THE POLYNUCLEOTIDE PHOSPHORYLASE OF THE EXTREME HALOPHILE *HALOBACTERIUM CUTIRUBRUM*

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ABBREVIATIONS

RNA	Ribonucleic acid.
DNA	Deoxyribonucleic acid.
RNase	Ribonuclease.
DNase	Deoxyribonuclease.
PNPase	Polynucleotide phosphorylase.
AMP, GMP, IMP, UMP, CMP	The 5'-phosphates of adenosine, guanosine, inosine, uridine, cytidine.
ADP, etc.	The 5'-diphosphates of adenosine, etc.
dADP, etc.	The 5'-diphosphates of deoxyadenosine, etc.
ATP, etc.	The 5'-triphosphates of adenosine, etc.
ApA	Adenylyl-(3'-5'-) adenosine.
ApApdA	Adenylyl-(3'-5'-) adenylyl-(3'-5'-) deoxyadenosine.
tRNA	Transfer RNA.
mRNA	Messenger RNA.
Poly A, etc.	Linear 3'-5' polymer of adenylic acid, etc.
Poly AC, etc.	Linear 3'-5' copolymer of adenylic acid and cytidylic acid, etc., in random sequence.
PGP	Phosphatidyl glycerylphosphate (diether analogue).
PG	Phosphatidyl glycerol (diether analogue).
PEP	Phosphoenol pyruvate.
NAD^+	Nicotinamide-adenine dinucleotide, oxidized.
NADH	Nicotinamide-adenine dinucleotide, reduced.

I. INTRODUCTION

A. POLYNUCLEOTIDE PHOSPHORYLASE

The first published report of the enzymic synthesis and breakdown of polynucleotides appeared in 1955. Grunberg-Manago and Ochoa, in the course of a study of biological phosphorylation mechanisms, found that extracts of Azotobacter vinelandii catalyzed a rapid exchange of ^{32}P-labelled orthophosphate with the terminal phosphate of ADP, IDP, UDP, CDP and GDP. Further, this activity when partially purified (40-fold) catalyzed the incorporation of nucleoside diphosphates into an acid precipitable ultraviolet absorbing material which remained at the origin in a standard chromatographic system for the separation of nucleotides. This material was identified as polyribonucleotide. The polymerization reaction was found to be readily reversible as, in the presence of the enzyme, P_i and Mg^{2+}, the polynucleotide was phosphorolyzed with the release of NDP. Since the reaction was conceived as being analogous to the reversible synthesis and breakdown of polysaccharides catalyzed by phosphorylase, the name polynucleotide phosphorylase was proposed for the enzyme. The name nucleoside diphosphate-polynucleotide nucleotidyltransferase EC 2.7.7.8 has been assigned to it by the Enzyme Commission.

Polynucleotide phosphorylase catalyzes the following reversible reactions:

$$nNPP \rightleftharpoons (NP)_n + nP_i \qquad (1)$$

$$NPP + {}^*P_i \rightleftharpoons NP{}^*P + P_i \qquad (2)$$

The polymerization reaction (Eq.1) can be used to prepare homopolymers by using a single nucleoside diphosphate as the substrate, or heteropolymers by using two or more. The reaction does not require a template and hence there is no ordering of the bases along the polynucleotide chain.

The reverse of equation 1, the phosphorolysis of polyribonucleotides in the presence of orthophosphate, yields the appropriate nucleoside diphosphate or diphosphates as the product. An exchange reaction (Eq.2) exists in which the terminal phosphate of an NDP exchanges with orthophosphate. A fourth reaction has been reported by Heppel, Singer and Hilmoe (1959) in which nucleoside monophosphate units are transferred from a polynucleotide donor to a polynucleotide acceptor without the intervention of orthophosphate or NDP:

$$pApApA + pApApA \rightleftharpoons pApApApA + pApA \qquad (3)$$

All these reactions require the presence of a divalent cation, usually magnesium.

Polynucleotide phosphorylase is specific with respect to the number of phosphates terminally esterified to the nucleoside and the

nature of the sugar moiety of the nucleotide. No reaction occurs with the nucleoside mono- or triphosphates as substrates, nor are they the products of the phosphorolysis reaction. Under usual conditions of incubation no reaction occurs when the deoxyribonucleoside diphosphates replace the ribonucleoside derivatives nor is DNA phosphorolyzed. However, Kaufmann and Littauer (1969) have shown that E. coli polynucleotide phosphorylase catalyzes the addition of one or two deoxyadenylyl residues to ApA. Further, the incubation of ApApdA with the enzyme in the presence of orthophosphate yields ApA and dADP. These incubations were carried out at 37° for 48 h and 24 h respectively.

Early workers found the enzyme to be widely distributed among aerobic and anaerobic bacteria: A. vinelandii, E. coli, Micrococcus lysodeikticus, Clostridium perfringens, Streptococcus faecalis, Bacillus cereus, Edbacterium sarcoginosenum, and Pseudomonas aeruginosa (Grunberg-Manago, 1963). Until recently the enzyme could not be detected in soluble extracts of Lactobacillus arabinosus. However, Thang, Dondon and Grunberg-Manago (1969) have now reported polynucleotide phosphorylase activity in both cell-free extracts and toluene treated cells. The lactobacillus enzyme catalyzes the phosphorolysis of oligonucleotides only and not that of high molecular weight poly A. The polymerization activity was very low being about 1% of that present in A. vinelandii. Thang et al. conclude that polynucleotide phosphorylase is present in all bacteria.

Hilmoe and Heppel (1957) detected a polynucleotide phosphorylase-like activity in guinea-pig liver nuclei. However, their results were equivocal

and Smellie (1963) concluded that all the published reports of the presence of the enzyme in animal and plant tissues were inconclusive. He considered that a combination of other enzymes such as adenylate kinase, phosphatases, RNA polymerases, etc., could have been responsible for the results and that no convincing evidence existed for the occurrence of polynucleotide phosphorylase in higher organisms. Recently, Fitt and See (1970) have isolated, partially purified and proven the specificity of a polynucleotide phosphorylase from guinea-pig liver nuclei. Successive extraction of liver nuclei with sucrose and then saline gave extracts with good phosphorolytic activity but no detectable polymerization activity. The phosphorolytic reaction yielded nucleoside diphosphates as its primary product, proving that the enzyme was indeed a polynucleotide phosphorylase. An exchange activity may have been due to another enzyme. A similar enzyme has now been prepared from rat liver nuclei and its specificity established (See & Fitt, 1970).

The purification of several polynucleotide phosphorylases has been reported. Kimhi and Littauer (1968) obtained a 700-fold purification of the E. coli enzyme in about 10% yield, with DEAE- Sephadex and Sephadex G-200 being the most advantageous steps. Klee and Singer (1968) reported the 400-fold purification in 5% yield of the M. lysodeikticus enzyme. A 50-fold purification of the Cl. perfringens enzyme was reported in 1969 by Fitt and Wille. Thang (1967) obtained a highly purified A. vinelandii enzyme, about 1000-fold purification, in 9% yield. His procedure was laborious involving many steps. Recently, Gajda and Fitt (1970)

have obtained a 2100-fold purification of the A. vinelandii enzyme in better than 75% yield. This good yield is achieved by maintaining the enzyme in the reduced state during preparation and purification with buffers containing β-mercaptoethanol.

The polyribonucleotides formed by the catalytic action of polynucleotide phosphorylase are linear polymers composed of chains of nucleotide units linked by the 3'-5' phosphodiester bonds typical of the nucleic acids. These polymers are of high molecular weight; a poly A with a molecular weight of 2,000,000 has been prepared with the M. lysodeikticus enzyme (Grunberg-Manago, 1963).

The availability of these high molecular weight polymers has made it possible to gain insight into the structure and physico-chemical properties of the nucleic acids. Studies concerning the interaction of poly A and poly U, and poly I and poly C agree with the base-pairing requirements of the double helix structure of DNA proposed by Watson and Crick. They were of help in demonstrating strand separation and specific recombination in DNA. They were also valuable tools for the investigation of the secondary structure of RNA and the hyperchromicity of polynucleotides. The homo- and heteropolymers formed by the action of the enzyme have been employed as synthetic messengers in in vitro systems for the elucidation of the genetic code (Crick, 1963).

All polynucleotide phosphorylases studied have shown an absolute requirement for a divalent metal cation. Grunberg-Manago (1963) stated

that the enzyme has an absolute requirement for Mg^{2+} and that it could not be replaced by Mn^{2+}. However, Babinet, Roller, Dubert, Thang and Grunberg-Manago (1965) made an extensive study of the metal ion requirements of the enzyme and showed that contrary to earlier reports, the E. coli enzyme was active in the presence of several divalent cations. Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} could all replace Mg^{2+} although the latter was the most effective with this enzyme. Mn^{2+} was found to be the most suitable metal for replacing Mg^{2+}.

An E. coli mutant deficient in polynucleotide phosphorylase was isolated by W. Gilbert using nitrosoguanidine on E. coli A19. Polynucleotide phosphorylase activity in this mutant, E. coli Q13 (Hfr, met^-, tyr^-, RNaseI$^-$, PNPase$^-$) was the subject of two interesting reports in 1967 (Hsieh and Buchanan; Thang, Thang and Grunberg-Manago). Hsieh and Buchanan found a manganese-dependent nucleoside diphosphate polymerizing activity. This activity which they purified 20-fold was completely Mn^{2+} dependent and did not show any polymerization activity in the presence of Mg^{2+} even up to 10mM. They could find no evidence of phosphorolytic activity with either Mn^{2+} or Mg^{2+}.

Thang et al. (1967), however, found the Q13 mutant to possess a Mg^{2+} dependent phosphorolytic activity. This moved faster during sucrose density gradient centrifugation than the 150,000 MW alcohol dehydrogenase marker. They also found a very low Mg^{2+} dependent polymerization activity which moved more slowly than the marker. This contrasted with a density

gradient centrifugation of the enzyme from E. coliB in which both the polymerization and phosphorolytic activity moved together at a density corresponding to a MW of about 2000,000.

To some extent the discrepancy between these two reports was resolved by Castles and Singer (1968). They worked with E. coli 11133, a mutant also developed in Gilbert's laboratory, and which only differed from its parent strain E. coli Q13 in having a heat-sensitive RNaseII. A Mn^{2+} dependent polymerization activity was demonstrated by polyacrylamide gel disc electrophoresis of the crude extract. This method is useful in the demonstration of small amounts of polymer. There was also a very faint band of polyribonucleotide formed when Mg^{2+} was used as the divalent cation. On further purifying the extract and separating the protein by gel filtration on Sephadex G-200 they obtained two peaks of polymerization activity. The faster moving (larger molecular size) peak had fifteen times more activity with Mn^{2+} than with Mg^{2+}. The slower moving or smaller molecules had a low polymerization activity with both Mn^{2+} and Mg^{2+}. Both peaks showed minimal phosphorolysis with either divalent cation. They suggested that their Peak A was the enzyme described by Hsieh and Buchanan with Peak B being the low molecular weight enzyme reported by Thang et al. This finding of a Mn^{2+} dependent polymerization activity in Q13 strains was confirmed in a detailed study of polynucleotide phosphorylase mutants of E. coli (Reiner, 1969).

The pH optima of polynucleotide phosphorylases were discussed in Grunberg-Manago's review (1963). She stated that with the A. vinelandii

enzyme the polymerization reaction has a plateau between pH 7.5 and 9, and the exchange reaction a sharp optimum at pH 8.1. With the M. lysodeikticus enzyme the polymerization reaction has an optimum between pH 9 and 9.5 and the phosphorolysis an optimum of pH 8.5. Hsieh and Buchanan (1967) reported a plateau between pH 8.0 and 9.0 for the Mn^{2+} dependent polymerization activity. Fitt and Gibbs (1969) found that the highly purified A. vinelandii enzyme has a pH optimum in the range pH 9 to 10 when glycine buffer was used in place of the Tris buffer used by other workers.

My discussion of the activation of polynucleotide phosphorylase by various small molecules is divided into two sections which deal respectively with

1. The primer requirement of the E. coli, A. vinelandii and M. lysodeikticus enzymes;
2. The stimulation of the Cl. perfringens enzyme by polybases and electrolytes.

1. Early workers found that the polymerization reactions of the partially purified E. coli and A. vinelandii enzymes have a lag phase which is overcome by certain oligonucleotides. The exchange reaction does not show this effect. These nucleotides have to be of the type pApA——pA with a phosphate monoesterified to the C-5' hydroxyl and free terminal C-2' and C-3' hydroxyl groups. These added oligonucleotides are incorporated into the developing polymer. C-3' esterified oligonucleotides of the type ApAp——Ap do not overcome the lag phase and sometimes even act as inhibitors (Grunberg-Manago, 1961). It was

found that with the A. vinelandii enzyme these 3' esterified oligonucleotides can accelerate the reaction but are not incorporated into the final product. This same anomaly is observed when polymers are added to the reaction mixture.

Further elucidation of the role of these primers in the reaction mechanism came with the report of Singer and Guss (1962) that, using the partially purified M. lysodeikticus enzyme, both the exchange and polymerization reactions are stimulated by oligonucleotides with a free terminal C-3' hydroxyl group. They stated that this might be due to the low nucleic acid contamination of their enzyme compared to that of the A. vinelandii preparation. Further they found that the amount of pApApA required for maximal stimulation of polymerization is ten times higher than that required for exchange. This finding is consistent with the oligonucleotide serving catalytically in exchange but as a substrate or chain initiator for polymerization. It is also consistent with the proposal that the exchange occurs when the reversible reaction 1. is operating at or near equilibrium conditions. It does not support Olmstead's (1958) suggestion that the exchange reaction reflects the reversible formation of an AMP-enzyme complex.

Olmsted and Lowe (1959) found that treating a crude preparation of the M. lysodeikticus enzyme with crude trypsin gave an enzyme specific for the polymerization of ADP. They found that their product did not catalyse the polymerization of CDP or UDP, nor the phosphorolysis of poly C.

Fitt and Fitt (1967) studied the effects both of limited and more extensive trypsin digestion on a partially purified, primer-dependent enzyme from M. lysodeikticus. Limited digestion resulted in a marked increase in the primer requirement for CDP-polymerization with no effect on the ADP-polymerization activity. More extensive digestion led to an increase in primer requirement for ADP-polymerization. The limited digestion caused little change in the physical properties of the enzyme molecule as shown by Sephadex G-200 gel filtration and polyacrylamide gel disc electrophoresis. More extensive digestion however resulted in an active product with an electrophoretic mobility greater than that of the native enzyme. (Fitt, Fitt and Wille, 1968b). The activity of the digested enzyme with or without primer could be restored by addition of β-mercaptoethanol to the reaction mixture. Partially purified E. coli polynucleotide phosphorylase showed a slight inhibition of polymerization activity by β-mercaptoethanol and certain aminothiols. (Bogoyavlenskaya et al., 1967).

Klee (1967) reported on a primer-independent enzyme isolated from M. lysodeikticus and partially purified. This could be converted to a primer-dependent form with an accompanying increase in electrophoretic mobility, by a variety of treatments including prolonged ultracentrifugation, trypsin digestion and treatment with 1M guanidinium-HCl. The primer-dependent form obtained after tryptic hydrolysis could be reconverted to the primer-independent enzyme by treatment with thiol reagents such as β-mercaptoethanol. (Klee and Singer, 1968). Thiol inhibitors restored

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primer-dependency. They concluded that tryptic hydrolysis unmasks a thiol group essential for polymerization when primer is not present.

Using a highly purified A. vinelandii enzyme, Gajda and Fitt (1969) found that the enzyme showed a reversible inactivation by oxidation or treatment with thiol inhibitors. The fully reduced enzyme was slightly inhibited by β-mercaptoethanol, but in the oxidized form all the lost activity was restored by the thiol reagent. As slow aerial oxidation proceeded there was a fall in the number of free thiol groups in the enzyme. This suggests the presence of free thiol groups essential for activity. However, though the fresh enzyme had no primer requirement for ADP-polymerization a primer requirement developed after two months storage in the presence of β-mercaptoethanol. Further, trypsin digestion of highly purified, reduced A. vinelandii enzyme leads to the development of a primer requirement without any increase in thiol requirement (Gajda & Fitt, 1970).

Therefore with the A. vinelandii enzyme a primer requirement develops which is not reversible with a thiol reagent. These results suggest that the need for a primer develops as the result of structural changes leading to a loss of chain-initiating ability rather than as the result of the oxidation of a thiol group as proposed by Klee and Singer (1968).

2. Early studies of the Cl. perfringens enzyme (Dolin, Godiniaux & Grunberg-Manago, 1961; Dolin, 1961 & 1962) showed that this polynucleotide phosphorylase differs considerably from others.

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The polymerization of ADP or GDP was markedly stimulated by polybases such as polylysine, polyornithine and polyvinylamine, whereas UDP and CDP polymerization were completely inhibited. This effect was studied in detail (Fitt, Dietz and Grunberg-Manago, 1968a), and it was found that both polylysine and inorganic salts increased the rate of formation of poly A. A lag phase existed in the reaction activated by salt. The reaction in the presence of polylysine was twice as fast as that with salt and there was no lag phase. The polylysine effect showed a sharp optimum at pH 8.2. Both polylysine and salt lower the K_m but only the former increases the V_{max}. With a more highly purified Cl. perfringens enzyme, Fitt and Wille (1969a & b) found that, on trypsin digestion, a rapid loss of the polylysine-stimulated activity occurred while the activity without the activator was affected more slowly. Also the purified enzyme did not separate into two components during sucrose density gradient centrifugation or Sephadex G-200 gel filtration. It was concluded that charge effects on the enzyme itself rather than the existence of two forms of the enzyme are responsible for the stimulation by polybases and salts. The effectiveness of these activators increases with chain length up to about 30 residues.

There is not a great deal known yet as to the precise localization of polynucleotide phosphorylase in the cell. Grunberg-Manago (1963) states that the enzyme is found in the soluble extracts after initial methods of breaking the cell walls which include grinding with alumina, preparation of an acetone powder and prolonged sonication. In 1962, Abrams and McNamara

presented data which showed the presence of the enzyme in the cell membranes of Streptococcus faecalis produced by the metabolic lysis of protoplasts. The activity of the washed pellets was ten times that of the soluble fraction. The ribosomal particles isolated from the soluble fraction contained no activity. Kimhi and Littauer (1967) studied the intracellular distribution of the enzyme in E. coli cells. The cells were broken in a French pressure cell at a pressure of 4000 psi. The major portion of the enzyme activity was in the soluble fraction (80%); washed ribosomes contained 10%; cell membranes (a 30,000g pellet) contained the remaining 10%. The specific activity of the membrane and soluble fractions was the same. The activity was found in 70S, 50S and 30S ribosomes.

The molecular weight of the polynucleotide phosphorylase of E. coli is about 200,000 (Williams and Grunberg-Manago, 1964). Castles and Singer (1968) using E. coli mutant 1113B demonstrated the separation on Sephadex G-200 of two peaks of activity with molecular weights of about 200,000 and 100,000. This was confirmed by them using sucrose density gradient centrifugation. Valentine, Thang and Grunberg-Manago (1969) reported the presence, in electron micrographs of the E. coli enzyme, of 85 Å molecules which showed a triangular profile with a clear central space. This size is appropriate for a molecular weight of 200,000. On the basis of unpublished observations by Thang et al. that polynucleotide phosphorylase separates into 30,000 MW subunits on treatment with urea, a dihedral molecule with point group 32 symmetry having six subunits arranged in two layers was proposed.

Thang, Thang and Grunberg-Manago (1969) isolated a polynucleotide phosphorylase with a molecular weight of 100,000 from E. coli Q13 mutant. On the basis of their unpublished results concerning the existence of 30,000 MW subunits, they suggested that the enzyme in this mutant occurs in two forms - a trimer of MW 100,000 having only phosphorolytic activity and a hexamer of MW 200,000 having both polymerization and phosphorolytic activity.

Dietz and Grunberg-Manago (1967) presented evidence that Cl. perfringens polynucleotide phosphorylase could be separated by sucrose density gradient centrifugation into two activities differing in molecular size. Fitt et al. (1968a) estimated the molecular weight of these two peaks to be 190,000 and 62,000. The partial conversion of the heavy species into the light one can be accomplished by a 30 min. incubation of 25° with β-mercaptoethanol (Guissani and Grunberg-Manago, 1969). However, Fitt and Wille (1969a) could not reproduce the results of Dietz and Grunberg-Manago and obtained a single peak of activity after sucrose density gradient centrifugation. They pointed out that with the Cl. perfringens enzyme spurious double peaks may be observed when a constant volume of enzyme solution is taken from each fraction for assay as was done by Dietz and Grunberg-Manago. This is due to the bell-shaped enzyme saturation curve typical of the Cl. perfringens enzyme when assayed with polylysine (Dolin, 1962).

The physiological role of polynucleotide phosphorylase in the cell has not yet been elucidated. The concentration of free orthophosphate in the cytoplasm indicates that the enzyme possibly acts as a catalyst in the

direction of phosphorolysis of polynucleotides. The roles which have been suggested are:

1. The degradation of mRNA (Grunberg-Manago, 1963). This is supported by the appearance of nucleoside diphosphates after the degradation of ribosomal RNA in E. coli Q_{14} (Natori & Mizuno, 1967).

2. The sequestering of ribonucleoside diphosphates as the precursors of the deoxyribonucleotides (Cohen, 1961).

3. The intranuclear degradation of rapidly-labelled nuclear RNA (Harris, 1963).

No conclusive evidence has yet been put forward to support any of these suggestions. In any case they are difficult to accord with the evidence of Abrams and McNamara that the S. faecalis enzyme is located in the membrane.

B. EXTREMELY HALOPHILIC BACTERIA

Halophilic bacteria are those which require at least 0.5 M salt for survival and growth. They are divided into two classes in the nomenclature of Baxter and Gibbons (1956).

1. Moderate halophiles which grow in concentrations of sodium chloride from 0.5 M to 3.5 M.

2. Extreme halophiles which will not grow unless the salt concentration is 2.5 M or higher and will grow even in saturated salt (about 5 M).

The extreme halophiles are of greater interest to the biochemist because of the limiting conditions in which their metabolic apparatus must function. Halophiles in general were reviewed by Larsen (1962), the

extreme halophiles being the subject of reviews by Larsen (1967) and Kushner (1968).

The extremely halophilic bacteria are found in salt lakes, such as the Great Salt Lake and the Dead Sea, in solar evaporation ponds of salt works and in salted products such as bacon, fish and hides where they cause spoilage. The spoiled products turn red as do the solar ponds due to the color from the many so-called "red halophiles" growing there.

The bacteriological classification of the extreme halophiles has been the subject of much controversy over the years due partly to the fact that their morphology changes readily in different cultural conditions. In Bergey's Manual, Seventh Edition (1957) the rod-shaped extreme halophiles are placed in the genus Halobacterium, family Pseudomonadaceae. There are five species: H. salinarium, H. cutirubrum, H. halobium, H. marismortui and H. trapanicum. This genus consists of rod-shaped obligate aerobes, Gram-negative and non-spore forming, which when motile have polar flagella. A minimum of 15% NaCl(2.5 M)is required for growth with optimum growth occurring at about 25% NaCl(4.3 M). Under favorable growth conditions the rods are mostly regular and slender but under less favorable conditions they assume club-shaped, irregular, sometimes even coccoid forms. The colonies are usually pigmented showing colors from orange to red. Colonies of H. halobium containing gas vacuoles are opaque whereas those without vacuoles are translucent, as are colonies of other halobacteria.

The halophilic cocci are placed in the family Micrococcaceae. (Bergey, 1957). There are three species: Micrococcus morrhuae, Sarcina littoralis and S. morrhuae. They are obligate aerobes, nonmotile and non-spore forming, occurring singly, in pairs or packets. They are Gram-negative or Gram-variable and the colonies are colored various shades of red. They are less extreme halophiles than the halobacteria requiring at least 12% NaCl(2 M)for growth with optimum growth occurring at about 20% NaCl(3.2 M).

Kushner (1968) discusses a reassessment by computer analysis of the taxonomy of the extremely halophilic bacteria. An analysis of 63 strains suggested that many of the species were identical. Only two genera were substantiated, Halobacterium and Micrococcus, the halophilic cocci which Larsen proposes should be given a generic rank and named Halococcus. Within the genus Halobacterium only the single species H. salinarium should be recognized and H. cutirubrum and H. halobium should be considered synonyms.

Moore and McCarthy (1969b) have assessed the genetic relationship between various strains of halophilic bacteria by DNA-DNA duplex formation and DNA-RNA hybridization. They conclude from their base sequence homology studies that the bacteria of the genus Halobacterium are closely related and that there is a more distant relationship between the halophilic rods and cocci.

The extremely halophilic bacteria grow slowly even under optimum conditions, having a generation time of about 7 hours. The cells are grown

at 37° with vigorous aeration which can be supplied by shaking or, in larger cultures, by bubbling air through the medium. Sehgal and Gibbons (1960) devised a medium which supports good growth and consists of casein hydrolysate, yeast extract, sodium citrate, magnesium sulfate, 0.2% KCl, 25% NaCl and ferrous ions 10 ppm. A chemically defined medium has been developed by Onishi, McCance and Gibbons (1965) which contains fifteen amino acids, two nucleotides, glycerol and 25% NaCl as well as several other inorganic salts. The cells for biochemical studies are usually harvested after 48 h growth, about middle log phase.

Abram and Gibbons (1961) first showed that the extreme halobacteria have an absolute requirement for salt not only for growth but for maintenance of the cell envelope. As the saline environment is diluted the rods become spheres and eventually lyse completely when the NaCl concentration reaches 1-1.5 M. NaCl and LiCl are much more effective than KCl or NH_4Cl in maintaining intact cells of H. cutirubrum. The halococci, on the other hand, do not lyse in hypotonic solutions. They require salt for growth but not for maintenance of integrity.

Kushner (1964) showed that NaCl protects the intact rods in the range from 4 M down to 1 M. In KCl and NH_4Cl solutions however, the intact cells start to lyse at a concentration of 3.5 M and are completely lysed at 2.5 M or less. All three salts are equally effective in maintaining the integrity of mechanically-prepared envelopes whose breakdown starts at 3 M salt and is nearly complete at 1 M. These results suggest that the exterior cell surface has certain sites where Na^+ acts to prevent leakage while the

interior surface is equally well maintained by all three monovalent cations. Further they suggest that the role of salts in maintaining the integrity of the intact cell is not solely osmotic.

Soo-Hoo and Brown (1967) studying 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 that order which is the same order as their hydrated volumes. They concluded that the effect of salt concentration on the integrity of the organisms is 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.

Magnesium salts are more effective than sodium salts in preventing lysis of intact cells (Kushner,1964; Soo-Hoo and Brown, 1967). For example 0.5 M $MgCl_2$ is more effective in preventing lysis of H. cutirubrum than is 1 M NaCl.

All these results show that there are two factors involved in the effect of salts on the integrity of halobacteria: the prevention by cations of the electrostatic disaggregation of the membrane and the maintenance of equal osmotic pressures externally and internally.

In electron micrographs the surface of whole cells of H. cutirubrum shows a textured structure with a regular hexagonal pattern (Kushner and Bayley, 1963). The center-to-center distance is between 120 and 150 Å. Brown and Shorey (1962) reported that H. halobium is bounded by a single lipoprotein membrane, the so-called "unit" membrane. However in 1967,

Cho, Doy and Mercer as well as Stoeckenius and Rowan, showed that by using improved fixation techniques a cell envelope in H. halobium consisting of an outer wall and an inner plasma membrane could be demonstrated. The latter authors also found that a stepwise reduction of the salt concentration in which cells were suspended caused a release of cell wall material at a higher concentration than that at which disintegration of the membrane occurs. However even after prolonged dialysis against distilled water some large membrane sheets remain.

Steensland and Larsen (1969), in a detailed study of the cell envelopes of H. salinarium, showed that when the NaCl concentration was lowered to 2.2 M the outer layer became "frayed" and a release of material occurred. In distilled water the outer layer dissolved completely and the membrane disintegrated into tiny sheets. An amino acid analysis of the envelope protein which was about 66% of the salt-free dry weight of the envelopes, was performed. Aspartic and glutamic acids (about 50% as asparagine and glutamine) formed about 25% of the protein. The basic amino acids formed only 5%. This confirmed earlier work by Brown (1963) on the acidic nature of the envelope protein. It is felt that the cations neutralize the negative charges from these acidic residues and that in the absence of salts mutual repulsion causes the envelopes to break up. Kushner and Onishi (1966) removed the protein from envelopes of H. cutirubrum by peptic digestion. The residues consisting of lipid and carbohydrate were stable in distilled water. By contrast removal of the lipids raised the requirement for both monovalent and divalent cations. It was concluded that the requirement for high salt to maintain integrity is due to mutual repulsion between negatively charged groups on proteins. The negatively charged phosphate

groups on the lipids probably act as binding sites for divalent cations such as Mg^{2+}.

The envelopes of halobacteria lack diaminopimelic acid and muramic acid. Since it therefore lacks the mucopeptide which provides mechanical rigidity in other bacteria it is not known how halobacteria maintain their rod shape.

McClare (1967) proposed a model for the binding of the lipid and protein in H. halobium in which a tetradentate inter-molecular chelate forms between groups on the protein, the Mg^{2+} ions and the phosphate groups of the lipids, together with an ionic link between the terminal phosphate and the guanidyl group of an arginine residue. An alternative model showed the terminal phosphate also binding with the magnesium which, in view of the low content of basic amino acids in the envelope proteins, would seem to be preferred. This chelate hypothesis accounts for the stability of the polar linkage in a high salt environment. It would also account for the observation that after extensive dialysis of H. cutirubrum membranes the lipid is extracted as a 1:1 lipid-magnesium complex.

The first intimation of the unusual nature of the lipids of the membranes of the halobacteria was given by Sehgal, Kates and Gibbons (1962) who reported that the lipids consist almost entirely of phosphatides whose side chains are long-chain alkyl groups joined by ether linkages to glycerol, rather than the usual fatty acid ester linkages. The lipids of H. cutirubrum have now been found to consist exclusively of derivatives of a long-chain diether of glycerol. The structure of the diether was established

as α,β-di-*O*-(3,7,11,15-tetramethylhexadecyl)-*L*-glycerol or α,β-di-*O*-phytanyl-*L*-glycerol (Kates, Sastry & Yengoyan, 1963; Kates, Palameta, Joo, Kushner & Gibbons, 1966). The two unusual features are the isoprenoid side chain joined by an ether linkage and *L*-configuration of the glycerol. The three asymmetric centers of each phytanyl group have the absolute configuration 3-*D*, 7-*D*, 11-*D* (3R, 7R, 11R) (Kates, Joo, Palameta & Shier, 1967).

The total lipids of *H. cutirubrum* consist of:

acetone-soluble lipids (including pigments)	10%
phosphatides	65%
glycolipid sulfate	25%

The major phosphatide, about 85% of the lipid-phosphate, is di-*O*-phytanyl-*D*-α-glycerylphosphoryl-α-glycerophosphate (phosphatidyl glycerophosphate, diether analogue). A minor phosphatide, about 10% of the lipid-phosphate, is di-*O*-phytanyl-*D*-α-glycerylphosphoryl-*L*-α-glycerol (phosphatidyl glycerol, diether analogue).

The glycolipid sulfate was found to contain glucose, mannose, galactose, sulfate and di-*O*-phytanyl glycerol in equimolecular proportions.

These unusual lipids have been found in all the species of extreme halophiles yet studied but appear to be absent from moderate or non-halophiles. Also, no fatty acids have yet been found in extreme halophiles. The lipids are associated almost entirely with the cell envelopes. The phosphatides occur in relatively high proportion as magnesium salts, supporting the

hypothesis that binding of the lipids and protein may occur through magnesium chelates.

The acetone-soluble lipids (non-polar) of H. cutirubrum were found to consist of red carotenoid pigments (43%) and squalenes (48%), with a small amount of a vitamin K-type quinone (Tornabene, Kates, Gelpi & Oro, 1969). The carotenoid pigments are situated in the cell envelope and their function seems to be to protect the organism against photochemical damage. A colorless mutant of H. salinarium had its growth rate strongly depressed when exposed to visible light from a tungsten source; the growth rate of the red wild type was not affected by these conditions (Larsen, 1962). The main pigment is bacterioruberine a which has the structure of a di-demethylated spirilloxanthin, as suggested by Lederer (1938) and confirmed by Liaaen Jensen (1960). It has absorption maxima of 460, 495 and 528 nm in methanol. The squalenes consist of the fully isoprenoid squalene ($C_{30}H_{50}$), dihydrosqualene ($C_{30}H_{52}$) and tetrahydrosqualene ($C_{30}H_{54}$) in the ratio 1:0.4:0.1.

The predominant pathway of lipid biosynthesis in H. cutirubrum involves mevalonic acid with the specific activity of the phytanyl chains being much higher when ^{14}C-mevalonate is used as the precursor than when ^{14}C-acetate is used (Kates, Wassef & Kushner, 1968). Rather unexpectedly fatty acids (once suggested as being contaminating lipids from the culture medium) are definitely formed with 1-^{14}C-acetate as the precursor, though no evidence of uptake of 2-^{14}C-malonate could be obtained.

The question also arises as to the source of the D-α-glycerophosphate (sn-glycero-1-phosphate) present in extreme halophiles. In studies of the stereospecificity of the glycerol kinase and the glycerophosphate dehydrogenase in H. cutirubrum Wassef, Sarner and Kates (1970) found them to be stereospecific for the formation of only sn-glycero-3-phosphate. The activity of these enzymes was associated with the cell envelope as well as the cytoplasm. Various other pathways for the synthesis of sn-glycero-1-phosphate were examined and found not to function. It was suggested that a stereospecific reduction of a phytanyl ether derivative of dihydroxyacetone might yield a glyceryl ether with the required configuration. What is at least clear is that the biosynthetic pathway for the phospholipids of H. cutirubrum must be different from that demonstrated in other organisms.

The requirement of the extreme halophiles for such a high salt concentration for growth and the maintenance of morphological integrity raises the question of the intracellular salt concentration of these bacteria. Do they possess a highly efficient osmotic pump or is their internal ionic strength comparable to that of their external environment? Evidence has accumulated that the intracellular salt concentration is indeed very high. Chloride determinations on packed cells (Gibbons and Baxter, 1953) gave the first indications of a high total salt content.

However the extremely high ratio of K^+ to Na^+ within the cell was demonstrated by Christian and Waltho (1962). They tested non-halophiles as well as moderate and extreme halophiles; a coccal and a rod form from

each group were examined. They determined the apparent intracellular concentration of Na^+, K^+, amino acids, Cl^- and inorganic phosphate, and corrected for interstitial space using phosphate as an indicator. Their data for the ions of the halococcus and halobacterium are:

	S. morrhuae	*H. salinarium*
	moles/kg cell water	moles/kg cell water
Na	3.17 ± 0.28	1.37 ± 0.21
K	2.03 ± 0.36	4.57 ± 0.12
Cl	3.66 ± 0.25	3.61 ± 0.07
NaCl in medium(M)	4.0	
KCl in medium(M)	0.032	

Though the figures are more striking for the rod than for the coccus, both these cells concentrate K^+ against a steep gradient and indeed in *H. salinarium* the concentration approaches the solubility limit for this salt. Evidently the water activity inside these cells must be exceptionally low.

The functioning of the metabolic apparatus of these cells in the presence of nearly saturated concentrations of salt was investigated by a number of early workers. The following enzymes were studied in crude extracts: aspartate-glutamate transaminase (Robinson and Katznelson, 1953), glycerol, isocitrate, succinate, malate and lactate dehydrogenases, cytochrome oxidase and cysteine desulfhydrase (Baxter and Gibbons, 1954, 1956, & 1957; Baxter, 1959), malate dehydrogenase (Holmes and Halvorson, 1965a), alkaline phosphomonoesterase and glucose dehydrogenase (Egami, 1955;

Maeno, 1957), nicotinamide adenine dinucleotide oxidase (Hochstein and Dalton, 1968). These enzymes required a high salt concentration (2-4 M) for optimum activity in sharp contrast to the corresponding ones from non-halophilic bacteria which were inhibited by salt concentrations over 0.3 M and were completely inhibited by 1 M salt. Egami (1955) proposed the term "halophilic" for these enzymes which was supported in Larsen's 1967 review. In testing the effect of a number of salts other than sodium chloride it was found that K^+ was as least as effective as Na^+ in maintaining activity, and sometimes doubled the activity (Baxter and Gibbons, 1956; Holmes and Halvorson, 1965b).

More recent studies on partially purified enzymes have shown that this requirement for salt is maintained as the enzyme is purified. A highly purified (760-fold) malate dehydrogenase of H. salinarium retains its requirement for at least 1 M NaCl or KCl for optimum activity (Holmes and Halvorson, 1965b).

A nearly crude preparation of H. cutirubrum catalase (5-fold purification) was used by Lanyi and Stevenson (1969) for a study of the effect of many salts and some organic solvents on the activity of the enzyme. They found that the salts affected the activity in the following order: KCl > NaCl > LiCl > NH_4Cl, with LiCl being most effective at a concentration of 0.5 M and the others at about 1-1.5 M. The effect of ethylene glycol, glycerol or dimethyl sulfoxide was similar to that of the salts with optimum activity between 3-5 M and inhibition at higher concentrations.

In studying the proteolytic enzymes from an extreme halophile Norberg and Hofsten (1969) found three activities - an extracellular caseinolytic activity, a cell-bound caseinolytic activity and a peptidase. All of these showed no activity below 1 M NaCl or KCl, with optima between 2 and 4 M of each of the salts.

Griffiths and Bayley (1969) studied the effect of NaCl and KCl on the activity of a crude preparation of H. cutirubrum aminoacyl transfer ribonucleic acid synthetases. They found that aminoacyl-tRNA formation dropped markedly at KCl concentrations below 3.8 M and was only 10% of maximum at 1 M KCl. NaCl would partially compensate for reduction of the KCl concentration but when KCl was completely replaced by NaCl, the activity was only 30% of maximum. Replacing K^+ and Na^+ does not affect the apparent K_m of the reaction but causes some reduction in the apparent V_{max}. In contrast, replacing K^+ with NH_4^+ considerably increases the apparent K_m without changing the apparent V_{max}. These data could be explained on the basis of the NH_4^+ ion being a competitive inhibitor of the enzyme or the enzyme-AMP complex with respect to the amino acid.

Aitken, Wicken and Brown (1970) published the results of a study of the salt relations and kinetics of a crude enzyme preparation of H. salinarium isocitrate dehydrogenase (NADP-specific). The velocity was maximal with either NaCl or KCl at about 1 M. The maximum was higher with NaCl. The apparent K_m for isocitrate was minimal at 0.75 M NaCl and 1.5 M KCl. The apparent K_m for NADP was minimal at 0.25 M concentration of either salt. At concentrations above 1 M both salts acted as linear non-competitive inhibitors of the enzyme.

In studying the electron transport chain of H. cutirubrum, Lanyi (1969a) tested the NADH oxidase system for NaCl dependence at pH 7.2 and 9.4. The crude extract was incubated 3 min in buffer containing various concentrations of NaCl before the reactions were started. All three enzymes - NADH oxidase, NADH dehydrogenase (FMN acceptor) and cytochrome oxidase showed no activity at zero salt with an optimum activity at 2 M and higher. The activity of only the NADH dehydrogenase was affected by the pH. At pH 7.2 it reached an optimum at about 1.5 M NaCl while at pH 9.4 it did not reach optimum until 3 M NaCl. The loss of activity of the enzymes at low salt concentrations was tested by exposure for only three minutes before being returned to high salt. Even with this extremely short exposure to low salt NADH oxidase and NADH dehydrogenase lost as much as 90% of their activity. Lanyi (1969b) reported the effects of monovalent and divalent cations and of polyamines on NADH dehydrogenase as assayed with menadione. The enzyme was prepared by spinning the crude extract for 72 h at 78,500g. A clear red viscous liquid between the pellet and the supernatant fluid, labelled the particulate fraction, was used without further purification. It showed no activity in the absence of salt, reaching an optimum at 2 M or higher. There was a reversible loss of activity at low salt as well as a time-dependent irreversible inactivation. The K_m for menadione was unchanged even when the activity was lowered by low salt.

In addition to monovalent salts other substances such as $MgCl_2$, $CaCl_2$ and polyamines have been shown to activate the halophilic respiratory enzyme NADH dehydrogenase as assayed with indophenol (Hochstein and Dalton,

1968). These multivalent ions are effective at much lower concentrations than is NaCl in activating the enzyme.

The effect of salt on the stability of halophilic enzymes has been reported by several investigators. Baxter (1959) concluded from his studies on crude lactic dehydrogenase of H. salinarium that whereas 3 to 4 moles of salt per mole of enzyme are required for activation, less is required for stabilization, about 2 moles. Holmes and Halvorson (1965b) reported that their highly purified malic dehydrogenase required 1 to 3 M NaCl or KCl for optimum activity whereas 4.3 M NaCl was needed for stabilization. Hubbard and Miller's results (1968) with crude isocitrate dehydrogenase are essentially the same, with the optimum salt concentration for activity being about one-quarter that needed for stability. This enzyme was also found to be stabilized by its substrate, 4 mM isocitrate and 10 mM $MgCl_2$ giving the same protection as 4 M NaCl. The crude NADH dehydrogenase studied by Lanyi (1969b), in contrast to all these showed optimum activity and good stability at the same salt concentration, namely 2 M NaCl. It is difficult to compare his results with those of others, however, as his stabilization studies were for a period of only 3 minutes.

In the first study on the regulation of a halophilic enzyme, Liebl, Kaplan and Kushner (1969) reported results on a crude (3-fold purification) preparation of H. cutirubrum aspartate transcarbamylase. This enzyme required 1.1 M NaCl plus 2.9 M KCl for optimum activity, and was highly sensitive to feedback inhibition by cytidine triphosphate. This inhibition was in itself salt-dependent, showing the largest percentage inhibition at 4 M salt with no inhibition at 2 M or lower.

The fact that halophilic enzymes are inactivated, usually irreversibly, at low ionic strength makes their purification and characterization difficult. Indeed their purification is so difficult that the plaints find their way into the scientific literature. To quote Holmes and Halvorson (1965a), "In the past, we have spent much time in attempts to purify halophilic proteins in the presence of high salt concentrations and such endeavours have met with little success". This statement came in their report of the first highly purified halophilic enzyme, the malate dehydrogenase (NAD-specific) of H. salinarium which they prepared 763-fold purified in 0.5% yield. They achieved this by taking advantage of the fact that this enzyme can be reactivated about 55% from the salt-free and inactive state by dialysis against 25% NaCl. In the salt-free state the enzyme could be purified by ammonium sulfate fractionation, ion exchange chromatography and so on. They suggested this as a possible method for the purification of many halophilic enzymes.

Egami (1955) reported the 50-fold purification of an alkaline phosphomonoesterase of a halophilic bacterium isolated in his laboratory. The purification was achieved by trypsin digestion, which the author states does not affect this enzyme, followed by fractionation with cold alcohol.

Almost nothing is known about the molecular weights of halophilic enzymes. Ingram (1947) theorized that "since in halophiles the enzymes resist salts even in vivo, they may perhaps be involved in smaller molecular aggregates than in the case of normal cells".

The catalases of H. cutirubrum and beef liver were chromatographed separately on a Biogel P-300 column (2.5 by .35 cm) in a 0.2 M NaCl-0.1 M $MgCl_2$-Tris buffer (Lanyi and Stevenson, 1969). Both enzymes emerged well after the void volume, the retention constants being 1.70 and 1.60 respectively. The beef liver enzyme is reported to have a molecular weight of about 240,000, and this gel filtration experiment suggests a close similarity in the molecular weights of these two catalases.

Norberg and Hofsten (1969) chromatographed a cell extract of H. salinarium on Sephadex G-100 in the presence of 25% NaCl. The activity peak for a peptidase which cleaves L-leucylglycine had a retention constant of about 1.9. The main protein peak emerged at the void volume, indicating that the peptidase has a lower molecular weight than the other soluble enzymes of this organism.

There is a small amount of data available at present on the nucleic acid content of the extremely halophilic bacteria, and almost none on their nucleic acid metabolism.

The base composition of bulk RNA and DNA of H. salinarium strain 1, has been determined chemically to be as follows (Larsen, 1967):

	Base composition (moles %)				
	Guanine	Adenine	Cytosine	Thymine or Uracil	$\frac{A+T}{G+C}$
DNA	31.0 ± 2.7	17.5 ± 0.8	34.5 ± 1.5	17.0 ± 1.7	0.53
RNA	33.4 ± 0.4	24.0 ± 0.5	22.0 ± 1.3	20.0 ± 0.9	0.16

The GC content of the DNA is about 65% which accords well with Marmur, Falkow and Mandel (1963) who give the GC content of the DNA of H. salinarium and H. cutirubrum as 66-68%.

The nucleic acids of the same strain were investigated further by ion exchange chromatography on DEAE- and ECTEOLA-cellulose columns, being eluted with a NaCl gradient. The elution patterns obtained for both DNA and RNA were identical to those of a non-halophilic pseudomonad. Melting curves on the DNA preparation gave T_m values which fitted well with the base data of the table. There is nothing to indicate that the nucleic acids of halobacteria differ in their fine structure from those of non-halophilic organisms.

The tRNA of H. cutirubrum does not precipitate at pH 5, from a 150,000g supernatant as is the case with non-halophiles (Griffiths and Bayley, 1969). Aminoacylation of this tRNA was dependent on 3.8 M KCl, 0.04 M magnesium acetate and ATP. Manganese could substitute for magnesium with the loss of about 25% of the activity.

The tRNA of H. cutirubrum was not inactivated by low salt concentration as these conditions were met with during the isolation. The tRNA was completely soluble in 3.4 M KCl plus 0.1 M magnesium acetate, and also in distilled water. In the salt solution it had an ultraviolet absorption spectrum with λ_{max} 258nm and λ_{min} 228nm; optical density 258/228=2.1-2.26; preparations contained 1-3% protein. This tRNA could replace E. coli tRNA in the aminoacylation system from E. coli and vice versa.

Generally the DNA molecules from any one species of bacteria show a unimodal distribution of base composition as shown by a single peak in a CsCl density gradient and a smooth absorbance vs. temperature curve. Minor secondary bands have been observed in cases of interspecies episomal infection. However in 1964, Joshi et al. reported the interesting finding of a bimodal peak in the distribution of a CsCl gradient of DNA isolated from H. salinarium, strain 1. The two bands were at positions corresponding to 58% and 67% GC content. The lower density band contained about 20% of the total DNA. To determine whether the presence of two DNA species is a general characteristic of extreme halophiles they performed CsCl density gradient studies on the DNA of H. cutirubrum and four other species of halotolerant bacteria which grow in but do not require high salt. The DNA of H. cutirubrum showed two components at nearly the same densities as those of H. salinarium, with the minor component representing about 10% of the total. The DNA from the four moderate halophiles exhibited only one component each, with GC contents varying from 55-64%.

Moore and McCarthy (1969a) enlarged this work to determine whether the presence of a satellite DNA represents a general property of the extreme halophiles. They tested three halobacteria, a halococcus, a photosynthetic extreme halophile and four other strains isolated in their laboratory and classified as extreme or moderate halophiles by Larsen's classification. The DNA from the extreme halophiles showed major DNA components with a base composition from 66% to 68% GC content and a minor DNA component with 57% to 60% GC content. The authors were unable to demonstrate the presence of a satellite component in the four strains of

moderate halophiles tested. The DNA from the photosynthetic extreme halophile showed only a single DNA component with a base composition of 70% GC. Purification of the bacterial cells in a CsCl density gradient and other strain purification procedures indicated that the presence of the satellite DNA is not a result of mixed cultures. These results suggest that the presence of a minor DNA component is a characteristic of all non-photosynthetic strains of extreme halophiles.

An assessment of the genetic relatedness among the various halophiles was made by determining the relative homology of base sequence in both DNA components of one of the extreme halophiles with the nucleic acids of other strains (Moore and McCarthy, 1969b). The technique used for comparing the sequences consisted of measuring the extent to which RNA or the separated strands of DNA of one organism are able to reanneal with the separated strands of DNA from another organism (DNA/RNA hybridization and DNA/DNA duplex formation). All of the extremely halophilic rods which were examined showed a close relationship to each other. Little or no homology was shown between a halobacterium and a halococcus, the photosynthetic extreme halophile, nor the two moderate halophiles.

Several lines of evidence suggested to the authors that the satellite DNA of extreme halophiles is not an episome of the usual size. The presence of the multiple copies of identical stretches of base sequences which would be required to account for this quantity of satellite DNA is excluded by the kinetics of DNA renaturation which were those expected for a simple non-repeating genome. On the other hand, an episomal element of a greater size could be a plausible explanation for the satellite DNA.

The ribosomes of H. cutirubrum were investigated by Bayley and Kushner (1964). They were found to exist as 70S particles if kept in an environment of 4 M KCl plus 0.1 M $MgCl_2$. If the concentration of either ion drops below these levels, the ribosomes dissociate into 52S and 31S subunits. This requirement for K^+ is specific, neither ammonium, caesium nor sodium being able to act as substitutes. On greater dilution of the salts the ribosomal subunits dissociate still further into soluble protein plus particles rich in RNA (about 80%). An examination of the proteins in the supernatant by starch-urea gel electrophoresis, performed in either acetate buffer pH 5.3 or veronal buffer pH 8.0, showed that most of the proteins are negatively charged.

The nucleic acids and the proteins of the ribosomes of non-halophilic bacteria are bonded predominantly by electrostatic linkages between the acidic groups of the RNA and the basic groups of the protein. This type of ribosome would dissociate in the high salt internal environment of H. cutirubrum. However the ribosomes of this bacteria are composed of two types of polyanions, the RNA and the negatively charged protein. These appear to remain associated only so long as their negative charges are neutralized by sufficient concentrations of monovalent cations, about 1 M.

There is a further specific requirement for much higher concentrations of potassium ions to maintain the integrity of the 70S ribosome. This may be related to a requirement for a hydrated monovalent cation of a precise size. An additional stabilizing factor may be the formation of chelates with the magnesium ions. Thus in the ribosomes of H. cutirubrum, potassium and magnesium ions appear to fill the neutralizing and linking roles

normally taken by the basic groups which their proteins lack.

With the development of a cell-free amino acid incorporating system from H. cutirubrum Bayley and Griffiths (1968a) were able to extend the previous work. The system requires 3.8 M KCl, 1M NaCl and 0.4 M NH_4Cl for maximum activity. It requires ATP, PEP, GTP and ribosomes and incorporates 10-100 nmol/mg of ribosomes of each of 17 amino acids into a hot trichloracetic acid insoluble material. The very specific requirement for K^+ relates to the integrity of the ribosomes.

This system was then used to study the codon assignments and fidelity of translation in this organism (Bayley and Griffiths, 1968b). The incorporation of 20 amino acids into hot trichloracetic acid insoluble material was measured in response to several synthetic polyribonucleotides added as messengers. These were poly U, poly C, poly CA, poly CU, and poly UG. It was shown that codons for nine of the amino acids have the same overall base composition as those of the established code.

The electron transport chain of H. cutirubrum was investigated by Lanyi (1968, 1969a). Five cytochromes were identified and their spectra determined. A possible scheme for the electron transfer system was proposed. In the absence of sodium chloride the complete electron transport chain shows no activity and a rapid and irreversible inactivation.

We see in the extreme halophiles therefore organisms which are fully adapted to life in a harsh environment. Some of the features which seem

to be unique to these interesting microorganisms are:

1. The cell envelopes of the halobacteria, though not the halococcus, require high salt for the maintenance of their integrity.

2. The lipids are almost exclusively isoprenoid derivatives, specifically phytanyl chains, joined by ether linkages to the glycerol moiety of the predominant phosphatides. Almost no fatty acids have been found. The glycerol of these lipids is of the L-configuration.

3. The intracellular salt concentration approaches saturation, with potassium being the main monovalent cation.

4. The enzymes are halophilic, requiring high salt both for activity and stability.

5. The DNA which is high in GC content shows a satellite band of lower density containing 10-20% of the total DNA.

6. The ribosomes require 3.4 M KCl for structural integrity.

7. The electron transport chain requires high salt for activity and stability.

C. PURPOSES OF THIS RESEARCH

It seemed that the study of the polynucleotide phosphorylase of H. cutirubrum might prove valuable in two ways. Some knowledge might be gained about polynucleotide phosphorylase enzyme-substrate and enzyme-substrate-primer interactions by examining these in conditions of limiting ionic strength. Further light might be cast on the phenomenon of halophilism by study of the nucleic acid enzymology of an extreme halophile. In addition it was deemed desirable to develop procedures for the purification of halophilic enzymes in conditions where their activity was fully maintained.

The aims of the studies described in this thesis include:

1. The demonstration of the existence of a polynucleotide phosphorylase in H. cutirubrum.

2. The isolation and partial purification of the enzyme.

3. The proof of its specificity for nucleoside diphosphates.

4. The study of its requirements for stability.

5. The establishment of conditions for optimal activity.

II. EXPERIMENTAL

A. MATERIALS

Intermediates and enzymes were purchased from the following suppliers:

^{32}P-orthophosphate and ^{14}C-labelled nucleoside mono-, di-, and triphosphates: Amersham-Searle, Des Plaines, Illinois, U.S.A.

RNA (Wheat germ): Calbiochem, Los Angeles, California, U.S.A.

Difco casamino acids and yeast extract: Canadian Laboratory Supplies, Montreal, Quebec.

Serva DEAE-cellulose and ApA: Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, New York, U.S.A.

Protein molecular weight markers (albumin, ovalbumin, chymotrypsinogen A, cytochrome C): Mann Research Laboratories, New York, U.S.A.

Poly A, Poly C, Poly G and Poly U: Miles Chemical Co., Elkhart, Indiana, U.S.A.

Sephadex^{R}, Blue Dextran^{R} and Ficoll^{R}: Pharmacia Fine Chemicals, Uppsala, Sweden.

Unlabelled nucleoside mono-, di-, and triphosphates: P-L Biochemicals, Inc., Milwaukee, Wisconsin, U.S.A.

DNA (Calf thymus): Sigma Chemical Co., St. Louis, Missouri, U.S.A.

DNase: Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.

B. METHODS

(i) Organisms

The strain of Halobacterium cutirubrum used in this work was the gift of Dr. D. J. Kushner. It was originally isolated by Lochead from salt bacon.

(ii) Media and growth conditions

The growth medium used was that of Sehgal and Gibbons (1960). It consists of the following:

Difco casamino acids	0.75 g
Difco yeast extract	1.0 g
Sodium citrate	0.3 g
Potassium chloride	0.2 g
Magnesium sulfate (hydrated)	2.0 g
Sodium chloride	25.0 g
Ferrous chloride	2.3 mg

in a total volume of 100 ml of aqueous solution.

The pH of the medium was adjusted to 6.5 with 20% NaOH before autoclaving. A small amount of precipitate forms in the medium after sterilization which does not affect growth.

Stock cultures were maintained on slants of Sehgal and Gibbons medium containing 2% agar in screw cap tubes. They were grown at 37° until a heavy growth was attained, about two to three weeks, and stored at 4°. Aeration was achieved during incubation by loosening the screw caps every three or four days. Transfers were made at 6-month intervals.

Culture flasks with Morton closures (125 ml; Bellco Glass Inc.) containing 50 ml of the liquid medium were inoculated from the stock culture and incubated at 37° for 48 h in a New Brunswick Incubator Shaker with rotary action. One of these flasks was then stored at 4° to use as the inoculum of the next preparation. Organisms stored in this way remain viable for 1 yr or longer.

The mass culture for enzyme studies was grown in 1 liter flasks containing 400 ml of liquid medium. These were inoculated with 20 ml of the culture from the 125-ml flasks and incubated in the same way. After 48 h the cells in middle log phase were harvested by centrifuging at 15,000g for 10 min at 4°. The supernatant was poured off and the unwashed cells used for the preparation of the crude extract [Section(iv),p.42].

A routine check was maintained on the purity of our mass cultures. Two agar slants of Sehgal and Gibbons medium were inoculated with about 1 ml of the liquid culture at the time of each transfer. These were incubated as described above and examined for freedom from contamination.

(iii) Sephadex G-200 gel filtration in high salt

For the successful use of Sephadex G-200 as a chromatography medium in conditions of 3-4 M salt, the dextran particles must be swollen in the high salt buffer for 4-6 weeks before being used. For a 2.5 cm X 100 cm column, 30 g of Sephadex G-200 were suspended in 2 l of a buffer consisting of 2.5 M KCl, 1 M NaCl and 0.01 M Tris-HCl, pH 8.5 (Buffer A), with sodium azide as a preservative, and stored at 4° for about one month. The bottom flow adapter was fitted to the column and about 30 ml of Buffer A

were poured in. Bubbles were removed by means of a long glass tube and the column was carefully levelled. The column was packed with the aid of a column extender and, after the top flow adapter was fitted, was run by downward flow under a hydrostatic head of not more than 5-10 cm for 18 h. The column was then connected for upward flow and the flow rate regulated at 10 ml/h by a Polystaltic Pump. After a stabilization period of 2 h, the sample (10 ml) was applied and elution was performed at 10 ml/h with Buffer A. The column effluent was monitored at 280 nm with a flow cell and recorder (LKB Uvicord II) and 4 ml fractions were collected (LKB Ultrorac fraction collector).

(iv) Enzyme preparation

All operations were carried out at 0-5° except where indicated. Both phosphorolysis and polymerization activities (Assays A and B, p.45) were determined at all stages of purification. The pH of buffers refers to the value at room temperature. Ammonium sulfate concentrations were calculated according to Green and Hughes (1955).

Step 1. Preparation of the cell-free extract.

The harvested cells were carefully suspended in Buffer A at a concentration of 1 g wet weight of cells to 3 ml of solution. This was stirred for about 1 h until a smooth suspension was obtained. It was then sonicated on a Bronwill Biosonik for 10 s at maximum intensity. The fragile cell walls of these bacteria are easily broken and 10 s proved to be sufficient. The disrupted cells were centrifuged at 15,000g for 20 min, the cell-free extract was decanted and the pellet discarded.

Step 2. 80,000g supernatant.

The cell-free extract was centrifuged for 60 min at 80,000g in a Beckman Model L2-65B Preparative Ultracentrifuge using a 65 rotor. The supernatant was carefully decanted and the small red pellet discarded.

Step 3. Ammonium sulfate fractionation.

The supernatant from Step 2 was dialysed against two changes of 0.01 M Tris-HCl buffer, pH 8.5(100 vol.) for 2 h to remove most of the salt. Ammonium sulfate (0.275 g/ml) was added to 45% saturation and the mixture stirred for 1 h. The suspension was centrifuged at 45,000g for 20 min and the pellet was discarded. Ammonium sulfate (0.3g/ml) was added to the supernatant to raise its concentration to 85% of saturation and stirring continued for 1 h. The suspension was centrifuged as previously described and the supernatant was discarded. The pellet was transferred to a small, glass hand homogenizer with the aid of an appropriate amount of Buffer A and uniformly dispersed with one or two strokes of a tight-fitting glass pestle. The protein dissolved readily and this fraction was then extensively dialyzed against Buffer A to remove all traces of ammonium sulfate and to ensure that the salt concentration is raised to the level required for the stability of the enzyme.

Step 4. Sephadex G-200 gel filtration.

A 2.5 cm x 85 cm column of Sephadex G-200 in Buffer A was prepared and run as described in Section (iii), p. 41. A 10 ml sample of the dialyzed material from Step 3 was applied to the column. Two protein fractions with polynucleotide phosphorylase activity (Peaks I and II, fig 3)

were resolved. The fractions of each peak with the highest specific activity were pooled separately and concentrated by dialysis against 30% Ficoll in Buffer A. The concentrated enzyme was then dialysed for 1 h against Buffer A and stored at 4°.

(v) Extraction and chromatography of lipids

Total lipids were extracted by the method of Bligh and Dyer (1959) using redistilled solvents. In this method, the lipids are initially extracted in a monophasic system of methanol, chloroform and water which is then converted to a biphasic system by the further addition of chloroform and water.

To 10 ml of column eluate 25 ml of methanol and 12.5 ml of chloroform were added. The mixture was shaken thoroughly and left 1 h at room temperature. A further 12.5 ml of chloroform and 12.5 ml of distilled water were added and mixed by inversion. The copious salt precipitate present in the monophasic system redissolves readily in the aqueous phase of the biphasic system. The chloroform phase was withdrawn, diluted with an equal volume of benzene and brought to dryness in a rotary evaporator at 45°. The residual lipid was dissolved in a mixture of 2 ml each of chloroform and benzene. This was blown down with nitrogen to dryness, 2 drops of chloroform were added and the extract was centrifuged briefly.

The extract was applied to silicic acid-impregnated Whatman 3MM paper and developed about 24 h in diisobutyl ketone-acetic acid-water (40:25:5,v/v)

according to the procedure of Marinetti (1962). Chromatograms were stained with Rhodamine 6G and viewed under ultraviolet light.

(vi) Standardization of G-200 columns

A 2.5 cm x 85 cm column of Sephadex G-200 in Buffer A was prepared and run as described in Section (iii), p. 41. The void volume and internal volume of the column were determined with Blue Dextran and adenosine respectively. The standard proteins and the Blue Dextran were dissolved in Buffer A (7mg in 1 ml) and allowed to stand overnight at 4° to allow any conformational changes due to the high salt to occur. Cloudy samples were centrifuged at 40,000g for 10 min and the clear supernatant used. The sample (0.75 ml) was applied and 4 ml fractions collected. The eluant was monitored at 280 nm. The individual fractions in the area of 280 absorbance were read in a Zeiss Spectrophotometer Model PMQII at 280 nm except for cytochrome which was read at 405 nm. The Blue Dextran and adenosine peaks were read at 280 nm and 257 nm respectively. The elution volume was determined at the maximum height of the peak (Fischer, 1969).

(vii) Enzyme assays

Polynucleotide phosphorylase activity was assayed by a modification of the standard procedures (Fitt and Fitt, 1967; Singer and Guss, 1962). Specific activity is defined as the number of enzyme units per mg of protein.

Assay A. Phosphorolysis assay

This assay measures the formation of ^{32}P-nucleoside diphosphates during the phosphorolysis of polyribonucleotides in the presence of ^{32}P

-orthophosphate. The reaction mixture contained: Tris-HCl, pH 9.8, 10 μmol,; EDTA, 0.03 μmol; poly A, 50 μg; $MnCl_2$, 2 μmol; $MgCl_2$, 1 μmol; KCl, 125 μmol; NaCl, 50 μmol; ^{32}P-orthophosphate, 1 μmol (about 50 counts/min/nmol); enzyme, in a final volume of 0.1 ml. The mixture was incubated at 37° for 15 min, and the reaction was stopped by the addition of 1 ml of a mixture containing 1 part of an aqueous 10% (w/w) suspension of acid-washed Norit A and 10 parts of 2.5% (v/v) $HClO_4$. The charcoal suspension was allowed to stand 10 min at 0°, with occasional mixing and was then collected on a 2.4 cm Whatman GF/C glass-fiber filter. The charcoal was washed four times with 10 ml of cold water, during which washing the salt precipitated by the perchloric acid dissolved. The filter was then placed upside down in a planchet, dried and counted.

One unit of phosphorolysis activity was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{32}P-orthophosphate into charcoal-adsorbable material per h in these conditions.

Assay B. Polymerization assay

This assay measures the incorporation of ^{14}C-nucleoside diphosphates into an acid-insoluble form. The reaction mixture contained: Tris-HCl, pH 7.6, 15 μmol; EDTA, 0.04 μmol; ^{14}C-NDP, 4 μmol (about 7 counts/min/nmol); ApA, 200 μg; $MnCl_2$0.5 μmol; $MgCl_2$, 0.5 μmol; KCl, 125 μmol; NaCl, 50 μmol; enzyme, in a final volume of 0.1 ml. The mixture was incubated at 37° for 60 min and the reaction was stopped by the addition of 0.1 ml of 7% (v/v) $HClO_4$. The mixture was allowed to stand 10 min at 0°, with occasional mixing. The precipitate was then collected on a Whatman GF/C filter, washed

four times with 2 ml of 1% (v/v) $HClO_4$ and once with 2 ml of 50% (v/v) ethanol. The filter was placed in a planchet, dried and counted.

One unit of polymerization activity was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{14}C-NDP into an acid-insoluble form per h in these conditions.

Assay C. Exchange assay

This assay measures the formation of ^{32}P-NDP during the exchange between the terminal phosphate of nucleoside diphosphate and ^{32}P-orthophosphate. The reaction mixture contained: Tris-HCl, pH 8.2, 10 μmol; EDTA, 0.03 μmol; NDP, 0.25 μmol; ^{32}P-orthophosphate, 0.35 μmol (about 100 counts/min/nmol); enzyme, in a final volume of 0.1 ml. The mixture was incubated at 37° for 15 min and the reaction was stopped with 1 ml of a mixture of charcoal and $HClO_4$ as in Assay A. The charcoal was collected, washed, dried and counted as in Assay A.

The amount of ^{32}P-orthophosphate incorporated into charcoal-adsorbable material was calculated from the expression (Grunberg-Manago, Ortiz & Ochoa, 1956):

$$\text{nmol of } {}^{32}P_i \text{ incorporated} = \frac{\text{counts/min incorporated}(\text{nmol } P_i + \text{nmol NDP})}{\text{counts/min in reaction}}$$

One unit of exchange activity was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of ^{32}P-orthophosphate into charcoal-adsorbable material in 60 min in these conditions.

Radioactivity for the assays was determined in a Nuclear Chicago low-background thin-window counter. Suitable enzyme and substrate controls were carried out on all assays and their value was subtracted from the assay values. The specific activity of the radioactive material was determined by placing a suitable portion of the solution directly on a planchet, then drying and counting in the usual manner. In the case of ^{32}P-orthophosphate, a GF/C disc was placed over the dried material before counting to allow for quenching due to the filter.

(viii) Specificity of phosphorolysis activity

Duplicate phosphorolysis assays (Assay A) and a control without enzyme, all at triple the scale of the standard assay, were incubated at 37° for 16 h. The reaction was stopped with cold charcoal-$HClO_4$(1 ml). The charcoal was washed 6 times with 1.25 mM KH_2PO_4(3ml) in 1% $HClO_4$. It was then washed 3 times with cold water (3 ml). Finally it was resuspended in 1 ml of cold water. Tiny columns were prepared in Pasteur pipettes with cotton batting and cellulose as a support bed. The charcoal suspension was layered on this, care being taken that the cellulose layer was thick enough that no charcoal escaped into the filtrate. The adsorbed nucleotides were eluted with 1 ml of 2% NH_4OH in 50% ethanol (v/v). The eluant was freeze-dried until a volume of about 0.05 ml remained, to which 10^{-2} mol of cold AMP, ADP and ATP were added. The total volume was spotted on a 5X20 cm TLC plate of Serva DEAE-cellulose and developed in 4% formic acid (w/v) (Fahn, Albers & Koval, 1965). The nucleotides were located under ultraviolet light. The radioactivity was located by scanning the plates in a Nuclear Chicago strip scanner, and then counted in a Nuclear Chicago Mark I liquid scintilla-

tion counter by scraping sections of cellulose off the plate and adding them directly to PPO-POPOP-toluene counting fluid. Quenching was determined by the two channels ratio method and the counts were corrected to 100% efficiency.

(ix) Protein determination

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

The high salt content of the enzyme preparations interfered with the biuret and Lowry procedures of protein determination.

III. RESULTS

A. PREPARATION OF CRUDE EXTRACT

The earliest preparations, in which the bacterial membranes were disrupted by a 5-10 s burst of sonication, yielded cell-free extracts which had both phosphorolytic (Assay A) and polymerization (Assay B) activity. Both these activities were at a low level. In attempts to improve the specific activity of the crude extract, the effect of varying periods of sonication was tested. As sonication time increased, the phosphorolytic activity increased somewhat until it reached a stable level, but the polymerization activity decreased until at 60 s sonication none of this activity remained (Table I).

During the course of the investigation the effect of disrupting the membranes by grinding with cold alumina, by osmotic shock or by an acetone powder preparation (Gunsalus, 1955) was also studied.

The disruption of the membranes of extreme halophiles by osmotic shock was particularly easy as these membranes need at least 1 M NaCl to maintain their integrity. The cells, suspended with rapid stirring in 0.02 M $MgCl_2$ at a concentration of 7 g wet weight in 100 ml solution, lysed rapidly. The suspension became extremely viscous due to the release of DNA. Deoxyribonuclease (0.8 mg/100 ml solution) was added and the stirring continued at room temperature for 15 min. The suspension was then centrifuged in the cold for 1 h at 15,000g and the pellet and supernatant tested (Brown, Shorey & Turner, 1965).

All these methods yielded crude extracts in which the phosphorolytic activity was no higher than in the 10 s sonication method, and in which there was no polymerization activity at all.

TABLE I

Effect of Sonication time on Activity of Crude Extract.

Time s	Specific Activity units/mg protein	
	Phosphorolysis	Polymerization
5	24	384
10	80	146
30	68	58
45	104	44
60	100	0

Finally I returned to the original method of cell disruption - a 10 s burst of sonication - as the only means of obtaining a preparation with both phosphorolytic and polymerization activity.

In the early preparations, the crude extracts were prepared in 4M NaCl, 0.01 M Tris-HCl pH 9.5 (Buffer B), in line with previous work on halophilic enzymes where the crude extracts were prepared in 4-5 M NaCl (Baxter & Gibbons, 1954; Holmes & Halvorson, 1965a). However the intracellular salt concentration of an extremely halophilic bacterium was reported as 4.6 molal K^+ and 1.4 molal Na^+ (Christian & Waltho, 1962). To test the activity of the enzyme in conditions approaching the physiological, it was prepared in 2.5 M KCl, 1 M NaCl, 0.01 M Tris-HCl pH 9.5 (Buffer C). These concentrations approach the solubility limit of a mixture of these two salts in aqueous solutions at 4°.

Crude extracts were prepared in Buffers B and C by a 10 s sonication of the suspended cells. After centrifugation, phosphorolysis and polymerization assays were performed on the supernatants, as shown in Fig. 1. The concentration of salts in the assay was half that of the buffers as indicated. The enzyme was more active when prepared and assayed in the mixture of salts than when NaCl alone was used.

Fig. 1

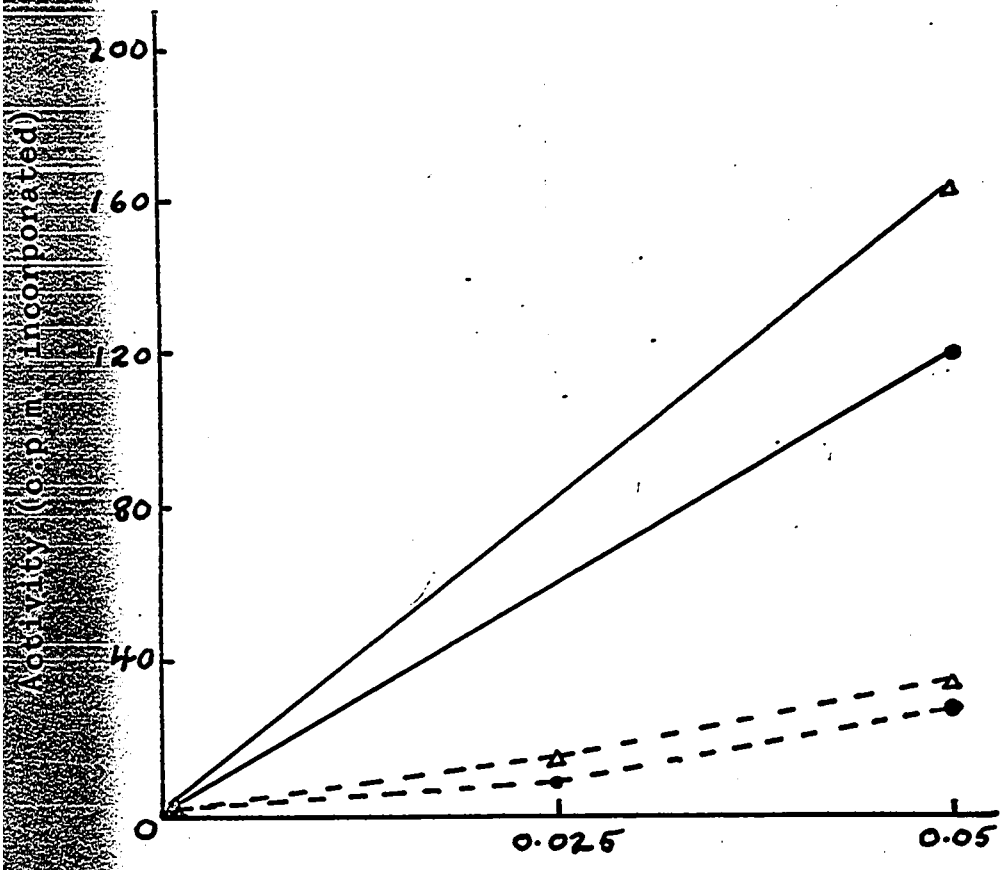
Effect of Different Salts on Activity of Crude Extract

1.3 M KCl, 0.5 M NaCl - Phosphorolysis

1.3 M KCl, 0.5 M NaCl - Polymerization

2 M NaCl - Phosphorolysis

2 M NaCl - Polymerization



B. 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. Aliquots (1 ml) of the active fraction from Step 3, p.43, were dialysed at 4° for 24 h against two changes of a series of 0.01 M Tris-HCl buffers pH 8.2 (125 vol.). One of these buffers was used without the addition of salt and the others contained the following: 4 M NaCl, 2 M NaCl, 3.6 M KCl or 2 M KCl. The enzyme was then stored at 4° and, at intervals over a three week period, samples were withdrawn and assayed for phosphorolytic activity at a salt concentration of 2 M NaCl in the assay. As shown in Fig. 2, the enzyme retained up to 50% of its activity for 3 weeks when in an environment of 4 M NaCl or 3.6 M KCl. However, in a 2 M NaCl or KCl the enzyme lost all its activity in 3 days and in a salt-free environment it lost 95% of its activity in 24 h and all of it in 48 h. This activity could not be restored even after dialysis against 4 M salt for 1 week.

Some indication of the effect of pH on the stability of the enzyme was obtained in the following way. Enzyme preparations carried out in salt solutions buffered at pH 9.5 resulted in a low yield (about 10% of the original activity) of purified enzyme after the Sephadex G-200 gel filtration (Step 4, p.43). After the pH of the standard buffer was changed to 8.5, the yield was much improved, now being 40% to 50% of the original activity.

Fig. 2

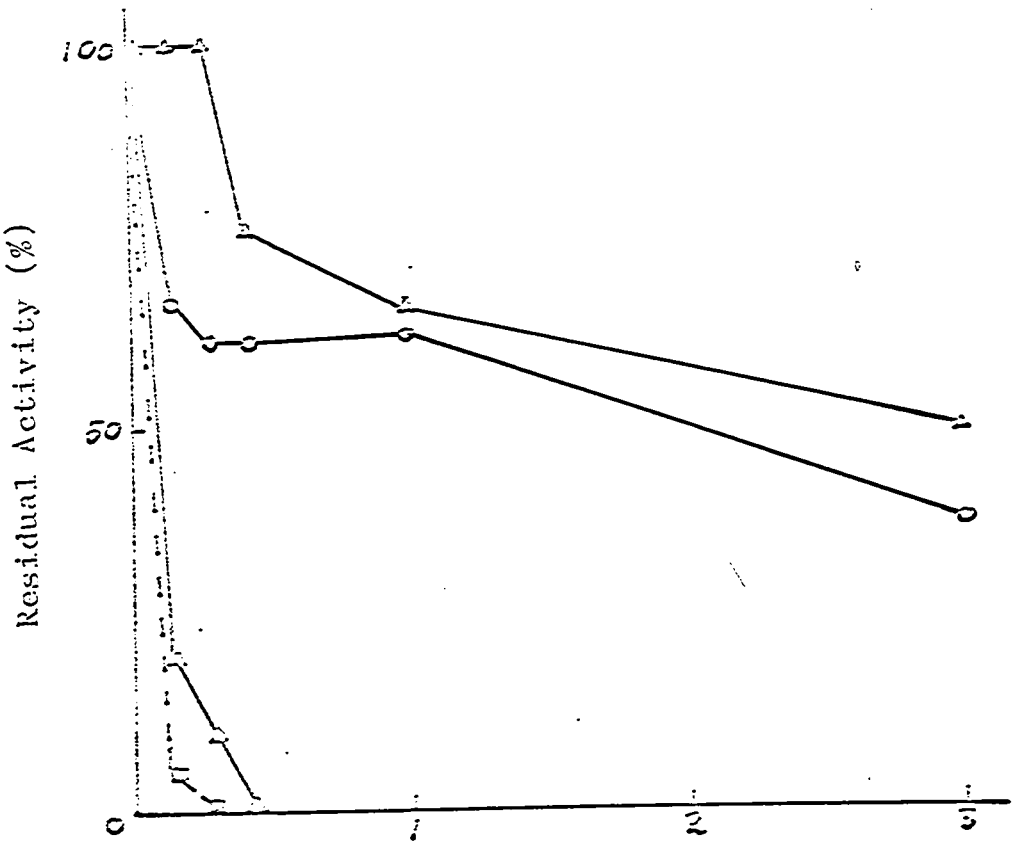
Enzyme Stability - Effect of Salt

△——△ 4 M NaCl

○——○ 3.6 M KCl

△- - -△ 2 M NaCl or KCl

□- - -□ No salt



6. PURIFICATION OF THE ENZYME

As a result of the experiments already described our standard buffer used to prepare the crude extract, for dialysis and as the eluant in gel filtration has the composition 2.5 M KCl, 1 M NaCl, 0.01 M Tris-HCl pH 8.5 (Buffer A).

Because of the instability of the enzyme in the absence of salt, it was necessary to use purification steps in which an environment of high ionic strength was maintained. The various procedures tried included acid precipitation, high speed centrifugation, calcium phosphate gel fractionation, bentonite adsorption and ammonium sulfate precipitation. High speed centrifugation at 80,000g for 1 h and ammonium sulfate fractionation proved to be the best preliminary steps. Though the preliminary dialysis in the ammonium sulfate step removed the enzyme from its high salt environment, the immediate addition of the dry salt afforded some protection.

The technique of running successful columns of Sephadex G-200 gel filtration in high salt was developed by trial and error. Initially the dextran particles were swollen in distilled water, used to pack the column and then equilibrated with the high salt buffer. These columns channelled badly. Later the gel was swollen in high salt for about 1 week and used to pack columns which were run by downward flow with a sample applicator at the top of the column. These columns shrank 2 to 3 cm during a 48 h run. Finally, it was realized that dextran particles swell so slowly in high salt that it was necessary to leave them 4 to 6 weeks before using them to pack a column. These columns were run by upward flow

using flow adapters, according to standard procedures (Fischer, 1969). It was found that such columns can be run reproducibly, did not shrink and gave void volume and internal volume values within normal limits when the latter were determined with Blue Dextran and adenosine respectively.

The enzyme has now been purified several times by the procedure described in the Methods section (p.42). The results of a typical purification are shown in Table II. The ammonium sulfate fractionation step shows the greatest loss of activity. After gel filtration on G-200, 40% of the original activity was recovered. These were divided into two peaks, with the material from the best fraction of Peak II being purified 217-fold in a yield of 26%.

Initially, the pooled active fractions from the Sephadex G-200 column were concentrated on a Sartorius collodion bag apparatus (BDH Canada, Ltd.). This resulted in a loss on concentration of 70% of the activity recovered from the column. When the fractions were concentrated by dialysing them against 40% Ficoll dissolved in Buffer A, no activity was lost.

D. SIZE AND NATURE OF MATERIAL IN SEPHADEX G-200 ACTIVITY PEAKS

When the 10 ml sample of the dialysed ammonium sulfate fraction was applied to the gel filtration column, there was a visible separation into a fast-moving pink band and a slower-moving pale brown band. The polynucleotide phosphorylase activity, as measured by phosphorolysis, separated into two peaks; Peak I emerging at or near the void volume and Peak II being highly retarded (Fig. 3). The Peak I activity was associated with the pink color in each preparation yet made.

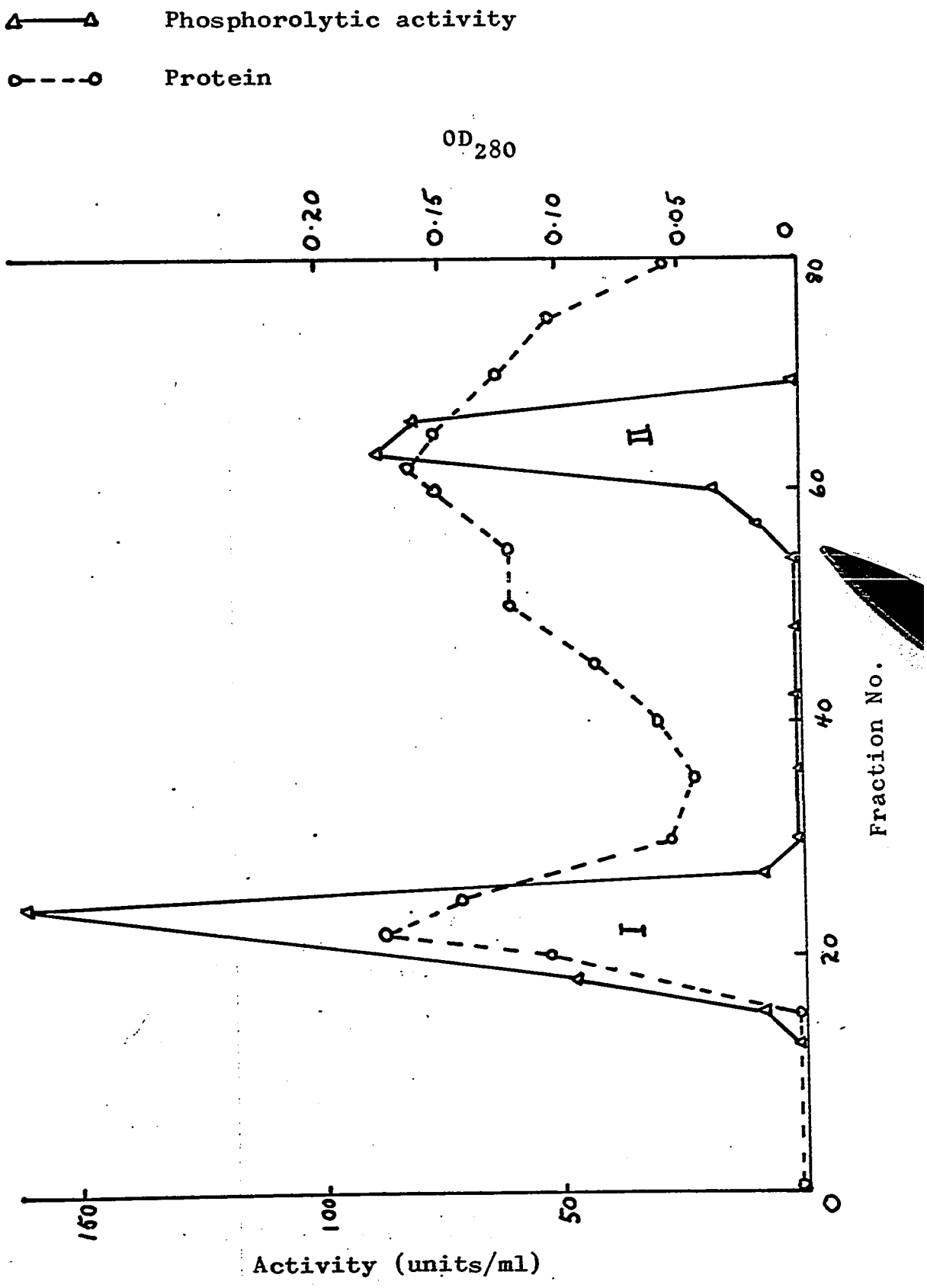
TABLE II

Purification of H. cutirubrum polynucleotide phosphorylase.

FRACTION	Total Activity units	S.A.	Purification	Yield %
Crude Extract	7680	9.6	1.0	100
80,000g supernatant	8400	12	1.3	109
Ammonium sulfate	5200	40	4.2	68
Sephadex G-200				
Total recovered	3172			41
Peak I best fraction		240	25	3
Peak II best fraction		2080	217	26

Fig. 3

Sephadex G-200 Gel Filtration



The average partition coefficient, K_{av}, was calculated for each peak from a series of runs. This is given by the equation

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

where V_o is the void volume of the column, V_e is the elution volume of the particular protein and V_t is the total column volume. These values and the distribution of activity between the two peaks are shown in Table III, the first three lines showing the results from the successive enzyme preparations. It will be noted that the variation between the partition coefficients for the Peak I material is twice as great as that between the values for the Peak II material. Also the distribution of activities between the two peaks varies in an arbitrary fashion. These two facts, together with the constant association of the Peak I activity with the pink color due to the pigments which are found in the cell membrane, led me to conjecture that Peak I may represent the enzyme molecule associated with membrane fragments of varying size, whereas Peak II consists of the enzyme molecule itself dissociated from the membrane by the method of disrupting the cell walls.

TABLE III

G-200 Peaks-Enzyme Size and Distribution of Activity

Prep No.	K_{av}		Activity	
	Peak I	Peak II	Peak I	Peak II
1	0.02	0.79	2400(73%)	900(27%)
2	0.06	0.81	1152(60%)	768(40%)
3	0.10	0.77	1056(48%)	1152(52%)
3 - Peak I Sonicated 10s	0.27	0.74	736(66%)	378(34%)

Two experiments were performed to test this hypothesis. Firstly, Peak I material was sonicated for 10 s as is done in the enzyme preparation, then subjected to gel filtration on a Sephadex G-200 column. Secondly the lipids associated with both peaks were extracted and identified.

The last line of Table III shows the result of the first of these experiments. The 1056 units of sonicated Peak I material emerged in two peaks of activity. Peak I now had a much higher K_{av} indicating the particles were of a smaller size. Peak II material emerged with a K_{av} only 0.04 smaller than the average of the four runs. There was a 100% recovery of activity.

The chromatogram of the lipids of Peak I and Peak II material, together with that of the total lipids of H. cutirubrum as standards, was performed as described in Methods, p. 44 . The results are shown in Table IV. Peak I had lipids which are characteristic of the membranes, namely neutral lipids which include the red carotenoid pigment, PGP, a sulfolipid and an unidentified phospholipid (X_2). Peak II had none of these lipids, but does contain an additional unidentified glycolipid not shown in the table.

The results of these two experiments strongly suggest that the polynucleotide phosphorylase of H. cutirubrum is membrane associated.

TABLE IV

G-200 Peaks - Chromatogram of Lipids

Standard *H. cutirubrum* total lipids	Peak I	Peak II
Neutral lipids	+	-
*Phosphatidyl glycerol	-	-
*Phosphatidyl glycero-phosphate	+	-
X_2	+	-
Glycolipid	-	-
Sulfolipid	+	-

*Diether analogue

E. PROPERTIES OF THE PURIFIED ENZYME

The activity of the polynucleotide phosphorylase of H. cutirubrum is low compared to that of the enzyme in E. coli and, because of the small amount of radioactive material incorporated, the effect of certain factors on the activity of the enzyme was, initially, difficult to assess. The phosphorolytic, rather than the polymerization, activity was chosen as the subject of detailed study of the properties of the purified enzyme for the following reasons:

1. The phosphorolytic activity was 4 to 5 times greater than the polymerization activity.
2. As the radioactive material of the phosphorolysis assay (^{32}P-orthophosphate) was cheaper than that of the polymerization assay (^{14}C-ADP), it was practicable to increase its specific activity and hence the sensitivity of the assay.

The combination of these two factors yielded significant results in the studies presented here, which were obtained initially with either the crude extract or the active ammonium sulfate fraction. All of them were confirmed on the purified enzyme, with both Peak I and Peak II material.

(i) Effect of salt on phosphorolytic activity.

An aliquot (1 ml) of enzyme was dialysed for two hours against two changes of 0.01 M Tris-HCl buffers pH 8.5 (500 vol.) to remove salt. It was then assayed in the conditions of Assay A, with the following change. Dry salt (NaCl or KCl) was weighed directly in the assay tubes to give the indicated concentration in the 0.1 ml total volume of the assay. Since no allowance was made for the volume of this salt, the final concentration in the assay was in fact slightly lower than those indicated. The required concentrations of NaCl with the A. vinelandii enzyme were obtained by adding the appropriate amount of 4 M NaCl in the assay. (This enzyme was essentially pure and was a gift from Mr. A. T. Gajda).

The results seen in Fig.4 showed that H. cutirubrum polynucleotide phosphorylase had no activity in the absence of salt. The activity increased with increasing concentration of either NaCl or KCl to an optimum at 2 M. At a concentration of 4 M NaCl or KCl the activity was still 90% of optimum.

This contrasted completely with the behavior of a non-halophilic polynucleotide phosphorylase from A. vinelandii. This enzyme had optimum activity in the absence of salt, dropping to 13% of optimum at 1 M NaCl and was totally inactive in 2 M NaCl.

Fig. 4

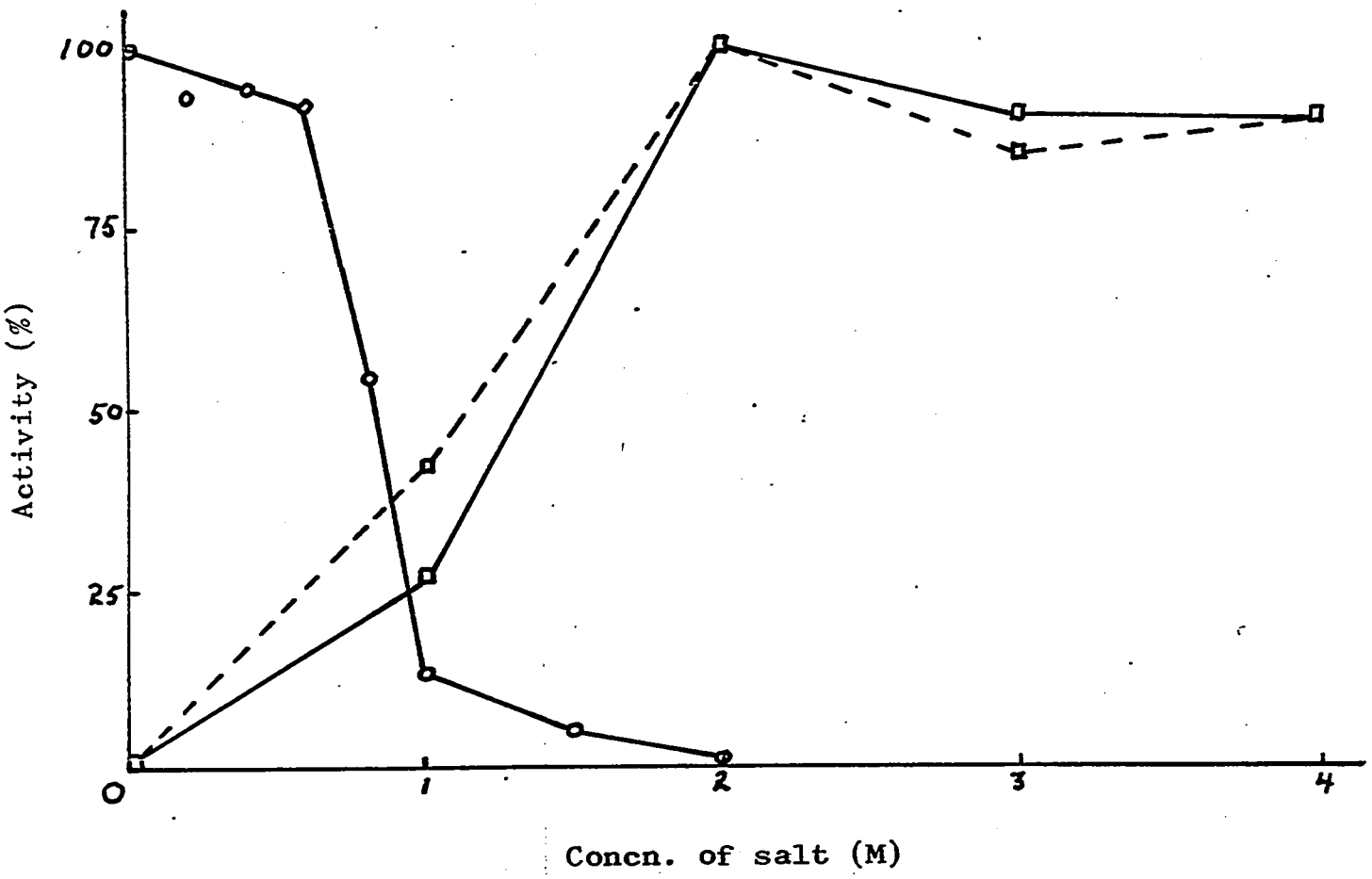
Phosphorolysis of Poly A

Effect of Salt

H. cutirubrum PNPase - NaCl

H. cutirubrum PNPase - KCl

A. vinelandii PNPase - NaCl



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(ii) Effect of divalent cations on the phosphorolytic activity.

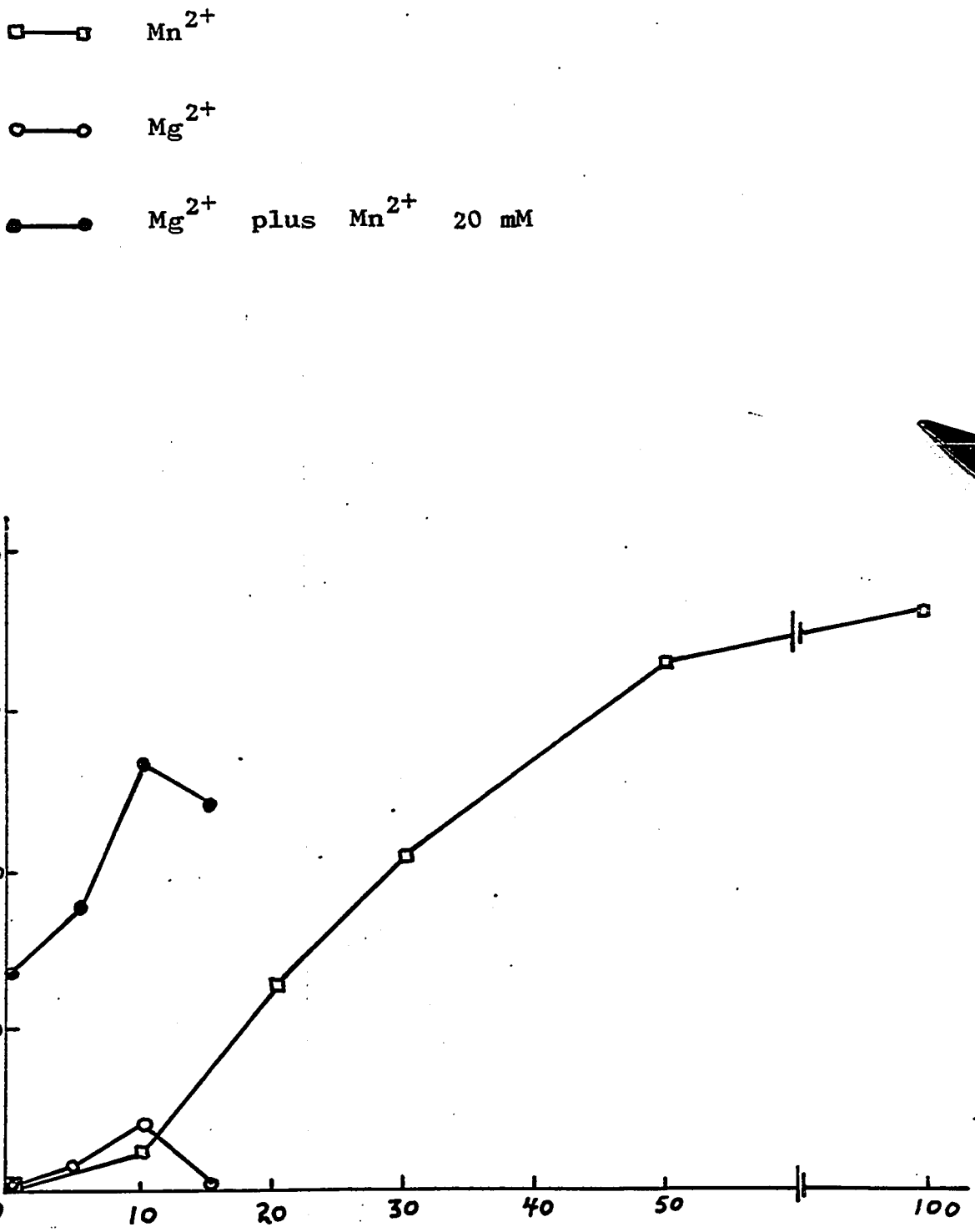
Since magnesium is the most effective divalent cation for all the polynucleotide phosphorylases except that of the mutant strain E. coli Q_{13}, it was used in the earliest assays. As the activity in these conditions was low, the effect of manganese ions on the phosphorolytic activity was examined (Fig.5). The assay conditions were those of Assay A, except for the indicated concentrations of Mn^{2+} and/or Mg^{2+}.

The enzyme showed a slight activity with Mg^{2+} up to a concentration of 10 mM but a complete inhibition at a concentration of 15 mM. However, with only Mn^{2+} present, the activity was sharply increased and a nearly linear relationship was evident in the concentration range of 10 to 50 mM. There was no significant increase in activity in the range from 50 to 100 mM Mn^{2+}. At these elevated concentrations, a brown precipitate of manganese oxides led to high and variable blanks. As shown in the figure, the presence of Mg^{2+} enhanced the effect of the Mn^{2+}, with the activity being doubled by the addition of 10 mM Mg^{2+} to 20 mM Mn^{2+}. Even though the conditions are sub-optimal, these concentrations of divalent cation were chosen for the standard assay as they resulted in a good activity together with a low background.

Fig. 5

Phosphorolysis of Poly A

Effect of Divalent Cations



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(iii) Effect of pH on phosphorolytic activity.

The assay conditions were those of Assay A with the following change: Tris/HCl buffer (100mM in the assay) at a series of pH's as indicated was used. As shown in Fig.6 the phosphorolytic activity of the enzyme showed a sharp pH optimum of 9.8. This contrasted with the broad plateaus between pH 8 and 10 reported for the enzyme in A. vinelandii (Grunberg-Manago, 1963) and in E. coli Q_{13} (Thang et al., 1969b).

(iv) Enzyme concentration curve.

The reaction mixtures were those of the standard Assay A. The enzyme solution was added to make a series of concentrations in the assay as indicated and the salt concentration was kept constant by adding an appropriate volume of a solution of 2.5 M KCl and 1 M NaCl to a final volume of 0.1 ml.

As can be seen in Fig.7, the phosphorolysis of Poly A by the enzyme was proportional to the enzyme concentration over the range measured. Higher enzyme concentrations could not be conveniently measured in this particular assay system.

Fig. 6

Phosphorolysis of Poly A

Effect of pH

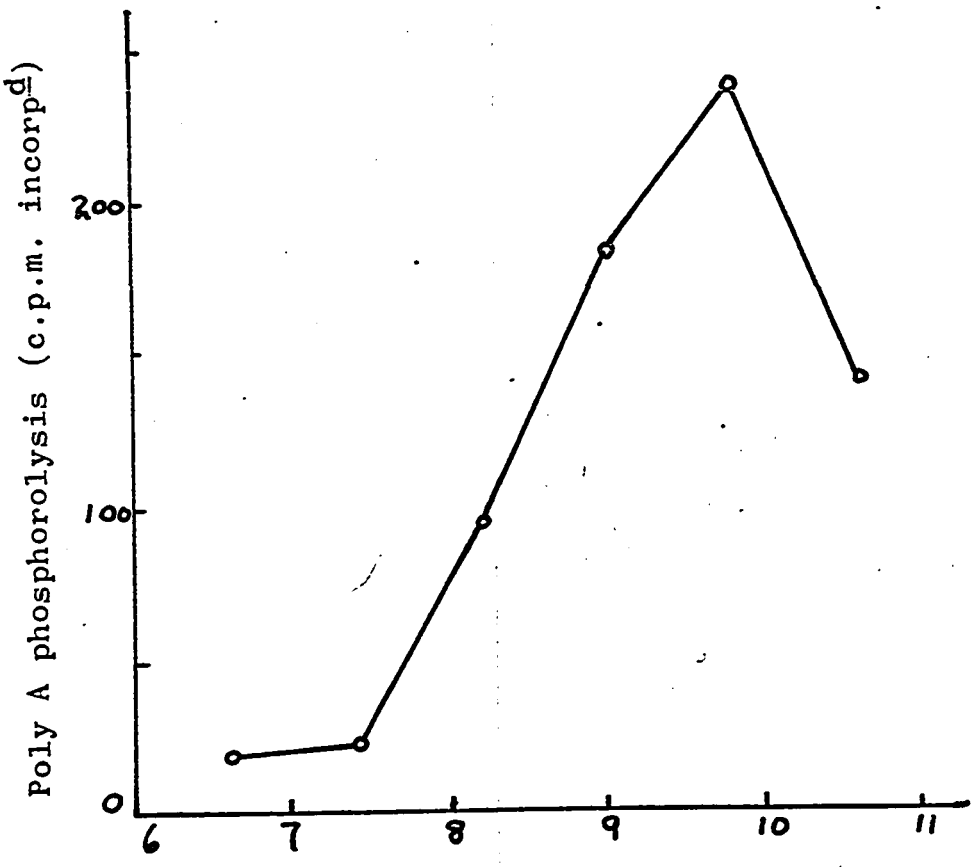
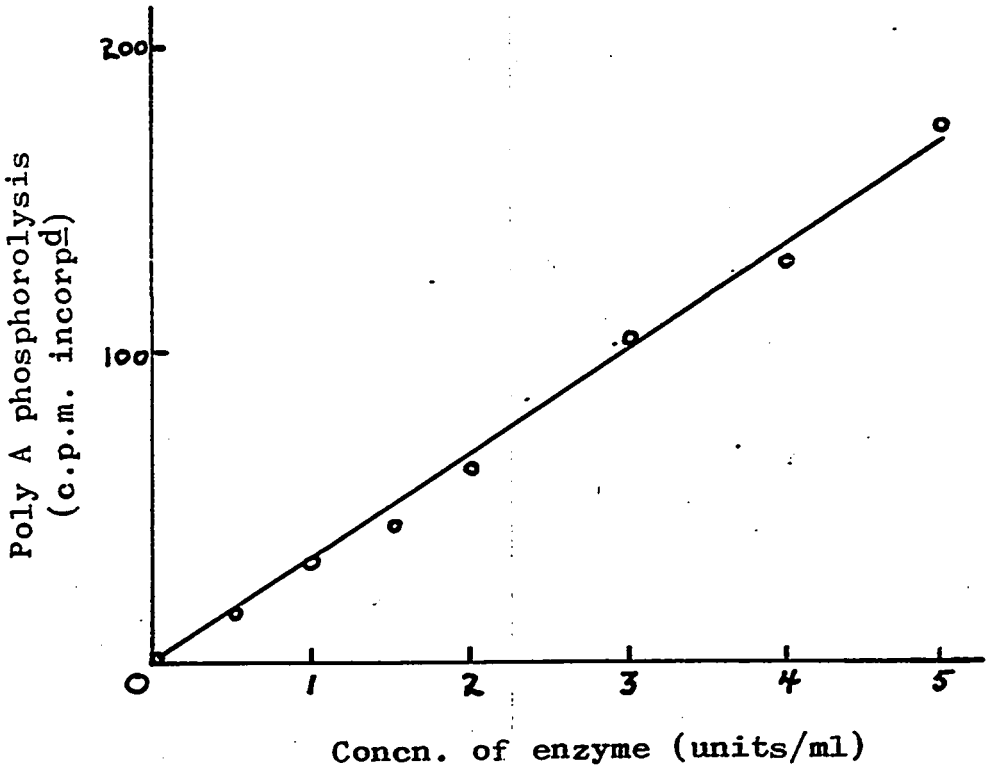


Fig. 7

Phosphorolysis of Poly A

Enzyme Concentration



(v) Substrate saturation curve - Poly A.

The reaction mixtures were those of the standard Assay A except for the indicated variations in the Poly A substrate concentration. There was a linear relationship between the rate of phosphorolysis of Poly A and its concentration up to 200μg/ml. (Fig.8) and thereafter a plateau to the highest concentration tested, 500μg/ml. The concentration of Poly A in the standard assay was chosen on the plateau section of the curve, at 500μg/ml.

The concentration shown may not be the true one as there was some difficulty in dissolving the Poly A in the assay mix because of the high salt concentration (1.25 M KCl, 0.5 M NaCl). This difficulty was more pronounced with freshly prepared batches of substrate, and lessened as the Poly A solution was subjected to daily freezing and thawing. There is a possibility that the improved solubility was due to a shortening of the polymer chains under these conditions. Shorter oligonucleotides could well prove a better substrate in these assay conditions but they were not available for testing.

(vi) Time curve.

The reaction mixture was that of Assay A. Sufficient assays were prepared to obtain duplicate and control values at each point in the curve (Fig.9). The reactions were stopped at the times indicated and treated in the usual manner.

The phosphorolysis of Poly A was proportional to the time of incubation up to 15 min and thereafter the rate decreased until a plateau was reached from 60 min to 120 min. This plateau suggested that the enzyme preparation was essentially free from phosphatases which are active in these conditions.

Fig. 8

Substrate Saturation Curve

Poly A

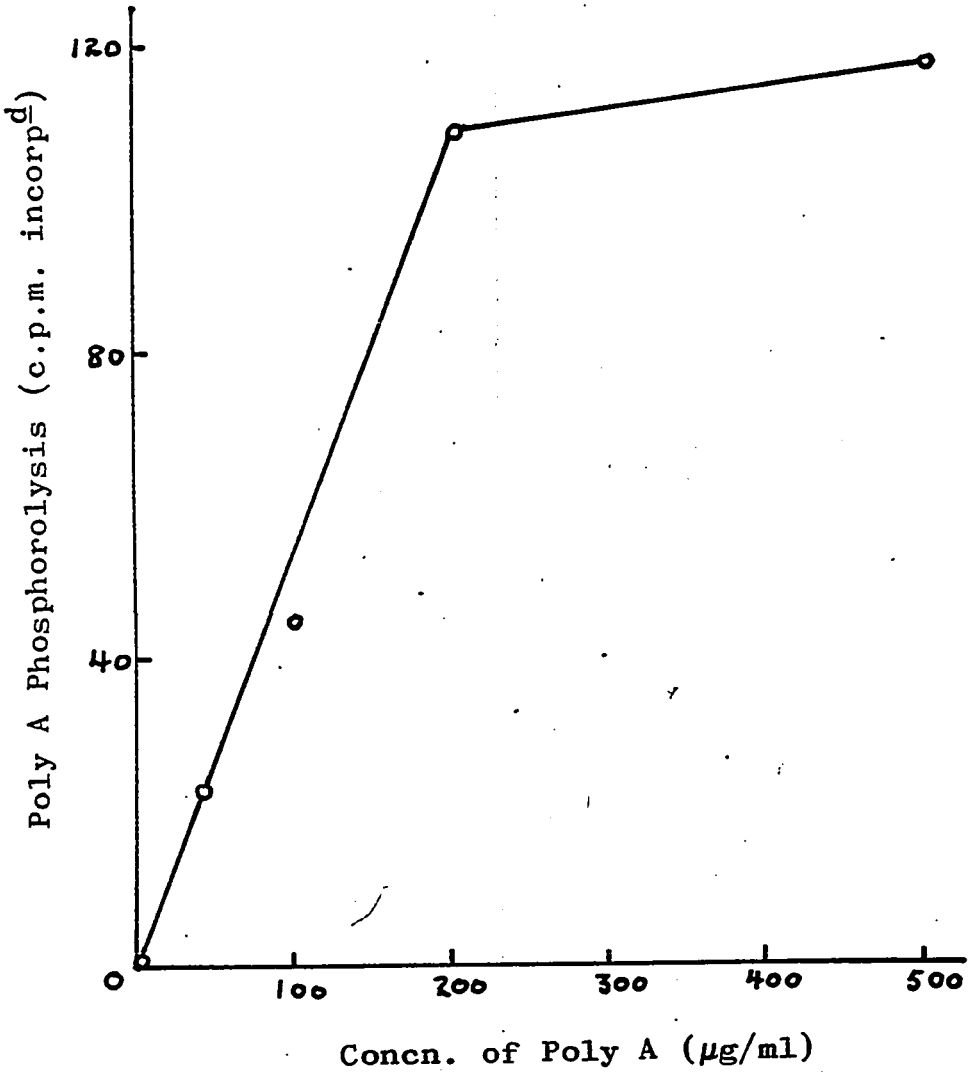
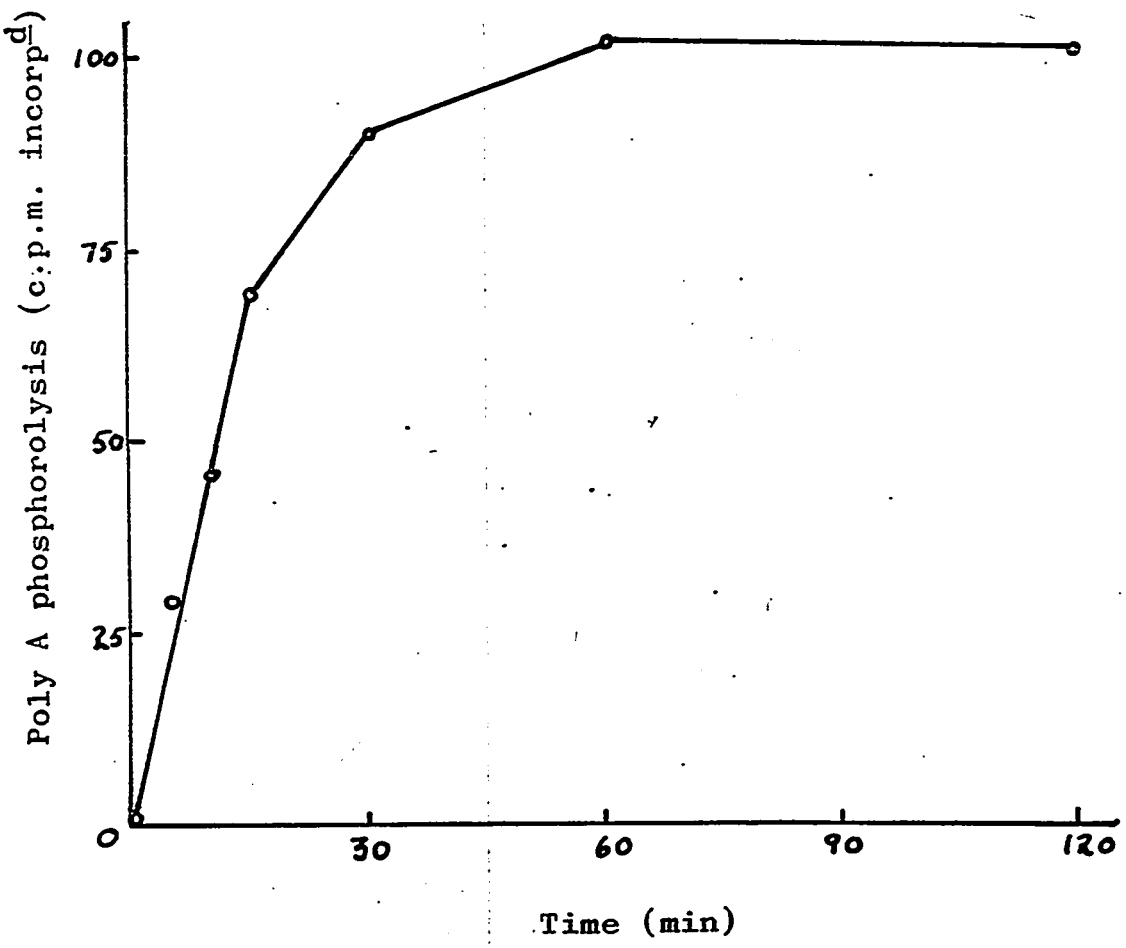


Fig. 9

Phosphorolysis of Poly A

Time Course of Reaction



(vii) The activity of the enzyme with various substrates.

The reaction mixtures were those of Assay A with the following changes:

> Poly C, Poly G, Poly U, RNA(Wheat germ) and DNA(Calf thymus) at a concentration of 500 μg/ml were each substituted for Poly A in a series of assays to assess the phosphorolytic activity of the enzyme with a variety of substrates. Otherwise the reaction was carried out in the conditions of Assay A.

The results of this experiment are shown in Table V. The enzyme phosphorolyzed Poly C three times faster than Poly A. Its activity toward Poly G, Poly U and RNA was one-half to one-third that toward Poly A. As expected for polynucleotide phosphorylase, it did not catalyze the phosphorolysis of DNA.

TABLE V

Effect of Various Polymers as Substrate

Polymer	c.p.m.
Poly A	300
Poly C	980
Poly G	152
Poly U	100
RNA	104
DNA	-

(viii) Phosphate exchange activity.

The earlier crude extracts showed a low ability to catalyze phosphate exchange (Assay C). These assays were done in the conditions established for the M. lysodeikticus enzyme - that is with Mg^{2+} as the divalent cation and at a pH of 8.2. This activity was investigated further and found to have optimum activity at a Mg^{2+} concentration of 30 mM and a pH of 7.4 (Table VI). However when the specificity of the activity was studied by testing its ability to catalyze exchange with AMP, ADP and ATP, it was found to have about 10% more activity with ATP as the substrate. This indicated the Mg^{2+} -dependent exchange activity was not due to polynucleotide phosphorylase.

Because of the manganese ion dependency of the phosphorolytic activity, phosphate exchange assays were then performed in the presence of Mn^{2+}. A Mn^{2+} -dependent exchange activity was found with an optimum at 5 mM Mn^{2+} and a pH of 8.2. However, though it showed slightly more activity with ADP than with ATP, these results were not convincing proof of the presence of polynucleotide phosphorylase. Later, with the highly purified enzyme, a high degree of specificity for nucleoside diphosphates was demonstrated for the Mn^{2+} -dependent exchange activity (Table VIII).

TABLE VI

Phosphate Exchange Activity
(expressed as c.p.m. of ^{32}P - orthophosphate incorporated)

A. Effect of divalent cation concentration at pH 8.2

	5 mM	10 mM	30 mM
Mg^{2+}	590	678	688
Mn^{2+}	281	48	16

B. Effect of pH

	6.5	7.4	8.2	9.0	9.0
Mg^{2+} (30 mM)	1141	1193	1006	552	435
Mn^{2+} (5 mM)	102	264	298	288	154

C. Specificity of Mg^{2+} -dependent exchange activity at pH 7.4

	Substrate (2.5 mM)		
	AMP	ADP	ATP
Mg^{2+} (30 mM)	387	935	1022

(ix) Polymerization activity.

The earliest crude extracts showed a low activity when measured by the incorporation of ^{14}C-ADP into an acid-insoluble product. This activity was not improved by the various methods tested for disrupting the membranes (Section A,p. 50). The polymerization activity has a Mn^{2+} optimum of 5 mM and a broad pH optimum from 8.2 to 9.0 (Table VII).. Under these conditions, the crude extract incorporated about twice as much radioactivity with ADP as the substrate than with ATP.

The polymerization activity of the crude extract was tested for the effect of added primer. The primer used was the dinucleotide ApA and it did not increase the low activity of the crude extract. However, when the effect of added ApA was tested on the purified enzyme, there was no activity in the absence of primer. Further, no activity was evident until the concentration of ApA in the assay reached 20 mg/ml. This activity was at the same low level as that found in the crude extract without added primer.

The purified enzyme had about three times more CDP-polymerization activity than ADP-polymerization activity.

The polymerization activity showed an absolute specificity for nucleoside diphosphates as the substrate (Table VIII).

TABLE VII

Polymerization Activity
(expressed as c.p.m. of ^{14}C-ADP incorporated)

A. Effect of Mn^{2+} - concentration at pH 9

2 mM	5 mM	7 mM
12	50	0

B. Effect of pH at 5 mM Mn^{2+}

6.5	7.4	8.2	9.0	9.8
5	26	37	40	26

C. Effect of primer on purified enzyme

ApA mg/ml

0	0.4	1.0	2.0	3.0
0	0	0	11	13

(x) Specificity of the activities of the enzyme for nucleoside diphosphates.

The results of an examination of the specificity of the purified enzyme for nucleoside diphosphates as opposed to mono- and triphosphates are shown in Table VIII.

The first part of the table shows the results of the analysis of the distribution of the radioactivity in the products of the phosphorolysis assay. As can be seen, both Peak I and Peak II material gave essentially the same results when the products were separated and counted as described in detail in METHODS, p. 48. The nucleoside diphosphate spot on the thin-layer chromatogram contained 92% to 95% of the radioactive material.

The reaction mixtures for the investigation of specificity in the polymerization and exchange reactions were the standard assays B and C, with substitution of the required concentrations of nucleoside mono- and triphosphates.

The results for the Mn^{2+} -dependent exchange activity and for the polymerization activity gave equally clear-cut results. The exchange reaction which is known to be the least specific of the three polynucleotide phosphorylase activities showed six times more activity with nucleoside diphosphate than with either the mono- or triphosphate. In the case of the polymerization activity there was no incorporation of radioactive material at all when NMP or NTP were used as the substrate.

These results show conclusively that the activity being investigated here is due to a polynucleotide phosphorylase.

TABLE VIII

Specificity of H. cutirubrum Polynucleotide Phosphorylase

A. Products of phosphorolysis of Poly A.

	NMP d.p.m.	NDP d.p.m.	NTP d.p.m.
Peak I	3(1%)	390(92%)	30(7%)
Peak II	0	250(95%)	13(5%)

B. Substrate for polymerization activity.

NMP nmol incorp[d]	NDP nmol incorp[d]	NTP nmol incorp[d]
0	4(100%)	0

C. Substrate for phosphate exchange activity.

NMP nmol incorp[d]	NDP nmol incorp[d]	NTP nmol incorp[d]
1(12%)	6(76%)	1(12%)

(xi) Molecular weight of the Peak II material.

The emergence of the Peak II material at an elution volume of about 360 ml from a column 2.5 cm by 90 cm contrasted markedly with the elution volume of about 180 ml obtained for the polynucleotide phosphorylases from A. vinelandii and M. lysodeikticus. It was also found that Peak I material contained lipids characteristic of the membrane of H. cutirubrum whereas Peak II did not and that Peak I material could be partially converted to Peak II by a 10 s sonication. As these facts strongly supported the theory that Peak II material was the enzyme itself dissociated from the membranes, it now became of interest to determine the molecular weight of this material as it seemed to be much smaller than the corresponding one from non-halophilic bacteria.

There was a possibility that the highly retarded peak was an artefact due to the anomalous behavior of G-200 gel filtration in high salt. We therefore first standardized our column by the technique described in detail in the Methods Section, p. 45. The standards were run individually and emerged at the void volumes shown in Table IX. These values were used to plot the average partition coefficient (K_{av}).

When K_{av} was plotted against log molecular weight on semi-logarithmic paper a straight line was obtained (Fig.10). This was in accordance with Andrews (1965) who stated that the elution position of a native protein can be correlated directly with its molecular weight, and showed that Sephadex G-200 behaves in a predictable manner in high salt.

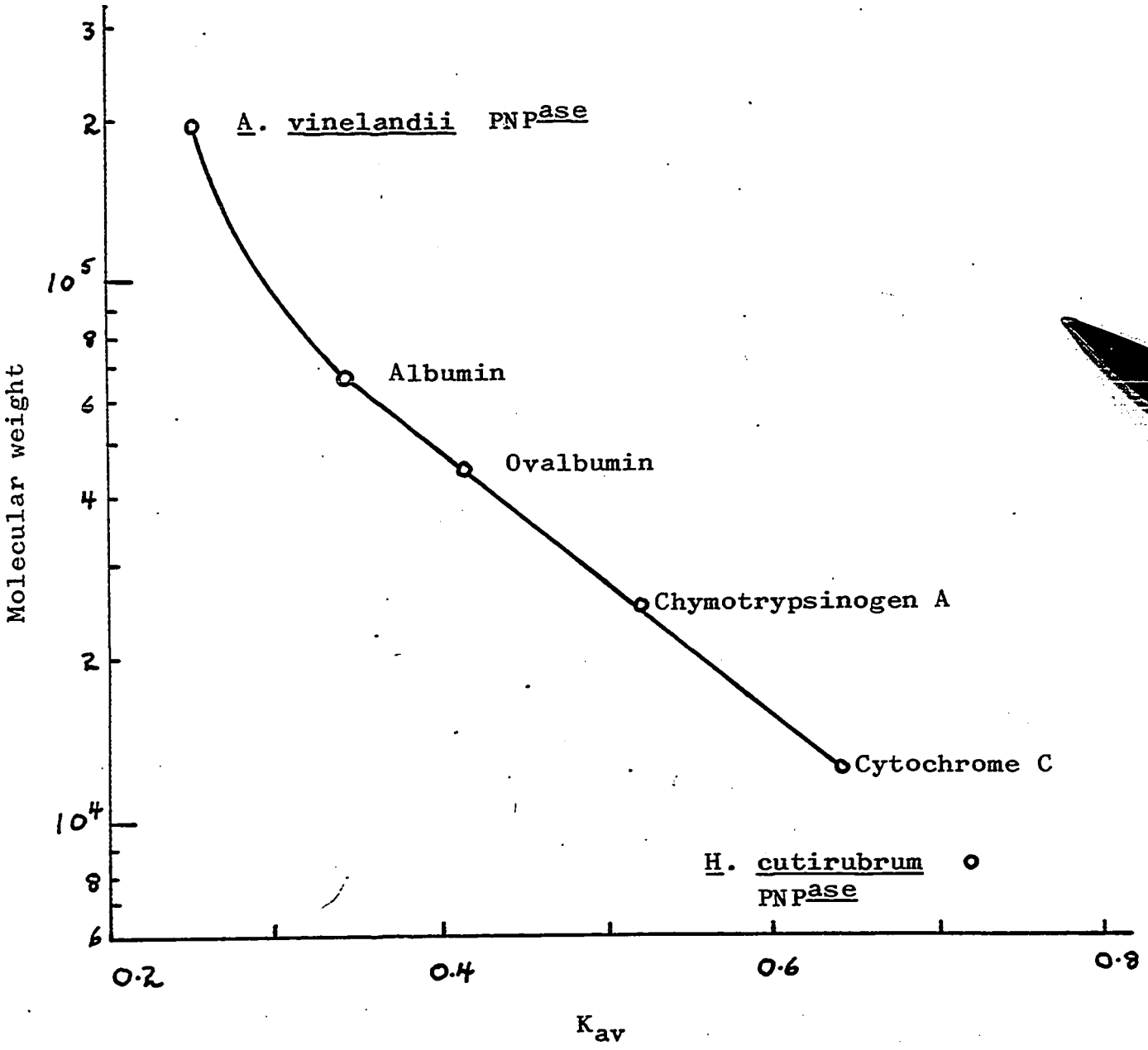
TABLE IX

Elution Volumes and Molecular Weights of Proteins

Protein	M.W.	V_e	K_{av}
Albumin	67,000	260	0.34
Ovalbumin	45,000	276	0.41
Chymotrypsinogen A	25,000	304	0.52
Cytochrome C	12,400	336	0.64
H. cutirubrum PNP^{ase}		356	0.72
A. vinelandii PNP^{ase}	200,000	240	0.25

Fig. 10

K_{av} vs Log Molecular Weight of Standard Proteins



When the elution volume of the enzyme was ascertained on the standardized column, its K_{av} was calculated as 0.72. This unfortunately was beyond the plot obtained with the standard proteins being a higher K_{av} than that obtained for cytochrome C. However it strongly suggested a molecular weight of less than 12,000 and certainly was in contrast with the K_{av} of 0.25 which was obtained on the standardized column for purified A. vinelandii polynucleotide phosphorylase, which has a molecular weight of about 200,000.

(xii) Activity in 6 M guanidinium hydrochloride.

It was thought it would be of interest to determine if the enzyme was active in 6 M guanidinium hydrochloride in the absence of salt. Protein molecular weight determinations in 6 M guanidinium-HCl have been suggested by Fish, Mann and Tanford (1969). These authors had located the standard proteins in the eluant by absorbance at 280 nm, which method can be used only if a pure protein is available. As future studies of the molecular weight of halophilic enzymes may include determinations by the method mentioned above, it would facilitate the location of the enzyme in the eluant if it were active in such conditions.

The results of this experiment are shown in Table X. The enzyme is about twice as active in 6 M guanidinium-HCl as in 2.5 M KCl, 1 M NaCl. However, after six days, the enzyme kept in the guanidine solution had lost 85% of its activity compared to a loss of only 20% when stored in high salt. Though it is relatively unstable it would retain its activity long enough to run columns in these conditions.

TABLE X

Phosphorolytic Activity in 6 M Guanidinium - HCl
(c.p.m. incorporated)

	No Salt	2.5 M KCl, 1 M NaCl	6 M Guanidinium - HCl
0 days	0	52	111
6 days	0	42	17

(xiii) Comparison of enzymological properties of Peak I and Peak II material.

Previous reports of the separation of polynucleotide phosphorylase activities during gel filtration or sucrose-density-gradient centrifugation have stated that certain properties such as relative abilities to phosphorolyze and/or polymerize, as well as divalent cation requirements, vary between the two peaks (Castles & Singer, 1968; Thang et al., 1969b; Guissani & Grunberg-Manago, 1969).

A comparison of some of the properties of the Peak I and Peak II material of this enzyme (Table XI) shows that the relative activities, as well as the pH optimum of the phosphorolysis reaction were essentially the same. Both the activities demonstrated excellent specificities for the production of nucleoside diphosphate in the phosphorolysis reaction. Furthermore, other studies showed the phosphorolysis activity of both Peak I and Peak II to be Mn^{2+} -dependent.

We conclude that the enzymological properties of Peak I and Peak II material are essentially similar, with the only difference being in the particle size and the lipid content.

TABLE XI

Enzymological Properties of Peak I and Peak II

A. Enzyme Activities (Units/mg Protein).

	Phosphorolysis	Polymerization	Exchange
Peak I	240	10	100
Peak II	200	12	120

B. Effect of pH on Phosphorolysis Activity

pH	6.6	7.4	8.2	9.0	9.8	10.6
Peak I (c.p.m. incorp^{d})	10	10	13	36	51	20
Peak II (c.p.m. incorp^{d})	15	16	15	26	35	17

C. Specificity of Phosphorolysis Assay

Nucleotides	NMP	NDP	NTP
Peak I (% incorp^{d})	1%	93%	6%
Peak II (% incorp^{d})	0	95%	5%

IV DISCUSSION

The enzymes of the extremely halophilic bacteria which have been studied previously include certain key enzymes of glycolysis, the tricarboxylic acid cycle and the electron transport chain, as well as some phosphatases and transaminases. The nucleic acid enzymology of these interesting bacteria had not yet been explored when the present study was started.

The data presented here show conclusively that polynucleotide phosphorylase is present in H. cutirubrum. The enzyme catalyzes all three characteristic reactions, namely, the polymerization of nucleoside diphosphates, the phosphorolysis of polyribonucleotides and the nucleoside diphosphate/orthophosphate exchange reaction. These activities have been shown to be specific for the nucleoside diphosphate, not the mono- or triphosphate. The enzyme, as is typical for polynucleotide phosphorylase, does not catalyze the phosphorolysis of polydeoxyribonucleotides.

Purification

A method was developed for the purification of H. cutirubrum polynucleotide phosphorylase in conditions of high ionic strength. This results in a product with a 200-fold increase in specific activity in about 25% yield. An interesting contrast with this purification procedure is afforded by the method for the purification of H. salinarium malate dehydrogenase described by Holmes and Halvorson (1965a). These authors lost 90% of their

original activity in the first purification step, while gaining a 7-fold increase in specific activity. Their method is based on reactivation of the enzyme by high salt after each purification step performed in the salt-free and inactive state. This results in a product of high purity but extremely low yield (700-fold purified, 0.5% yield). They conclude that it is probable that a considerable fraction of any contaminating protein in their purified product consists of inactivated enzyme.

Kinetic studies done on a purified enzyme of this nature lose some of their validity. There may be changes of an unspecified nature in the protein which still retains catalytic activity. The large excess of inactive protein may still retain its capacity to bind substrate even though it is unable to catalyze the reaction (Dixon & Webb, 1964) and this would result in a false value for the apparent K_m of the reaction. A purification procedure for halophilic enzymes carried out in conditions where full activity is maintained was needed.

Furthermore, many halophilic enzymes are so unstable in an environment of low ionic strength that their reactivation by salt is not possible (Robinson & Katznelson, 1953; Baxter, 1959; Lanyi, 1969b). The present work deals with an enzyme which also is irreversibly inactivated in the absence of salt, and it was necessary to work out procedures where the purification was done in a high salt concentration.

There is certainly a great need to develop methods which can be used to obtain a pure protein in good yield from extreme halophiles. Many

aspects of protein study are of doubtful validity unless performed on pure material. For example, amino acid analyses and sequencing, the effect of monovalent cations on protein conformation and detailed kinetic studies all await the availability of pure proteins. The purification of halophilic enzymes in conditions of high ionic strength seems to be a method that may be of general use towards this end.

There are other purification procedures than those used in this research which can be carried out at a concentration of 2 M or higher salt, and which may prove to be of value for further work in this area. Sucrose-density-gradient centrifugation of nucleic acids has been carried out in 2 M NaCl (Loeb, 1967). The same group have used agarose and polyacrylamide gel filtration columns for the separation of DNA from proteins where the salt concentration is 2 M (Loeb & Chauveau, 1969). Polyacrylamide gel filtration on Biogel P60 and sucrose-density-gradient centrifugation, both in 2.5 M KCl, 1 M NaCl, have now been used successfully in the purification of H. cutirubrum RNA polymerase (Louis & Fitt, 1970).

Adsorption chromatography on hydroxylapatite columns in 25% (4.3M) NaCl has been successfully used as a purification step for an extracellular halophilic protease (Dr. P. Norberg, personal communication). Acid precipitation at pH 5 in 3.4 M KCl was used by Bayley and Griffiths (1968a) in their preparation of crude aminoacyl-tRNA syntheses. This was also used successfully by me in an investigation of possible initial purification steps but was discarded in favor of ones more advantageous for this particular enzyme.

One must also keep in mind the possibility of using ion-exchange columns. If conditions can be found in which the enzyme is adsorbed to the resin, e.g. DEAE-cellulose, QAE-Sephadex, then elution by a pH gradient could be performed. Even though it may seem unlikely that proteins would be absorbed to ion-exchangers in conditions of high ionic strength, this is an unproven assumption so far as halophilic enzymes are concerned.

Activation and Stabilization

Halophilic enzymes previously studied have been shown to exhibit differences in the salt concentration required for activity and for stability (Baxter, 1959; Holmes & Halvorson, 1965b; Hubbard & Miller, 1968). Lanyi (1969b) claimed that the NADH oxidase of H. cutirubrum exhibited a direct relationship between enzyme activity and stability as affected by salt concentration, in contrast to other halophilic enzymes. Specifically, his enzyme showed optimum activity and good stability in 2 M NaCl. However, in his stability experiments the enzyme was in a low-salt environment for a maximum of 3 minutes. It is therefore not possible to compare his results with those of the other authors who were testing stability during considerably longer periods of time in a low-salt or salt-free environment.

I found that the purified halophilic polynucleotide phosphorylase showed an optimum activity at 2 M NaCl or KCl. Its requirement for stability was considerably higher, however, as maximum protection was afforded by 4 M NaCl. It was therefore very similar in these respects

to the results obtained with purified malic dehydrogenase (Holmes & Halvorson, 1965b) and contrasted with the varying effects seen when crude enzyme preparations are used for this type of study.

The protein conformation which results in optimum enzyme activity would, in general, also result in maximum stability. Yet, here, twice as much salt is needed to stabilize the enzyme as to activate it. Hubbard and Miller (1968) showed that the substrate of H. cutirubrum isocitrate dehydrogenase can stabilize the enzyme, with a mixture of 4 mM isocitrate and 10 mM $MgCl_2$ giving the same protection as 4 M NaCl. Though the effect of the divalent cation is difficult to evaluate, it is well known that combination with its substrate often protects an enzyme from denaturation (Dixon & Webb, 1964). The fact that twice the number of moles of salt is needed to stabilize the enzyme than is needed to keep the enzyme in its active conformation, may well be due to the protection afforded by the substrate in the assay mix, with the substrate tending to hold the chains together.

There are two ways in which a monovalent cation could affect the activity of an enzyme (Suelter, 1970).

1. The ion could exert its role by maintaining a specific protein conformation necessary for optimum catalytic efficiency.
2. The monovalent cations could actively participate in the catalysis forming a ternary complex with the substrate and enzyme.

Baxter (1959) suggested the possibility that the first effect was operative in halophilic enzymes. He proposed that salts decreased the electrostatic repulsion between ionized groups in the molecule and thus allowed it to assume its enzymatically active conformation. Christian and Waltho (1962) determined that the intracellular Na^+ and K^+ concentrations are 1.4 and 4.6 molal respectively whereas the Cl^- concentration is 3.6 molal. This would indicate an excess of positive charges and it would follow that the proteins of the halophilic organism might be electrostatically negative. I know of no reference to the amino acid analysis of a halophilic enzyme. However, Steensland and Larsen (1969) reported the results of an amino acid analysis of a membrane protein. It showed an excess of acidic over basic amino acids of about 20 mol % (5 mol % when corrected for amide). Protein extracted from ribosomes was shown to be negatively charged at both pH 5.3 and 8.0 in gel electrophoresis studies (Bayley & Kushner, 1964). Both the cell envelopes and the ribosomes require 2 to 4 M salt for structural integrity in a manner analogous to the requirement of the enzymes for high salt for activity. The suggestion that the halophilic enzymes in general are acidic may prove to be true.

The second proposal that the requirement for monovalent cations may be due to the formation of an active ternary complex is difficult to support or reject for halophilic enzymes at the present level of knowledge. However, it might be argued that such an effect would have a much more

specific requirement for a monovalent cation of a certain size than the halophilic enzymes seem to exhibit. My data, for example, showed that K^+ and Na^+ were equally effective in the stimulation of activity of halophilic polynucleotide phosphorylase. Hochstein and Dalton (1968) found the same to be true for a halophilic NADH oxidase. In contrast, Suelter (1970) found a quite specific monovalent cation requirement in certain non-halophilic enzymes which are activated by monovalent cations. He presents data to show that this activation is probably due to a ternary complex formation.

The data for the activation of halophilic enzymes by salt are, I think, more consistent with the first hypothesis, namely, that the monovalent cations are required to keep the enzyme in its active conformation. The problem will not be settled until much more is known of the primary and tertiary structure of halophilic enzymes and until highly purified enzymes are available for meaningful kinetic studies.

Polynucleotide phosphorylases require a divalent metal cation for activity (Grunberg-Manago, 1963). The assay conditions for the phosphorolysis reaction as given in her review show $MgCl_2$ at a concentration of 7 mM. The M. lysodeikticus enzyme is assayed for phosphorolytic activity at a Mg^{2+} concentration of 5 mM (Fitt & Fitt, 1967). The Cl. perfringens enzyme is assayed in the same conditions (Fitt & Wille, 1969a). The guinea-pig liver nuclei enzyme, which exhibits only phosphorolytic activity, shows optimum activity at 4 mM Mg^{2+}, and exhibits a marked inhibition at higher concentrations (See & Fitt, 1970).

The contrast between these levels and the divalent metal cation requirements for the phosphorolytic activity of the H. cutirubrum enzyme is striking. Even the feeble Mg^{2+} -dependent activity shows an optimum at 10 mM, followed by complete inhibition at 15 mM. The Mn^{2+} -dependent activity reaches a plateau at 50 mM, and shows no inhibition even at 100 mM. The assay conditions chosen for routine work have a total divalent cation concentration of 30 mM, Mg^{2+} being 10 mM and Mn^{2+} being 20 mM.

There has been little investigation in general of the divalent cation requirements of halophilic enzymes. The crude NADH dehydrogenase as assayed with menadione was reported by Lanyi (1969b) as having negligible activity at 2 mM $MgCl_2$ and showing an optimum activity at 200 mM $MgCl_2$. The same author (Lanyi & Stevenson, 1969) showed that a partially purified H. cutirubrum catalase had optimal activity at 100 mM $MgCl_2$ or $CaCl_2$.

The extreme halophiles require an unusually high concentration of magnesium ions (0.1 to 0.5 M) in the culture medium for good growth (Larsen, 1967). This seems to be related to some need of the cell envelopes for Mg^{2+}, because the rod-shaped halobacteria take on a coccoid form when grown in a medium low in magnesium. However, there is a possibility that the intracellular content of divalent cations may also be higher in halophiles than in non-halophiles. This point has never been investigated as far as I know, but in view of the requirements of some enzymes at least, for a high concentration of divalent cation for optimum activity, it warrants consideration.

The only previous observation on the effect of denaturing agents on halophilic enzymes is Baxter's report (1959) that the lactic dehydrogenase of H. salinarium shows much lower activity when 4 M urea is present, than in its absence. This contrasts with my finding that the polynucleotide phosphorylase is about twice as active, though less stable, in 6 M guanidinium - HCl as in 2.5 M KCl, 1 M NaCl.

Nature of two activity peaks.

The level of activity of H. cutirubrum polynucleotide phosphorylase both for the phosphorolysis and polymerization activities is about the same as that reported by Thang et al.(1967) for the E. coli Q_{13} mutant. This mutant has about 25% of the phosphorolytic activity and about 1% of the polymerization activity as those in E. coli B.

There have been several reports of the separation of polynucleotide phosphorylase activities upon gel filtration chromatography or sucrose-density-gradient centrifugation. The enzyme in E. coli 1113B separated into two activity peaks on a Sephadex G-200 column (Castles & Singer, 1968). The phosphorolysis activity was the same in both peaks, but the Mn^{2+} -dependent polymerization activity of Peak I was four times that of Peak II, whereas the Mg^{2+} -dependent polymerization activity was one-quarter that of Peak II. The enzyme of E. coli Q_{13} separated into two peaks on sucrose-density-gradient centrifugation (Thang et al., 1969). The lower molecular weight material catalyzed the phosphorolysis of short chain oligonucleotides only, and had no polymerization activity. The higher molecular weight

material had a Mn^{2+} -dependent polymerization activity. Dietz and Grunberg-Manago (1967) described the separation of the Cl. perfringens enzyme into two components by sucrose-density-gradient centrifugation. One component was specific for ADP-incorporation and was totally dependent on polylysine and β-mercaptoethanol. However, these results may be due to spurious double peaks of activity due to failure to ensure proportionality of enzyme with polylysine in the assay of the fractions (Fitt & Wille, 1969a).

These previous findings of differing enzymological properties between polynucleotide phosphorylases of differing molecular size from the same organism, led me to look for such differences when the activities first separated on Sephadex G-200 gel filtration. However no differences in the activities of the two peaks could be detected. They had essentially similar levels of polymerization, phosphorolysis and exchange activities with the same divalent cation requirements. The pH optima of the phosphorolysis activities were identical. We conclude that there is no evidence for the existence in H. cutirubrum of two polynucleotide phosphorylases having different catalytic abilities.

Polynucleotide phosphorylase - like activities can be due to various combinations of other enzymes such as phosphatases, phosphokinases, etc. Hence it is essential to demonstrate unequivocally the production of nucleoside diphosphates in the phosphorolysis reaction or the substrate specificity for the diphosphate in the polymerization reaction. Both these specificities

were clearly demonstrated, together with a substrate specificity in the Mn^{2+} -dependent exchange reaction.

It is interesting to note the presence of Mg^{2+} -dependent ATP/orthophosphate exchange activity present both in the crude extracts and the ammonium sulfate fraction and hence active in high salt. This could be the object of a further study to compare it with the Na^{+}/K^{+} -activated adenosine triphosphatases of non-halophilic organisms.

Intracellular localization

The evidence we have presented strongly suggests that the polynucleotide phosphorylase of H. cutirubrum is associated with the membranes in the whole organism. The enzyme seems not to be firmly bound, as a 10 second burst of ultrasound suffices to release some of it from the membranes. The lipids with which it is associated in the membrane are not essential to its activity.

A non-specific association of membrane fragments with some of the enzyme molecules is militated against by the fact that the Peak I activity and a peak of absorbance at 280 nm due to proteins and lipids (due, at least partly, to the Vit.K-type quinone of the neutral lipids, Kates et al., 1969) showed an exact agreement each time a Sephadex G-200 gel filtration was done. Also the visible pink color in the fraction collection tubes, due to the red carotenoid pigment, commenced, reached maximum intensity and disappeared in coincidence with the enzyme activity. If the association was fortuitous, one would not expect to find this good agreement of activity and lipids each time.

More convincing evidence of the association of the enzyme with the cell membrane awaits the preparation and assay of isolated membranes.

The only previously reported polynucleotide phosphorylase in bacterial membranes was isolated by Abrams and McNamara (1962) from S. faecalis protoplasts prepared with lysozyme. The washed membranes showed ten times the activity of the soluble fraction. No attempt was made by these authors to release the activity from the membranes.

However, it has been possible to isolate a typical soluble polynucleotide phosphorylase from an acetone powder of S. faecalis (Fitt & Gibbs, 1969). It is possible that the enzyme is located in the membranes in other species, and has been solubilized by the methods used to prepare the cell-free extracts. An investigation of the intracellular localization of bacterial polynucleotide phosphorylases seems to be indicated.

The mammalian polynucleotide phosphorylase of guinea-pig liver nuclei (See & Fitt, 1970) has lipids characteristic of mammalian membranes associated with it. It may prove to be located on the nuclear membrane.

If polynucleotide phosphorylase is located in the bacterial cell membrane it is difficult to visualize it as performing the physiological roles that have been proposed for it, for example, the degradation of mRNA located on the ribosomes. Hendler (1968) showed evidence that the active ribosomes of bacteria are mainly located within the cells at membrane sites. If his view is correct, the membrane location of polynucleotide phosphorylase and its possible physiological role in the degradation of mRNA could be reconciled.

Molecular weight

The evidence that is presented here shows the molecular weight of *H. cutirubrum* polynucleotide phosphorylase to be less than 12,000. This was based on its being more highly retarded on a standardized column of Sephadex G-200 than cytochrome C which has a molecular weight of 12,400.

This result has four possible alternative explanations: (1) The enzyme is truly of a low molecular weight. (ii) Sephadex G-200 particles do not exhibit a true molecular sieving effect when in high salt concentrations. (iii) Proteins are adsorbed on dextran particles in high salt. (iv) Halophilic enzymes are of abnormally high density. The second possibility is excluded by the behavior of the standard proteins. A linear relationship existed between their average partition coefficient and the log of their molecular weight. This demonstrates gel chromatography is the operative principle even when the dextran particles are in a high salt environment (Fischer, 1969).

If the third possibility, an adsorption on the dextran particles, were the cause of the highly retarded peak, this effect would be even more obvious on a more highly cross-linked gel. The average partition coefficients (K_{av}) of *H. cutirubrum* RNA polymerases have been determined on Sephadex G-200 and on standardized Biogel P-60 columns (Mr. B. G. Louis & Dr. P. S. Fitt, personal communication). The two determinations were in agreement showing that for these enzymes, at least, there is no adsorption to the gel particles.

The fourth possible explanation, that of an abnormally high density for halophilic enzymes has been excluded by sucrose-density-gradient centrifugation studies on thesesame RNA polymerases. These enzymes are also of low molecular weight, and the results obtained by this method gave agreement, within the limits of experimental error, with the molecular weight as determined by gel filtration.

Thus the results obtained with the standardized G-200 column show that H. cutirubrum polynucleotide phosphorylase is of low molecular weight, less than 12,000.

Future investigation of the molecular weight of the enzyme should include gel chromatography on a more highly cross-linked gel, such as Sephadex G-50, as well as sucrose-density-gradient centrifugation studies.

The molecular weight of bacterial polynucleotide phosphorylases is reported to be 200,000 for the E. coli, A. vinelandii and Cl. perfringens enzymes and 230,000 for the M. lysodeikticus enzyme (Thang, 1969). Various lighter species have been reported. The E. coli Q_{13} mutant contains an enzyme with a molecular weight of 100,000 (Thang et al., 1969). The Cl. perfringens enzyme can be partially converted to a 70,000 MW species (Guissani and Grunberg-Manago, 1969). Further, Grunberg-Manago and her colleagues proposed a polynucleotide structure consisting of 30,000 MW monomers (Thang et al., 1969). The trimer (MW 100,000) would possess only an active center for phosphorolysis and a binding site for

polynucleotides. The hexamer (MW 200,000) would possess also the active center and binding sites necessary for the polymerization reaction.

On the basis of the results presented here it seems that the H. cutirubrum enzyme does not have a comparable structure. This small molecule possesses all three activities of the enzyme, though the polymerization activity is at a very low level. The active site of an enzyme which catalyzes a reaction in one direction must of necessity catalyze the reverse reaction. However, an enzyme may or may not possess a binding site for a particular substrate, and this may explain the lack of, or low level of activity of, the reverse of a given reaction. It is possible that the H. cutirubrum enzyme binds polynucleotides well, but binds nucleoside diphosphates poorly, if at all. If this were so, it would explain the low level of polymerization ability which the enzyme exhibits.

Thus the polynucleotide phosphorylase of H. cutirubrum, with a molecular weight of less than 12,000, is in contrast with the light species of other enzymes which possess partial or differing activities from those of the native enzyme (Thang, 1969). Indeed, we conclude that the Peak I activity consists of the enzyme bound to membrane fragments of varying size (hence the variation in K_{av} of this material) and the Peak II material consists of the enzyme, the protein molecule itself, separated from the membrane by the method used to disrupt the cell walls. If this enzyme were to be prepared in a pure form it would be small enough

to make the determination of its amino acid sequence feasible. It could thus prove a valuable aid in the studies of the active center and binding sites of polynucleotide phosphorylase.

This is the first experimental evidence, as far as I know, to support Ingram's suggestion (1948) that halophilic enzymes would prove to be small molecules. He presented evidence to indicate that the effect of concentrated solutions of salts in depressing bacterial respiration could be explained in terms of salting-out. With halophilic bacteria, if the proteins involved in respiration were of high molecular weight, they would be precipitated at the high salt concentrations involved. He therefore concluded that "as the enzymes resist salts even *in vivo*, they may perhaps be involved in smaller molecular aggregates".

Separable RNA-dependent and DNA-dependent RNA polymerases (Fitt & Louis, 1970) have now been shown by gel chromatography and sucrose-density-gradient centrifugation to have molecular weights of about 17,000 (Mr. B. G. Louis, personal communication). The DNA-dependent RNA polymerase of *E. coli* has a molecular weight of about 370,000 (Maitra & Hurwitz, 1967).

In contrast to these enzymes of nucleic acid metabolism, *H. cutirubrum* catalase showed essentially the same retention constant as beef liver catalase (MW 240,000) on a Biogel P-300 column. It will be of great interest to study other enzymes of nucleic acid metabolism, both synthetic and degradative, in order to determine if such enzymes are smaller on the average than other halophilic enzymes. The studies reported in this thesis are a start in this direction.

V SUMMARY

1. A polynucleotide phosphorylase has been isolated from H. cutirubrum.

2. The enzyme has been purified 200-fold in 25% yield in a high concentration of salt. In these conditions full activity is maintained.

3. The enzyme has been shown to be a typical polynucleotide phosphorylase in that it catalyzes all three characteristic reactions. It is specific for nucleoside diphosphates and does not phosphorolyze DNA.

4. The enzyme has a rather low activity.

5. The enzyme exists in association with the membranes in the intact cell. It can be released by gentle methods without losing its activity.

6. The molecular weight of the enzyme is less than 12,000.

7. The two activity peaks which separate during gel chromatography on Sephadex G-200 in high salt have similar enzymological properties and differ only in molecular size and lipid content.

8. The purified enzyme possesses the following characteristics:
   - Mn^{2+} -dependent
   - unstable in less than 4 M salt
   - requires at least 2 M salt for optimum activity
   - the phosphorolysis reaction is optimal at pH 9.8

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