevented by  $Mg^{2+}$ . The halococci, on the other hand, do not lyse in hypotonic solutions. They require salt for growth, but not for maintenance of integrity.

Experimental evidence has accumulated showing that the intracellular salt concentration of the Halobacteria is indeed very high and reaches values comparable to that in the medium. the principal internal cation is  $K^+$  (Brown and Gibbons, 1955; Christian, 1956); this finding was rather unexpected since  $K^+$  is only a minor component of the media in which the cells are normally cultured. The very high intracellular  $K^+$  content of H. salinarium has been thoroughly confirmed by Christian and Waltho (1962), who found that when the bacterium was grown in a medium containing 4M  $Na^+$  and 0.032 M  $K^+$  the intracellular concentrations of  $Na^+$  and  $K^+$  were 1.4 M and 4.6 M, respectively. More recently, Lanyi and Silverman (1972) found intracellular concentrations of 1.63 M  $Na^+$  and 2.94 M  $K^+$  in H. cutirubrum cells grown in a medium containing 3.5 M NaCl and 0.027 M KCl. These differences between the internal and external  $K^+$  concentrations in these two halobacteria suggest that the cells possess active transport systems as well as the possibility of some selective permeability of their membranes towards different ionic species. The high intracellular concentration of potassium ion is required to maintain the structural integrity of the

ribosomes and for protein synthesis, and  $K^+$  is also one of the ions able to prevent the loss of the activity of the soluble enzymes of extreme halophiles.

The exterior cell surface of halobacteria has certain sites where  $Na^+$  acts most effectively in preventing leakage through the cell envelope, while the interior surface is equally well maintained by  $Na^+$ ,  $K^+$ , or  $NH_4^+$  (Christian and Waltho, 1962; Kushner, 1964; Soo-Hoo and Brown, 1967). Soo-Hoo and Brown (1967) indicated that the effects of  $Li^+$ ,  $Na^+$ ,  $K^+$  and  $NH_4^+$  on the morphological integrity of H. halobium showed that the monovalent cations protect the organisms with decreasing efficiency in the order mentioned. They also indicated that the effect of salt concentration on the integrity of the organisms is due to the sum of the electrostatic and osmotic effects, with the differential effect of these ions being due to their differing ability to penetrate the cell membrane.

The high extra and intracellular salt concentrations required by these organisms raises questions about the integrity of the cell envelopes and macromolecule components. The proteins of the extreme halophiles appear to be quite acidic in nature. This was first pointed out by Brown (1963) who found an excess of acidic over basic amino acids of about 15 mol % for the envelope protein of H. halobium. Such findings were confirmed by others (Kushner et al., 1964; Kushner and Onishi, 1966) by accurate analysis of the amino acid composition of the envelope of H. cutirubrum. Their data indica-

ted an excess of acidic over basic amino acids of about 14 mol % in the envelope of H. cutirubrum. Others (McClare, 1967; Marshall et al., 1969; Stoeckenius and Kunau, 1968; Steensland and Larsen, 1969) also confirmed the acidic nature of the cell envelopes of the genus Halobacteriaceae.

This phenomenon could be one of the explanation for the halophilic character of the proteins of these organisms in that the monovalent cations are needed to neutralize the excess of negative charges on the protein.

A similar picture has been found in an interesting study of the halobacterium ribosomes. The ribosomes of the halobacteria have much the same properties as the ribosomes of non-halophilic bacteria, and certainly the same functions. However, KCl (4M) and  $Mg^{++}$  (0.1M), are essential for the stability of the halobacterium organelles and the ribosomal proteins are also strongly acidic (Bayley and Kushner, 1964; Bayley, 1966; Vinsentin et al., 1972). Even though the amino acid composition of pure enzymes has not been shown to be strongly acidic, the behavior of the enzymes in salt gives a hint that one might also find many of these to be characteristically acidic. Analysis of the bulk protein isolated from the cytoplasmic, soluble, fraction of whole cells of various extreme halophiles indicated that it is strikingly more acidic than those from non-halophiles (Reistad, 1970).

Based on these findings the hypothesis was proposed that the dependence on or protective effect of salt is due to the shielding of negative charges of the acidic amino acid side chains in the enzyme or envelope proteins. It was presumed that, in the absence of salt, charge repulsion caused unfolding and loss of conformation or activity in the case of proteins or of integrity in the case of the envelopes.

Even though there is considerable evidence to support this view, Lanyi (1974) considered that the charge screening hypothesis alone was inadequate in several respects. Indeed, the salt concentration required by the cells or their isolated proteins is too high to support a charge shielding alone, because the latter should be complete with only a few tenths of molarity, as found in the case of well-studied charged molecules such as DNA (Inman and Jordan, 1960) and polyaminoacids (Ciferri et al., 1968; Wada, 1960). Therefore, without additional assumptions, the requirements of several molar salt concentration for the integrity of halophilic macromolecules or cells is not predicted by the counter ion hypothesis. Secondly, the specificity of the various cations, e.g. the greater effectiveness of  $Mg^{++}$  ion in the preserving the integrity of halophilic cells compared to

$\text{Na}^+$  , cannot be explained on the basis of charge shielding alone. Thirdly, in a highly charged macromolecule, such as polyglutamic acid at  $\text{pH} > 5$  , the screening effect of the added cation is more than counteracted by the lowering of the  $\text{pK}$  of the acid groups at high salt concentration resulting in destabilization of the secondary structure (Ciferri et al, 1968). As these arguments suggest, effects other than charge screening must also be considered if the phenomenon of halophilic behaviour is to be understood. Lanyi concluded in his review that, at high concentrations of salt, new hydrophobic interactions are formed, which had insufficient stability in water, and the molecule assumes a more tightly folded conformation. Finally local residues appear to be influenced by salt, both through changes in the overall structure and through direct effects on the residues.

One of the unique features of the halobacteria is their cell walls. Unlike the cell walls of other familiar organisms, those of the halobacteria do not contain diaminopimelic acid or muramic acid, which normally form the rigid back bone of these structures, but are composed largely of lipoproteins. Furthermore, the lipids are mostly non-saponifiable phospholipids and glycolipid derivatives of a glycerol diether. The di-ether linkage is unusual, and may be one of the unique features of these bacteria, as is the presence of dihydrophytol groups rather than fatty acids (Kates et al., 1966;

Kates, 1972). Ether-linked lipids in Thermoplasma acidophilum have been reported and may be an adaptation to extreme environments (Bergey's Manual, 1974).

Generally, halophilic bacteria have an aerobic oxidative metabolism. Nevertheless, under conditions of oxygen starvation, when the normal source of metabolic energy in the cell is cut off, halobacteria can use light energy by the special functions of the purple membrane formed in these conditions as specialized patches integrated within the cell membrane. These patches contain bacteriorhodopsin and function as a light driven proton pump (Stockenius and Rowen, 1967; Oesterhelt and Stoeckenius, 1973; Kushwaha et al., 1974) capable of providing a chemiosmotic gradient for oxidative phosphorylation. Thus, these bacteria possess a photophosphorylating apparatus some what parallel to the well-known chlorophyll based system of green plants and photosynthetic bacteria that provides the sustaining power for the life on earth. Further details of the purple membrane will be discussed later.

Lipid synthesis is by the mevalonate pathway rather than the familiar malonate pathway (Kates et al., 1968) and leads to the synthesis of isoprenoid chains, which may fill the same function as the unbranched hydrocarbon chains in other organisms. A very low, or possibly zero, content of higher fatty acid residues therefore seems to be a common feature of the cell envelope of the extreme halophiles which differ in this respect from other bacteria.

The DNA of these organisms consists of a major and minor component. The latter may comprise as much as 10 - 30% of the total cellular DNA and this high proportion of satellite DNA appears to be unique to the halobacteria. The G + C content of the major and minor components are in the range 66 - 68 mol. % and 57 - 60 mol. %, respectively, based on buoyant density measurements (Joshi et al., 1963; Moore and McCarthy, 1969 a).

The better studied extreme halophiles are chemoorganotrophs that satisfy their energy requirement by the metabolism of amino acids. Extreme halophiles obtain most of their energy by aerobic oxidation of amino acid side chains and not by a fermentative one. Carbohydrates are used only slightly in most cases, if at all, and acid is not produced in sugar-containing media, which become alkaline as a result of the deamination and or decarboxylation of amino acids. Some carbohydrates, and related compounds such as glycerol, were reported to stimulate growth under certain conditions (Gochnauer and Kushner, 1969). Recently (Tomlinson et al., 1974; Tomlinson and Hochstein, 1976), a carbohydrate metabolizing extremely halophilic bacterium was reported. This organism was isolated from a mixture of mud and brine obtained from a saltern in the southern San Francisco Bay and the name Halobacterium

saccharovorum was proposed for it. The organism was shown (Tomlinson et al., 1974) to degrade glucose to gluconic acid and then to pyruvate and 3-phosphoglyceraldehyde via a modified Entner-Doudoroff pathway and appeared to lack both glucose-6-phosphate and 6-phosphogluconate dehydrogenases. However, no evidence is available to indicate if this pathway is present in other extreme halophiles.

It has been shown that the enzymes of the extremely halophilic bacteria are extremely salt-tolerant or in most of the cases even strikingly halophilic. With the knowledge that the total salt concentration inside the cells is close to saturation, many investigators have attempted to explore the function of the metabolic systems of these organisms. Egami (1955) proposed that the enzymes that require high salt concentrations for activity and stability be called halophilic and this terminology is now generally used. The following enzymes are amongst those that have been studied; aspartate-glutamate transaminase (Robinson and Katznelson, 1953), glycerol, isocitrate, succinate, malate, and lactate dehydrogenases, cytochrome oxidase and cysteinedesulphydrase (Baxter and Gibbons, 1954, 1956 and 1957; Baxter, 1959), malate dehydrogenase (Holmes and Halvorson, 1965 a,b; Mevarech et al., 1977; Mevarech and Neumann, 1977), nicotinamide adenine dinucleotide oxidase (Hochstein and Dalton, 1968, 1973; Hochstein, 1975), aminoacyl t-RNA synthetases (Griffiths and Bayley, 1969),

cytochrome oxidase (Cheah, 1970), malic enzyme and citrate synthase (Cazzulo, 1973), polynucleotide phosphorylase (Peterkin and Fitt, 1971), DNA-dependent RNA polymerase (Louis and Fitt, 1971 a,c, 1972, a,b; Fitt et al., 1975). RNA-dependent RNA polymerase (Louis and Fitt, 1971 b, 1972 c) and alkaline phosphatase (Fitt and Peterkin, 1976), etc. Thus over 30 different enzymes have been studied, from both the halobacteria and halococci and in most of the cases studied they are found to function at NaCl concentration approaching saturation (Larsen, 1973).

Baxter and Gibbons (1954, 1956, 1957) tested the effect of a number of salts other than NaCl on the enzymes of H. salinarium. It appeared that several monovalent cations other than  $\text{Na}^+$  could activate halophilic enzymes and that  $\text{Cl}^-$  could be replaced by  $\text{Br}^-$  and  $\text{NO}_3^-$ . They also found that, in many cases, the enzymes were about twice as active in the presence of KCl as in the presence of NaCl at the same molar concentrations. The observation that many extreme halophile enzymes are more strongly activated by KCl than by NaCl fits nicely with the finding that  $\text{K}^+$  is the dominant cation inside the cells and thus can be postulated to be an activator of the enzymes in vivo. In contrast, Norberg and Hofsten (1969) have shown that the extracellular protease of H. salinarium, that has to act in the culture medium containing a concentration of Na much higher than that of  $\text{K}^+$ , is preferentially activated by NaCl.

The function of the sodium and potassium chloride is not limited to activation of the enzymes. They also fulfill an essential function as stabilizers. It has been shown in numerous experiments that when salt is removed from the enzyme preparations, they tend to become irreversibly denatured quite rapidly (Baxter and Gibbons, 1954, 1957, 1959; Kushner, 1968; Cazzulo, 1975).

Holmes and Halvorson (1963, 1965 a) showed that H. salinarium malate dehydrogenase that had been inactivated by dialysis against water could not be reactivated by addition of solid NaCl to the solution. However, if the salt-free preparations were dialysed against 25% NaCl, so that they were gently exposed to an increase in the NaCl concentration, the enzyme regained activity partially, even if it had been kept in a salt free environment for a relatively long period of time (Holmes and Halvorson, 1965 a). Hubbard and Miller (1969) found that the isocitrate dehydrogenase of H. cutirubrum could be reactivated in a similar manner but this property is not typical of extreme halophile enzymes, which are frequently inactivated irreversibly at low ionic strengths in the absence of their substrates. Other studies indicated that inactivation by removal of the salt can be prevented, in some cases at least, by the presence of substrates or cofactor- (Holmes & Halvorson, 1965 b; Hubbard & Miller, 1969; Cazzulo, 1973, 1975; Higa and Cazzulo, 1975). The inactivation of halophilic enzymes at low ionic strengths may expose essential thiol groups.

Hubbard and Miller (1970) indicated that over 90% of the initial activity can be restored by dialyzing the inactivated isocitrate dehydrogenase against 4 M NaCl, provided that the exposed sulfydryl groups were protected with dithiothreitol and Holmes and Halvorson (1965 b) found that the inactivated malate dehydrogenase was more sensitive to thiol-specific reagents than the native enzyme.

The slow recovery of activity during dialysis suggests that the reactivation is not a simple process and that it may reflect a salt-dependent conformational change in the molecule. Presumably, some critical amino acid residues in the secondary structure of protein play a role. This view is supported by the observed protection of the inactive enzymes by thiol compounds or substrates and by the pH dependence of the reactivation process (Cazzulo, 1975).

There are a few reports of exceptions to the general rule that the enzymes from the extreme halophile are inactive at low ionic strengths (Larsen, 1962; Pugh et al., 1971). In particular, Pugh et al. (1971) found that the fatty acid synthetase of H. cutirubrum was strongly inhibited by high salt concentrations. They also found that the malonyl-Co A; acyl carrier protein transacylase, which catalyzes the first step in the fatty acid biosynthetic pathway, was also strongly inhibited by salt. These results agree with their previous studies (Kates et al., 1968; Kates et al. 1970) showing that these bacteria contain very little fatty acid.

A very interesting feature of some enzymes isolated from H. cutirubrum is their unusually low molecular weight. Fitt et al. have described four such cases: polynucleotide phosphorylase, mol. wt. 11500 - 12500 (Peterkin and Fitt, 1971); DNA-dependent RNA polymerase, with two subunits each of mol. wt. about 18000 (Louis and Fitt, 1971 a, c; 1972 a,b; Fitt et al., 1975); RNA-dependent RNA polymerase, mol. wt. 17000 - 18000 (Louis and Fitt, 1971 b, 1972 c) and an alkaline phosphatase, mol. wt. about 15500 (Fitt and Peterkin, 1976). These mol. wts. are from one quarter to one twentieth those of the corresponding non-halophile enzymes and, in the case of the first three enzymes, were determined by analytical gel filtration and sucrose density gradient centrifugation at high ionic strength (Louis et al., 1971). These results provided the first evidence in support of the suggestion made by Ingram (1947) that extreme halophile enzymes might have to be unusually small in order for their enzymes to be soluble at the high ionic strength found in the bacteria.

Many investigators have shown that the halobacteria have a textured surface with a regular hexagonal pattern (Houwink, 1956; Mohr and Larsen, 1963; Kushner and Bayley, 1963; Kushner et al., 1964). A report (Brown and Shorey, 1962) based on a study of thin sections of H. halobium indicating that the cell

was surrounded only by a unit membrane without a rigid tough outer wall and that at low salt concentrations this cell membrane disintegrated into identical subunits of lipoprotein was controversial. Subsequently, Stoeckenius and Rowen (1967) found that the H. halobium do not lack a cell wall, but they confirmed the existence of the identical subunits. They found that the way the membrane fragmented was determined primarily by the proteins in it rather than by the lipids, and that fact suggested some interesting possibilities for exploring the relation between structure and function in the membrane (Stoeckenius and Rowen, 1967; Stoeckenius and Kunau, 1968).

It is now widely accepted that three distinct main fractions of halobacterium membrane can be recognized by their colors: a low density orange-red fraction, a denser purple one and a still denser yellow one.

The red fraction, which constitutes the bulk of the membrane, contain fragments that differ widely in size and composition. Its color was due to a high content of 50-carbon carotenoids together with a pigment bacterioruberin, that apparently protects the cells against the lethal effect of the high intensity blue light to which they are exposed in their natural environment. The red fraction also contains cytochromes, flavo-proteins and other components of the respiratory chain.

The yellow fraction consists almost entirely of the walls of the gas vacuoles of H. halobium, small gas filled sacs in the cytoplasm that presumably help the cell to float at a certain depth. It also includes bits of the flagella that propel the bacterium, which are attached as two bundles at the ends of the cell.

The purple fraction turned out to be mostly protein; its low lipid content, only 25%, accounted for its high density. Electron micrographs and x-ray diffraction studies (Blaurock and Stoeckenius, 1971) indicated that the purple membrane material had a well defined structure, approaching that of protein crystals in regularity, and that the fraction was not distributed throughout the membrane, but was present in the intact cell as discrete patches set into and continuous with the cell membrane. Finally Oesterhelt and Stoeckenius (1971) demonstrated that the purple membrane contained only one species of polypeptide with an apparent molecular weight of 26,000 and that the characteristic purple color was due to retinal, vitamin A aldehyde, bound in a 1 : 1 ratio via a Schiff base linkage between its aldehyde group and an amino group on certain amino acid residues, specifically lysine, of the protein.

The purple color shift of the supposedly colorless retinal was explained by the particular conditions under which the retinal is complexed with its protein: the amino acids to which

it is joined appear to form a hydrophobic pocket that shields the Schiff's base from the aqueous environment. Thus, it was confirmed that when this special conformation of the protein is destroyed by a solvent or a detergent, the absorption maximum does shift from about 570 nm to 370 nm, and then the retinal is released from the protein. (Oesterhelt and Hess, 1973; Oesterhelt and Stoeckenius, 1973; Stoeckenius and Lozier, 1974).

Finally, Oesterhelt and Stoeckenius (1973) proposed that the purple membrane has a definite function as a light-driven proton pump that creates an electrochemical gradient across the membrane which can be used by the cells for ATP synthesis, along the lines of Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1966). The large concentration of purple membrane in the cells provides further proof that it drives some metabolic process, rather than merely triggering a sensory response such as phototaxis. Under optimal growth conditions, nearly half of the total surface area of the cells may be occupied by purple membrane (Oesterhelt and Stoeckenius, 1973).

Therefore, a firm belief has been established that the purple membrane functions as a light-energy transducer on the following basis:

- 1) Cells synthesize purple membrane when their normal supply of metabolic energy is interrupted, e.g. when they are illuminated in anaerobic conditions (Oesterhelt and Stoecknius, 1971).

2) Protons are released from purple membrane sheets when they are bleached to the 412 nm form by light and the cyclic light-driven reaction occurs over and over again until the light is switched off (Oesterhelt and Hess, 1973).

3) Light reduces the pH of suspensions of cells containing purple membrane and uncouplers abolish the effect (Oesterhelt 1972).

4) Oxygen consumption by respiring cells is reduced by light if the cells contain purple membrane (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Krippahl, 1973).

Further support for the proposed function of the purple membrane has accumulated steadily, so that the role of bacteriorhodopsin as a light-driven proton pump seems to be proven beyond doubt (Henderson, 1977). Purple membrane fractions from several other pigmented extremely halophilic bacteria including H. cutirubrum have been reported (Gochnauer et al., 1972; Kushwaha and Kates, 1973; Kushwaha et al., 1974; Kushwaha et al., 1975).

The bacteria grow well in complex media such as that devised by Sehgal and Gibbons (1960) and subsequently improved by Gochnauer and Kushner (1969). In addition, several attempts were made in the past to establish chemically defined media (Dundas et al., 1963; Onish; et al., 1965; Gochnauer and Kushner, 1969; Grey and Fitt, 1975).

Weber (1949) found that gelatine hydrolyzate was required for growth. Katznelson and Lochhead (1952) found that growth

was stimulated by yeast extract and its ash, but that vitamins, purines or pyrimidines were not required for the growth of red halophiles. Brown and Gibbons (1955) showed that the stimulation by yeast extract ash was the result of its potassium content. Sehgal and Gibbons (1960), in further growth studies, found that ferrous ions, 20-50 ppm, were stimulatory, but that ferric ions had very little effect. They also demonstrated that trace amounts, 0.05 ppm, of manganous ion stimulated growth, but that excess  $Mn^{2+}$ , 0.5 ppm - 2 ppm, inhibited growth. Dundas et al., (1963) tried to devise a better, chemically - defined medium. They found that valine, methionine, isoleucine and leucine were essential amino acids among 10 amino acids they studied for the growth. Onishi et al (1965) then developed a synthetic medium containing 15 amino acids, adenylic and uridylic acids, glycerol, ammonium chloride and various salts. They concluded that almost all the 18 amino acids were required for good development. The lag phase prior to rapid growth could be reduced by the addition of either asparagine (1-2%), glutamine (1-2%) or ammonium chloride (0.5%). They speculated that the requirement for a high concentration of asparagine, glutamine or inorganic ammonium ion even in the presence of glutamic acid or aspartic acid indicated that they might play a role in the synthesis and organization of the envelopes. The enhancement of growth by ammonium ion is very interesting in connection with the subject of

this thesis, since L-alanine dehydrogenase catalyses L-alanine formation from pyruvate and ammonium ion.

The stimulatory effect of glycerol is another interesting nutritional characteristic of halophilic bacteria. Dussault and Lackance (1952) noted that glycerol caused both an increased growth rate and a deeper pigmentation of the cells. Glycerol is incorporated readily into the diphosphatidyl, glycerol of the cell envelope. They also indicated that four nucleotides, adenylic, guanylic, uridylic and cytidylic acid stimulated growth.

Grey and Fitt (1975) developed a synthetic growth medium for H. cutirubrum which contains 15 amino acids along with  $\text{Ca}^{++}$ ,  $\text{Cu}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{K}^+$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{KNO}_3$ ,  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Na}^+$ ,  $\text{Zn}^+$ , sodium citrate and glycerol. This synthetic medium, which was developed by modification of the medium of Onishi et al (1965), differs from the latter in the following respects:

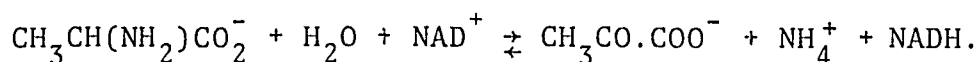
The potassium concentration is that recommended by Gochnauer and Kushner (1969) while the pH of 6.6 is close to that used by these authors, as opposed to the value of 6.2 for the original medium of Onishi et al.; the phosphate concentration is three times higher; only L-amino acids are used; and  $\text{NH}_4\text{Cl}$  and nucleotides are omitted. According to the results of Grey & Fitt (1975), nucleosides or nucleotides have no significant effect on the growth of H. cutirubrum

in the presence of sufficiently high concentration of orthophosphate. They claimed that the nucleotides used by previous authors merely served as an additional source of phosphate in media deficient in this component. They also indicated that there was a lag phase of several hours before the start of exponential growth when the initial pH was 6.2.

Thus the findings of Grey & Fitt (1975) support the claim of Katznelson and Lochhead (1952) that the extreme halophiles do not require vitamins, purines, or pyrimidines and the demonstration by Gochnauer and Kushner (1969) that a relatively high potassium concentration increase stimulates their growth. However, the findings of Gochnauer and Kushner that effective growth is promoted by galactose, glucose, lactate, succinate, and the vitamins thiamine, folic acid, biotin and B12 merit further study, since Grey & Fitt (1975) found that growth in their medium which lacks these components was as good as in the complex medium of Gochnauer & Kushner (1969).

#### D. Alanine dehydrogenase

Alanine dehydrogenase (L-alanine: NAD oxidoreductase (deaminating) E.C. 1.4.1.1.) was first isolated from Bacillus subtilis (Wiame & Piérard, 1955; Piérard & Wiame, 1960) and shown to catalyse the reversible NAD<sup>+</sup>-dependent, oxidative deamination of L-alanine:



Subsequently, the enzyme has been detected in Mycobacterium tuberculosis (Goldman, 1959), Bacillus cereus (O'Connor & Halvorson, 1960), Neurospora crassa (Bürk & Pateman, 1962), Halobacterium salinarium (Holmes et al. 1965), Aspergillus niger mycelium (Kretovich et al., 1964, Demina et al., 1966) Bacillus subtilis (Yoshida & Freese, 1964), Lactobacillus plantarum (Galyas et al., 1966), Desulphorribrio desulphuricans (Germano & Anderson, 1968), Chlorella pyrenoidosa (Shatilov et al., 1968), Streptomyces erythreus (Roszkowski et al., 1969), Lemna Minor (Joy, 1969), Spinach chloroplasts (Tsukamoto, 1970), Chlorella (Kretovich et al., 1970), Bacillus licheniformis (Meers & Pedersen, 1972), blue-green algae (Neilson & Duodoroff, 1973; Batt & Brown, 1974) and Myxococcus xanthus (Kottel et al., 1974).

The substrate specificity of the 60-fold purified B.cereus L-alanine dehydrogenase has been studied (O'Connor & Halvorson, 1961) and the molecular weight of the 200-fold purified enzyme was shown to be 248000 (McCormick & Halvorson, 1964). Yoshida

& Freese (1964) purified the B. subtilis enzyme to homogeneity and crystalized it: it had a molecular weight of 228000 and appeared to consist of six identical subunits (Yoshida, 1965). The enzymic properties of B. subtilis L-alanine dehydrogenase were very similar to those of the B. cereus enzyme (Yoshida & Freese, 1965). The alanine dehydrogenase from Desulfovibrio desulfuricans was purified approx. 56-fold and it was found to be absolutely specific for L-alanine and NAD (Germano et al., 1968).

The alanine dehydrogenase of H. salinarium (Holmes et al., 1965) has not been purified, but during the course of the work described in this thesis, Keradjopoulos & Wulff (1974) reported the interesting observation that in crude extracts the enzyme appeared to have an unusually high optimum reaction temperature and they referred to it as thermophilic. They also indicated that their crude enzyme could be reactivated by dialysis to high ionic strength following inactivation at low ionic strength, provided 2-mercaptoethanol was always present during both inactivation and reactivation.

The enzyme appears to play an important role in alanine utilization or nitrogen assimilation by various organisms.

Fairhurst et al. (1956) studied amino acid biogenesis, specifically the synthesis of alanine from pyruvate and ammonia, in Bacillus subtilis. They observed that alanine was more rapidly formed when ammonia itself rather than aspartate or glutamate was the amino donor and that neither oxaloacetate nor

$\alpha$ -ketoglutarate were as rapidly aminated as pyruvate. They considered the possibility that the conversion of pyruvate to alanine might proceed directly rather than via transamination. They also pointed out that a wide range of organisms, when grown on glucose, liberate alanine, but no other amino acid, into the medium.

O'Connor & Halvorson (1960) indicated that the germination of bacterial spores has emerged as one of the best characterized examples of trigger mechanisms in biology. They also pointed out that the germination process appears to have an enzymic basis as Hills (1949) found that contact with L-alanine leads to rapid germination of aerobic spores. They demonstrated that the conversion of L-alanine to pyruvate and  $\text{NH}_3$  during the initial stage of germination of spores of Bacillus cereus strain T, and the disappearance of L-alanine during the germination, could be dependent upon oxidative metabolism. In a subsequent paper (O'Connor & Halvorson, 1961), they indicated that the broad specificity of alanine dehydrogenase from the spores of Bacillus cereus strain T suggest that this enzyme may play an important part in the synthesis and, in certain cases, the oxidation of aliphatic amino acids. They conceded the fact that the unfavorable equilibrium for the deamination of pyruvate catalysed by alanine dehydrogenase would suggest that this reaction might not be expected to contribute to amino acid oxidation. Thus, the identification of alanine dehydrogenase as the primary route of L-alanine deamination is

a paradox since alanine and glutamic acid dehydrogenases are generally believed to be the primary route of  $\text{NH}_3$  assimilation in microorganisms. However, they pointed out that the system capable of maintaining a high  $\text{NAD}/\text{NADH}_2$  ratio that results from recycling of NADH by NADH oxidase in spores can overcome this unfavourable equilibrium and may be sufficient to drive the deamination reaction. They indicated that spores of B. cereus strain T have an active NADH oxidase stimulated by the addition of dipicolinic acid to crude extracts and that this system stimulates the rapid attainment of an equilibrium condition in which the alanine can be deaminated. They also indicated that intact spores deaminated seven amino acids, all of which are substrates of alanine dehydrogenase while thirteen non-substrates of alanine dehydrogenase are not deaminated. McCormick & Halvorson (1963) found that the alanine dehydrogenase activity in spores could be increased by growth in a sporulation medium to which L-alanine was added. In spite of the fact that L-alanine dehydrogenase favors L-alanine synthesis and in vivo this enzyme undoubtedly serves as the primary route of L-alanine synthesis, they found that enzyme level is increased by the addition of L-alanine to the growth medium. Therefore, they stated that the apparent physiological product of the reaction, L-alanine, induces (or derepresses) enzyme synthesis rather than represses its formation. They found that the effect of alanine is expressed during the sporulation process but does not appear to influence during vege-

tative growth.

Freese et al. (Freese, E. & Oosterwyk, J. 1963; Freese, E. Park, S.W. & Cashel, M. 1964) also suggested that a factor restricting the excess formation of L-alanine seems to be the concentration of NADH. They indicated that, even though the reaction constants of L-alanine dehydrogenase greatly favor the formation of L-alanine from pyruvate, internal repression of alanine dehydrogenase appears useful to the cell for two reasons: first it prevents the excessive drainage of pyruvate into non-energy-producing pathways, second it avoids the accumulation of L-alanine which, for some unknown reason, inhibits growth.

Berberich et al. (1968) also suggested that the physiological role of the enzyme is to catabolize Bacillus subtilis L-alanine to pyruvate and ammonia. They showed the induction of L-alanine dehydrogenase by L-alanine and its analog amino acids. According to their experimental results, a mutant deficient in alanine racemase, neither L-alanine nor any of the other L-amino acid inducers can induce L-alanine dehydrogenase. However with the above mutant, L-ADH enzyme could be induced by D-alanine or other D-amino acid inducers. They speculated that the L-amino acids induce L-alanine dehydrogenase only if they can be converted first L-alanine to D-alanine by alanine racemase. The pattern of control observed illustrates a new form of end product regulation, whereby D-alanine regulates its own biosynthesis via alanine racemase by the induction of L-alanine dehydrogenase.

They concluded L-alanine dehydrogenase catabolizes L-alanine, and thereby limits the amount of L-alanine available to alanine racemase for the synthesis of D-alanine.

In contrast, Germano et al. (1968) stated that the reductive amination of pyruvate is significant in that it leads to alanine biosynthesis and provides a means of incorporating free ammonia into cellular protein.

Meers & Pedersen (1972) studied nitrogen assimilation by Bacillus licheniformis cells growing in chemostat cultures. They found that when excess nitrogen as ammonium ion, was given to the cultures as a pulse, rapid increase in the concentration of glutamine and alanine occurred. Thus, they concluded that during ammonia-limited growth alanine dehydrogenase was involved in ammonia assimilation and would probably be repressed; under these conditions the specific activity of ADH was small. ADH was, however, synthesized to a greater extent when the organisms were carbon limited and highest specific activity for this enzyme was measured in organisms provided with alanine as the sole energy source for a carbon limited culture. Further, they found that the activity of ADH was furthermore subject to severe end-product inhibition by pyruvate, but not by ammonia. Thus, they also concluded that the essential physiological function of ADH is to catabolize L-alanine to produce pyruvate which can

then readily be used as a carbon and energy source.

McCowen & Phibbs (1974) also studied the regulation of alanine dehydrogenase in Bacillus licheniformis and they found that high specific activities were found in extracts of cells throughout growth cycles only when alanine served as the primary source of carbon or carbon and nitrogen.

From these studies, alanine dehydrogenase is viewed as an enzyme that play a role of catabolism of L-alanine to pyruvate during sporulation and initial vegetative growth of bacteria.

On the other hand, there have been claims that L-alanine dehydrogenase might play an anabolic role in plants and blue-green bacteria (algae). Fraustadt (1959) found that Mucor racemosus produced more alanine from pyruvate in the presence of fluoroacetate than in its absence and in anaerobic conditions than in aerobic conditions and proposed that alanine formation might be occurring by direct synthesis instead of transamination. Joy (1969) claimed that alanine dehydrogenase was induced in Lemna minor by  $\text{NH}_4^+$  and suggested that in the organism the enzyme might be involved in primary nitrogen assimilation. Finally, Batt and Brown (1974) reported the Anabaena cylindrica a blue-green bacterium that fixes nitrogen, contains high levels of alanine dehydrogenase.

Thus although the weight of evidence at present favours the view that L-alanine dehydrogenase is primarily involved in alanine utilization in vivo, a possible role in alanine for-

mation is not excluded, in some organisms at least.

III EXPERIMENTAL

A. MATERIALS

Materials were purchased from the following suppliers:

Standard AR grade chemicals: Fisher or Canlab, Ottawa  
Ontario, Canada.

Sepharose 6B: Pharmacia Fine Chemicals, Uppsala, Sweden,  
Pharmacia (Canada) Ltd., Montreal, P.Q.

Protein molecular weight Standards:

Human  $\gamma$ -globulin, bovine serum albumin,  
ovalbumin.

Schwarz-Mann (Picker Nuclear Engineering  
Co., Ottawa, Ontario, Canada).

Cofactors, Substrates and Tris (Trizma base):

Sigma Chemical Co., St. Louis, Mo., U.S.A.

B. METHODS

1) Organisms

Halobacterium cutirubrum strain NRC 34001, which was obtained from Dr. D.J. Kushner, was used in this study. The strain was originally isolated by Lochhead (1934) from salted buffalo hides and salted bacon.

2) Growth condition and culture medium

H. cutirubrum was grown as described by Peterkin and Fitt (1971) in the complex medium of Gochnauer and Kushner (1969). The latter has the following composition per 100 ml:

Difco yeast extract	1.00 g
Difco casamino acids	0.75 g
Sodium citrate	0.30 g
Potassium chloride	0.20 g
Magnesium sulfate heptahydrate	2.00 g
Sodium chloride	25.00 g
Ferrous chloride heptahydrate	5 mg

The pH was adjusted to 6.5 - 6.6 with 5% NaOH and the medium was autoclaved for 15 minutes at 121°C. The small amount of precipitate formed in the medium during sterilization did not affect growth and redissolved slowly during storage of the medium at room temperature.

Stock cultures which were grown on agar slants at 37°C for 2 - 3 weeks until a heavy growth was attained. These were stored at 4°C and transferred at 6 month intervals.

Bellco Glass (Vineland, N. J., U.S.A.) 125 ml culture flasks with Morton closures were used for the growth of the starter culture. Flasks of sterilized medium (60 ml) were inoculated from a stock liquid culture and incubated at 37°C and 225 r.p.m. in a New Brunswick Scientific Co. (New Brunswick, N. J., U.S.A.) gyratory shaker incubator for 48 hours. One of these flasks was then stored at 4°C to use as the inoculum of the next preparation. The remainder of the flasks were used for the inoculation of mass cultures. Fresh stock liquid cultures were prepared by loop inoculation at regular intervals of not more than three months.

The mass cultures for enzyme isolation were grown in 2 liter flasks containing 600 ml of sterile medium. These were inoculated with 30 ml of a 48 h starter culture and incubated as described above.

After 72 h of growth, the cells were in late log phase and were harvested by centrifugation at 27000g max. for 15 min at 4°C. The supernatant was poured off and the cells were resuspended in 4 M NaCl/10mM Tris/HCl, pH 8.5 buffer for the preparation of the crude extract (See Section 3i).

As a routine check on the purity of the mass culture medium, two agar slants were inoculated with about 1 ml of li-

quid medium at the time of each preparation. These were incubated for about one week at 37°C and examined for freedom from contamination.

H. halobium contamination was also examined from the agar plate inoculation with 0.2 ml of 1:1×10<sup>5</sup> th dilution of the harvested homogeneous mass culture by visual observations of viable count, after incubation intervals of one, two and four weeks at 37°C.

### 3) Purification of the enzyme

All procedures were carried out at 0 - 5°C except where indicated. Enzyme activities were determined at all stages of purification using optimum conditions unless otherwise indicated.

The pH of buffers refers to the value at room temperature except where indicated.

Ammonium sulfate concentrations were calculated according to Green and Hughes (1955).

#### i) Preparation of cell-free extracts.

The freshly harvested cells were suspended in 4M NaCl/10mM Tris/HCl buffer, pH 8.5, at a concentration of 1 gram wet weight of cells per 3 ml of buffer solution, by slow addition of the buffer while the cells were stirred carefully with a bent glass rod so as to prevent clumping.

a) Suspension of cells in homogeneous phase

The suspension, about 60 ml, was transferred to a 100 ml beaker. The centrifuge bottles were rinsed with a further 5 ml of buffer, which was then added to the suspension in the beaker. A teflon-coated stirring bar was placed in the beaker and the cell suspension was stirred magnetically for 1 - 2 hours until a smooth suspension was obtained.

b) Ultrasonic desintegration of the cells.

The bacteria were ruptured using a Biosonic II ultrasonic disintegrator (Bronwill Scientific Co., Rochester, N. Y. U.S.A.) fitted with the large probe. The latter was positioned 1 - 2 cm above the bottom of the beaker during sonication, which was performed at 80% of maximum intensity. Normally, the suspension changed color from a milky pinkish red to darker clear red within 1 minute. In order to ensure complete cell wall disruption without heat denaturation, the suspension was cooled in ice and two successive 2 minutes sonications were used with a 1 minute pause between them.

c) Centrifugation

Relatively large fragments of cell debris were removed by centrifuging the lysed cell suspension for 30 minutes at 46000g max. The supernatant was then centrifuged at 300,000g max in the Ti-60 rotor of a Beckman L-2 65B preparative ultracentrifuge for a further 1 - 2 hrs. Both pellets were discarded and the final cell-free supernatant was the crude extract.

The bulk of the crude extract was kept at  $-20^{\circ}\text{C}$  for up to 2 months without significant loss of activity and portions were thawed overnight at  $4^{\circ}\text{C}$  as required. Unfrozen crude extract was used within two days.

ii) Ammonium sulfate fractionation

Ammonium sulfate precipitation by the conventional methods could not be used since they require preliminary removal of the NaCl by dialysis to avoid precipitation of  $\text{Na}_2\text{SO}_4$  and led to a loss of activity at low ionic strength. Instead small portions of crude extract were diluted with large volumes of concentrated, buffered ammonium sulfate, this simultaneously increasing the ammonium sulfate concentration while reducing the concentration of sodium chloride.

The crude extract was cooled in ice and stirred while, 9 volumes of 4.2 M  $(\text{NH}_4)_2\text{SO}_4$ /10mM imidazole HCl buffer, pH 6.8 were added slowly to give a final ammonium sulfate saturation of approximately 72%. After a further 10 minutes of stirring, the precipitate (approx. 35% w/w of the total protein in the extract) was removed by centrifugation at 46000g max for 30 minutes and discarded.

iii) pH precipitation.

the pH of supernatant solution was adjusted to between 3.5 and 4.0 by slow and cautious addition of conc. HCl and stirring was continued for 5 minutes. Further cautious additions of 1N HCl were made to lower the pH to 3.0. After the

pH remained at  $3.0 \pm 0.1$  for 10 minutes at  $0^{\circ}\text{C}$ , the suspension was centrifuged at 46000g max, for 30 minutes. The precipitate, which contained most of the L-alanine dehydrogenase activity, was dissolved in a volume of 4M NaCl/10mM Tris/HCl buffer, pH 8.5, equivalent to 5 times that of the original crude extract taken. The solution was then dialyzed for 4 h against two changes of 100 volumes of the same buffer (referred to below as 4M NaCl buffer) to give the  $(\text{NH}_4)_2\text{SO}_4$  pH<sub>3</sub> fraction.

iv) Sepharose 6B gel filtration

The  $(\text{NH}_4)_2\text{SO}_4$  pH<sub>3</sub> fraction was purified further by Sepharose 6B gel filtration. A 2.5 cm x 85 cm column of Sepharose 6B was prepared and equilibrated with 4M NaCl buffer for at least one week prior to the application of the sample. A maximum of 4.5 ml of the concentrated  $(\text{NH}_4)_2\text{SO}_4$  fraction could be applied to the column without affecting the resolution. Elution was performed by upward flow with 4M NaCl buffer. The flow rate was carefully controlled at 12 ml per hour by means of an LKB perpex pump throughout the packing of the column and elution of the fractions, and the hydrostatic pressure was maintained at 10 cm.

Fractions (4 ml) were collected in the 7 ml test tubes that had been washed with glass distilled water and dried using an LKB Ultrorac fraction collector equipped with an LKB UV scan monitor and recorder. The protein concentration and

the enzyme activity of each fraction was determined as soon as possible using the method discussed below.

The L-alanine dehydrogenase activity emerged as a well-defined peak in a total volume of less than 50 ml. An overall purification of about 100-fold was consistently achieved by this method.

Similar results were obtained by using 3M KCl buffer instead of 4M NaCl of same composition and pH. Owing to the relative insolubility of KCl at 0 - 5°C, it is inconvenient to use KCl at concentrations above 3M, because of its tendency to crystallize during the column chromatography, and the enzyme is also less stable in the lower ionic strength buffer.

#### 4) Enzyme assay

L-alanine dehydrogenase activity was assayed using a Zeiss PMQ II spectrophotometer equipped with automatic zero, cell changer, transmission-extinction converter, a servogor recorder and a thermostatically-controlled cell holder.

Two standard assay procedures used to determine the reductive amination (Assay I) and oxidative deamination (Assay II) activities measured respectively the rates of decrease in  $A_{340}$  during  $\text{NH}_4^+$  and pyruvate dependent oxidation of NADH and of increase in  $A_{340}$  during L-alanine-dependent reduction of  $\text{NAD}^+$ .

i) Assay system I

The reductive amination reaction medium (1 ml) contained:

Tris/HCl, pH 9	0.1 M
NaCl	2.4 M
NH <sub>4</sub> Cl	0.5 M
Pyruvate	10 mM
NADH	0.2 mM

ii) Assay system II

The oxidative deamination reaction medium ( 1 ml) contained:

Tris/HCl, pH 9	0.1 M
KCl	1.8 M
L-alanine	50 mM
NAD	3 mM

In both cases, the final pH of the mixture was 9.0 and incubation was at 37°C ± 0.05°C. Up to 10 units of enzyme could be assayed without affecting the linearity of either assay.

The reactions were initiated by the addition of the pyruvate or L-alanine, respectively, to the preincubated mixture after a stable baseline was obtained on the recorder. The linear change in absorbance between 5 and 10 minutes after initiation of the reaction was used for the calculation of

the enzyme activity.

A unit of enzyme activity was defined as that amount of enzyme catalysing the transformation of 1 nmol/min of NADH or  $\text{NAD}^+$  in the conditions of standard assay systems I and II, respectively.

The extinction coefficient of NADH was taken to be  $6.22 \times 10^3$  liter  $\text{mol}^{-1} \text{cm}^{-1}$  (Horecker and Kornberg, 1948). Spectrophotometric accuracy was checked with a Holmium Oxide filter (A.H. Thomas Co.) and  $\text{K}_2\text{Cr}_2\text{O}_7$  NBS spectrophotometric standard solution.

#### iii) Malic dehydrogenase assay

Malic dehydrogenase activity was measured in the conditions of assay I with 10 mM oxaloacetate as substrate instead of pyruvate and without  $\text{NH}_4^+$ .

#### 5) Protein assay

Protein concentration was determined spectrophotometrically by the method of Warburg and Christian (1942). The absorbance of the solution was read in Zeiss spectrophotometer Model PMQ II and the protein concentration was calculated from the  $A_{280 \text{ nm}}/A_{260 \text{ nm}}$  ratio (Layne, 1957).

#### 6) Standardization of Sepharose column.

A Sepharose 6B column (approx, 2.5 cm x 85 cm) in 4M NaCl buffer was prepared and run as described in Section 3-iv (p 44). The void volume of the column as determined with 0.75

mg/ml Blue Dextran in 4M NaCl buffer. The standard protein were dissolved at 10 mg/ml in 4M NaCl buffer with slight warming. The standard proteins and Blue Dextran solutions were allowed to stand overnight at 4°C and any precipitate formed was removed by centrifugation at 40000 g for 10 min. Samples (1 ml) of the clear supernatants were applied to the columns and 4 ml fractions were collected during elution with 4M NaCl buffer. The eluant was monitored at 280 nm and the fractions with detectable absorbance were read at 280 nm with a Zeiss Model PMQ II spectrophotometer. The elution volume was taken at the maximum height of the peak (Fisher, 1969).

#### 7) Molecular weight determination

The molecular weight of H. cutirubrum L-alanine dehydrogenase was determined by analytical gel filtration on a column 2.5 x 85 cm of Sepharose 6B which was equilibrated with 4M NaCl buffer as described for preparative chromatography (p 44). The column was calibrated as described above with the following protein standards:

Apo-Ferritin	Molecular weight	480,000
Human gamma globulin	" "	160,000
Bovine Serum Albumin	" "	67,000
Egg Ovalbumin	" "	45,000

These protein standards were selected, after preliminary gel filtration experiments with various standards and preparative chromatography of H. cutirubrum L-alanine dehydrogenase,

so that their elution volume should bracket those of L-alanine dehydrogenase and malic dehydrogenase of H. cutirubrum.

The average partition coefficient,  $K_{av}$ , for each protein was calculated according to the following equation (Fisher, 1969):

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where  $V_o$  is the void volume of the column determined equivalent to the elution volume of Blue Dextran;  $V_e$  is the elution volume of the corresponding enzyme or standard proteins; and  $V_t$  is the total bed volume, calculated from the dimensions of the packed and equilibrated column.

Louis et al. (1971) showed by a combination of gel filtration and sucrose density gradient centrifugation studies at high ionic strength of three highly-purified H. cutirubrum enzymes that gel filtration was a valid method of the determination of the approx. mol. wts of extreme halophile enzymes.

#### IV RESULTS AND DISCUSSION

##### A. PURIFICATION AND MOLECULAR WEIGHT OF H. CUTIRUBRUM L-ALANINE DEHYDROGENASE.

###### 1) Purification of the enzyme

A typical purification of H. cutirubrum L-alanine dehydrogenase by the method described in the experimental section is summarized in Table 1. The enzyme activity emerged from the Sepharose 6B column in a sharply defined peak that was well separated from the bulk of the protein, so that the best fractions, containing 40% of the original activity, were purified approximately 100-fold.

The partially purified enzyme was free from the glutamic dehydrogenase activity present in the crude extract, but was not completely separated from malic dehydrogenase (Holmes and Halvorson, 1965; Higa and Cazzulo, 1973) during the Sepharose 6B gel filtration step of the purification procedure. However, the best fractions of the H. cutirubrum L-alanine dehydrogenase were free from malic dehydrogenase and the best fractions of the malic dehydrogenase was also free from L-alanine dehydrogenase as shown in Fig. 1.

Thus, the two enzymes are close in size, but their distribution during gel filtration (Fig. 1) proves that the two activities are associated with distinctly different proteins.

Table 1. Purification of *H. cutirubrum* L-alanine dehydrogenase

A crude extract (110 ml) was prepared from 50g wet wt. of bacteria and portions (0.9 -1 ml) were purified as required by the method described in the Experimental section. The bulk crude extract could be stored at -20°C with little change in activity for up to 6 months. Enzymic activity was determined by assay I (reductive amination of pyruvate).

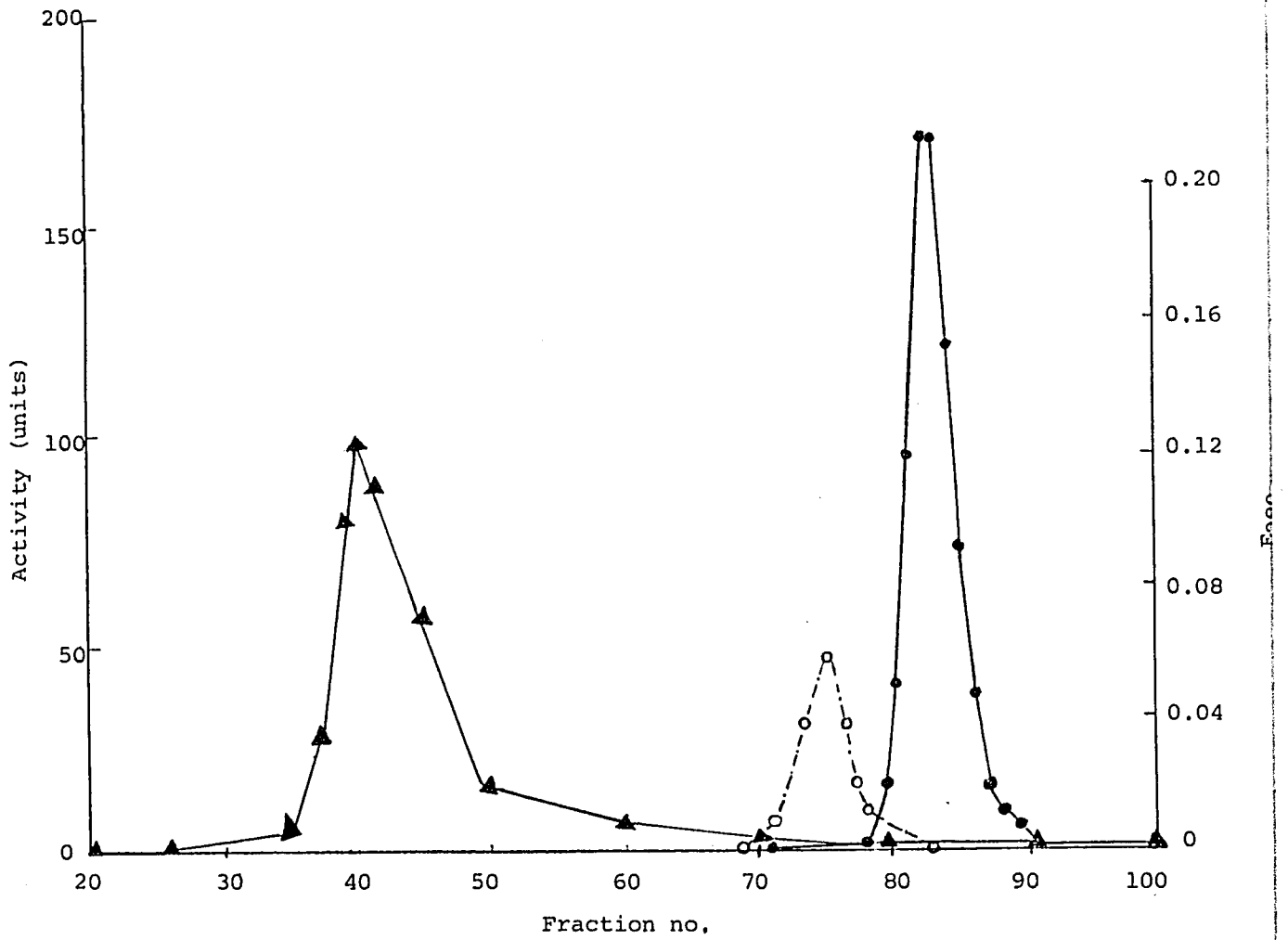
Table 1.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Sp. activity (units/mg)	Yield (%)
Crude extract	0.9	4.5	7605	1690	100
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , pH 3	4.5	1.35	5445	4033	72
Sepharose 6B: (a) total	48		4604		61
(b) Three best fractions	12	0.019	3192	168000	42

Fig. 1 Separation of *H. cutirubrum* L-alanine dehydrogenase and malate dehydrogenase by gel chromatography

A sample of an '(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 3' fraction from a standard purification was passed through a column (2.5cm x 85cm of Sepharose 6B prepared and run as described in the Experimental section. Fractions (4 ml) were collected and assayed for L-alanine dehydrogenase (assay I) and malic dehydrogenase activities (see the Experimental section). E<sub>280</sub>, ▲: alanine dehydrogenase activity, ●; malate dehydrogenase activity, ○.

Fig. 1



In general, about 100 ml of crude extract was prepared from each batch of harvested H. cutirubrum cells, which amounted to about 50g wet weight. Small portions of crude extract of about 1.0 ml were diluted with 9 ml of 4.2M  $(\text{NH}_4)_2\text{SO}_4$ /10mM imidazol/HCl buffer, pH 6.8. This simple step removed 35% of the proteins lacking L-alanine dehydrogenase or malic dehydrogenase activity. It reduced the NaCl concentration of the crude extract to 0.4M while adding ammonium sulfate salt, thus protecting the enzyme and permitting effective fractionation of the crude extract at a final ammonium sulfate saturation of approx. 72%. Empirically, this ammonium sulfate concentration was found to give the best purification of the alanine dehydrogenase during salting-out.

Further purification of the 72% ammonium sulfate supernatant was achieved by acidification to pH 3. The resulting precipitate contained 70% of original L-alanine dehydrogenase activity. This precipitate was redissolved in 5 ml of 4M NaCl buffer and ammonium sulfate was removed by extensive dialysis against the same buffer. The combined ammonium sulfate fraction-pH precipitation gave a two-fold purification. The enzyme could then be purified approx. fifty-fold further by Sepharose 6B gel filtration in 4M NaCl buffer as described in the methods section to give an overall purification of approx. 100-fold with a 42% recovery of the original activity.

Even though some halophile enzymes have been successfully

Table 2. Attempted purification of *H. cutirubrum* L-alanine dehydrogenase by ammonium sulfate fractionation at low ionic strength.

The crude extract was dialyzed twice for 1 h against 100 vol. of 10 mM 2-mercapto ethanol/10 mM Tris/HCl buffer pH 8.5 (one hour each).

The dialysed crude extract was then fractionated by addition of solid,  $(\text{NH}_4)_2\text{SO}_4$  to the indicated saturations (Green and Hughes, 1955). Each precipitate was redissolved in the initial volume 4M NaCl/10 mM Tris/HCl buffer, pH 8.5 and the solutions were dialyzed twice for 1 h against 100 vol. of the same buffer prior to assay of enzyme activity (Assay I) and protein concentration. Total recovery of protein was 78% and of enzyme activity 56%.

Ammonium sulfate fraction	Total Activity	Specific Activity	Yield	Purification
Crude Extract	2,773	118.6	100%	1.0
25% AS precipitate	399.2	146.8	14.4%	1.2
40% "	588.4	173.6	21.2	1.5
60% "	385.4	123.7	14.0	1.0
90% "	174.0	58.0	6.3	0.5
90% AS supernatant	0.0	0.0	0.0	0.0

reactivated by redialysis into high salt after an appreciable period of exposure to low ionic strength during purification (see Introduction P20 ) classical ammonium sulfate fractionation at low ionic strength gave poor results with the L-alanine dehydrogenase.

During the development of the purification procedure outlined above, several different methods of ammonium sulfate precipitation were tried, either after complete or partial removal of salt from the extract. An example of such an experiment is shown in Table 2, in which the distribution amongst the fractions from an ammonium sulfate precipitation of the activities of a sample of a crude extract, previously dialysed for 2 h against 10 mM Tris/HCl buffer, pH 8.5 is shown. It can be seen that all the fractions contained activity and that there was no appreciable increase in specific activity. Moreover the enzyme was not only spread throughout the fractions, but there was also an overall loss of activity of 44%. The data indicate that once H. cutirubrum L-alanine dehydrogenase was exposed to a low ionic strength environment, it was no longer salted out in a classical manner (Dixon and Webb, 1961) at well-defined ammonium sulfate saturations. The loss of activity is also in agreement with the statement by Holmes and Halvorson (1965 a) that a considerable fraction of any contaminating protein in the malate dehydrogenase they obtained by reactivation after purification at low ionic strength consist of inactivated enzyme.

We also made numerous attempts to purify this enzyme by other methods, such as adsorption chromatography on hydroxylapatite columns, ultrafiltration. However, they were all unsatisfactory compared to the procedure finally adopted.

The advantage of the method is that it is simple and quick, while providing a considerable purification and a reasonable overall recovery of the activity. The principal disadvantage is that the method is limited to a small scale, since no more than 4-5 ml of the  $(\text{NH}_4)_2\text{SO}_4$ -pH 3 fraction can be placed on the gel filtration column without a loss of resolution - this was, however, not a problem in the work discussed in this thesis.

2) Molecular weight of H. Cutirubrum L-alanine dehydrogenase and malic dehydrogenase.

The Blue Dextran and standard proteins were run individually by the method described in the experimental section. The four standard proteins all emerged in narrow and well defined peaks and were chosen so that their elution volumes were appropriately distributed around those of L-alanine dehydrogenase and malic dehydrogenase. The void volume ( $V_0$ ) of the column, determined with Blue Dextran, the calculated total column volume ( $V_t$ ) and the elution volumes ( $V_e$ ) of the standards and the two H. cutirubrum enzymes are shown in Table 3.

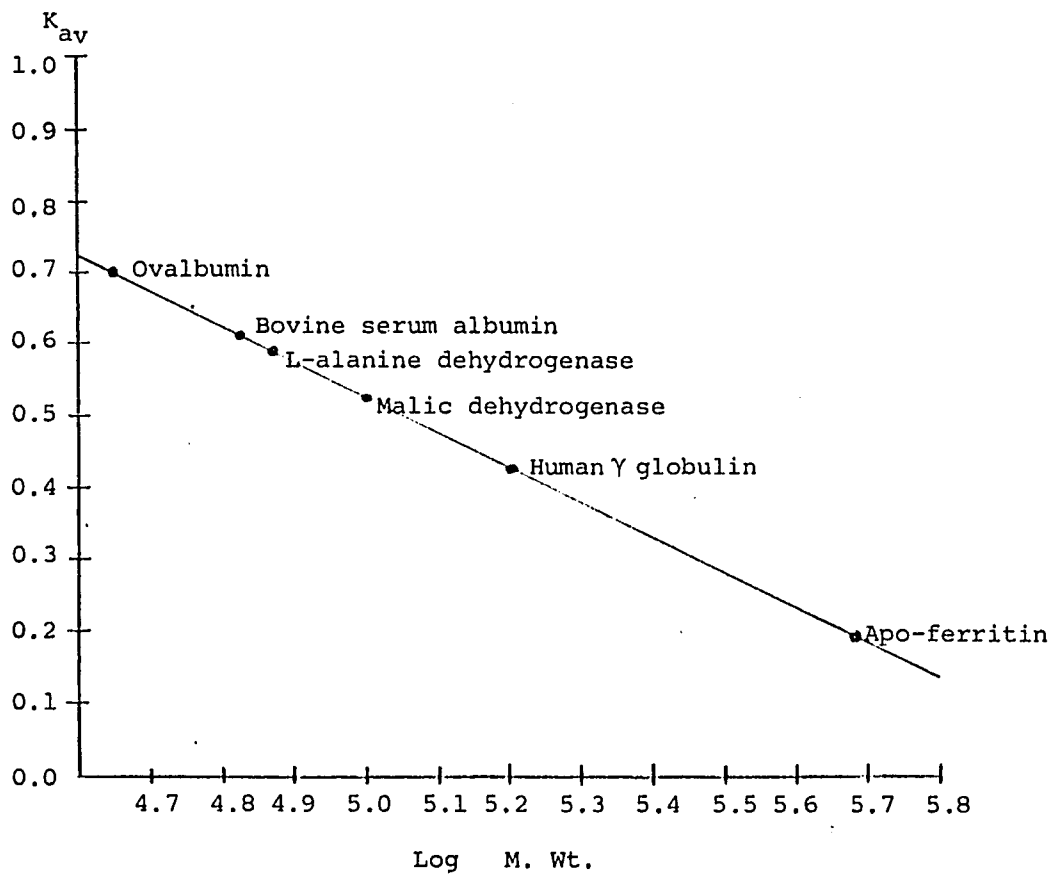
The average partition coefficients ( $K_{av}$ ) of the proteins were calculated according to the formula described in the Methods section (p.49) from the pertinent data obtained from

Table 3. Molecular weight of H. cutirubrum L-alanine dehydrogenase

The molecular weight of H. cutirubrum L-alanine dehydrogenase was determined by analytical gel filtration on a column, 2.5 cm x 91 cm, of Sepharose 6B prepared and run in 4M NaCl/10 mM Tris/HCl, pH 8.5 buffer as described for preparative chromatography in the experimental section. The column was calibrated with the standard protein standards as indicated.  $K_{av}$ s were calculated from the void volume, the calculated total bed column volume and the measured elution volumes by the standard method (Fisher, 1969). Details of the method and calculations were described in the experimental section.

Protein	$V_e$	$K_{av}$	Log M.W.	Molecular weight
Blue dextran ( $V_o$ 186 ml)				
Human $\gamma$ -Globulin	298	0.43	5.204	160,000
Bovine Serum Albumin	346	0.61	4.826	67,000
Ovalbumin	364	0.69	4.653	45,000
Total column bed ( $V_t$ 447 ml)				
H. cutirubrum Malic dehydrogenase	324	0.53	5.000	100,000
H. cutirubrum L-alanine dehydrogenase	340	0.59	4.860	72,500

Fig. 2  $K_{av}$  vs. Log Molecular Weight of Standard  
Proteins and enzymes.



the calibrated columns (Fisher, 1969). As shown in Fig. 2, a straight line was obtained for the plot of  $K_{av}$  vs.  $\log$  (mol. wt.) of the protein standards in agreement with the observations of Andrews (1965). The  $K_{av}$  values for the H. cutirubrum L-alanine and malic dehydrogenases were 0.59 and 0.53 respectively, between those of bovine serum albumin and human gamma globulin, corresponding to molecular weights of 72,500 and 100,000, respectively.

These results were highly reproducible both in analytical and preparative experiments, and in the latter were the same in the presence of 4M NaCl and 3M KCl, so that they were not affected by changes in ionic strength within this range. Louis et al. (1971) showed that the mol. wt. obtained for three highly-purified H. cutirubrum enzymes by both gel filtration and sucrose density gradient centrifugation at an ionic strength of 3.5 were the same. If the enzymes had been adsorbed to the gels in these conditions or if they were abnormally dense, a large difference between the results obtained by these two methods would be expected. There is therefore good reason to believe that gel filtration at ionic strengths within the range used in the work described in this thesis is a valid method for the determination of approximate values for the mol. wt. of halophilic enzymes.

The observation that the mol. wt. of the H. cutirubrum L-alanine dehydrogenase is about 72,000 provides a further example of an H. cutirubrum enzyme smaller than the corresponding enzyme from non-halophilic bacteria. The molecular

weight of the L-alanine dehydrogenase of B. cereus was determined to be 248,000 by McCormick and Halvorson (1964) and Yoshida and Freeze (1964) demonstrated that the molecular weight of the B. subtilis enzyme was 228,000. Therefore, the H. cutirubrum L-alanine dehydrogenase appears to be significantly smaller than these two well-characterized, non-halophile L-alanine dehydrogenases, even though the difference is not so great as that between the molecular weights of certain extreme halophile enzymes, RNA-dependent RNA polymerase, DNA-dependent RNA polymerase and poly nucleotide phosphorylase (Louis et al., 1971), and those of their non-halophile counterparts. However, a similar difference has been observed in the case of H. cutirubrum alkaline phosphatase, whose mol. wt. is approximately a quarter that of E. coli alkaline phosphatase (Fitt and Peterkin, 1976).

Yoshida (1965) found that B. subtilis L-alanine dehydrogenase consists of six identical subunits, so if sufficient of the H. cutirubrum enzyme could be purified, it would be interesting to establish if it contains more than one polypeptide chain. A comparison of the subunits structure and peptide finger prints of the halophile and non-halophile L-alanine dehydrogenase could be a valuable aid in identifying those parts of alanine dehydrogenase that are essential for activity and for the binding of substrates and cofactors, since there should be some similarity between the non-halophile enzyme and its smaller halophile counterpart.

B. ENZYMIC PROPERTIES OF *H. CUTIRUBRUM* L-ALANINE DEHYDROGENASE

A detailed study of the requirements of the 100-fold purified enzyme was carried out for both the reductive amination and the oxidative deamination reactions.

The factors investigated included the influence on these activities of pH, mono- and divalent-cations, ionic strength and reaction temperature. In addition, the substrate and coenzyme specificities and thermal stability of the enzyme were studied.

1) Effect of pH

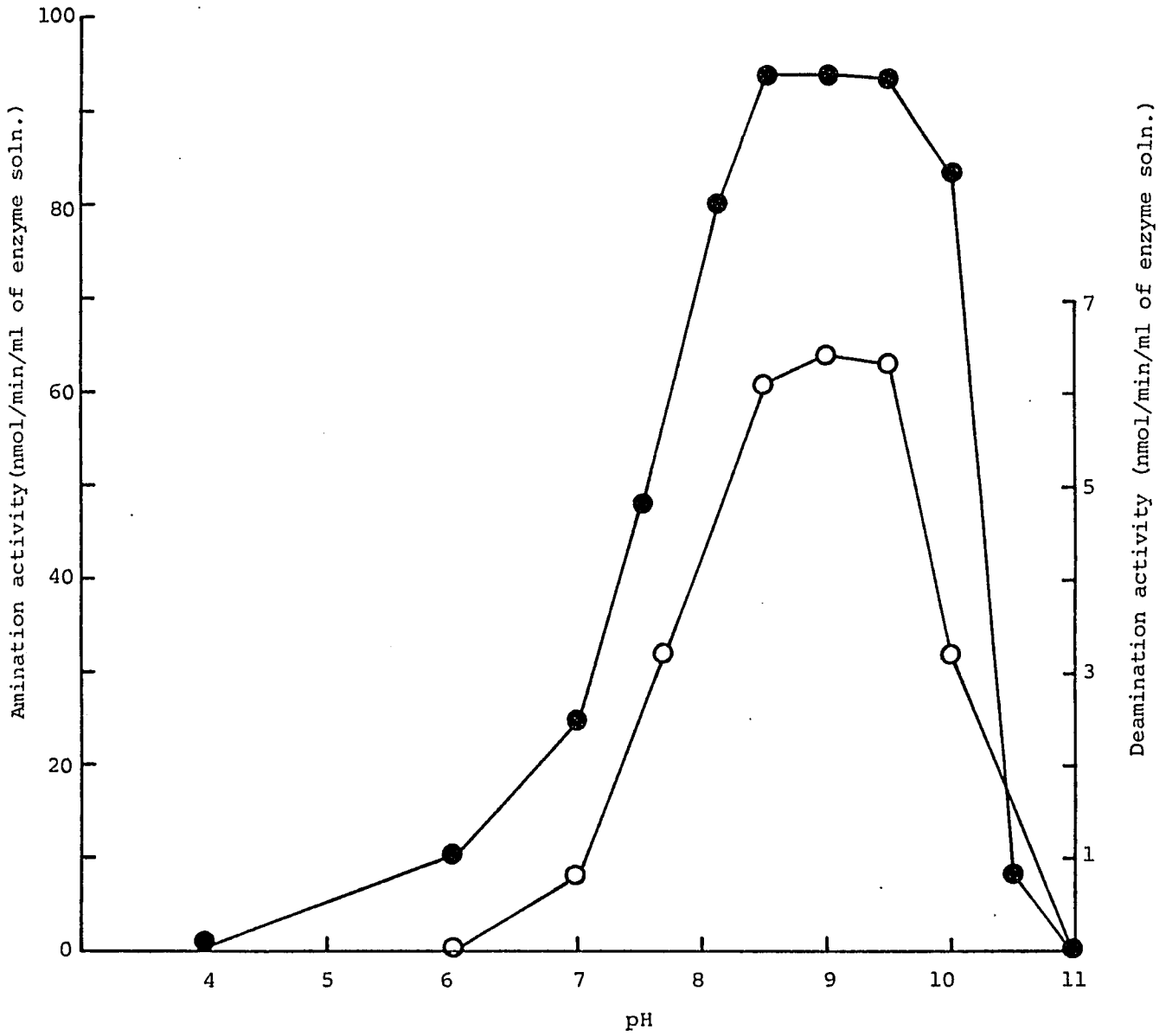
Changes in pH of the assay medium had a similar effect on both the oxidative deamination and reductive amination reactions. The enzyme is active only in an alkaline medium even though it could be safely precipitated in acid conditions in  $(\text{NH}_4)_2\text{SO}_4$ , as described in the Method section.

As shown in Fig. 3, there is a well defined pH optimum around 9 for both reductive amination and oxidative deamination. This is in good agreement with those previously reported for the amination reaction with the enzyme from other bacteria, but significantly lower than the values of pH 10 - 10.5 found for the deamination reaction by earlier workers (O'Connor and Halvorson, 1960; Yoshida and Freese, 1965; Germano and Anderson, 1960). It should also be noted that the reaction rate of reductive amination is more than ten times that of oxidative amination in the standard assay conditions we used. Furthermore

Fig. 3 Effect of pH on activity of H. cutirubrum L-alanine dehydrogenase

The activity of L-alanine dehydrogenase in the reductive amination of pyruvate (assay I, ) and the oxidative deamination of L-alanine (assay II, ) was determined as a function of pH . The reaction conditions were those described in the Experimental section with 6.4 units of enzyme activity in assay I and 98.8 units in assay II. The pH values of the assay mixtures were checked before and after the measurements. (N.B. the scales for the two activities differ.).

Fig. 3



the true difference is 2 - 3 times greater than indicated because an optimum  $\text{NH}_4^+$  concentration cannot readily be used (see p. 91 ).

## 2) Enzyme stability

The stability of the enzyme in various concentrations of sodium and potassium chloride, as well as in the absence of salt, was examined.

In common with other halophile enzymes, H. cutirubrum L-alanine dehydrogenase has an absolute requirement for a high ionic strength for stability. Dialysis of the enzyme solution against 10 mM-Tris/HCl buffer, pH 8.5, led to a complete loss of the activity of the enzyme, as measured in the standard conditions at high ionic strength.

As with some other halophilic enzymes (see Introduction), H. cutirubrum L-alanine dehydrogenase can be reactivated after exposure to a low ionic strength by dialysis against concentrated salt solutions.

Partial reactivation of the enzyme in crude extracts occurred during prolonged dialysis of the salt-free solution against 4M NaCl/10 mM Tris/HCl buffer, pH 8.5 (Table 4). However, it can be seen that, in the absence of a thiol compound, only 35% of the original activity could be recovered, whereas nearly complete reactivation occurred if 10 mM 2-mercaptoethanol was present at all times during the return of the enzyme to a high ionic strength environment. In addition, it is possible that some, slight additional reactivation

Table 4. Reactivation of *H. cutirubrum* L-alanine dehydrogenase after low salt inactivation of the enzyme.

Inactivating dialysate	Reactivation dialysate	Specific activity after reactivation	
		Crude	Purified
0.01 M Tris Buffer only	4.0M NaCl buffer only	35%	16%
"	" & 10 mM BME*	92%	53%
"	3.5M KCl buffer only	3%	3%
"	" & 10 mM BME	3%	3%
0.01 M Tris & 10 mM BME	4.0M NaCl buffer only	47%	18%
"	" & 10 mM BME	98%	53%
"	3.5M KCl buffer only	3%	3%
"	" & 10 mM BME	4%	3%

\*BME : 2-mercaptoethanol

The samples (1-2ml) of either the crude extract or the purified enzyme were dialysed for 4h against two changes of 100 vol. of the indicated inactivation buffer and then for 12h against 100 vol. of the appropriate reactivation buffer. Enzyme activity was determined by the standard assay I.

could occur if the thiol was also present during the initial removal of the salt. These results are in good agreement with those obtained by Keradjopoulos and Wulff (1974) with the crude H. salinarium enzyme. In contrast, the purified H. cutirubrum L-alanine dehydrogenase was much less stable and only a maximum of 53% of its original activity could be recovered in conditions where the crude extract could be reactivated to the extent of 92 - 98%.

Another interesting phenomenon that can be seen from Table 4 is that dialysis against 3.5M KCl did not reactivate either the crude or purified enzyme, even when 2-mercaptoethanol was present all times during the two dialyses. Such differences of mono valent cations are very interesting in view of the specific nature of  $K^+$  ion in the oxidative deamination reaction which will be discussed later.

The enzyme, crude or purified, in 4M NaCl buffer retained up to 70% of its original activity over the 3 week period at 4°C. The crude extract retained 90% of its original activity for 6 months at -20°C, but the purified enzyme was much less stable and lost 30% of its activity in these conditions.

### 3) Monovalent cation requirements

In the reductive amination reaction, an ionic strength of approximately 1.2 M was required for full activation of the enzyme by either  $K^+$ ,  $Na^+$ , or  $NH_4^+$ . As shown in Fig. 4, a plateau of activity was attained at any given  $NH_4Cl$  concentration when the sum of the  $NH_4Cl$  and  $NaCl$  or  $KCl$  concentrations was approximately 1.2 M, but this plateau was displaced to progressively higher values as the  $NH_4Cl$  concentration increased.

Thus  $NH_4^+$  is at least as effective an activator of H. cutirubrum L-alanine dehydrogenase  $K^+$ ,  $Na^+$ , in addition to being an essential cosubstrate in reductive amination reaction.

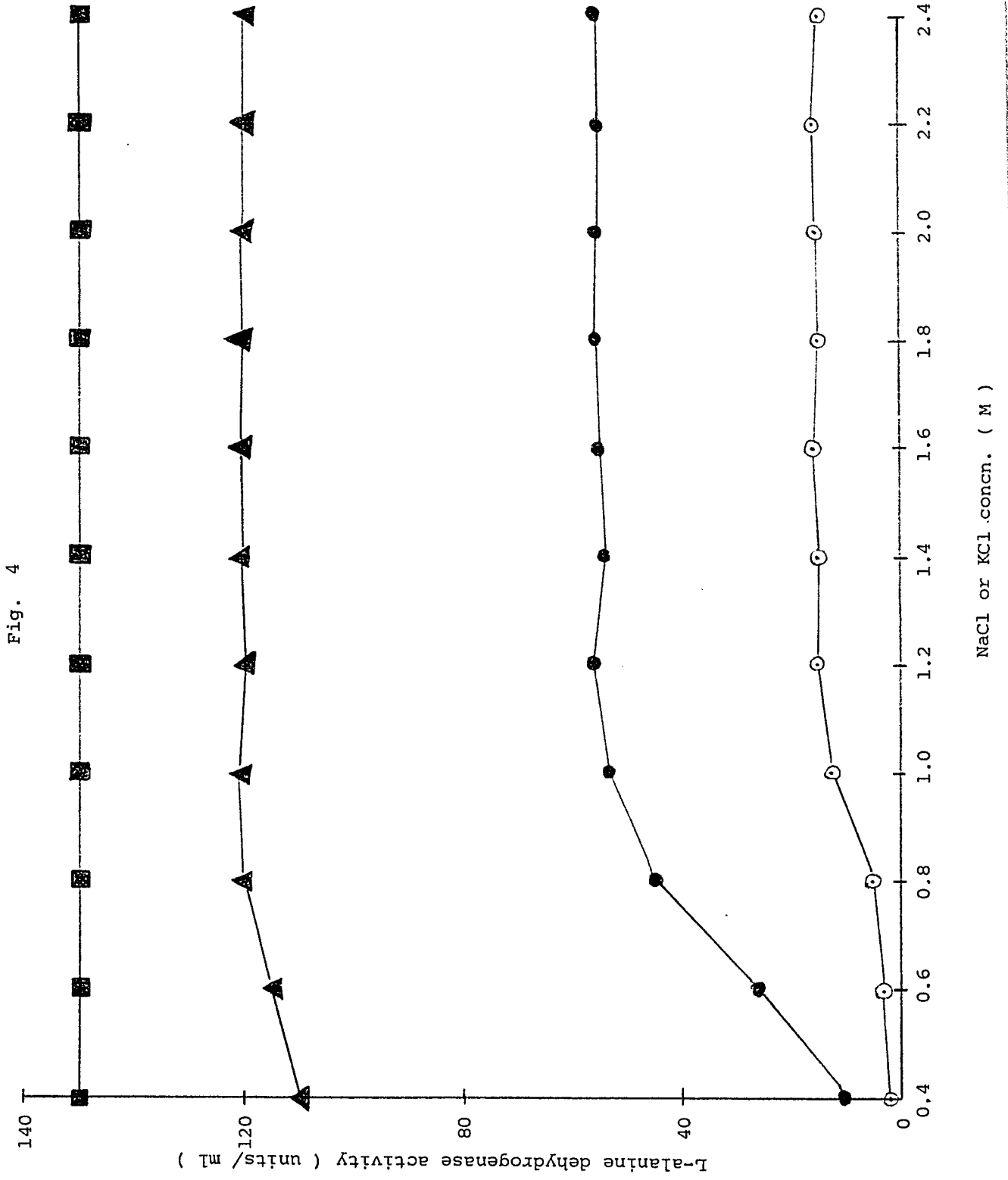
The fact that during the purification, as discussed in the Methods section, replacement of  $NaCl$  by  $(NH_4)_2SO_4$  at high concentration permitted precipitation at pH 3 with a good recovery of activity also suggests that  $NH_4^+$  is a good stabilizer of the enzyme, again in agreement with its role as a substrate.

When in the standard assay conditions, 1.5 M  $NaCl$  was replaced by 1.5 M  $CsCl$  or 1.5 M  $LiCl$ , 86% and 64% of the activity in the presence of  $NaCl$  was obtained, respectively. Therefore, the requirement of a monovalent cation during amination is relatively non-specific.

In contrast to the amination reaction, the enzyme had a remarkable specificity for  $K^+$  in the oxidative deamination of

Fig. 4 Effect of  $K^+$ ,  $Na^+$  and  $NH_4^+$  concentration on the rate of reductive amination of pyruvate by *H. cutirubrum* L-alanine dehydrogenase

The rate of the reductive amination reaction was determined as described under assay I (see the Experimental section) at the indicated concentrations of KCl, NaCl and  $NH_4Cl$ .  $NH_4Cl$  concentration: ○, 0.1 M; ●, 0.25 M; ▲, 0.5 M; ■, 0.75 M.



L-alanine. As shown in Fig. 5, the enzyme was completely inactive with  $\text{Na}^+$  or  $\text{NH}_4^+$  alone and  $\text{K}^+$  was required absolutely for the reaction to proceed. Further,  $\text{Na}^+$  did not inhibit the reaction in the presence of  $\text{K}^+$  and neither  $\text{Cs}^+$  nor  $\text{Li}^+$  could replace the latter.

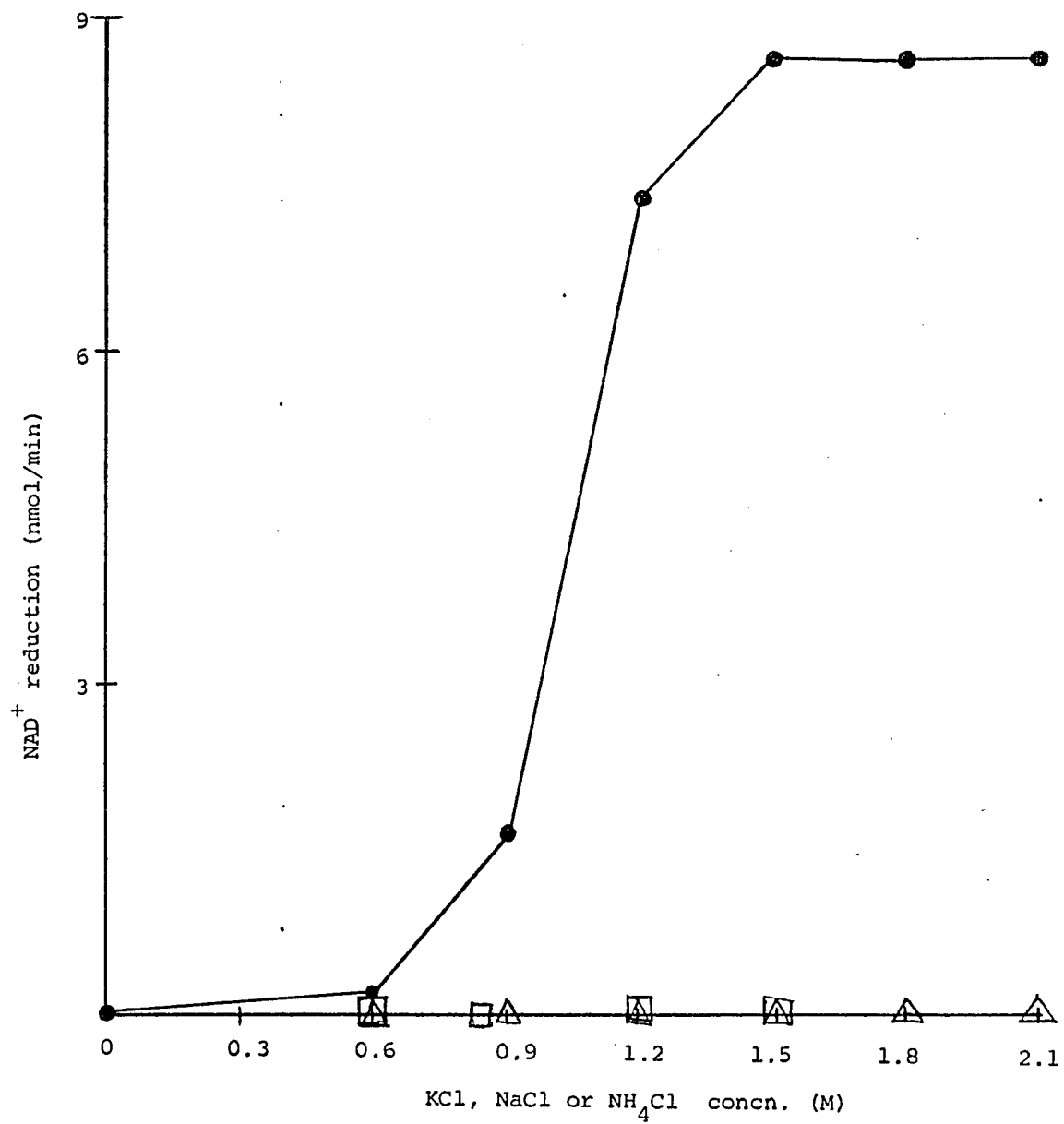
Although Baxter and Gibbons (1957) have described a cysteine desulphydrase from an extreme halophile that was only 12% as active with  $\text{NaCl}$  as with  $\text{KCl}$  at their respective optimum concentrations and Cazzulo and Vidal (1972) found that the crude malic enzyme of *H. cutirubrum* was inactive with  $\text{NaCl}$  and active in the presence either  $\text{KCl}$  or  $\text{NH}_4\text{Cl}$ , to our knowledge this is the first case of an enzyme from an extreme halophile that has been shown to have different monovalent cation requirements for the forward and backward reactions. This phenomenon is difficult to explain on the basis of the available information, but a direct effect on the catalytic process seems unlikely, since it would contravene the principle of microscopic reversibility (Fersht, 1977). A more probable explanation would be an effect of  $\text{K}^+$  on the binding of the alanine to the enzyme.

As discussed in the previous section, the lack of reactivation in the presence of  $\text{K}^+$  and the absolute requirement for this ion in oxidative deamination are very interesting subjects for possible future study of this enzyme, with particular

Fig. 5 Effect of  $K^+$ ,  $Na^+$  and  $NH_4^+$  concentration on the rate of oxidative deamination of L-alanine by *H. cutirubrum* L-alanine dehydrogenase .

The rate of the oxidative deamination reaction was measured as described under assay II (see the Experimental section) at the indicated concentrations of KCl ( $\bullet$ ), NaCl ( $\Delta$ ) and  $NH_4Cl$  ( $\square$ ).

Fig. 5



reference to the mechanism of the reaction and physiological role of L-alanine dehydrogenase in extreme halophiles.

#### 4) Substrate specificity and optimum concentration

The substrate specificity of H. cutirubrum L-alanine dehydrogenase and the optimum concentrations of the substrates used in the standard conditions were studied for both the amination and deamination reactions.

The  $K_m$  (apparent) values of H. cutirubrum L-alanine dehydrogenase for (i) pyruvate,  $\text{NH}_4^+$  and NADH in the reductive amination reaction (assay I) and (ii) L-alanine and  $\text{NAD}^+$  in oxidative deamination (assay II) were determined from plots (Dixon and Webb, 1964) of  $[s]/v$  versus  $[s]$  using standard methods (Fig. 6;7,8).

The results were:

Pyruvate	0.8 mM
NADH	0.2 mM
L-alanine	7.0 mM
$\text{NAD}^+$	0.5 mM
$\text{NH}_4^+$	0.82 M

The values are similar to those reported for the B. cereus and B. subtilis enzymes (O'Connor and Halvorson, 1960; Yoshida and Freese, 1965) except in the case of  $\text{NH}_4^+$ . The  $K_m$  value for the latter is about twenty times that reported for the other two alanine dehydrogenases, a difference that is presumably related in some as yet unknown way to the halophilic

Fig. 6 Determination of the  $K_m$ s of *H. cutirubrum* L-alanine dehydrogenase for NADH and pyruvate

The rate of the reductive amination reaction was measured in the conditions of assay I (Experimental Section) as a function of (a) NADH and (b) pyruvate concentration. The appropriate  $K_m$  was then determined from a plot of  $[s]/v$  versus  $[s]$ , as shown.

Fig. 6

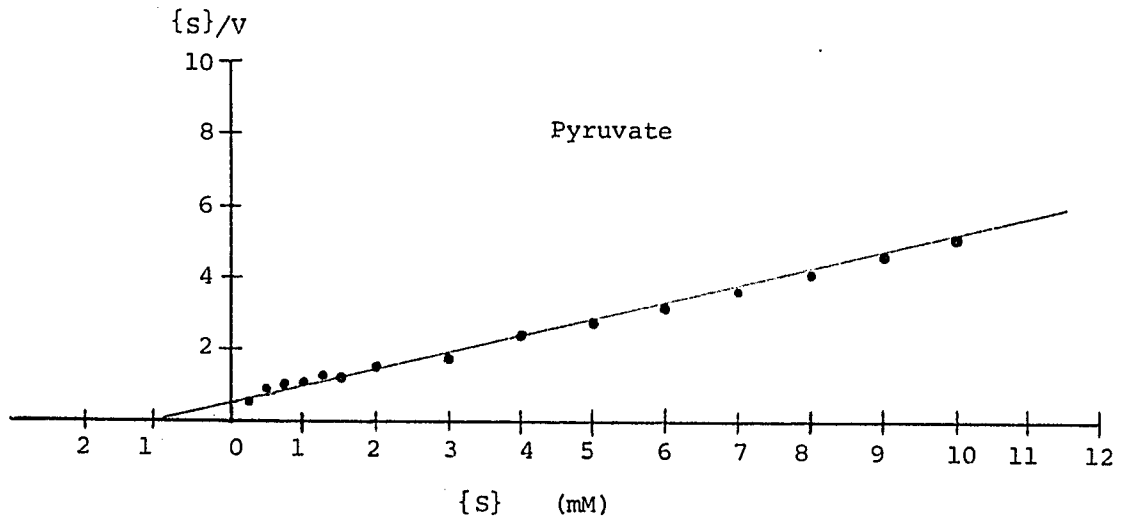
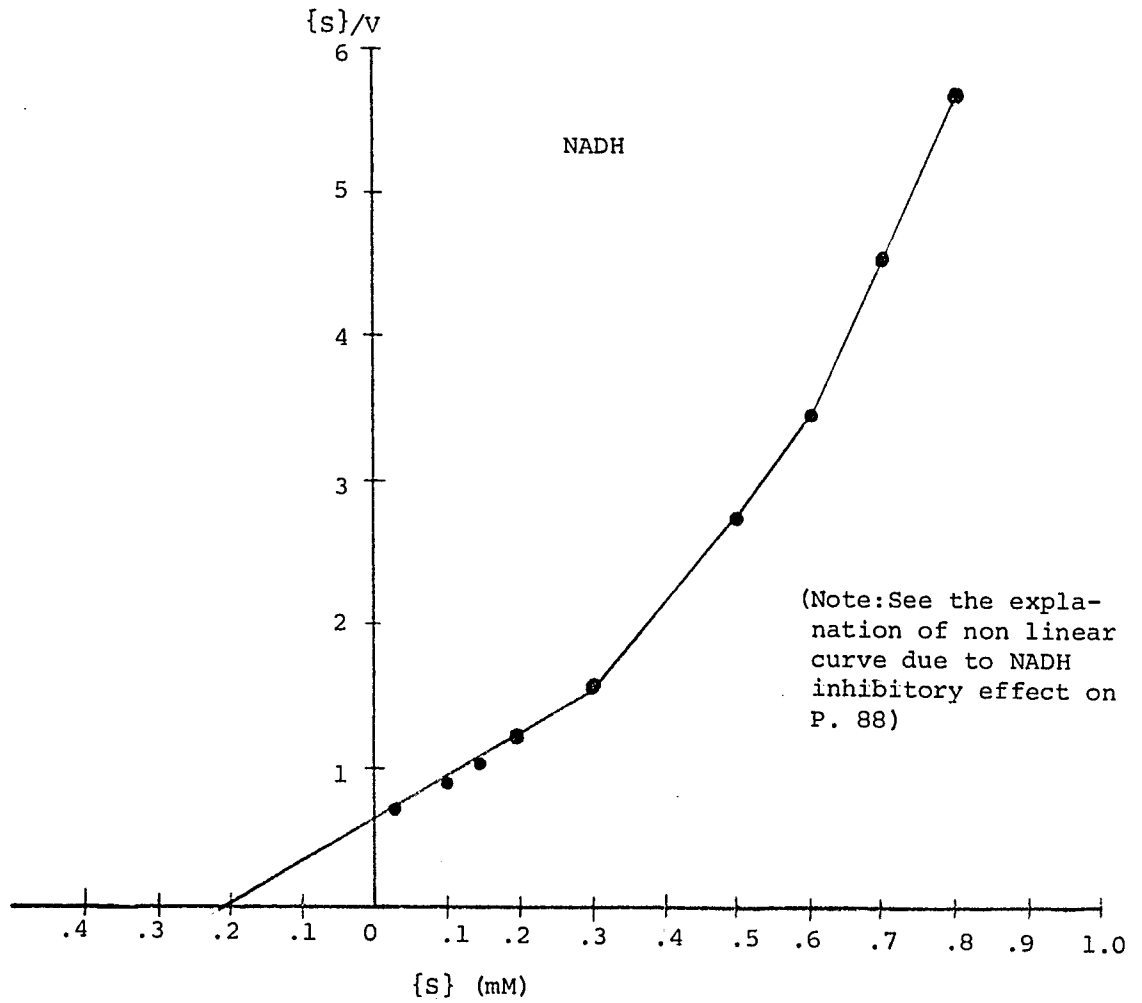


Fig. 7 Determination of the  $K_m$ s of *H. cutirubrum* L-alanine dehydrogenase for  $\text{NAD}^+$  and L-alanine

The rate of the oxidative deamination reaction was measured in the conditions of assay II (Experimental section) as a function of (a)  $\text{NAD}^+$  and (b) L-alanine concentration. The appropriate  $K_m$  was then determined from a plot of  $[s]/v$  versus  $[s]$ , as shown.

Fig. 7

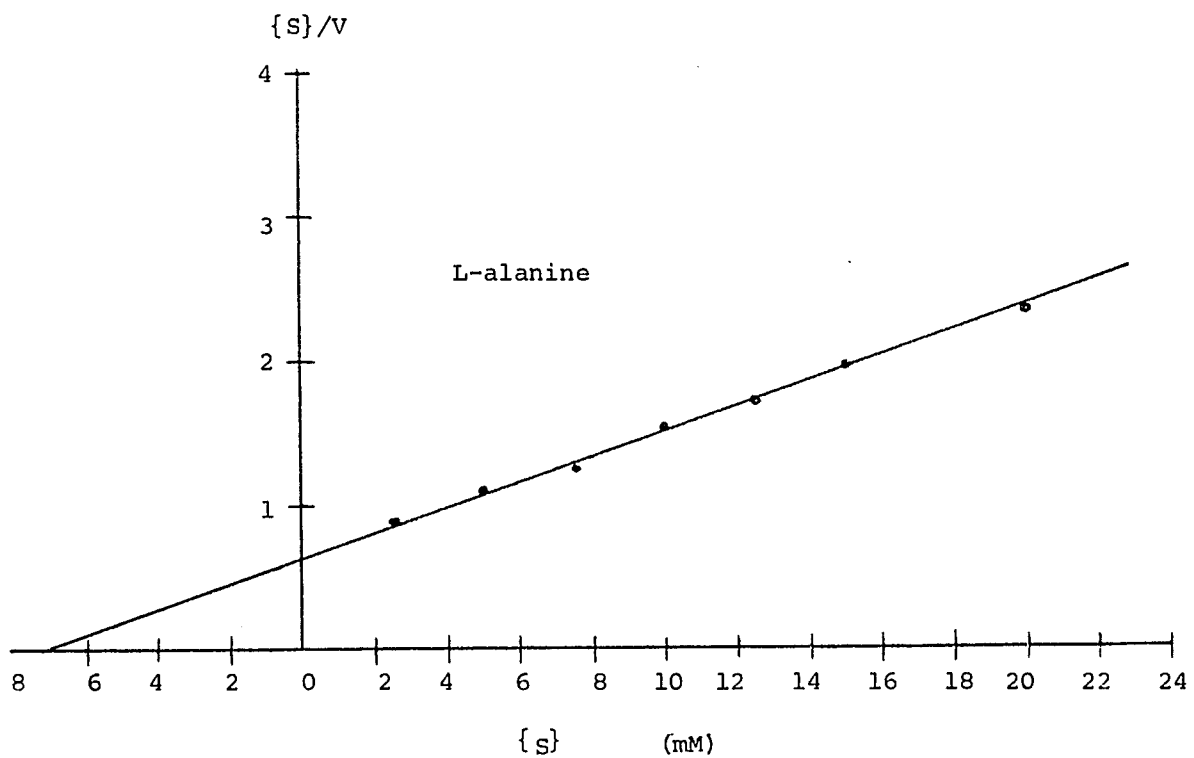
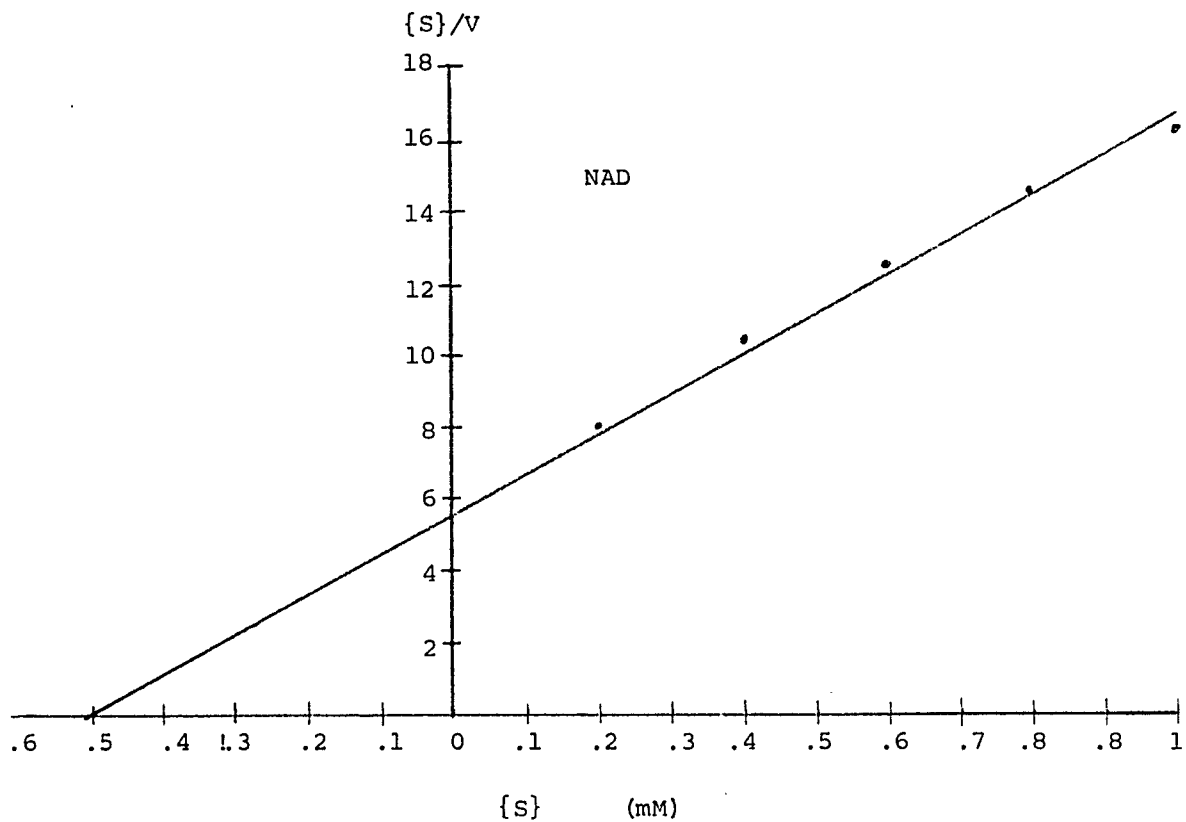
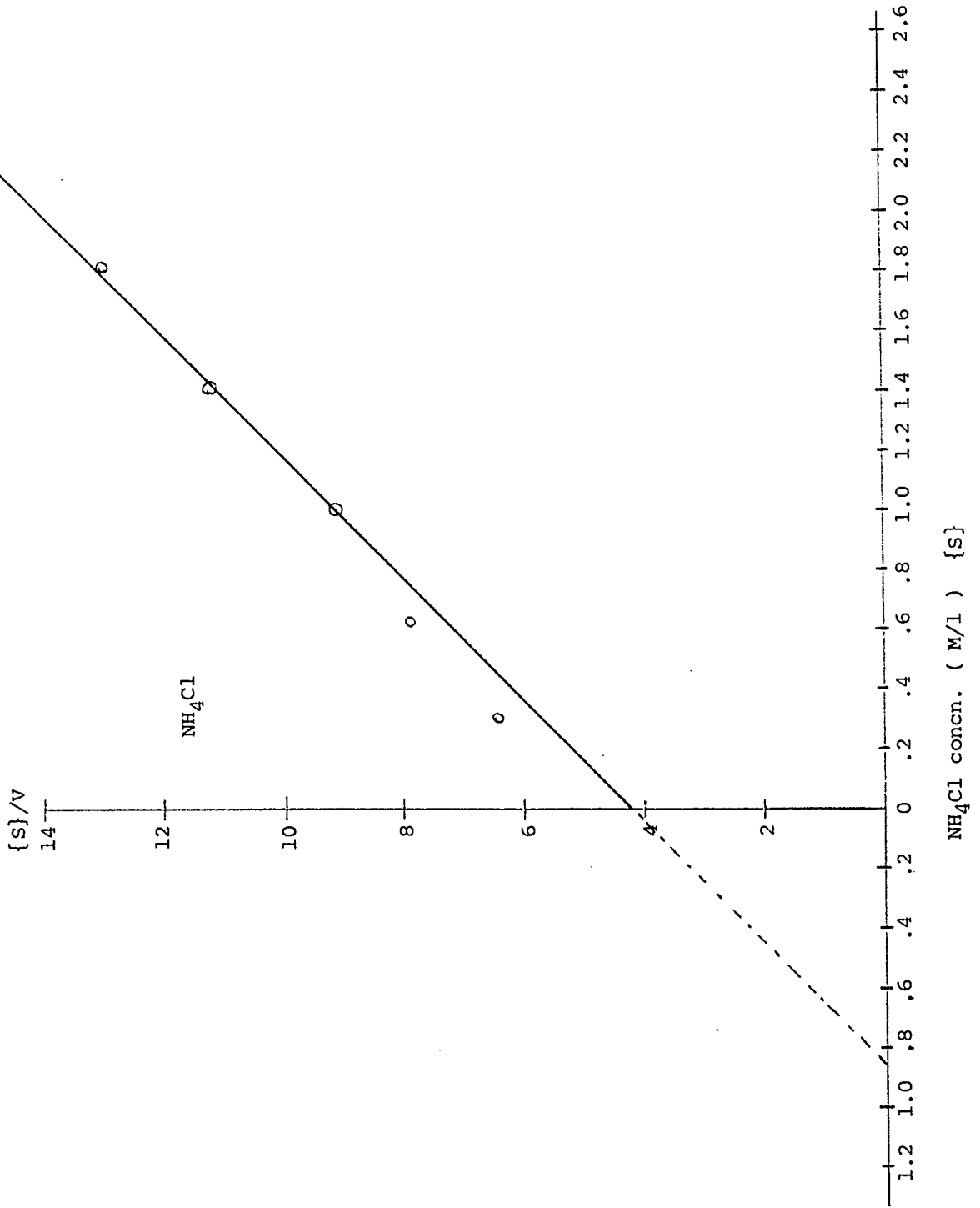


Fig. 8 Determination of the  $K_m$  of *H. cutirbrum* L-alanine dehydrogenase for  $\text{NH}_4^+$

The rate of reductive amination of pyruvate was measured as a function of  $\text{NH}_4\text{Cl}$  concentration in the conditions of assay I (Experimental Section). The  $K_m$  of the enzyme for  $\text{NH}_4^+$  was then determined from a plot of  $[s]/v$  versus  $[s]$ , as shown.

Fig. 8



character of the H. cutirubrum enzyme. Reductive amination of pyruvate was approximately ten times as fast as oxidative deamination of L-alanine, as measured in the conditions of assays I and II, respectively, using equal amounts of partially-purified enzyme.

In the oxidative deamination reaction only L-alanine and L- $\alpha$ -aminobutyric acid were found to be substrates (Table 5); there was no reaction with the D forms of these compounds, but they inhibited deamination of the corresponding L forms by about 10% when added to the appropriate reaction mixtures at a concentration of 50 mM. However, 20 mM pyruvate did not inhibit the deamination reaction of L-alanine by the enzyme. Therefore, product inhibition is minimal in the deamination reaction.

However, in the reductive amination reaction, the product inhibition is somewhat greater than the deamination reaction. In the standard assay I conditions with 20 mM pyruvate, the enzyme was inhibited 40% by the presence of 50 mM D- or L-alanine. Yet the same concentration of D- or L-aminobutyric acid did not have any inhibitory effect.

The enzyme had a slightly broader substrate specificity in the reductive amination compared to the oxidative deamination reaction as shown in the Table V, but it should be noted that  $\alpha$ -ketoglutarate was not a substrate. This latter fact provides additional evidence that the enzyme was free from the glutamate dehydrogenase. The reaction with oxaloacetate

occurred only when  $\text{NH}_4^+$  was present, and therefore could not be due to contamination by malate dehydrogenase. Absence of the latter was further confirmed by the failure of either L- or D-malate to serve as a substrate in the conditions of the deamination reaction (Table 5). As discussed below, the reaction with oxaloacetate is probably caused by conversion of the latter into pyruvate by a contaminating oxaloacetate decarboxylase, followed by the normal reductive amination of the pyruvate.

The nature and rates of the reductive amination reactions with pyruvate and oxaloacetate were studied quantitatively, using an amino acid analyser (Table 6). In both cases, L-alanine was the only product detected. The amount formed from pyruvate agreed well with the expected yield calculated from the amount of NADH oxidized during the reaction. When the substrate was oxaloacetate, there was also good agreement with the yield of L-alanine predicted on the assumption that the measured oxidation of NADH was due solely to reactions leading to alanine formation. Two reaction sequences could explain the formation of alanine from oxaloacetate in these conditions:

(1) the reductive amination of oxaloacetate to aspartate, by alanine (or an aspartate) dehydrogenase, followed by decarboxylation of aspartate to alanine by aspartate decarboxylase (Tate and Meister, 1971);

(2) formation of pyruvate from oxaloacetate catalysed by oxaloacetate decarboxylase (Plaut and Lardy, 1949; Horton and Kornberg, 1964), followed by reductive amination of the

Table 5. Substrate specificity of H. cutirubrum L-alanine dehydrogenase

The substrate specificities of H. cutirubrum L-alanine dehydrogenase in the reductive amination and oxidative deamination reactions were determined in the conditions of assay I and assay II (Experimental section), respectively. The substrate concentrations were 10 mM for reductive amination and 50 mM for oxidative deamination. The results are expressed as a percentage of the activity observed with the appropriate standard substrate (pyruvate or L-alanine).

Table 5.

Substrate	Activity (%)
<u>(a) Oxidative deamination</u>	
L-alanine	100
D-alanine	0
L- $\alpha$ -aminobutyric acid	170
D- $\alpha$ -aminobutyric acid	0
L- $\&$ D-malic acid	0
L- $\&$ D-aspartic acid, L-glutamic acid, L-glycine, L-isoleucine, L-leucine L-lysine, L-methionine, L-serine, L-threonine, L-tryptophan, L-valine	0
<u>(b) Reductive amination</u>	
Pyruvic acid	100
$\alpha$ -ketobutyric acid	220
$\alpha$ -ketovaleric acid	11
glyoxylic acid	4
oxaloacetic acid	50
$\alpha$ -ketoglutaric acid	0

Table 6. Amino acid analysis of reductive amination reaction mixtures containing pyruvate or oxaloacetate as substrates

Individual assay I reactions mixtures (3ml) containing pyruvate (Expt. 1) or oxaloacetate (Expt. 2) and the partially purified enzyme (138 units/assay) were incubated as usual (see the Experimental section) and the amount of NADH oxidized was determined spectrophotometrically. At zero time and after 5 min and 10 min of incubation, 20% (w/v) sulphosalicylic acid (0.2 ml) was added to the appropriate reaction mixture. The mixtures were then cooled in ice for 10 min. and centrifuged to remove precipitated protein. Portions (0.7 ml) of the supernatants were mixed with 1 mM-norleucine (0.2 ml) and the mixtures were analysed with a Technicon TSM amino acid analyser. Actual yields of alanine in the reaction mixtures were calculated from the ratio of alanine to norleucine in the samples and the known concentration of the latter. Theoretical yields of alanine were calculated from the measured consumption of NADH. No product other than alanine could be detected in either case.

Table 6.

Substrate	Time (min)	Alanine yield Theor.	( $\mu\text{mol/ml}$ ) Found
Expt. 1, pyruvate	0	0.00	0.00
	5	0.38	0.35
	10	0.61	0.65
Expt. 2, oxaloacetate	0	0.00	0.00
	5	0.29	0.32
	10	0.51	0.49

pyruvate to alanine by alanine dehydrogenase.

Since L-aspartate was not a substrate for the partially purified enzyme (Table 5), and no aspartic acid could be detected by amino acid analysis of the reaction mixture, the second alternative is a more probable explanation.

These results show that H. cutirubrum L-alanine dehydrogenase has a restricted substrate specificity compared with those of the B. cereus (O'Connor and Halvorson, 1961) or B. subtilis (Yoshida and Freese, 1965) enzymes and, in this respect, is more like the D. desulphuricans enzyme that was reported by Germano and Anderson (1968) to be highly specific for L-alanine in the deamination reaction.

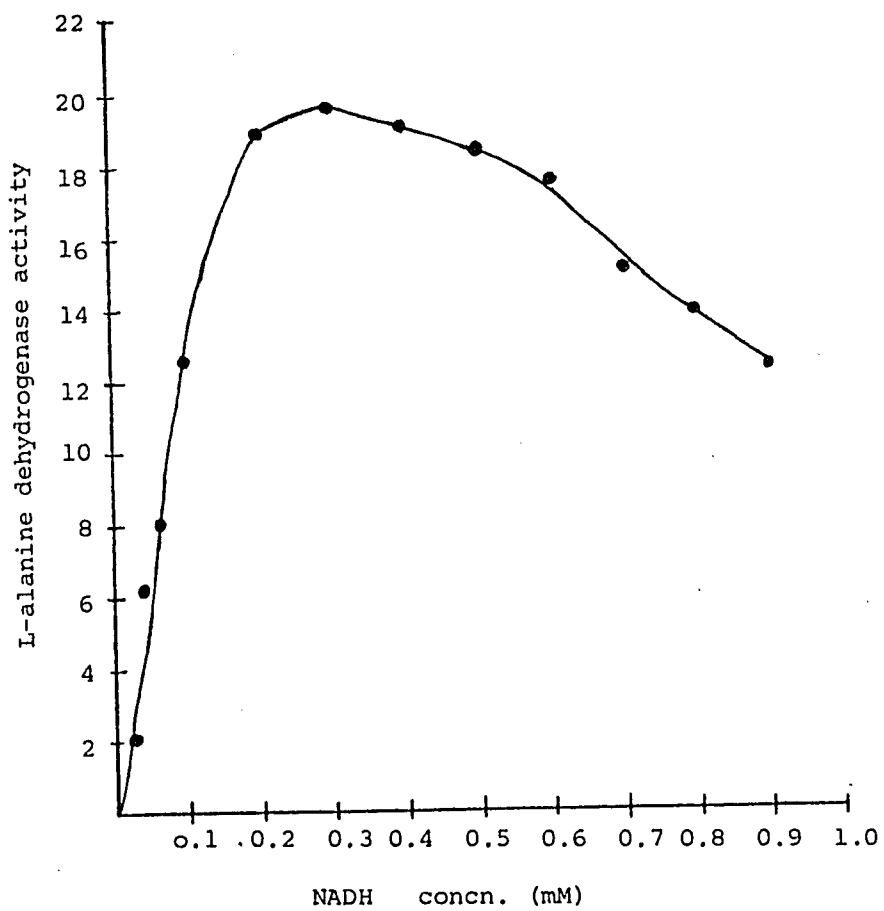
#### 5) Coenzyme specificity

The enzyme was absolutely specific for  $\text{NAD}^+$  or NADH, according to the direction of the reaction, and they could not be replaced by  $\text{NADP}^+$  or NADPH respectively. The kinetics of alanine deamination appeared to be Michaelis-Menten with an apparent  $K_m$  for  $\text{NAD}^+$  of 0.5 mM. Similarly, the rate of pyruvate amination increased with NADH concentration up to a short plateau at 0.2 - 0.4 mM NADH, but higher concentrations of this co-enzyme were inhibitory as shown in Fig. 6a and 9.

Fig. 9 Effect of NADH concentration on the rate of the re-  
ductive amination reaction

The rate of reductive amination of pyruvate was determined as a function of NADH concentration in the conditions of the standard assay I (see Experimental Section).

Fig. 9



6) Requirement for ammonium ion.

Assimilation of nitrogenous compounds other than  $\text{NH}_4^+$  ion was investigated. None of the following compounds could be utilized by this enzyme: methylamine, dimethylamine, and diethylamine.

The role of  $\text{NH}_4^+$  as an activating univalent cation in reductive amination has been discussed before. However, it is also an essential substrate in this reaction and it is clear from Fig. 4 that at KCl or NaCl concentrations above 1.2 M the rate of amination was dependent upon  $\text{NH}_4^+$ . A detailed study of this requirement was made in the presence of 1.5 M NaCl and Fig. 10 shows that the enzyme was not fully saturated with  $\text{NH}_4^+$  even when the concentration of the latter was 3.4 M. In these conditions, there was a considerable loss of  $\text{NH}_3$  from the reaction mixture and the baseline extinctions on the recorder were high; it was also inconvenient to achieve high concentrations of both  $\text{Na}^+$  and  $\text{NH}_4^+$ : 0.5 M  $\text{NH}_4^+$  was therefore used in the routine assay I, which measured only about one third of the full activity as a result (Fig. 4 and 10).

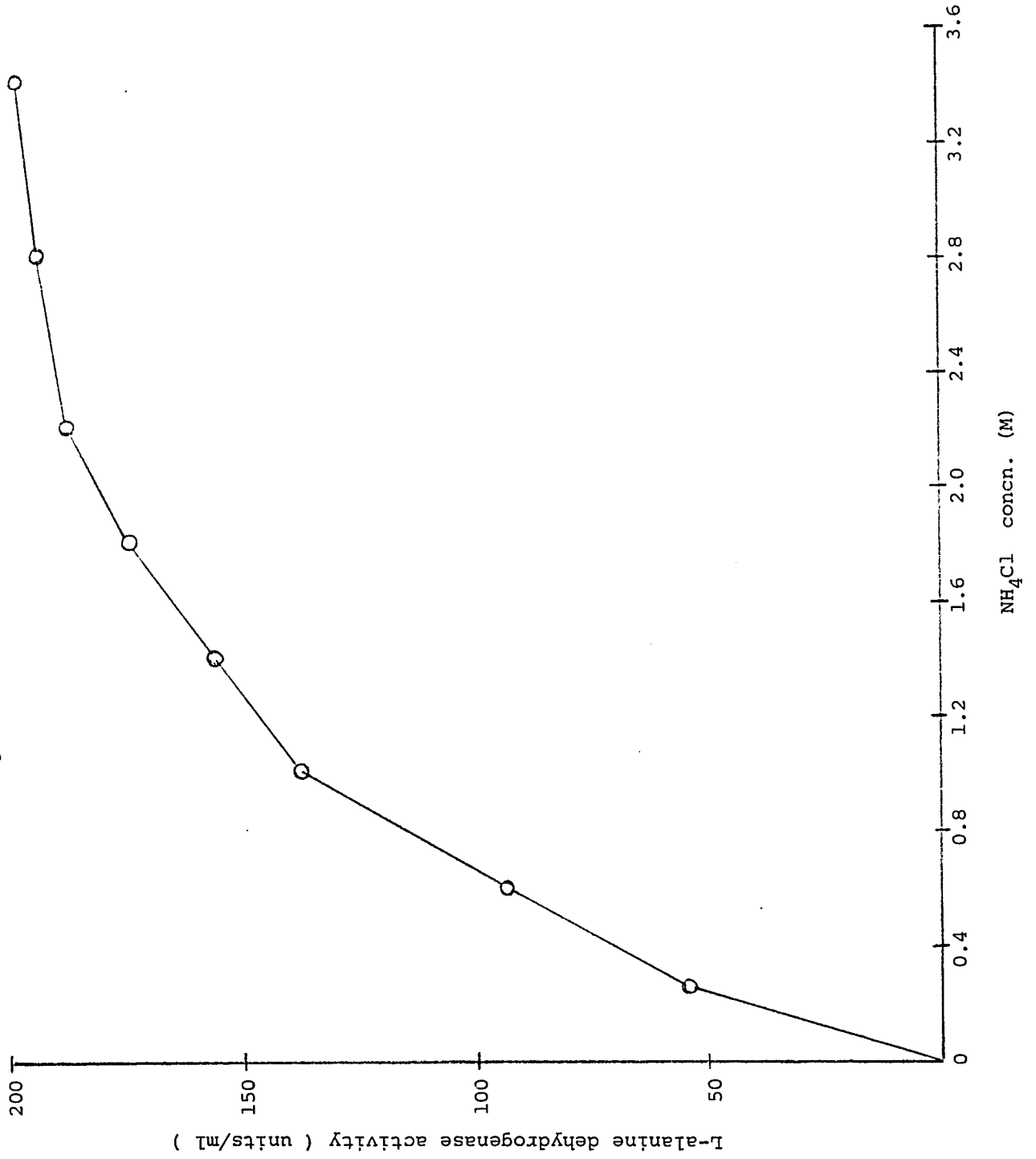
7) Divalent cation requirements

The activity of the enzyme in either the amination or deamination reactions was unaffected by the presence of (i)  $\text{Mg}^{2+}$  (5, 10 or 20 mM) or (ii) EDTA- $\text{Na}_2$  (1, 2 or 4 mM).

Fig. 10 Effect of  $\text{NH}_4^+$  concentration on the rate of the reductive amination reaction in the presence of 1.5 M-NaCl

The rate of the reductive amination reaction was determined in the presence of 1.5 M-NaCl and with the indicated  $\text{NH}_4\text{Cl}$  concentrations as described under assay I (see the Experimental section). In order to attain final  $\text{NH}_4\text{Cl}$  concentrations  $\geq 1$  M conveniently, all the other components of the reaction were dissolved in a solution of the enzyme in 3 M-NaCl/0.2 M-Tris/HCl, pH 9. Equal volumes of the latter and of  $\text{NH}_4\text{Cl}$  solutions, pH 9, of double the required concentration were warmed to  $37^\circ\text{C}$  and mixed in the spectrophotometer cuvette immediately before assay. Each point on the graph represents the average of five successive determinations of the rate, because the loss of  $\text{NH}_3$  from the solutions and the high baseline values on the recorder at high concentrations of  $\text{NH}_4\text{Cl}$  caused variations of up to  $\pm 10\%$  in individual results.

Fig. 10



The effect of several other cations on the reductive amination reaction only was also studied in the conditions of assay I at a final concentration of 10 mM.

<u>Divalent Cation</u>	<u>Activation</u>	<u>Inhibition</u>
Ca <sup>++</sup>	no effect	no effect
Mg <sup>++</sup>	" "	" "
Ca <sup>++</sup>		36%
Co <sup>++</sup>		60%
Cu <sup>++</sup>		60%
Fe <sup>++</sup>		85%
Fe <sup>+++</sup>		85%
Mn <sup>++</sup>		60%
Ni <sup>++</sup>		60%

As shown above, none of the divalent cation studied could enhance the activity. In contrast, many could inhibit the enzyme. This observation could be of interest for possible future study on the regulation of the enzyme in the organism and on the nature of its binding sites.

8) Thermal stability and temperature optimum of H. cutirubrum L-alanine dehydrogenase

Keradjopoulos and Wulff (1974) found that the crude L-alanine dehydrogenase of H. salinarium, an organism closely related to H. cutirubrum (Kushner, 1968), had an optimum reaction temperature of 70°C in 3.4 M KCl and 3.4 M NaCl. They considered the enzyme to be thermophilic, but did not

present any evidence that the protein was thermally stable in the absence of its substrates.

We have now determined the optimum reaction temperature (Fig. 11) and examined the thermal stability (Fig. 12) of the 100-fold purified H. cutirubrum L-alanine dehydrogenase. The results in Fig. 11 confirm Keradjopoulos and Wulff's (1974) observation that the optimum reaction temperature of the enzyme is high. The optimum temperature appeared slightly higher with  $K^+$  than with  $Na^+$  as the principal monovalent cation in the assay mixture in one series of experiments, but no difference was observed on another occasion (Fig. 11): this variability was due to the fast thermal inactivation of the enzyme at higher temperatures (see below), with a consequent imprecision in the measurement of initial velocities arising from the short time of reaction. However, Fig. 12 shows that the thermal stability of H. cutirubrum L-alanine dehydrogenase is in fact not significantly greater in the absence of substrate than that of most well-studied enzymes from mesophiles (e.g. Escherichia coli) and quite unlike the remarkable resistance to heat inactivation of truly thermophilic enzymes from organisms such as the moderate thermophile Bacillus stearothermophilus (e.g. Amelunxen and Lins, 1968; Saunders et al, 1969) or the extreme thermophile Thermus aquaticus (e.g. Freeze and Brock, 1970; Higa and Ramaley, 1973).

Fig. 11 Effect of assay temperature on the rate of the reductive amination reaction

The rate of reductive amination of pyruvate by H. cutirubrum L-alanine dehydrogenase was determined at the indicated temperatures in the conditions of assay I (Experimental section) in the presence of either 2.6 M KCl or 2.75 M NaCl. The broken and solid lines show the results of experiments performed on separate occasions with different samples of the enzyme. The results are expressed as a percentage of the activity at 37°C, NaCl concn., ●; KCl concn., Δ; Expt. 1, —; Expt. 2. ---.

Fig. 11

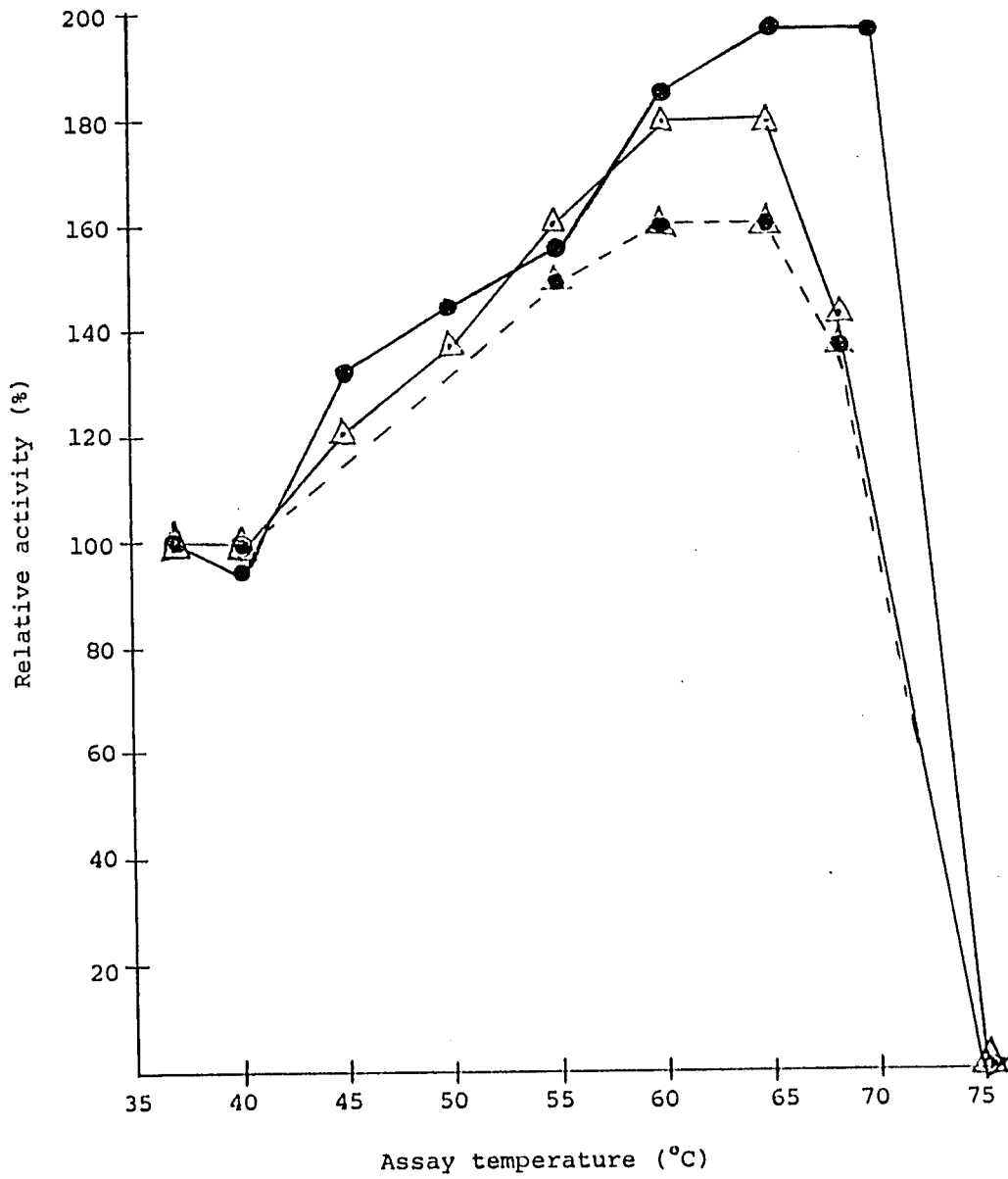
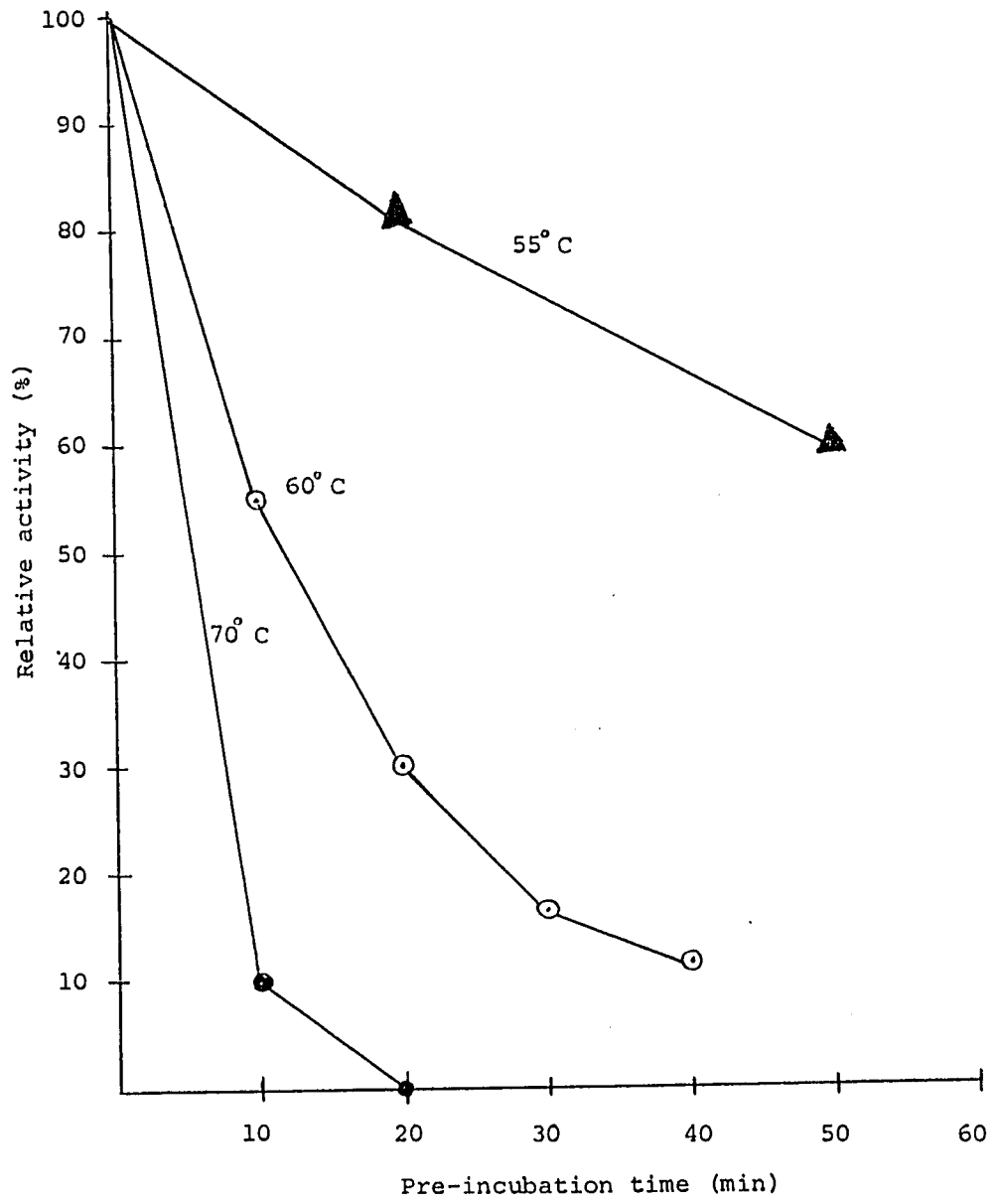


Fig. 12 Thermal stability of H. cutirubrum L-alanine de-  
hydrogenase

Samples of a solution of the enzyme in 3 M-KCl-0.01 M Tris-HCl pH 8.5 were heated at 55°C ( $\blacktriangle$ ), 60°C (0) or 70°C ( $\odot$ ) for the indicated time. They were then cooled in ice and assayed for reductive amination activity at 37°C in the conditions of assay I (Experimental section). The results are expressed as a percentage of the activity of the unheated enzyme assayed in the same conditions.

Fig. 12



### V Summary

The results presented above show that H. cutirubrum contains an active L-alanine dehydrogenase that requires a high ionic strength for optimum activity and stability. Its activity in the standard reductive amination assay increases with temperature up to about 70°C, but the enzyme itself is not thermostable.

The molecular weight of the enzyme is approximately 72500. Thus, although it is not an unusually small protein, it is less than one third the size of the two well-studied B. cereus and B. subtilis L-alanine dehydrogenases and is the fifth enzyme from an extreme halophile found to be smaller than its non-halophile counter parts.

H. cutirubrum L-alanine dehydrogenase has a much more restricted substrate specificity than the corresponding B. cereus and B. subtilis enzymes, especially in the deamination reaction. However, its most remarkable property is its absolute requirement for  $K^+$  for oxidative deamination compared with its ability to catalyse the reverse reaction, reductive amination, equally well in presence of  $K^+$ ,  $Na^+$  or  $NH_4^+$ , and to a lesser extent with  $Cs^+$  or  $Li^+$ , as the monovalent cation. This is the first case to be described of an enzyme from an extreme halophile whose alkali metal cation specificity differs for the forward and backward reactions.

VI Bibliography

- Abram, D. & Gibbons, N.E. (1961) Can. J. Microbiol. 7 741.
- Amelunxen, R. & Lins, M. (1968) Arch. Biochem. Biophys. 125  
765.
- Baas-Becking, L.G.M. (1928) Tijdschr. Ned. Dierkund. Ver. Sr.  
III 1 6.
- Batt, T. & Brown, D.H. (1974) Planta 116 27.
- Baxter, R.M. & Gibbons, N.E. (1954). Can. J. Biochem. Physiol.  
32 206.
- Baxter, R.M. & Gibbons, N.E. (1956) Can. J. Microbiol. 2  
599.
- Baxter, R.M. & Gibbons, N.E. (1957) Can. J. Microbiol. 3  
461.
- Baxter, R.M. (1959) Can. J. Microbiol. 5 47.
- Bayley, S.T. & Kushner, D.J. (1964) J. Mol. Biol. 9 654.
- Bayley, S.T. (1966) J. Mol. Biol. 15 420.
- Berberich, R. Kaback, M. & Freese, E. (1968) J. Biol. Chem.  
243 1006.
- Bergey's Manual of Determinative Bacteriology (7th Ed.)  
(1957) Ed. by Breed, R.S., Murray, E.G.D. & Smith, N.R.  
Baltimore; Williams & Wilkins Co.
- Bergey's Manual of Determinative Bacteriology (8th Ed.)  
(1974) Ed. by Buchanan, R.E. & Gibbons, N.E. Williams &  
Wilkins, Co. Baltimore.
- Blaurock, A.E. & Stoeckenius, W. (1971) Nature New Biology  
233 152.

- Breed, R.S., Murray, E.G.D. & Smith, N.R. (1957) Bergey's Manual.
- Brown, A.D. & Gibbons, N.E. (1955) Can. J. Microbiol. 1 486.
- Brown, A.D. & Shorey, C.D. (1962) Biochim. Biophys. Acta 65 258.
- Brown, A.D. (1963) Biochim. Biophys. Acta 75 425.
- Brown A.D. (1964) Bacteriol. Rev. 28 296.
- Bürk, R.R. & Pateman, J.A. (1962) Nature 196 450.
- Cazzulo, J.J. & Vidal, M.C. (1972) J. Bacteriol. 109 437.
- Cazzulo, J.J. (1973) FEBS Lett. 30 339.
- Cazzulo, J.J. (1975) Rev. Assoc. Arg. Microbiol. 7 68.
- Cheah, K.S. (1970) FEBS Lett. 7 301.
- Christian, J.H.B. (1956) Ph.D. Thesis, Cambridge University.
- Christian, J.H.B. & Waltho, J.A. (1962) Biochim. Biophys. Acta 65 506.
- Ciferri, A., Puett, D., Rajagh, L., & Herman, J. (1968) Biopolymers. 6 1019.
- Cohen-Bazire, G. and Pfennig, N. (1969) J. Bacteriol 100 1049.
- Colwell, R.R. & Liston, J. (1961) J. Bacteriol. 82 1
- Colwell, R.R. (1963) Taxonometrics 3 1
- Colwell, R.R. & Gochnauer M.B. (1963) Bacteriol Proc. P. 40.
- Colwell, R.R. & Gibbons, N.E. (1978) Int. J. Sys. Bact. in the press.
- Dawson, R.M.C., Elliot, D.C., Elliot, W.H. & Jones, K.M. (1969) Data for Biochemical Research, 2nd Ed. p. 196. Clarendon Press Oxford.

- Dannon, A. & Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71 1234.
- Demina, A.S. Yokovleva, V.I. & Kretovich, W.L. (1966) Mikrobiologiya 35 767.
- Dixon, M. & Webb, E.C. (1961) Adv. Protein Chem. 16 197.
- Dixon, M. & Webb, E.C. (1964) Enzymes, 2nd Ed., Academic press. Inc. N.Y.
- Dundas, I.D., Srinivasan, V.R., & Halvorson, H.O. (1963) can. J. Microbiol. 9 619.
- Dussault, H.P. & Lackance, R.A. (1952) J. Fish, Res. Board. Can. 9 157.
- Egami, F. (1955) Bull. Soc. Chim. Biol. 37 207.
- Farlow (1880): Kocur & Hodgkiss (1973) Int. J. Syst. Bacteriol. 23 151.
- Fersht (1977) Enzyme Structure & Mechanism, W.H. Freeman & Co. San Francisco.
- Fairhurst, A.S. King, H.K. & Sewell, C.E. (1956) J. Gen Microbiol. 15 106.
- Fisher, L. (1969) in Laboratory Techniques in Biochemistry and Molecular Biology Vol. I p.157-396. (Work, T.S. & Work, E. Ed. New York, John Wiley & Sons Inc.).
- Fitt, P.S. & Peterkin, P.I. Barua, N.N. (1975) Biochem. J. 149 719.
- Fitt, P.S. & Peterkin, P.I. (1976) Biochem. J. 157 161.
- Flannery, W.L. (1956) Bacteriol Rev. 20 49.
- Fraustadt, M. (1959) Flora oder Allg. Bot. Zeit. 148 203.

- Freese, E. & Oosterwyk, J. (1963) *Biochem. J.* 2 1212.
- Freese, E., Park, S.W., & Cashell, M. (1964) *Proc. N.A.S.*  
51 1164.
- Freeze, H. & Brock, T.D. (1970) *J. Bacteriol.* 101 541.
- Galyas, E., Yakovleva, V.I. & Kretovich, V.L. (1966)  
*Biokhimica* 31 636.
- Germano, G.J. & Anderson, K.E. (1968) *J. Bacteriol.* 96 55.
- Gibbons, N.E. & Baxter, R.M. (1953) *Proc. Inter. Congr.*  
*Microbiol.* 6th Congr. Rome. I. 20.
- Gibbons N.E. (1957) *Can. J. Microbiol.* 3 249.
- Gibbons, N.E. (1969) In *Methods in Microbiology* Vol. 3B.  
p. 169 J.R. Norris & P.W. Ribbons Ed. Academic Press. N.Y.
- Gochnauer, M.B., & Kushner D.J. (1969) *Can. J. Microbiol.* 15  
1157.
- Gochnauer, M.B, Kushwaha, S.C., Kates, M. & Kushner, D.J.  
(1972) *Arch. Mikribiol.* 84 339.
- Goldman, D.S. (1959) *Biochim. Biophys. Acta* 34 527.
- Green, A.A. & Hughes, W.L. (1955) *Methods Enzymology* 1,  
67.
- Grey, V. & Fitt, P.S. (1975) *Can. J. Microbiol.* 22, 440.
- Griffiths, E. & Bayley, S.T. (1969) *Biochemistry* 8, 541.
- Harrison, F.C. & Kennedy, M.E. (1922) *Trans. Roy. Soc. Can.*  
V. 16 101.
- Henderson, R. (1977) *Ann. Rev. Biophys. Bioeng.* 6 87.
- Higa, E.H. & Ramaley, R.F. (1973) *J. Bacteriol.* 114 556.
- Higa, A.I. & Cazzulo, J.J. (1973) *Experientia* 29 1081.

- Hilderbrand, E. & Dencher, N (1975) Nature, 257, 46.
- Hills, G.M. (1949) Biochem. J. 45, 363.
- Hochstein, L.I. & Dalton, B.P. (1968) J. Bacteriol. 95, 37.
- Hochstein, L.I. & Dalton, B.P. (1973) Biochim. Biophys. Acta, 302, 216.
- Hochstein, L.I. (1975) Biochim. Biophys. Acta 403, 58.
- Hof, T. (1935) Rec. Trav. Botan. NeeHandais 32, 92.
- Holmes, P.K., Dundas, I.E.D. & Halvorson, H.O. (1965) J. Bacteriol. 90, 1159.
- Holmes, P.K. & Halvorson, H.O. (1965a) J. Bacteriol. 90, 312.
- Holmes, P.K. & Halvroson, H.O. (1965b) J. Bacteriol. 90, 316.
- Horecker, B. & Kornberg, A. (1948) J. Biol. Chem. 175, 385.
- Horton, A.A. & Kornberg, H.L. (1964) Biochem. Biophys. Acta. 89, 381.
- Houwink, A.L. (1956) J. Gen. Microbiol. 15, 146.
- Hubbard, J.S. & Miller, A.B. (1968) Bact. Proc. 68, 117.
- Hubbard, J.S. & Miller, A.B. (1969) J. Bacteriol. 99, 161.
- Hubbârd, J.S. & Miller, A.B. (1970) J. Bacteriol. 102, 677.
- Ingram, M. (1947) Proc. R.Soc. B. 134, 181.
- Ingram, M. (1957) Symp. Soc. Gen. Microbiol. 7, 90.
- Inman, R.B. and Jordan, D.O. (1960) Biochim. Biophys. Acta 42, 421.
- Joshi. J.G. Guild, W.R. & Handler, P. (1963) J. Mol. Biol. 6, 34.
- Joy, K.W. (1969) Plant Physiol. 44, 849.

- Kates, M., Palameta, By Joo, C.N., Kushner, D.J., & Gibbons, N.E. (1966) *Biochemistry* 5 4092.
- Kates, m., Wassef, M.K. & Kushner, D.T. (1968) *Can. J. Biochem.* 46 971.
- Kates, M. Wasset, M.K. and Pugh, E.L. (1970)
- Kates, m. (1972) *Techniques of Lipidology* p. 345 North Holland publishing Co., Amsterdam & London.
- Kayushin, L.P. Skulachev. Y.P. (1974) *FEBS Lett.* 39 39.
- Katznelson, H. and Lochhead, A.G. (1952) *J. Bacteriol.* 64 97.
- Kellerman (1915) *Zentrabl. Bakteriolog. Parasitenk. Infektionskr. Hyg. Abt. II* 42 398.
- Keradjopoulos, D. & Wulff, K. (1974) *Can. J. Biochem.* 52 1033.
- Klebahn, H. (1919) *Mitt. Inst. Allgem. Botan.Hamburg*, 4 11.
- Kluyver, A.J., Hof, T. & Boezaardt, A.G.J. (1939) *Enzymologia* 7 257.
- Kottle, R.R., Orłowski, M., White, D. & Grutsch, J. (1974) *J. Bacteriol.* 119 650.
- Kretovich, V.L., Demina, A.S. & Yakovleva, V.I. (1964) *Doklady Acad. Nauk. S.S.R.* 159 1169.
- Kretovich. W.L., Evstigneeva, Z.G. & Tomova, N.G. (1970) *Can. J. Botany* 48 1179.
- Kushner, D.J. & Bayley, S.T. (1963) *Can. J. Microbiol.* 9 53.
- Kushner, D.J. (1964) *J. Bacteriol.* 87 1147.
- Kushner, D.J., Bayley, S.T., Boring, J., Kates, m. & Gibbons, N.E. (1964) *Can. j. Microbiol.* 10 483.
- Kushner, D.J. & Onishi, H. (1966) *J. Bacteriol.* 91 653.
- Kushner, D.J. (1968) *In Advances in Applied Microbiology*

Vol. 10, p. 73 Ed. By Umbreit, W.W. New York. Academic Press.  
Inc.

Kushwaha, S.C. & Kates, M. (1973) *Biochim. Biophys. Acta*  
316, 235.

Kushwaha, S.C., Gochnauer, M.B., Kushner, D.J. & Kates, M.  
(1974) *Can. J. Microbiol.* 20 241.

Kushwaha, S.C., Kates, M. & Martin, W.G. (1975) *Can. J.*  
*Biochem.* 53 284.

Lanyi, J.K. & Silverman, M.P. (1972) *Can. J. Microbiol.* 18  
993.

Lanyi, J.K., Yearwood-Drayton, V. & Macdonald, K.E. (1976)  
*Biochemistry* 15 1595.

Lanyi, J.K. (1974) *BBA* 356 245.

Lanyi, J.K. (1974) *Bacteriol. Rev.* 38 272.

Larsen, H. (1962) In *the Bacteria*, Vol. 4, p. 297 Ed. by  
Gunsalus, I.C. & Stanier, R.Y. New York: Academic Press Inc.

Larsen, H. (1967) In *Advances in Microbial Physiology* Vol. 1,  
p. 97 Ed. By Rose, A.H. & Wilkinson, J.F. New York: Academic  
Press Inc.

Larsen, H. (1973) *Antonie Van Leeuwenhoek* 39 383.

Layne, E. (1957) In *Methods in Enzymology*, Vol. 3, p 447.  
Ed. by Colowick, S.P. & Kaplan V.O. New York: Academic Press  
Inc.

Lochhead, A.G. (1934) *Can. J. Res.* 10 275.

Louis, B.G. & Fitt, P.S. (1971a) *Biochem. J.* 121 621.

Louis, B.G. & Fitt, P.S. (1971b) *Biochem. J.* 121 629.

Louis, B.G., Peterkin, P.I. & Fitt, P.S. (1971) *Biochem. J.*  
121 635.

- Louis, B.G. & Fitt (1971c) FEBS Lett. 14, 143.
- Louis, B.G. & Fitt, P.S. (1972a) Biochem. J. 127, 69.
- Louis, B.G. & Fitt, P.S. (1972b) Biochem. J. 127, 81.
- Louis, B.G. & Fitt, P.S. (1972c) Biochem. J. 128, 755.
- MacLeod, R.A. (1965) Bacteriol. Rev. 29, 9.
- Maeno, H. (1957) J. Biochem. 44, 99.
- Marshall, C.L., Wicken, A.J. & Brown, A.D. (1969) Can. J. Biochem. 47, 71.
- Meers, J.L. & Pedersen, L.K. (1972) J. Gen. Microbiol. 70, 277.
- Mevarech, M. Eisenberg, H. & Neumann, E. (1977) Biochemistry 16, 3781.
- McClare, C.W.F. (1967) Nature 216, 766.
- McCormick, N.G. & Halvorson, H.O. (1964) J. Bacteriol. 87, 68.
- McCowen, S.M. & Phibbs, P.V. (1974) J. Bacteriol. 118, 590.
- Miller, J.E., & Hochstein, L.I. (1972) Bacteriol. 112, 656.
- Mitchell, P. (1961) Nature 191, 144.
- Mitchell, P. (1966) Biol. Rev. 41, 445.
- Mitchel, H. & Oesterhet, D. (1976) FEBS Lett 65, 175.
- Mohr, V. & Iarsen, H. (1963) J. Gen. Microbiol. 31, 267.
- Moore, R.L. & McCarthy, B.J. (1969a) J. Bacteriol. 99, 248.
- Moore, R.L. & McCarthy, B.J. (1969b) J. Bacteriol. 99, 255.
- Neilson, A.H. & Duodoroff, M. (1973) Arch. Microbiol. 89, 15.
- Norberg, P. and von Hofsten, B. (1969) J. Gen. Microbiol. 55, 251.

- O'Connor, R.J. & Halvorson, H.O. (1959) J. Bacteriol 78, 844.
- O'Connor, R.J. & Halvorson, H.O., (1960) Arch. Biochem. Biophys. 91, 290.
- O'Connor, R.J. & Halvorson, H.O. (1961) Biochim. Biophys. Acta. 48, 47.
- Oesterhelt, D., & Stoeckenius, W. (1971) Nature New Biol. 233, 149.
- Oesterhelt, D. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1554.
- Oesterhelt, D. & Krippahl, G. (1973) FEBS Lett. 36, 72.
- Oesterhelt, D. & Hess, B. (1973) Eur. J. Biochem. 37, 316.
- Oesterhelt, D. & Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2853.
- Oesterhelt, D. (1975) Ciba Foundation Symp. Energy Transform. Biol. Systems. 31, 147.
- Onishi, H., McCance, M.E., & Gibbons, N.E. (1965) C.J.M. 11, 365.
- Petter, H.F.M. (1932) Doctor's thesis, Utrecht. (1931) Kon. Akad. Wentenshap. Amsterdam. Proc. 34, 1417.
- Peterkin, P.J. & Fitt, P.S. (1971) Biochem. J. 121, 613.
- Plaut, G.W.E. & Lardy, H.A. (1949) J. Biol. Chem. 180, 13.
- Piérard, A. & Wiame, J.M. (1960) Biochim. Biophys. Acta 37, 490.
- Poulsen (1879) Vidensk Medd Naturhist Forenjöhenhaven 80 231.

- Pugh, E.L. Wassef, M.K., and Kates, M. (1971) *Can. J. Biochem.* 49, 953.
- Qualing, C., and Colwell, R.R. (1964) *Develop, Ind. Microbiol.* 5, 151.
- Reistad, R. (1970) *Arch. Microbiol.* 71, 353.
- Robinson, J. & Katznelson, H. (1953) *Nature* 172, 672.
- Roszkowski, J., Rajalski, A. & Raczynska-Bojanowska, K. (1969) *Acta Microbiol. Pol. Ser. B. Microbiol. Appl.* 1, 59.
- Schoop, G. (1935) *Deut. Tierärztl. Wochenschr* 43, 817.
- Saunders, P.P., Wilson, B.A. & Saunders, G.F. (1969) *J. Biol. Chem.* 244, 3691.
- Sehgal, S.N. & Gibbons, N.E. (1960) *Can. J. Microbiol.* 6, 165.
- Shatilov, V.R., Evstigneeva, Z.G. & Kretovich, V.L. (1968) *DORI. Akad. Nauk. S.S.R.* 178, 482.
- Soo-Hoo, T.S. & Brown, A.D. (1967) *Biochim. Biophys. Acta* 135, 164.
- Steensland, H. & Larsen, H. (1969) *J. Gen. Microbiol.* 55, 325.
- Stoeckenius, W. & Rowen, R. (1967) *J. Cell Biol.* 34, 365.
- Stoeckenius, W. & Kunau, W. H. (1968) *J. Cell Biol.* 38, 337.
- Stoeckenius, W. (1976) *Sci. Am.* 234, 38.
- Stoeckenius, W. & Lozier, R.H. (1974) *J. Supramol. Struct.* 2, 769.
- Sturges, W.S. & Heideman, A.G. (1924) *Abstr. Bacteriol* 8, 14.
- Tate, S.S. & Meister, A. (1971) *Adv. Enzymol.* 35, 503.
- Tomlinson, G.A., Koch, T.K. & Hochstein, L.I. (1974) *Can. J. Microbiol.* 20, 1085.

- Tomlinson, G.A. & Hochstein, L.I. (1976) *Can. J. Microbiol.* 22, 587.
- Tsukamoto, A. (1970) *Plant Cell Physiol* 11, 221.
- Vinsentin, L.P. Chow, C., Matheson, A.T., Yaguchi, M., & Rollin, F. (1972) *Biochem. J.* 130, 103.
- Volcani, B.E. (1940) Doctor's thesis, Hebrew Univ. Jerusalem.
- Wada, A. (1960) *Mol. Physics* 3, 409.
- Walsby, A.E. (1972) *Bacteriol. Rev.* 36, 1.
- Warburg, O. & Christian, W. (1942) *Biochem. Z.* 310, 384.
- Wiame, J. M. & Piérard, A. (1955) *Nature* 176, 1073.
- Weber, M.M. (1949) *Biol. Review, City College of N.Y.* 1, 9.
- Yoshida, A. (1965) *Biochim. Biophys. Acta* 105, 70.
- Yoshida, A. & Freese, E. (1964) *Biochim. Biophys. Acta* 92, 33.
- Yoshida, A. & Freese, E. (1965) *Biochim. Biophys. Acta* 96. 248.