Nucleic Acid Enzymology of Extremely Halophilic Bacteria

HALOBACTERIUM CUTIRUBRUM POLYNUCLEOTIDE PHOSPHORYLASE

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1. Polynucleotide phosphorylase was purified 200-fold from *Halobacterium cutirubrum*. 2. It is membrane-associated and can be solubilized by sonication. 3. The purified enzyme requires a high ionic strength for both stability and activity. 4. It is Mn^{2+} -dependent, has all three typical polynucleotide phosphorylase activities and is specific for nucleoside diphosphates. 5. The enzyme is of low molecular weight.

Extremely halophilic bacteria grow best in salt concentrations close to saturation and are interesting examples of organisms adapted to life in harsh environments (Kushner, 1968; Larsen, 1962, 1967). Baxter & Gibbons (1954, 1956, 1957) were the first to demonstrate that the enzymes of the extreme halophiles are active at salt concentrations that usually inhibit or inactivate similar enzymes from non-halophilic organisms and that they generally require high salt concentrations for stability. The structural basis for the unusual properties of these enzymes is clearly of great interest and might afford insight into the molecular basis of adaptation to life in extremely saline environments.

Although studies have been reported of the glycerol, isocitrate, succinate, malate and lactate dehydrogenases, cytochrome oxidase and cysteine desulphydrase (Baxter & Gibbons, 1954, 1956, 1957), alkaline phosphomonoesterase (Egami, 1955), aminoacyl synthetases (Griffiths & Bayley, 1969), malate dehydrogenase (Holmes & Halvorson, 1965a), catalase (Lanyi & Stevenson, 1969) and an extracellular protease (Norberg & von Hofsten, 1969) of extremely halophilic bacteria, in all but one of these studies crude extracts or enzymes purified to a very limited extent were used. The exception is the work of Holmes & Halvorson (1965a), who were able to achieve a 763-fold purification of *Halobacterium salinarium* malate dehydrogenase. They found that although this enzyme is totally inactive in the absence of salt it can be partially reactivated by prolonged dialysis against buffer containing a high concentration of sodium chloride. It was therefore possible to purify the inactive salt-free enzyme by using standard low-salt methods and recover some activity by returning it to a high-salt environment. However, the overall yield of purified enzyme was only 0.5%. Further, the approach is not generally applicable to the purification of enzymes from extreme halophiles,

since many are irreversibly denatured in low-ionic-strength environments (Baxter, 1959; Lanyi, 1969). Holmes & Halvorson (1965a) stated that their attempts to purify such proteins in the presence of high concentrations of salt had been unsuccessful.

We wished to examine the polynucleotide phosphorylase (nucleoside diphosphate-polyribonucleotide nucleotidyltransferase; EC 2.7.7.8) of *Halobacterium cutirubrum*, in the hope that it might prove of value in studies of the mechanism of action of this enzyme in limiting conditions of ionic strength. It has been suggested (Thang, Dondon & Grunberg-Manago, 1969) that polynucleotide phosphorylase is of universal occurrence in bacteria, and the first conclusive proof of its existence in animal cells has recently been published (Fitt & See, 1970; See & Fitt, 1970), but its enzymic properties are still only partly understood and its biological role remains uncertain.

In the present paper, we describe the 217-fold purification, in conditions of high ionic strength and in good yield, of *H. cutirubrum* polynucleotide phosphorylase. Details of its enzymic properties and preliminary evidence that it is membrane-associated and of low molecular weight are also presented. A short summary of some of this work has been published (Peterkin & Fitt, 1970). DNA-dependent and RNA-dependent RNA polymerases have also been isolated and purified from *H. cutirubrum* (Fitt & Louis, 1970; Louis & Fitt, 1971a,b) and the molecular weights of all three enzymes have been determined by gel filtration and sucrose-density-gradient centrifugation (Louis, Peterkin & Fitt, 1971).

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: ortho- $[^{32}P]$ phosphate and ^{14}C -labelled nucleoside mono-, di- and tri-phosphates, Amersham/Searle, Don Mills, Ont., Canada; wheat-germ

RNA, Calbiochem, Los Angeles, Calif., U.S.A.; Difco casamino acids and yeast extract, Canadian Laboratory Supplies Ltd., Montreal, P.Q., Canada; ApA and Serva DEAE-cellulose for t.l.c., Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A.; poly(A), poly(C), poly(G) and poly(U), Miles Chemical Co., Elkhart, Ind., U.S.A.; Sephadex G-200, Blue Dextran and Ficoll, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; unlabelled nucleoside mono-, di- and tri-phosphates, P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; calf thymus DNA, Sigma Chemical Co., St Louis, Mo., U.S.A.; pancreatic deoxyribonuclease, Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Highly purified *Azotobacter vinelandii* polynucleotide phosphorylase (Gajda, Zaror de Behrens & Fitt, 1970) was kindly supplied by Mr A. T. Gajda.

Growth of bacteria. The organisms used were *H. cutirubrum* strain no. 9, a gift of Dr D. J. Kushner. This strain was originally isolated from salted buffalo hides by Lochhead (1934). Stock cultures were maintained on slants of Sehgal & Gibbons (1960) medium containing 2% agar in screw-cap tubes. They were grown at 37°C for 2–3 weeks and stored at 4°C. Aeration was achieved during incubation by loosening the caps every 3–4 days. Transfers were made at 6-monthly intervals.

Large-scale cultures were grown under sterile conditions in 11 culture flasks, with Morton closures (Belleco Glass Inc., Vineland, N.J., U.S.A.), containing Sehgal and Gibbons medium (400 ml). The cultures were incubated at 37°C with vigorous shaking in a New Brunswick Rotary Incubator Shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) and were harvested by centrifugation at 5°C after 48 h of growth (middle exponential phase). The freshly harvested cells were used at once.

A routine check was maintained on the purity of the cultures. Two agar slants of Sehgal and Gibbons medium were each inoculated with about 1 ml of liquid culture. After incubation as described above, they were visually inspected for contamination by *Halobacterium halobium*.

Sephadex G-200 gel filtration. Sephadex G-200 was allowed to swell for 4–6 weeks at 4°C in 2.5 M-KCl–1 M-NaCl–10 mM-tris–HCl buffer, pH 8.5 (buffer A). Gel allowed to swell for less than 4 weeks gave unstable columns and irreproducible results, since dry Sephadex G-200 regains water slowly in the high-ionic-strength buffer A. It was also found that the water regain of the gel was about 30% less in buffer A than in water; more gel was therefore needed to pack a column of a given size. The void volume and internal volume of columns packed with Sephadex G-200 fully swollen in buffer A differed reproducibly from those of similar columns packed with gel that had been swollen in water (Louis *et al.* 1971).

Solutions (0.75 mg/ml) of Blue Dextran in buffer A were left overnight at 4°C before use and any insoluble material was removed by centrifugation.

Gel filtration was performed in a cold-room (2–5°C) with Sephadex K25/100 chromatography columns fitted with upper and lower flow adaptors (Pharmacia Ltd.). The hydrostatic pressure was not allowed to exceed 10 cm at any time during either preparation or use of the column. The columns were poured in one step by using a column extender and then run by downward flow for 12–18 h. They were then connected for upward flow and the flow of buffer A was maintained at 10 ml/h for the duration of

the experiment by means of a peristaltic pump. Samples (10 ml of enzyme solution; 1 ml in all other cases mentioned) were applied via a three-way valve to avoid interruption in the flow of buffer. Elution was performed with buffer A. The E_{280} of the effluent was monitored continuously with an LKB Uvicord II system and 4 ml fractions were collected. The extinction of all fractions in the peak areas was determined accurately with a Zeiss PMQ II spectrophotometer at (i) 405 nm for cytochrome *c*, (ii) 280 nm for other proteins and Blue Dextran and (iii) 257 nm for adenosine. The void and internal volumes of columns were determined by using Blue Dextran and adenosine respectively. Elution volumes were determined at the maximum height of the peak (Fischer, 1969).

Preparation of *H. cutirubrum* polynucleotide phosphorylase. All operations were carried out at 0–5°C, except where indicated. The $(\text{NH}_4)_2\text{SO}_4$ concentrations were calculated as described by Green & Hughes (1955).

Freshly harvested *H. cutirubrum* cells were suspended in 2.5 M-KCl–1 M-NaCl–10 mM-tris–HCl buffer, pH 8.5 (buffer A; 3 ml/g wet wt. of cells), and the suspension was stirred slowly until homogeneous (about 1 h). It was then sonicated for 10 s with a Bronwill Biosonik II ultrasonic disintegrator set at maximum intensity. The mixture was centrifuged at 27 000 g for 20 min and the pellet was discarded. The cell-free supernatant (crude extract, Table 1) was centrifuged at 80 000 g for 1 h and the small red pellet was discarded. The 80 000 g supernatant was dialysed for a total of 2 h against two changes of 10 mM-tris–HCl buffer, pH 8.5 (100 vol.), to remove most of the salt. Solid $(\text{NH}_4)_2\text{SO}_4$ was added and the protein fractions insoluble between 45 and 85% of saturation were collected by centrifugation at 27 000 g for 20 min. The pellet was transferred to a small glass hand homogenizer with the aid of a suitable amount of buffer A (about 5 vol.) and uniformly dispersed with one or two strokes of a tight-fitting glass pestle. The solution was extensively dialysed against buffer A and was then purified by Sephadex G-200 gel filtration as described above to give two active fractions (peaks I and II, Table 1) that were concentrated separately by dialysis against 30% (w/v) Ficoll in buffer A. The concentrated enzyme was dialysed for 1 h against buffer A (50 vol.) and stored at 4°C.

Enzyme assay. Polynucleotide phosphorylase activity was assayed by the following procedures.

Assay A. This assay measures the formation of [^{32}P]-ADP during the phosphorolysis of poly(A) in the presence of ortho[^{32}P]phosphate (Fitt & Fitt, 1967; Grunberg-Manago, Ortiz & Ochoa, 1956). The reaction mixture (0.1 ml) 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 ; ortho[^{32}P]phosphate, 1 μmol (about 50 000 c.p.m./ μmol); enzyme. Incubation was at 37°C for 15 min and the reaction was stopped by the addition of 1 ml of a mixture containing 1 part of an aq. 10% (w/w) suspension of acid-washed Norit A and 10 parts of 2.5% (w/v) HClO_4 . The charcoal suspension was left for 10 min at 0°C, with occasional mixing, and was then collected on a 2.4 cm Whatman GF/C glass-fibre filter. The charcoal was washed four times with 10 ml of cold water; during the washing the salt precipitated by the HClO_4 redissolved. The filter was then placed upside down in a planchet, dried and its radioactivity counted. One unit of phosphorolysis activity was defined as the

amount of enzyme catalysing the incorporation into charcoal-adsorbable material of 1 nmol of ortho[^{32}P]-phosphate/h in these conditions. Unless otherwise indicated all specific enzymic activities are expressed in terms of units of phosphorolysis activity per mg of protein.

Assay B. This assay measures the incorporation of ^{14}C -labelled nucleoside diphosphates into an acid-insoluble form (Fitt & Fitt, 1967; Littauer & Kornberg, 1957). The reaction mixture (0.1 ml) contained: tris-HCl, pH 7.6, 15 μmol ; EDTA, 0.04 μmol ; ^{14}C -labelled nucleoside diphosphate, 4 μmol (about 70000 c.p.m./ μmol); ApA, 200 μg ; MnCl_2 , 0.5 μmol ; MgCl_2 , 0.5 μmol ; KCl, 125 μmol ; NaCl, 50 μmol ; enzyme. Incubation was at 37°C for 60 min and the reaction was stopped by the addition of 0.1 ml of 7% (w/v) HClO_4 . The mixture was allowed to stand for 10 min at 0°C, with occasional mixing. The precipitate was then collected on a Whatman GF/C filter, washed four times with 2 ml of 1% (w/v) HClO_4 and once with 2 ml of 50% (v/v) ethanol. The filter was placed in a planchet, dried and its radioactivity counted. One unit of polymerization activity was defined as the amount of enzyme catalysing the incorporation into acid-insoluble material of 1 nmol of ^{14}C -labelled nucleoside diphosphate/h in these conditions.

Assay C. This assay measures the exchange between nucleoside diphosphate and ortho[^{32}P]phosphate (Grunberg-Manago *et al.* 1956). The reaction mixture (0.1 ml) contained: tris-HCl, pH 8.2, 10 μmol ; EDTA, 0.03 μmol ; nucleoside diphosphate, 0.25 μmol ; MnCl_2 , 0.5 μmol ; KCl, 125 μmol ; NaCl, 50 μmol ; ortho[^{32}P]phosphate, 0.35 μmol (about 100000 c.p.m./ μmol); enzyme. Incubation was at 37°C for 15 min. The reaction was stopped with a charcoal- HClO_4 suspension (1 ml) and the charcoal collected, washed and its radioactivity counted as described for the phosphorolysis assay (assay A).

Radioactivity assay. Radioactivity was determined in a Nuclear-Chicago low-background thin-window counter, except for some specificity studies when a Nuclear-Chicago mark I scintillation counter was used. Suitable controls lacking (i) enzyme or (ii) substrate were carried out in all cases and the assay values corrected accordingly.

Protein assay. Protein was determined spectrophotometrically by the method of Warburg & Christian (1942).

RESULTS AND DISCUSSION

Purification of *H. cutirubrum* polynucleotide phosphorylase. The results of a typical preparation

of the enzyme are shown in Table 1. The greatest loss of activity occurred during ammonium sulphate fractionation, probably due to the preliminary 2 h dialysis needed to remove the bulk of the salt. After gel filtration on Sephadex G-200, 40% of the original activity was recovered. This was divided between two peaks: the first (peak I) emerged at or near the void volume, whereas the second (peak II) was highly retarded. The material from the best fraction of peak II was purified 217-fold in 26% yield.

The stability of the ammonium sulphate fraction was examined at various concentrations of sodium chloride and potassium chloride. As shown in Fig. 1, the enzyme retained 40–50% of its activity for 3 weeks in 4M-sodium chloride or 3.6M-potassium chloride. However, in 2M-sodium chloride or -potassium chloride all the activity was lost in 3 days and in a salt-free buffer 95% of the activity was lost in 24 h and 100% in 48 h. The inactive enzyme could not be reactivated by dialysis against 4M-salt for as long as 1 week. It was therefore not possible to purify *H. cutirubrum* polynucleotide phosphorylase by the method used by Holmes & Halvorson (1965a) for the malate dehydrogenase of *H. salinarium*.

In previous work on the isolation of enzymes from the extremely halophilic bacteria, the cell-free extracts were prepared in 4.3M-sodium chloride (25%, w/v) (Holmes, Dundas & Halvorson, 1965). However, the intracellular salt concentration of these organisms has been reported to be 4.6 molal K^+ and 1.4 molal Na^+ (Christian & Waltho, 1962). The phosphorolytic and polymerization activities of polynucleotide phosphorylase were assayed in crude extracts of *H. cutirubrum* prepared in buffer containing either 4M-sodium chloride or 2.5M-potassium chloride–1M-sodium chloride. The activities were 25% higher with the latter, where the potassium chloride/sodium chloride ratio was closer to the physiological value.

The following methods of cell disruption were tried: ultrasonic disintegration for various times; grinding with cold alumina; osmotic shock (Brown,

Table 1. Purification of *H. cutirubrum* polynucleotide phosphorylase

The enzyme was prepared as described in the Experimental section. One unit is the amount of enzyme that catalyses incorporation of 1 nmol of [^{32}P]P_i into ADP/h in the conditions of the polyA phosphorolysis assay.

Fraction	Units	Sp. activity (units/mg)	Relative purification	Yield (%)
(1) Crude extract	7680	9.6	1.0	100
(2) 80000g supernatant	8400	12	1.3	109
(3) Ammonium sulphate ppt.	5200	40	4.2	68
(4) Sephadex G-200				
(a) Total recovered	3172			41
(b) Peak I: best fraction	230	240	25	3
(c) Peak II: best fraction	1997	2080	217	26

Fig. 1. Effect of salt on the stability of *H. cutirubrum* polynucleotide phosphorylase. Samples (1 ml) of the $(\text{NH}_4)_2\text{SO}_4$ fraction (Table 1) were dialysed at 4°C for a total of 24 h against two changes (125 vol. each) of 10 mM-tris-HCl buffer, pH 8.2, containing the appropriate concentrations of NaCl or KCl, and the enzyme was then stored at 4°C. Samples (10 μl) were withdrawn at the indicated times and their activity was determined in the standard phosphorolysis assay, 2 M-NaCl being used in place of 1.25 M-KCl-0.5 M-NaCl. The extra salt present in the samples was not allowed for because it did not affect the activity (see Fig. 2). Δ , 4 M-NaCl; $\circ$, 3.6 M-KCl; $\blacktriangle$, 2 M-NaCl or -KCl; $\square$, no salt.

Shorey & Turner, 1965); preparation of an acetone-dried powder (Gunsalus, 1955). Of these, only ultrasonic disintegration for 10 s yielded a crude extract with the three activities typical of polynucleotide phosphorylase, i.e. polyribonucleotide phosphorolysis, nucleoside diphosphate polymerization and nucleoside diphosphate-orthophosphate exchange.

Enzymic properties and substrate specificity of *H. cutirubrum* polynucleotide phosphorylase. It should be noted that the activities of *H. cutirubrum* polynucleotide phosphorylase are much lower than those of the typical, well-studied polynucleotide phosphorylases of non-halophilic bacteria such as *A. vinelandii*, *Escherichia coli* and *Micrococcus lysodeikticus*. Further, its phosphorolysis activity was always considerably higher than either its exchange or incorporation activities.

The enzyme was inactive in the absence of salt (Fig. 2). Its activity increased with increasing sodium chloride or potassium chloride concentration to a maximum in 2 M salt. In 4 M-sodium chloride or -potassium chloride the activity was still 90% of maximum. This contrasted markedly with the effect of salt on the activity of a highly purified polynucleotide phosphorylase from a non-

Fig. 2. Effect of salt on the phosphorolysis activity of *H. cutirubrum* polynucleotide phosphorylase. A sample (1 ml) of the purified enzyme was dialysed for a total of 2 h against two changes of 10 mM-tris-HCl buffer, pH 8.5 (500 vol.). Phosphorolysis activity was measured by assay A. Sufficient dry salt (NaCl or KCl) was weighed into the assay tubes to give the indicated final concentrations. The phosphorolysis activity of *A. vinelandii* polynucleotide phosphorylase was assayed by the standard procedure (Gajda *et al.* 1970) with the appropriate concentration of NaCl. $\blacksquare$, *H. cutirubrum* polynucleotide phosphorylase with NaCl; $\blacktriangle$, *H. cutirubrum* polynucleotide phosphorylase with KCl; $\bullet$, *A. vinelandii* polynucleotide phosphorylase with NaCl.

halophile, *A. vinelandii*. The latter was most active without salt, had only 13% of its optimum activity in 1 M-sodium chloride and was totally inactive in 2 M-sodium chloride. These results support the view that the enzymes from the extremely halophilic bacteria are in themselves strongly halophilic (Egami, 1955; Larsen, 1967), but nevertheless this is not a general property of all enzymes from these organisms (Louis & Fitt, 1971a,b).

The enzyme requires a much higher concentration of sodium chloride for maximum stability (Fig. 1) than for maximum activity (Fig. 2) and is similar in this respect to *H. salinarum* malate dehydrogenase (Holmes & Halvorson, 1965b). This difference may be due to the presence of substrate in the assay medium, since it is well known (Dixon & Webb, 1964) that an enzyme can be protected from denaturation by its substrate. Further, Hubbard & Miller (1968) showed that a mixture of 4 mM-isocitrate and 10 mM-magnesium chloride was as effective in stabilizing *H. cutirubrum* isocitrate dehydrogenase as was 4 M-sodium chloride.

Since Mg^{2+} is the most effective bivalent cation for all known bacterial or animal polynucleotide phosphorylases (Babinet, Roller, Dubert, Thang & Grunberg-Manago, 1965; See & Fitt, 1970) except the *Escherichia coli* Q 13 enzyme (Hsieh & Buchanan, 1967), it was used in our early studies. However, the activity in the presence of Mg^{2+} was low, so the effects of Mn^{2+} and Mg^{2+} on the phosphorolytic

Fig. 3. Effect of bivalent cations on the phosphorolytic activity of *H. cutirubrum* polynucleotide phosphorylase. Enzymic activity was determined in the standard phosphorolysis assay (assay A) with the indicated concentrations of $MnCl_2$ or $MgCl_2$, and purified enzyme. ●, $MgCl_2$; ■, $MnCl_2$; ○, $MgCl_2 + 20\text{ mM-MnCl}_2$.

activity were compared (Fig. 3). The enzyme had a slight activity in the presence of low concentrations of Mg^{2+} , but was totally inhibited when the concentration reached 15 mM-magnesium chloride. In contrast, the activity was markedly increased by Mn^{2+} with a plateau in the range 50–100 mM-manganese chloride. Unfortunately, high and variable blanks were obtained with saturating concentrations of manganese chloride, but as shown (Fig. 3) Mg^{2+} enhanced the effect of Mn^{2+} , the activity being doubled by the use of 10 mM-magnesium chloride in the presence of 20 mM-manganese chloride. Although these concentrations of bivalent cations were suboptimum, they were used for the standard assay, since they resulted in a good activity together with reproducible low control values. It is of interest to compare the high Mn^{2+} optimum of this enzyme with the requirement of *H. cutirubrum* NADH dehydrogenase for 200 mM-magnesium chloride for optimum activity (Lanyi, 1969).

The phosphorolytic activity of the enzyme had a sharp optimum at pH 9.8 in the standard assay conditions and was proportional to enzyme concentration over the range tested (up to 50 units/ml).

The effect of changes in poly(A) concentration on the phosphorolytic activity is shown in Fig. 4. There was a linear relationship between the rate of reaction and the polymer concentration up to 200 $\mu\text{g/ml}$, and a plateau in the range 200–500 $\mu\text{g/ml}$: 500 μg of poly(A)/ml was therefore used in the standard assay. It should be noted that the true concentrations of polymer may have been somewhat less than indicated, especially at the higher values, because the high salt concentration lowered the solubility of poly(A) in the assay medium. This difficulty was

Fig. 4. Effect of substrate concentration on the poly(A) phosphorolysis activity of *H. cutirubrum* polynucleotide phosphorylase. The enzymic activity was measured in the standard assay with the appropriate concentration of poly(A).

Table 2. Substrate specificity of *H. cutirubrum* polynucleotide phosphorylase

Phosphorolysis activity was measured in the standard assay A with the indicated substrate (500 $\mu\text{g/ml}$).

Substrate	P_i incorporated (nmol/h)
Poly(A)	8
Poly(C)	24
Poly(G)	4
Poly(U)	3
RNA	3
DNA	0

more pronounced with fresh solutions of poly(A) and lessened after they had been frozen and thawed repeatedly. It is therefore possible that oligonucleotides might be better substrates in these assay conditions. In the standard assays, the reaction was linear up to 15 min and reached a stable plateau after 1 h. The amount of radioactive product formed was unchanged after a further 1 h, suggesting that the enzyme preparation was free from phosphatases active in these conditions.

The enzyme catalyses phosphorolysis of poly(A), poly(G), poly(C), poly(U) and RNA (Table 2). DNA is not a substrate, as expected. The product specificity of poly(A) phosphorolysis catalysed by purified *H. cutirubrum* polynucleotide phosphorylase was

determined as described by Fitt & See (1970); 95% of the incorporated [^{32}P]P_i was found to be present in ADP, none in AMP and 5% in ATP. This result, together with the specificity of the polymerization and Mn^{2+} -dependent exchange activities (see below) for ADP as substrate, proves that the enzyme is a polynucleotide phosphorylase.

Polynucleotide phosphorylases catalyse exchange between the terminal phosphate residue of a nucleoside diphosphate and orthophosphate (Grunberg-Manago, 1963). Crude *H. cutirubrum* extracts had two such activities. The first was Mg^{2+} -dependent with a Mg^{2+} optimum of 30mM and a pH optimum of 7.4: this activity was 10% greater with ATP than with ADP, showing that it

could be due only in part to polynucleotide phosphorylase. The second was Mn^{2+} -dependent with a Mn^{2+} optimum of 5mM and a pH optimum of 8.2 and was specific for nucleoside diphosphates. The specificity was determined with the purified enzyme by using AMP, ADP or ATP in the standard assay C (see the Experimental section) and the rate of reaction with ADP was over six times those with the other two substrates.

H. cutirubrum polynucleotide phosphorylase was also found to have a low but reproducible incorporation activity with a Mn^{2+} optimum of 5mM in the presence of 5mM-magnesium chloride and a pH optimum in the range 8.2–9.0. ADP incorporation by the crude extract was not stimulated by addition of a primer (ApA) to the assay medium, but the purified enzyme had an absolute requirement for ApA (20 mg/ml). The purified enzyme polymerized CDP about three times as fast as ADP and had an absolute requirement for nucleoside diphosphates in the conditions of assay B (see the Experimental section). The peak II fraction had a specific activity of ADP incorporation of 960 units/mg.

No significant difference was found in the enzymic properties of the peak I and peak II fractions from the gel-filtration step (Table 1 and Fig. 5). Their relationship is discussed in the next section.

Gel-filtration studies and lipid content of the enzyme. The peak I fraction from the Sephadex G-200 gel-filtration step was barely retarded (Fig. 5) and was associated with a pink colour each time the enzyme was prepared; the peak II material was highly retarded (Fig. 5) and had no visible colour. Table 3 shows the average partition coefficients of the two peaks and the distribution of activity between them in three consecutive preparations. It can be seen that the K_{av} for peak II was extremely reproducible whereas that of peak I was more variable and rose as the percentage of the activity in peak II increased. When the peak I material from the third preparation was sonicated for 10s,

Fig. 5. Sephadex G-200 gel filtration of *H. cutirubrum* polynucleotide phosphorylase. A sample (10ml) of the ammonium sulphate fraction (Table 1) was fractionated by Sephadex G-200 gel filtration in conditions of high ionic strength, as described in the Experimental section. The fractions (4ml) were assayed for phosphorolysis activity (assay A) and their extinction at 280nm was measured. $\blacktriangle$, Phosphorolysis activity; $\bullet$, E_{280} (protein distribution).

Table 3. Comparison of the K_{av} and enzymic activity of the two Sephadex G-200 peaks

Sephadex G-200 gel filtration of the ammonium sulphate fraction was performed as described in the Experimental section. The average partition coefficient (K_{av}) of the two enzyme fractions was calculated by the formula $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_0 was the void volume of the column, V_t the total column volume and V_e the elution volume (Fischer, 1969). The phosphorolysis activity was measured by the standard assay A and the numbers in parentheses refer to the percentage of the total activity present in each of the two peaks.

Preparation no.	K_{av}		Activity (units)	
	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 after 10s sonication	0.27	0.74	736 (66)	378 (34)

Table 4. *Extraction and chromatography of the lipids of the two Sephadex G-200 peaks*

Total lipids were extracted by the method of Bligh & Dyer (1959), with redistilled solvents. Methanol (25 ml) and chloroform (12.5 ml) were added to a sample (10 ml) of the enzyme solution. The mixture was shaken thoroughly and left for 1 h at room temperature. Additional chloroform and water (12.5 ml of each) were added and the phases mixed by inversion of the tube. The chloroform phase was withdrawn, diluted with an equal volume of benzene and evaporated to dryness at 45°C with a rotary evaporator. The residual lipid was dissolved in a mixture of chloroform and benzene (2 ml of each). This solution was evaporated to dryness at room temperature in a stream of N₂. The residue was dissolved in 2 drops of chloroform and the solution was centrifuged briefly. The whole sample was applied to silicic acid-impregnated Whatman 3MM paper and developed for 24 h in di-isobutyl ketone-acetic acid-water (8:5:1, by vol.) by the procedure of Marinetti (1962). Total lipids of *H. cutirubrum* extracted by the same procedure were used as standards. Chromatograms were stained with Rhodamine 6G and viewed under u.v. light (Kates *et al.* 1966).

Standard	Peak I	Peak II
Neutral lipids	+	—
Phosphatidylglycerol*	—	—
Phosphatidylglycerophosphate*	+	—
Unidentified phospholipid	+	—
Glycolipid	—	—
Sulpholipid	+	—

* Diether analogue.

as in the preparation of the crude extract, and then rechromatographed on Sephadex G-200 (Table 3), two peaks were once again obtained. The first had a higher K_{av} than the original peak I material, whereas the second had essentially the same K_{av} as the peak II material from the three earlier experiments. The recovery of activity was quantitative.

The above results suggested that peak I consisted of the enzyme associated with membrane fragments containing the typical carotenoid pigments of the *H. cutirubrum* cell membrane, whereas peak II contained the enzyme dissociated from the membrane by sonication. Accordingly, lipid extractions were performed on each of the two peaks and the extracts were examined by paper chromatography (Table 4). Peak I contained lipids characteristic of the membrane of *H. cutirubrum* (Kates, Palameta, Joo, Kushner & Gibbons, 1966), whereas peak II contained none of these lipids.

The results of these experiments suggest strongly that *H. cutirubrum* polynucleotide phosphorylase is membrane-associated in the intact cell and is partially released from the membranes during preparation of the crude extract by sonication. Although most bacterial polynucleotide phosphor-

ylases are thought to be in the soluble fraction of the cell or attached to the ribosomes (Grunberg-Manago, 1963; Kimhi & Littauer, 1967), Abrams & McNamara (1962) showed that the *Streptococcus faecalis* enzyme was present almost exclusively in the cell membrane. Further, guinea-pig liver nuclei polynucleotide phosphorylase is apparently associated with the nuclear membrane (See & Fitt, 1970).

The high K_{av} of the peak II Sephadex G-200 fraction suggested that *H. cutirubrum* polynucleotide phosphorylase was of unusually low molecular weight. A detailed study of the behaviour of this enzyme, and of the *H. cutirubrum* RNA polymerases (Louis & Fitt, 1971a,b), during gel filtration and sucrose-density-gradient centrifugation was therefore undertaken (Louis *et al.* 1971). Both methods indicated that *H. cutirubrum* polynucleotide phosphorylase, possessing all three activities typical of bacterial polynucleotide phosphorylases, is smaller than cytochrome *c* and has mol.wt. 11 000–12 000. This should be contrasted with the mol.wt. (approx. 200 000) generally accepted for the fully active polynucleotide phosphorylases from *A. vinelandii*, *Clostridium perfringens*, *E. coli* and *M. lysodieticus* (Thang, 1969). If this result is confirmed after further purification of the *H. cutirubrum* enzyme, it will be the molecule of choice for studies of the structure and mechanism of action of polynucleotide phosphorylase. In addition, this is the first experimental evidence to support Ingram's (1947) suggestion that the enzymes of extreme halophiles would prove to be small molecules.

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