Isolation and Properties of Highly Purified *Halobacterium cutirubrum* Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase

By B. GREGORY LOUIS and P. S. FITT

Department of Biochemistry, University of Ottawa, Ottawa, Ont. K1N 6N5, Canada

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1. The subunits α and β of *Halobacterium cutirubrum* DNA-dependent RNA polymerase have been purified to electrophoretic homogeneity. Both have mol.wt. 18000 and they are required in equimolar amounts for optimum activity. 2. The instability of the complete enzyme, $\alpha\beta$, in the absence of salt is due to the rapid inactivation of the β subunit in these conditions. 3. Nearest-neighbour analysis of the product formed on poly[d(A-T)] as template shows that the enzyme copies the latter accurately. 4. The enzyme initiates new chains with purine nucleoside triphosphates exclusively. 5. The product obtained in the standard assay conditions contains some high mol.wt. (>16S) material, but consists primarily of short chains, of average length 70–80 nucleotide units. 6. The template specificity of the complete enzyme has been studied at high and low ionic strength. Its extreme dependence on salt concentration is unrelated to the gross overall base composition of the DNA used. 7. T₇ DNA is transcribed asymmetrically and the enzyme selectively copies the T₇ 'early' genes. 8. Preliminary amino acid analyses of α and β subunits show that their overall content of acidic, basic and neutral amino acids does not differ appreciably from that of *Escherichia coli* RNA polymerase.

We have described (Fitt & Louis, 1970; Louis & Fitt, 1971a,b) the isolation from the extreme halophile *Halobacterium cutirubrum* of DNA-dependent RNA polymerase [nucleoside triphosphate–RNA nucleotidyl transferase (DNA-dependent) EC 2.7.7.6] and RNA-dependent RNA polymerase. These enzymes were readily separable and both are of unusually low molecular weight (Louis *et al.*, 1971).

Louis & Fitt, (1971c) showed that the DNA-dependent enzyme could be separated into two components, designated proteins α and β , of similar molecular weight. They were required in equimolar amounts for optimum enzymic activity. Further, it was found that the template-specificity of the DNA-dependent RNA polymerase was affected by the ionic strength of the assay medium: *H. cutirubrum* DNA was a template only at high ionic strength; calf thymus DNA, only at low ionic strength; and poly[d(A-T)] was effective at both high and low salt concentrations. Coliphage T₄ DNA was not accepted as a template at all.

In the present paper, we describe in detail the purification to electrophoretic homogeneity of the proteins α and β that together form the active *H. cutirubrum* DNA-dependent RNA polymerase. It is shown that: (i) the enzyme initiates new chains and copies the template accurately; (ii) coliphage T₇ DNA is copied asymmetrically, with preferential transcription of the T₇ 'early' genes; (iii) the α and β subunits have similar amino acid compositions; (iv) the inactivation of the complete polymerase ($\alpha\beta$) at low salt concentrations is due to the instability of

protein β in these conditions. In the accompanying paper (Louis & Fitt, 1972) studies of the roles of the two proteins in the mechanism of action of the enzyme are discussed. A preliminary report of some of these results has been published (Louis & Fitt, 1971d).

Experimental

Materials

Chemicals and substrates were purchased from the following suppliers: ¹⁴C- and ³H-labelled nucleoside triphosphates, Amersham/Searle Corp., Don Mills, Ont., Canada; sodium dodecyl sulphate (specially pure), BDH (Canada) Ltd., Toronto, Ont., Canada; Bio-Gel HTP hydroxyapatite powder and P-60 (100–200 mesh) polyacrylamide gel, Bio-Rad Laboratories, Richmond, Calif., U.S.A.; cyclohexylaminopropane sulphonic acid, tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid and tris(hydroxymethyl)methylaminopropane sulphonic acid, Calbiochem, Los Angeles, Calif., U.S.A.; Cyanogum 41 and NNN'-tetramethylethylenediamine, E-C Apparatus Corp., Philadelphia, Pa., U.S.A.; *Escherichia coli* DNA, *Bacillus subtilis* DNA and poly[d(A-T)], General Biochemicals, Chagrin Falls, Ohio, U.S.A.; Triton X-100, Hartman-Leddon Co., Philadelphia, Pa., U.S.A.; ³²P-labelled nucleoside triphosphates, ICN Chemical and Radioisotope Division, Irvine, Calif., U.S.A.; Sepharose 4B, Blue Dextran and Ficoll, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; unlabelled nucleoside triphosphates, P-L Biochemicals Inc., Milwaukee, Wis.,

U.S.A.; calf thymus DNA, salmon sperm DNA, glycine and Trizma base (tris), Sigma Chemical Co., St. Louis, Mo., U.S.A.; pancreatic ribonuclease, Worthington Chemical Corp., Freehold, N.J., U.S.A. Dr. W. C. Summers kindly provided coliphage T₇ DNA, the separated chains of T₇ DNA and the labelled and unlabelled T₇-induced RNA species. *Halobacterium halobium* Verhoeven NRC 34020 DNA, prepared by the method described below, was a gift from Mrs. P. I. Peterkin.

Buffers. The pH of buffers refers to the value at 25°C. High-salt buffer had the following composition: 2.5M-KCl - 1M-NaCl - 10mM-tris - HCl, pH 8.6.

Cell culture. *H. cutirubrum* NRC 34001 cells were grown as described by Peterkin & Fitt (1971) except that mass cultures were harvested after 66-72h of growth, i.e. in late exponential or early stationary phase. (We previously referred to this strain as no. 9.)

Methods

Purification of *H. cutirubrum* DNA-dependent RNA polymerase subunits. All operations were done at 0°-5°C.

The bacteria were harvested by low-speed centrifugation and about 8-10g (wet wt.) of cells was obtained/l of culture medium. The pellets of bacteria (40g wet wt. in the preparation described in Table 1) were suspended in high-salt buffer (2ml/g) by slow addition of liquid while the cells were stirred gently with a bent glass rod. (If the buffer is added rapidly a homogeneous suspension cannot be obtained and the yield of enzyme is very low.) The mixture was then stirred gently with a magnetic stirrer for about 1h to give a smooth, rusty-red suspension. The latter was sonicated for 30s with a Bronwill Biosonik II sonic disintegrator (dial setting: 70). Sonication was complete when the suspension appeared to clear slightly and its colour changed to dark red. The mixture did not become viscous and cell debris could be removed readily by centrifugation at 27000g (International B-20; no. 870 rotor) to give a clear, dark-red solution (about 2ml/g wet wt. of bacteria). This solution was centrifuged at 78000g for 2h (Beckman L2-65B; type 30 rotor) to yield an orange-gold supernatant designated 'crude extract' in Table 1 (the small red pellet was discarded).

The pH of the crude extract was adjusted to 4.5 with 1M-HCl and the suspension was stirred for 10min before further cautious addition of acid to pH 4. (If the pH falls below 4, the DNA-dependent polymerase is precipitated, as well as being partially inactivated, and a clean separation from the RNA-dependent enzyme is not obtained.) The suspension was centrifuged immediately (20min at 27000g) and the pH of the supernatant readjusted to 8.6 without delay by addition of 1M-NaOH. The solution

was concentrated by overnight dialysis against 1 vol. of 30% (w/v) Ficoll in high-salt buffer.

The enzyme fraction (20ml) was purified by upward-flow gel filtration through a column (5cm × 85cm) of Bio-Gel P-60 polyacrylamide gel. The gel was preswollen in high-salt buffer for at least 1 month and the column was packed as described by Louis *et al.* (1971). High-salt buffer was passed through the column at 40ml/h (LKB Perplex peristaltic pump) and 10ml fractions were collected. The void volume of the large column, measured with Blue Dextran, did not change after proper stabilization of the bed and the column could be used many times over a period of 6 months before repacking became necessary. The active fractions (total vol. about 120ml) were concentrated to about 5ml by dialysis against 50% (w/v) Ficoll in high-salt buffer.

A column (0.9cm × 15cm) of hydroxyapatite was poured from a slurry of Bio-Rad HTP powder (5g) in high-salt buffer (25ml). The column was run by downward flow (5ml/h) and the bed was washed with a further 50ml of high-salt buffer. The concentrated P-60 fraction was applied and the column was washed with high-salt buffer (20ml), 2ml fractions being collected. During this washing step, two protein peaks were obtained. The second contained one of the polymerase subunits (protein β) and the corresponding fractions were concentrated by Ficoll/high-salt buffer dialysis to 1ml. The column was then eluted with 0.1M-sodium phosphate in high-salt buffer to give the other subunit (protein α); the pooled fractions containing protein α were concentrated to 1ml by Ficoll-high-salt buffer dialysis.

Protein α obtained by the above procedure was electrophoretically homogeneous in polyacrylamide gels (see below), but protein β usually contained a minor contaminant. The latter was then removed by batchwise treatment with hydroxyapatite. The solution of protein β (1ml) was diluted to 4ml with high-salt buffer and hydroxyapatite powder (1g) was added. The suspension was stirred for 30min and then centrifuged for 5min at 2000g. The supernatant was discarded and the pellet was resuspended in 0.1M-sodium phosphate in high-salt buffer (1ml). The hydroxyapatite was removed by centrifugation and the supernatant, containing electrophoretically homogeneous protein β , was dialysed against high-salt buffer to remove phosphate. The solutions of the purified subunits in high-salt buffer could be stored at 0-5°C for 2 months without loss of activity, but after storage for 5 months only 25% of the original activity remained.

Isolation of *H. cutirubrum* nucleic acids. A new procedure was developed for the isolation of both DNA and RNA from *H. cutirubrum* cells. The method is essentially a combination of part of the DNA isolation method of Marmur (1961) with an agarose gel-filtration step (Loeb & Chauveau, 1969). All

operations were performed at 0–5°C, except where indicated.

The nucleic acids were prepared either from freshly harvested bacteria or from a powder obtained by freeze-drying a suspension of the bacteria in 0.15M-NaCl (2ml/g wet wt.). The freeze-dried powder could be used for this purpose after storage for over 18 months at –20°C (although freeze-drying inactivated the RNA polymerase). The bacteria (9g wet wt.), or an equivalent weight of freeze-dried powder, were suspended in a mixture of 0.15M-NaCl–0.1M-EDTA, pH8 (75ml) and 25% (w/v) sodium dodecyl sulphate (6ml). The suspension was incubated at 75°C for 10min and then cooled to room temperature. 5M-NaClO₄ was added to a final NaClO₄ concentration of 1M and the mixture was divided into three equal parts. Each sample was swirled for 30min at 125rev./min on a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) with an equal volume of chloroform–3-methylbutan-1-ol (24:1, v/v). The mixtures were centrifuged for 5min at 7500g in stainless-steel centrifuge tubes and the aqueous phases were removed. Ethanol (2vol.) was added to the combined aqueous phases and the mixture cooled to –20°C. The precipitate was dissolved in 15mM-NaCl–1.5mM-sodium citrate, pH7 (27ml) and 3mM-sodium acetate–1mM-EDTA, pH7 (3ml) was added. The nucleic acids were separated by dropwise addition of propan-2-ol (16.2ml) to the rapidly stirred solution. The precipitated DNA was collected by centrifugation, washed with ethanol and stored at –20°C. The supernatant was mixed with ethanol (2vol.) and cooled to –20°C. The RNA (approx. 10mg) was collected by centrifugation, then washed with ethanol, and dissolved in 0.15M-NaCl–10mM-tris–HCl buffer, pH7.5 (10ml) and stored at 4°C; it contained 0.3% (w/w) of DNA and no detectable protein.

A column (5cm×35cm) was packed with a suspension of Sepharose 4B agarose gel in 2M-NaCl. The bed was washed by downward flow with 2M-NaCl (1 litre) at a hydrostatic pressure of 20cm. A portion (20mg) of the DNA from the propan-2-ol-precipitation step was dissolved in 15mM-NaCl–1.5mM-sodium citrate, pH7 (10–15ml). The solution was mixed with an equal volume of 4M-NaCl and the sample was applied to the column (20mg is the maximum amount of DNA that can be run on a column this size). The DNA was eluted with 2M-NaCl, at 30ml/h, and emerged as the first peak on the u.v. monitor. Fractions (10ml) were collected and their *E*₂₆₀ was determined with a Zeiss PMQII spectrophotometer. The fractions containing DNA were combined and the latter was precipitated with ethanol (2vol.). The precipitate was stored at –20°C. Before use, it was dissolved in 0.15M-NaCl–10mM-tris–HCl buffer, pH7.5 and the solution (2mg/ml) was stored

at 0–5°C. The DNA (18mg) contained 0.5% (w/w) of RNA and 0.7% (w/w) of protein. It had *T*_m values of 88 and 84.5°C, in agreement with the molar percentage of G+C of 66 and 57% of the major and minor components, respectively (Moore & McCarthy, 1969a), and its thermal denaturation curve was very similar to that previously reported for *Halobacterium salinarium* DNA (Joshi *et al.*, 1963).

Enzyme assays. *H. cutirubrum* DNA-dependent RNA polymerase activity was measured as described by Louis & Fitt (1971a) except that 0.1M-glycine–NaOH buffer, pH9.5, was substituted for the 0.1M-tris–HCl buffer, pH8.6, in some of the experiments (see below). The assay medium (0.1ml) then contained: glycine–NaOH buffer, pH9.5, 10μmol; native *H. cutirubrum* DNA, 30μg; [¹⁴C]ATP, 5nmol (about 10000d.p.m./nmol); GTP, CTP and UTP, 15nmol each; MgCl₂, 10μmol; MnCl₂, 1μmol; KCl, 150μmol; NaCl, 60μmol; enzyme, up to 3 units.

H. cutirubrum RNA-dependent RNA polymerase activity was measured by the standard assay (Louis & Fitt, 1971b), with substitution (see below) of cyclohexylaminopropanesulphonic acid for tris. The assay medium (0.1ml) contained: cyclohexylaminopropanesulphonic acid–NaOH buffer, pH9.5, 10μmol; high-mol.-wt. RNA, 75μg; [¹⁴C]ATP, 5nmol (about 10000d.p.m./nmol); CTP, GTP and UTP, 20nmol each; MgCl₂, 10μmol; MnCl₂, 1μmol; KCl, 150μmol; NaCl, 60μmol; enzyme, up to 3 units.

For both assays, incubation was at 37°C for 1h. Samples (up to 85μl) were then streaked on strips (1.5cm×3cm) of Whatman no. 3 filter paper. The latter were collected in 7% (w/v) HClO₄ (10ml/strip) and swirled for 7min. The acid was aspirated and the washing procedure repeated with 7% (w/v) HClO₄, 1% (w/v) HClO₄ and ethanol. The strips were air-dried and their radioactivity was determined in a Beckman LS-230 liquid-scintillation counter, with 0.8% (w/v) 2,5-diphenyloxazole in toluene (10ml/strip) as scintillation fluid. Suitable control experiments were performed in all cases and the results were corrected for quenching as described by Louis & Fitt (1971a). A unit was defined as the amount of enzyme catalysing the incorporation of 1nmol of [¹⁴C]ATP into acid-insoluble material/h.

During these studies, an unexplained difficulty arose because of rapid oxidation of Mn²⁺, and consequent high blanks, when using tris–HCl buffers in the assays for the two polymerases as previously described (Louis & Fitt, 1971a,b). In the case of the DNA-dependent RNA polymerase, this problem was eliminated by any of the following three methods: (i) use of glycine–NaOH buffer, pH9.5; (ii) use of tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid–NaOH buffer, pH8.6; (iii) addition of 2-mercaptoethanol (50mM) to the assay medium containing tris. In all three cases, the results of detailed

studies were the same as those previously obtained with tris buffer apart from the higher pH optimum with glycine-NaOH buffer. Similarly, the RNA-dependent enzyme may be assayed satisfactorily with either tris(hydroxymethyl)methylaminopropanesulphonic acid- or cyclohexylaminopropanesulphonic acid-NaOH buffers, pH 9.5, instead of tris-HCl buffer, pH 9.5.

Nucleic acid assays. DNA and RNA were determined by their respective H_2SO_4 -cysteine hydrochloride reactions (Dische, 1955).

Protein assays. Protein in nucleic acid preparations was determined by the micro-biuret method (Zamenhof, 1957), with α -chymotrypsinogen as standard. Enzyme protein concentration was assayed spectrophotometrically (Warburg & Christian, 1942).

Micro-disc polyacrylamide-gel electrophoresis. To conserve the highly purified proteins α and β , polyacrylamide-gel electrophoresis of *H. cutirubrum* DNA-dependent RNA polymerase subunits was performed by the micro-disc technique (Neuhoff, 1968; Neuhoff *et al.*, 1968, 1970a). Electrophoresis was performed in 5 μ l capillary tubes with a precision bore of 0.450 mm diam. (Drummond Microcaps, Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.).

Separating gels containing 8, 10 and 12% (w/v) polyacrylamide, and 5% (w/v) stacking gels, were prepared from the following stock solutions: (A) 10 ml of 10 mM- $MgCl_2$ -20 mM- NH_4Cl -10 mM-tris-acetate buffer, pH 7.0, 8.0 or 9.4, plus *NNN'*-tetramethylethylenediamine (63 μ l); (B) Cyanogum 41 (20 g), $K_3Fe(CN)_6$ (3.75 mg), water to 37.5 ml; (C) ammonium persulphate (7 mg), 4% (v/v) Triton X-100 (2.5 ml), water (2.5 ml); (D) tris (5.98 g) in water (50 ml), plus 1 M- H_3PO_4 to pH 6.7, plus *NNN'*-tetramethylethylenediamine (0.46 ml), plus water to 100 ml; (E) ammonium persulphate (10 mg), plus water (0.5 ml), plus 4% (v/v) Triton X-100 (0.5 ml); (F) tris (2.85 g) in water (30 ml), plus 1 M- H_3PO_4 to pH 6.7, plus water to 50 ml.

The separating gels were produced as follows: 0.5 ml of A was mixed with (i) B (0.6 ml) and water (0.9 ml) for 8% gels; or (ii) B (0.75 ml) and water (0.75 ml) for 10% gels; or (iii) B (0.9 ml) and water (0.6 ml) for 12% gels. Finally, 1 ml of the resulting solution was replaced with 1 ml of C. The capillaries were filled with the appropriate separating gel mixtures to a maximum of two-thirds of their length by capillary action and stuck in a plasticine block to seal the lower ends. The upper ends were filled with water by means of a finely drawn Pasteur pipette and the gels were left for not less than 10 h or more than 3 days in a moist chamber at room temperature.

Immediately before use, a layer of 5% (w/v) stacking gel, pH 6.7, was added to the capillaries. A dilute acrylamide mixture was prepared: B (0.5 ml) was mixed with D (0.5 ml) and water (1 ml); 1 ml of

the mixture was replaced with D (1 ml); finally, 1 ml of the second mixture was replaced with water (0.8 ml). Water was removed from the capillaries with the finely drawn Pasteur pipette, and the height of the separating gel marked with a waterproof, fine-point, felt marker. E (0.2 ml) was added to the dilute acrylamide mixture and a 2-3 mm layer of the resulting solution was added to each capillary, without the introduction of air bubbles. The capillaries were refilled with water. When polymerization was complete, the upper surfaces of the 5% gels were rinsed with a 1:8 (v/v) dilution of F and the position of the surfaces was marked as before. The capillaries were filled to the top with the samples [protein concentration about 100 μ g/ml in 1:8 (v/v) F].

Rubber plugs (Thomas 2319-B; Arthur H. Thomas Co.) were placed in the holes of the upper electrode bath of a 12-gel Buchler Polyanalyst (Buchler Instruments, Inc., Fort Lee, N.J., U.S.A.). The sharp end of a 17 ga hypodermic needle, the lower end of which had been blocked with solder, was inserted through one of the rubber plugs and a capillary placed in its bore. The length of the needle was such that one-fifth of the capillary projected above the tip. When the needle was withdrawn from the plug, the capillary remained in place. This process was repeated until a capillary had been inserted in each plug. The electrode buffer was prepared by dissolving tris (9 g) and glycine (43.2 g) in water (1500 ml). Bromophenol Blue (2% w/v, 0.5 ml) was added to the electrode buffer in the upper (cathode) chamber and electrophoresis was performed at 80 V (starting current about 100 μ A/gel), with a constant-voltage power supply capable of good regulation in the range 0-100 V (Model LL905, Lambda Electronics Corp., Melville, L.I., N.Y., U.S.A.), until the Bromophenol Blue band had traversed 4 mm of the separating gel. The gels were extruded from the tubes by water pressure into the staining solution. The protein bands were stained with either 0.5% (w/v) Amido Black 10B in 7.5% (w/v) acetic acid or 0.1% (w/v) Coomassie Brilliant Blue R250 in 12.5% (w/v) trichloroacetic acid and the gels were destained in the appropriate dilute acid lacking the dye.

Results and Discussion

Purification of H. cutirubrum DNA-dependent RNA polymerase subunits

Table 1 shows the results of a typical purification performed as described in the Experimental section. The use of bacteria that were in late-exponential or early-stationary phase and of a thicker bacterial suspension for sonication compared with that previously used (Louis & Fitt, 1971a) gave approximately the same yield of units (measured with *H. cutirubrum* DNA as template in the presence of salt)

Table 1. *Purification of H. cutirubrum DNA-dependent RNA polymerase*

The enzyme was purified from 40 g (wet wt.) of bacteria as described in the Experimental section and the standard assay was used to determine activity. Numbers in parentheses represent units of RNA-dependent RNA polymerase activity determined by the assay described in the Experimental section.

Fraction	Activity (units)	Sp. activity (units/mg)
Crude extract	2123 (319)	2.8 (0.4)
pH4 supernatant	2041 (0)	11.7 (0)
P-60 concentrated pool	1910 (0)	2760 (0)
Hydroxyapatite column		
Protein α , electrophoretically homogeneous		
Hydroxyapatite adsorption		
Protein β , electrophoretically homogeneous		
$\alpha + \beta$, equimolar	1820 (0)	14440 (0)

in the crude extract as our earlier procedure, but the enzyme was much easier to purify without significant loss of activity, particularly at the critical pH4 precipitation step. Further, use of a 5 cm diam. column of Bio-Gel P-60 polyacrylamide gel not only increased the possible through-put, but also gave a much greater resolution than could be obtained on a 2.5 cm diam. column, so the purification at that step was correspondingly increased.

The hydroxyapatite gel-column step permitted resolution of the two subunits of the *H. cutirubrum* DNA-dependent RNA polymerase and their purification to electrophoretic homogeneity. However, the conditions for exploiting the very slight retardation of protein β were critical and it was usually necessary to perform the second batchwise hydroxyapatite purification of that protein.

Polyacrylamide-gel electrophoresis of proteins α and β was performed, after removal of salt by dialysis, by the micro-disc method (Neuhoff, 1968; Neuhoff *et al.*, 1968) at pH 7.0, 8.0 and 9.4 in 8, 10 and 12% (w/v) polyacrylamide separating gels. Single bands were observed in each case. About 200 ng of protein was run on each gel, and the limit of detection by Coomassie Blue staining and visual inspection was about 4–6 ng of protein per band, so the two subunits were at least 97% pure with respect to other proteins. The E_{280}/E_{260} ratio was 1.16 for the P-60 fraction, indicating a nucleic acid content of not more than 2% (w/w).

Both protein α and protein β have a mol.wt. of approx. 18000 (Louis & Fitt, 1971c). As the unseparated subunits have the same apparent molecular weight (Louis *et al.*, 1971), the α and β subunits do not associate in high-salt buffer. The conditions in which association takes place are described in the accompanying paper (Louis & Fitt, 1972).

The preparative procedure described in the present

paper permits the isolation of the two subunits of *H. cutirubrum* DNA-dependent RNA polymerase free from impurities detectable by polyacrylamide-gel electrophoresis in nine different combinations of pH and gel concentration. Further, the use of high ionic strength buffers at all times avoids losses due to inactivation of the enzyme and leads to high overall recoveries of activity. If the method can be scaled up to yield milligram quantities of the two proteins, they should prove valuable for structural studies on account of their small size (Louis *et al.*, 1971; Louis & Fitt, 1971c), since even the active $\alpha\beta$ complex (Louis & Fitt, 1972), with a mol.wt. 36000, is significantly smaller than the T_3 - and T_7 -induced polymerases, mol.wt. 107000–110000 (Chamberlin *et al.*, 1970; Dunn *et al.*, 1971) or the *Neurospora crassa* mitochondrial enzyme, recently reported by Kuntzel & Schäfer (1971) to have a mol.wt. of 64000–75000.

Effect of ionic strength on the stability of proteins α and β

Both the purified subunits of *H. cutirubrum* DNA-dependent RNA polymerase may be stored for at least 60 days in high-salt buffer at 0–5°C with only a slow loss of activity. Removal of the salt by dialysis caused inactivation of the α and β subunits, but at markedly different rates, the potential activity of each protein being measured in the presence of an equimolar amount of a sample of the other protein that had been stored exclusively in high-salt buffer.

Protein α was fully active after dialysis against salt-free buffer for periods of 4–24 h and retained 50% of its original activity after storage for 20 days in the absence of salt. In neither case was any change in its electrophoretic mobility observed.

Protein β , on the other hand, was extremely unstable in the absence of salt, and was thus clearly responsible for the instability of the complete enzyme ($\alpha\beta$) in these conditions. As removal of salt was necessary before the routine electrophoretic analysis used to assess the purity of the subunits, and was achieved by dialysis against salt-free buffer for 24 h, a detailed study was made of the inactivation of the β protein in these conditions. Samples of protein β were dialysed against salt-free buffer for 4, 10 and 24 h. Their activity was assayed in the presence of protein α and their electrophoretic mobility in micro-disc polyacrylamide gels was determined. A progressive change in mobility from a slower- to a faster-moving form was observed and the activity of the samples appeared closely related to the amount of protein present in the slower-moving band. It is therefore likely that the latter is the true active species. After 24 h of dialysis, protein β was irreversibly inactivated and the gel electrophoretograms contained the faster-moving band exclusively (within the limits of detection of the staining procedure). It should be noted that the electrophoretic mobility of protein α was less than that of either the active or inactive forms of protein β .

Template specificity of *H. cutirubrum* RNA polymerase

Our earlier studies (Louis & Fitt, 1971c) have shown that the ionic strength of the assay medium profoundly influences the template specificity of *H. cutirubrum* DNA-dependent RNA polymerase. In particular, *H. cutirubrum* DNA was a template only at high ionic strength, calf thymus DNA only at low

ionic strength and T₄-coliphage DNA at neither high nor low ionic strength. Poly[d(A-T)] was equally effective both in the absence and presence of salt.

Table 2 shows the results of a more extensive survey of the ability of DNA from different sources to act as template in the standard assay conditions in the presence and absence of 1.5M-KCl-0.6M-NaCl (i.e. 2.1 M total salts). It can be seen that all the DNA species tested were templates in 2.1 M salt and not at low ionic strength, except for those from calf thymus and salmon sperm, where the reverse is true. As before, poly[d(A-T)] was a template with and without salt: this result, together with those for *B. subtilis* and salmon sperm DNA species which both contain 44% of G+C, shows that the effect of ionic strength on the ability of the enzyme to accept a given DNA as template is not related to the G+C-content of the latter.

Fig. 1 shows the results of a detailed study of the activity of *H. cutirubrum* RNA polymerase as a function of increasing KCl or NaCl concentration in the presence of either *H. cutirubrum* or calf thymus DNA. It can be seen that a salt concentration between 2 and 2.5M is required for highest activity with the *H. cutirubrum* DNA, and the rate of reaction falls rapidly as the salt concentration is decreased from 2M, whereas with calf thymus DNA the enzyme is barely active at salt concentrations of 0.5M and above. (Total salt concentrations higher than 2.1M cannot be used conveniently in the standard assay, as they require that solid KCl and/or NaCl be weighed directly into each assay tube.)

With the highly purified enzyme ($\alpha\beta$) incorporation was linear for 2 h in the standard assay conditions and the lag phase observed with the partially purified enzyme (Louis & Fitt, 1971a) was eliminated.

Table 2. Template specificity of *H. cutirubrum* RNA polymerase

H. cutirubrum DNA-dependent RNA polymerase was assayed by the standard method (Experimental section) with the appropriate DNA (30 μ g) both with and without 1.5M-KCl-0.6M-NaCl. The values for molar percentage of G+C content of the DNA are from the following sources: major and minor DNA components of *H. cutirubrum* and *H. halobium*, Moore & McCarthy (1969a); all other DNA species, Davidson (1969). Values in parentheses indicate the percentage (w/w) of the DNA present as the minor component in the two halobacteria (Moore & McCarthy, 1969a).

DNA	G+C (%)		ATP incorporated (nmol)	
	major	minor	+ salt	- salt
<i>H. cutirubrum</i>	66	57 (11%)	1.25	0.05
<i>H. halobium</i>	67	59 (30%)	0.56	0.04
<i>E. coli</i>	51	—	1.19	0.10
<i>B. subtilis</i>	44	—	1.34	0.08
T ₇ coliphage	41	—	0.92	0.06
Calf thymus	40	—	0.16	0.73
Salmon sperm	44	—	0.10	0.69
poly[d(A-T)]	0	—	0.89	0.56

Chain initiation and template transcription by *H. cutirubrum* DNA-dependent RNA polymerase

Our earlier studies (Louis & Fitt, 1971a) showed that the incorporation of ribonucleoside triphosphates into an acid-insoluble form by the presumed *H. cutirubrum* RNA polymerase was dependent on the presence of all four common ribonucleoside triphosphates and a native DNA template. Further, the product was destroyed by ribonuclease and the reaction was inhibited by deoxyribonuclease.

The results in Tables 3, 4 and 5 show that the enzyme catalyses the initiation of new polyribonucleotide chains and that chain elongation involves accurate copying of the template. Together they constitute formal proof that the enzyme is a DNA-dependent RNA polymerase.

Fig. 1. Effect of salt concentration on the template activity of calf thymus and *H. cutirubrum* DNA in the standard assay

Each point represents the average of two results that differed by less than 10%. ● and ○, KCl; ■ and □, NaCl; ○ and □, calf thymus DNA; ● and ■, *H. cutirubrum* DNA.

Chain initiation was measured by the method of Travers & Burgess (1969), which is based on the principle that the first nucleoside triphosphate is incorporated intact into the growing chain (Maitra *et al.*, 1965). Both ATP and GTP are equally effective as chain initiators (Table 3), whereas the two pyrimidine nucleoside triphosphates are not incorporated at the beginning of the polymer chain. Thus, *H. cutirubrum* RNA polymerase resembles the *E. coli* (Maitra & Hurwitz, 1965) and *Azotobacter vinelandii* (Krakow & Horsley, 1967) enzymes in this respect. The elongation activity/initiation activity ratio was 37 with ATP and 39 with GTP, in agreement with the value of about 18–20 observed (Louis & Fitt, 1971c) when both γ - ^{32}P -labelled purine nucleoside triphosphates were present simultaneously in the assay medium. Both elongation and chain initiation were linear for 2 h, in these conditions, so the elongation/initiation ratio was constant in contrast with the ten-fold increase over a period of 1 h observed by Maitra & Hurwitz (1965) with the *E. coli* enzyme. However, this difference is probably due to the low elongation/initiation ratio of the *H. cutirubrum* enzyme, as a value of 18–20 indicates an average chain length of only 70–80 nucleotide units. A preliminary sucrose-density-gradient centrifugation study of the product (isolated by phenol extraction followed by ethanol precipitation) showed that a significant amount of high-molecular-weight (>16S) RNA was formed, but that the bulk of the new chains made in the standard assay conditions were short. Further study will be needed to determine why the *H. cutirubrum* polymerase produces large numbers of small polynucleotides, but the following are possible explanations: (i) the length of the product is affected critically by the reaction conditions (e.g. $[\text{K}^+]/[\text{Na}^+]$, $[\text{Mn}^{2+}]/[\text{Mg}^{2+}]$, pH, etc.); (ii) some additional protein or non-protein factors may be required and are removed during purification of the enzyme; (iii) the enzyme preparation contains a high proportion of early quitter polymerase molecules (see Mueller & Bremer,

Table 3. Incorporation of γ - ^{32}P -labelled nucleoside triphosphates into RNA

The rate of initiation of new RNA chains by *H. cutirubrum* RNA polymerase was measured by the method of Travers & Burgess (1969) by including the appropriate γ - ^{32}P -labelled nucleoside triphosphates in an assay mixture containing $[\text{C}^{14}]\text{ATP}$ (see the Experimental section) and 15 nmol of each of the four nucleoside triphosphates. The template was *H. cutirubrum* DNA and the results were corrected for quenching, and also for ^{32}P counted in the ^{14}C -channel of the liquid-scintillation counter.

$[\gamma\text{-}^{32}\text{P}]\text{NTP}$	Chain elongation ($[\text{C}^{14}]\text{ATP}$ incorporation) (nmol)	Chain initiation ($[\text{P}^{32}]\text{ATP}$ incorporation) (nmol)	$^{14}\text{C}/^{32}\text{P}$ ratio
ATP	1.29	0.039	37
GTP	1.26	0.032	39
CTP	1.47	0.005	—
UTP	1.46	0.005	—

1969). A further possibility, namely that the templates used are extensively nicked, is unlikely, as the results obtained with T₇-DNA as template (elongation/initiation ratio = 22) are very similar to those with *H. cutirubrum* DNA, and the sample of T₇-DNA used had less than two nicks per molecule (W. C. Summers, personal communication).

A study of the incorporation of UTP, CTP and GTP, relative to that of ATP, in the presence of all four nucleotides and with poly[d(A-T)] as template showed (Table 4) that equivalent amounts of ATP and UTP were incorporated in the standard conditions and that very little incorporation of CTP or GTP occurred. When only UTP and ATP were present, the rate of reaction was the same as that observed in the presence of all four nucleotides and equal amounts of the two nucleotides were incorporated. However, with CTP or GTP instead of

UTP, the rate of incorporation of ATP was decreased to 11% of the maximum value. It should be noted that with only one nucleoside triphosphate no significant incorporation occurs (Louis & Fitt, 1971a).

The above results are those expected with a DNA-dependent RNA polymerase, and final confirmation that the enzyme copies the poly[d(A-T)] template accurately to produce a polyribonucleotide containing alternating AMP and UMP units was obtained by nearest-neighbour analysis by standard procedures (Table 5). The product of a reaction performed in the usual conditions in the presence of all four nucleoside triphosphates, including α -³²P-labelled ATP, with poly[d(A-T)] as template, was purified and hydrolysed with alkali and more than 95% of the radioactivity incorporated was recovered in 2'(3')-UMP after separation of the nucleotides by paper chromatography.

Table 4. Fidelity of transcription of poly[d(A-T)] by *H. cutirubrum* RNA polymerase

The ratio of the incorporation of the indicated [³H]NTP to the incorporation of [¹⁴C]ATP in the standard assay (see the Experimental section) with poly[d(A-T)] as template was determined: (i) with all four nucleotides present (15 nmol of each) (Expt. A); (ii) with only the two radioactive nucleotides present (Expt. B). The results were corrected for quenching and for simultaneous counting of the two isotopes by the external standardization method. The values in parentheses in the last column refer to the expected ratio of incorporation of the two isotopes.

Expt.	[³ H]NTP	[¹⁴ C]ATP incorporation (nmol)	[³ H]NTP incorporation (nmol)	³ H/ ¹⁴ C ratio
A	CTP	3.38	0.46	0.13 (0)
	GTP	3.37	0.41	0.12 (0)
	UTP	3.00	2.98	0.99 (1)
B	CTP	0.34	0.05	0.14 (0)
	GTP	0.34	0.06	0.18 (0)
	UTP	2.99	2.86	0.96 (1)

Table 5. Nearest-neighbour analysis of the product of poly[d(A-T)]-directed RNA synthesis by *H. cutirubrum* RNA polymerase

A large scale (0.5 ml) assay mixture containing poly[d(A-T)] as template and all four nucleoside triphosphates, with [α -³²P]ATP instead of [¹⁴C]ATP, was incubated at 37°C for 2 h. A sample (25 μ l) was withdrawn and counted for radioactivity on paper (see the Experimental section) to determine the total incorporation. Carrier poly(A) and poly(U) (0.5 mg of each in 0.5 ml of water) was added to the remaining bulk solution. The [³²P]RNA was isolated by the phenol procedure, as described by Bolton (1966), precipitated with ethanol in the presence of 0.1 M-NaCl and washed three times with ethanol saturated with unlabelled ATP. The RNA was hydrolysed by incubation for 14 h at 37°C with 0.2 M-KOH (0.1 ml). The nucleotides were then separated by paper chromatography as described by Litvak *et al.* (1970) and the positions of the individual 2'(3')NMP determined under u.v. light by comparison with standards. The chromatogram was cut up and the radioactivity present in each nucleotide determined by liquid-scintillation counting as in the standard assays.

Total incorporation of 5'-[³² P]AMP units	0.639 nmol (100%)
Recovery of 2'(3')-[³² P]AMP	0.020 nmol (3.2%)
Recovery of 2'(3')-[³² P]UMP	0.609 nmol (95.1%)
Total recovery of 2'(3')-[³² P]AMP and UMP	0.629 nmol (98.3%)

Asymmetric transcription of T₇ coliphage early genes by H. cutirubrum RNA polymerase

As *H. cutirubrum* DNA-dependent RNA polymerase is the smallest enzyme of this type yet described (Louis & Fitt, 1971a,c; Louis *et al.*, 1971) but still appears to have catalytic properties very similar to those of *E. coli* RNA polymerase, it was of interest to determine if it would recognize the same initiation signals as the latter and if it catalysed asymmetric transcription of a native, double-stranded DNA template. The DNA from coliphage T₇ provides a suitable system for studying this, as the two strands may be separated and only one, the r strand, is copied by the *E. coli* enzyme (Summers & Szybalski, 1968). Further, the genome of T₇ coliphage is divided into early and late genes, and one of the former, gene 1, codes for a specific T₇ RNA polymerase that transcribes the late genes (Chamberlin *et al.*, 1970). As the transcription of the late genes is blocked by addition of chloramphenicol to the medium that contains the T₇-infected *E. coli* (Summers & Siegel, 1969), it is possible to obtain early-gene-specific RNA for hybridization competition experiments. It was thus possible to determine in this system if the *H. cutirubrum* RNA polymerase catalyses asymmetric transcription of the entire r strand or of either the early or late genes selectively.

Hybridization was performed by a modification (W. C. Summers, personal communication) of the

method of Siegel & Summers (1970) and the conditions were chosen to yield about two-thirds of the input radioactivity as a ribonuclease-resistant hybrid when using radioactive RNA synthesized by the *H. cutirubrum* enzyme.

Table 6 shows the results of hybridization with the separated strands of T₇ DNA of [¹⁴C]RNA formed by the *H. cutirubrum* polymerase on a native, double-stranded T₇ DNA template, together with controls with authentic radioactive total RNA (T₇⁺ RNA) and early gene RNA (T₇CM RNA) from chloramphenicol-treated, T₇-infected *E. coli*. (Because of the low specific radioactivity of the ¹⁴C-labelled T₇CM RNA, the sensitivity of the control experiment in which it was used was decreased.) The product hybridized exclusively with the r strand of T₇ DNA, proving that *H. cutirubrum* DNA-dependent RNA polymerase catalyses asymmetric transcription of double-stranded T₇ DNA.

The results of hybridization competition experiments are shown in Table 7. The addition of unlabelled RNA to preliminary control experiments that lacked DNA resulted in no increase in the amount of non-specific binding of [¹⁴C]RNA to the membrane filters.

Unlabelled T₇⁺ (whole genome) RNA competed effectively for r strand in all cases, as expected. The pattern of competition displayed by the RNA from chloramphenicol-treated, infected bacteria, however, varied with the labelled RNA: T₇⁺RNA was relatively

Table 6. Hybridization efficiency of RNA species with T₇ DNA strands

The hybridization mixtures (100 μl) contained: T₇ DNA, r or l strand, 1 μg; ¹⁴C-labelled RNA, 1000–6500 c.p.m.; 0.3 M-NaCl–0.03 M-sodium citrate buffer, pH8. Control experiments lacked DNA. The mixtures were incubated for 5 h at 60°C and then cooled to room temperature. To each was added 0.45 M-NaCl–0.045 M-sodium citrate buffer, pH8 (2 ml) containing pancreatic ribonuclease (20 μg/ml), which had been previously incubated at 100°C for 5 min to inactivate deoxyribonuclease and then cooled. After 1 h at room temperature, the mixtures were diluted to 10 ml with 0.45 M-NaCl–0.045 M-sodium citrate buffer, pH8, and filtered slowly (2 min) through nitrocellulose filters (BAC-T-Flex Membrane Filters, B-6, Schleicher & Schuell, Inc., Keene, N.H., U.S.A.). Each filter was washed with 0.45 M-NaCl–0.045 M-sodium citrate buffer, pH8 (50 ml) and then dipped in a beaker containing 50 ml of the same solution as a final rinse. The discs were blotted and air-dried, and the radioactivity determined by liquid-scintillation counting as in the standard enzyme assay. [¹⁴C]RNA from the following sources was used: (i) synthesis *in vitro* by *H. cutirubrum* RNA polymerase with native T₇ DNA as template (*H. cutirubrum* RNA); (ii) *E. coli* cells infected with T₇ coliphage (T₇⁺ RNA); and (iii) chloramphenicol-treated *E. coli* cells infected with T₇ coliphage (T₇CM RNA).

DNA	Source ...	[¹⁴ C]RNA added (c.p.m.)		
		<i>H. cutirubrum</i> (1064)	T ₇ ⁺ (6120)	T ₇ CM (4033)
		Hybridized (c.p.m.)		
None		0	0	0
l strand		0	0.2	4.3
r strand		702.7	2761.3	262.0
% of total radioactivity hybridized with r strand		66.2	45.4	6.5

Table 7. *Hybridization competition*

Hybridization was performed as described in Table 6. The mixtures contained: T₇ DNA r strand (1 µg) and the appropriate labelled and unlabelled RNA species (as indicated). The sources of the RNA species were as given in Table 6 and the results are calculated as a percentage of the radioactivity hybridized (Table 6) in the absence of competing unlabelled RNA.

Unlabelled RNA	[¹⁴ C]RNA ...	Hybridization (%)		
		<i>H. cutirubrum</i>	T ₇ ⁺	T ₇ CM
None		100	100	100
T ₇ ⁺ , 5 µg		85.3	85.2	80.6
T ₇ ⁺ , 10 µg		71.7	79.5	83.6
T ₇ ⁺ , 25 µg		50.1	53.2	71.8
T ₇ ⁺ , 50 µg		28.7	34.6	35.7
T ₇ CM, 5 µg		57.6	107.0	68.1
T ₇ CM, 10 µg		46.8	82.0	61.4
T ₇ CM, 25 µg		27.9	93.9	35.6
T ₇ CM, 50 µg		4.1	86.1	6.4

Table 8. *Preliminary amino acid analysis of proteins α and β*

A sample of each protein, the product of one preparation, was hydrolysed in 6M-HCl at 110°C for 22h and the hydrolysate was chromatographed on a Beckman Model 120C amino acid analyser. Peaks were manually integrated by the half-height approximation and compared with the results obtained with standard mixtures of amino acids. No correction was made for amide N or for tryptophan. The results for the *E. coli* B RNA polymerase are from Nicholson (1971).

Amino acid	Amino acid composition (mol/100mol)		
	<i>H. cutirubrum</i> protein α	<i>H. cutirubrum</i> protein β	<i>E. coli</i> polymerase
Asp	7.0	6.3	10.2
Thr	5.3	5.0	5.3
Ser	15.9	16.1	6.7
Glu	14.2	13.4	13.6
Pro	4.2	4.1	3.4
Gly	15.6	17.8	7.4
Ala	8.0	8.2	8.8
Val	3.6	3.6	6.7
Met	0.6	0.5	2.9
Ile	1.8	2.2	5.6
Leu	3.1	4.0	10.9
Tyr	1.8	1.9	2.6
Phe	1.4	1.8	2.8
Lys	11.1	9.0	5.4
His	3.1	3.4	1.6
Arg	3.2	2.7	5.8

unaffected by the presence of T₇CM RNA, whereas the unlabelled T₇CM RNA displaced the labelled T₇CM RNA, as expected. The RNA synthesized by *H. cutirubrum* polymerase was also displaced very efficiently by unlabelled T₇CM RNA, indicating that this enzyme transcribes the early genes of T₇ coliphage preferentially, as is the case with the RNA poly-

merase from *E. coli* (Summers & Siegel, 1969). These studies therefore show that the enzymic properties of the small *H. cutirubrum* RNA polymerase resemble those of the large (mol.wt. 400000; Burgess, 1969) *E. coli* enzyme rather than those of the small T₃- and T₇-induced polymerases. Additional support for this conclusion comes from the demonstration (Louis &

Fitt, 1972) that the functions of the *H. cutirubrum* enzyme, like those of *E. coli* RNA polymerase (Zillig *et al.*, 1970; Burgess, 1971) are divided among its subunits and are not properties of the complete enzyme only, as is necessarily the case with the single chain T₃- and T₇-induced polymerases (Chamberlin *et al.*, 1970; Dunn *et al.*, 1971) and *N. crassa* mitochondrial enzyme (Küntzel & Schäfer, 1971). Thus, the size of an RNA polymerase is not necessarily directly related to the complexity of the genome it has to copy, as it is considered (Moore & McCarthy, 1969b) that the genome size of extreme halophiles is similar to that of *E. coli*. However, it is possible that distribution of the functions necessary for specific template recognition, template binding, chain initiation and chain elongation among different subunits does permit a flexibility in the overall mechanism of polymerization unavailable with the single chain enzymes, which are known (Dunn *et al.*, 1971; Küntzel & Schäfer, 1971) to have a highly restricted template specificity *in vitro*.

Preliminary amino acid analyses of proteins α and β

Amino acid analyses of *E. coli* RNA polymerase have been published by several authors (Maitra & Hurwitz, 1967; Preiss & Zillig, 1967; Neuhoﬀ *et al.*, 1970b; Nicholson, 1971). It was of interest to compare the amino acid composition of proteins α and β of the *H. cutirubrum* DNA-dependent RNA polymerase with that of the *E. coli* enzyme, as no composition of any enzyme from an extreme halophile has yet been reported. Proteins of the cell envelope (Brown, 1963; Kushner & Onishi, 1966; McClare, 1967; Marshall *et al.*, 1969; Steensland & Larsen, 1969) and of ribosomes (Bayley, 1966) of extreme halophiles are somewhat acidic with respect to their non-halophilic counterparts and the bulk protein of some extreme halophiles is also slightly more acidic than that of some non-halophiles (Reistad, 1970). It has been suggested (Brown, 1963, 1964; Kushner & Bayley, 1963) that the conformation of halophile proteins in strong salt solutions depends on interactions of negatively charged groups in the proteins with cations, and it is known, for example, that removal of salt causes unfolding of *H. cutirubrum* isocitrate dehydrogenase (Hubbard & Miller, 1969).

The small amounts of proteins α and β so far available have prevented us from performing complete analyses, but our preliminary results are shown in Table 8 together with those published by Nicholson (1971) for the *E. coli* B enzyme. Insufficient material was available for cysteine or tryptophan analysis, and the results are uncorrected for amide N or for minor peaks due to unhydrolysed oligopeptides. It can be seen that proteins α and β have very similar compositions that both differ in detail from that of *E. coli* RNA polymerase. In particular, the glycine and

serine content of the α and β proteins is much higher than that of the *E. coli* polymerase. However, it should be noted that the total acidic, total basic and total neutral amino acids do not differ by more than a few per cent for the complete *H. cutirubrum* ($\alpha\beta$) and *E. coli* enzymes.

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