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Isolation of Deoxyribonucleic Acid and Ribosomal Ribonucleic Acid from Bacteria

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1. Nucleic acids were released from *Escherichia coli* by lysing with tri-isopropylnaphthalene sulphonate and 4-aminosalicylate and then extracting with a phenol–cresol mixture. 2. Nucleic acids were similarly released from *Bacillus subtilis* after initial treatment with lysozyme. 3. DNA was sedimented after careful precipitation with *m*-cresol or 2-butoxyethanol (0·1–0·12 vol.) in the presence of 20% sodium benzoate. 4. Contaminating ribosomal RNA was removed by precipitation in the presence of 4 M-sodium chloride or by extracting DNA with an acetate–butyrate mixture, in which RNA is insoluble. 5. The DNA from *B. subtilis* has a transforming ability of 0·3–0·6% for the tryptophan marker. 6. Ribosomal RNA was then precipitated with rapidly labelled RNA by the addition of an equal volume of 2-butoxyethanol. 7. There was good separation of the nucleic acids from protein and polysaccharides.

The separation of nucleic acids from bacteria usually requires the use of a nuclease to remove either the DNA or the RNA (Marmur, 1961) and the separations from protein and polysaccharide are not always achieved. A method that separated DNA and RNA without the use of enzymes has been published (Kirby, 1964), but the solubilization of DNA proved difficult and the yield of ribosomal RNA was poor. The present methods give a good separation of RNA from DNA and the products were free from protein and polysaccharide when the bacteria used were *Escherichia coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive), but different techniques were used to separate small amounts of ribosomal RNA associated with DNA.

MATERIALS

E. coli strain B and *B. subtilis* strain 168 were used for these experiments.

Sodium tri-isopropylnaphthalenesulphonate (technical) was obtained from Kodak Ltd., Kirkby, Liverpool. The phenol–cresol mixture (Kirby, 1965) was made by adding water (55 ml.), *m*-cresol (70 ml.) and 8-hydroxyquinoline (0·5 g.) to 500 g. of phenol (detached crystals).

All radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks.

Deoxyribonuclease (free of ribonuclease) was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Lysozyme and ribonuclease were obtained from Armour Pharmaceutical Co., Eastbourne, Sussex.

METHODS

Preparation of DNA. The bacteria were grown from a small inoculum in about 80 ml. of medium (Anagnostopoulos & Spizizen, 1961) with slow aeration in a condenser maintained at 37°. The bacteria, in stationary phase, were then dropped automatically into 1·6 l. of medium that was well aerated at 37°. *E. coli* was in exponential growth phase 3 hr. later and the cells were collected by centrifugation (3000*g* for 20 min. at 5°). The sediment was usually 15–18 g. and was dispersed gently with some large glass beads and 100 ml. of a solution of 1% (w/v) sodium tri-isopropylnaphthalenesulphonate and 6% (w/v) sodium 4-aminosalicylate to each 100 ml. of which was added 3 ml. of the phenol–cresol mixture. Then 200 ml. of the same mixture was added and the viscosity increased when the mixture was shaken by hand in a stoppered flask. Then 300 ml. of the phenol–cresol mixture was added and the mixture was again shaken by hand until thoroughly mixed and then by a mechanical shaker for a further 20 min. at 20°. The phases were separated by centrifugation (12000*g* for 20 min. at 5°), and the top phase was carefully poured off, made 3% with respect to NaCl and mixed with 0·5 vol. of the phenol–cresol mixture (10 min. at 20°). The phases were again separated by centrifugation (17000*g* for 10 min. at 5°). The aqueous phase was carefully poured off, made 20% with respect to sodium benzoate (when the solution becomes somewhat less opalescent) and the DNA was precipitated by very careful addition of *m*-cresol (0·1–0·12 vol.). The viscosity of the mixture increases during the addition and then falls when the DNA suddenly coagulates. The coagulation is difficult to see and is best observed by pouring the mixture. Considerable care and practice are required for an efficient separation. After centrifugation (700*g* for 10 min. at 5°), the DNA separates as a gel that partly sediments and partly floats on the surface. The total gel was combined and mixed with 0·1 M-sodium acetate buffer, pH 6·0 (15 ml.). Solution is best obtained by careful shaking, centrifuging off the undissolved gel (500*g* for 2 min.), pouring off the supernatant solution and adding 0·1 M-sodium acetate buffer, pH 6·0 (5–10 ml.), to the gel. The operation was

repeated until the DNA was completely dissolved. The solution was then made 3% with respect to NaCl and 20% with respect to sodium benzoate, and the DNA was precipitated with *m*-cresol (0·15–0·18 vol., as no phenol was present in the mixture). The solution of DNA (in 0·1 M-sodium acetate buffer, pH 6·0) was made 4 M with respect to NaCl and the solution was stored at 0° for 16 hr., during which time ribosomal RNA was precipitated. The precipitate was removed by careful layering over 6 M-NaBr (4 ml.) and then centrifuging (80000*g* for 1 hr. at 5°). Centrifugation of the RNA from the 4 M-NaCl through 6 M-NaBr was essential to obtain a good separation from the viscous solution of DNA, which floated over and in the top layer of NaBr solution while the RNA sedimented to the bottom. The clear DNA solution was poured off and dialysed against 0·1 M-sodium acetate buffer, pH 6·0, or 5 mM-KF ($KF,2H_2O$; British Drug Houses Ltd., Poole, Dorset). The yield, estimated from the E_{260} value, was 25–30 mg. DNA was kept at 2° in sodium acetate buffer, pH 6·0 (0·15 M), containing NaN_3 (10 mM).

Preparation of ribosomal RNA. The clear solution left after the first removal of DNA was mixed with an equal volume of 2-butoxyethanol (purified by distillation over 2,4-diaminophenol hydrochloride, b.p. 72°/15 mm. Hg; Kirby, 1960). The resulting gel was collected by centrifugation (700*g* for 10 min. at 5°), washed twice with ethanol–water–sodium acetate (75:25:2, v/v/w), dissolved in cold 0·15 M-sodium acetate buffer, pH 6·0, and then made 4 M with respect to NaCl. The precipitate that formed overnight at 0° was centrifuged off (700*g* for 10 min. at 5°), washed twice with 3 M-sodium acetate buffer, pH 6·0, and twice with ethanol–water–sodium acetate (75:25:2, v/v/w) and the precipitate kept at 0° over this mixture. The yield was about 25–35 mg.

Two types of radioactive RNA were isolated, one from cells that were exposed to a short-term labelling (30 sec.), when the rapidly labelled RNA was isolated with, but could be distinguished from, the ribosomal RNA, and another from cells grown in medium in which radioactive precursors were present during the exponential growth phase (when the yield of bacteria was usually less), when ribosomal RNA only was highly labelled. A labelling of 30 sec. duration was achieved in *E. coli* by adding [^{3}H]uridine (100–200 μc/1·6 l. of medium) in exponential growth phase and stopping the reaction by the addition of NaN_3 (final concn. 10 mM). Azide could not be used with *B. subtilis* as it interfered with the lysozyme reaction, so the uridine was diluted by addition of 10–20 mg. of unlabelled uridine in 1–2 ml. of water.

A long-term labelling was effected by mixing [^{14}C]uracil (60 μc), sodium [^{14}C]formate (20 μc) and [^{14}C]adenine (30 μc) with 800 ml. of medium for *B. subtilis* and twice the amount of radioactivity for *E. coli* and growing to exponential phase.

Preparation of nucleic acids from B. subtilis. The experiments were basically the same as those described for *E. coli* except that the cells took about 5 hr. to grow into exponential phase although approximately the same yield was obtained. The cells (15 g.) were then suspended in 75 ml. of a solution of 0·15 M-NaCl–15 mM-sodium citrate to which lysozyme (10 mg.) was added and the mixture was stirred gently at 37° for 15 min. Sodium tri-isopropylnaphthalenesulphonate (2·25 g. in 6·75 ml. of phenol–cresol mixture and and 6·75 ml. of water) and sodium 4-aminosalicylate (13·5 g.) were then added and then 150 ml. of 0·15 M-NaCl–15 mM-sodium citrate followed by 225 ml. of the phenol–cresol mixture. The mixture was shaken by hand for 2–3 min. and then centrifuged. The procedures for the isolation of the nucleic acids were similar to those already described except that DNA from *B. subtilis* can with advantage be precipitated by 0·1–0·15 vol. of 2-butoxyethanol in the presence of 20% benzoate rather than *m*-cresol. The volume required is almost the same. Occasionally if the DNA was of lower molecular weight more 2-butoxyethanol was required to effect a complete precipitation, in which case more ribosomal RNA separated with the DNA. After removal of the DNA, RNA was precipitated from the supernatant solution with 1 vol. of butoxyethanol.

Small amounts of RNA always remained with the DNA when the 4 M-NaCl technique, used for the separation of nucleic acids from *E. coli*, was applied. A successful separation was achieved by extraction with a solution of sodium acetate trihydrate (4 g.) and sodium butyrate (18 g.) in a solution of water (100 ml.) adjusted to pH 7·2 with 20% (v/v) acetic acid.

The fractionation was most practicable if carried out on the fibrous DNA that had been precipitated by 2-ethoxyethanol, rather than the gelatinous DNA that had been precipitated by 2-butoxyethanol. The DNA was therefore dissolved in 0·1 M-sodium acetate buffer, pH 6·0, and the solution made 0·3 M with respect to sodium acetate. Precipitation was achieved with ethoxyethanol (1 vol.) and the precipitate extracted with the acetate–butyrate mixture (20 ml.). RNA was centrifuged off (10000*g* for 10 min. at 5°). The supernatant solution was poured off and DNA was precipitated by the addition of ethoxyethanol (1 vol.). The precipitate was re-extracted with the same acetate–butyrate mixture and centrifuged and the DNA was again precipitated by addition of ethoxyethanol. The precipitate was dissolved in 0·1 M-sodium acetate buffer, pH 6·0, and the solution dialysed against the same solution or against 5 mM-KF.

The RNA that was precipitated with 1 vol. of butoxyethanol could be purified by extraction with 3 M-sodium acetate buffer, pH 6·0, or with the same acetate–butyrate mixture described above. In both cases the DNA (of lower molecular weight) was soluble and ribosomal RNA (with rapidly labelled RNA) remained insoluble.

The yield of DNA was 10–15 mg. and that of RNA was 25–30 mg.

Other methods. Tests for the presence of protein and polysaccharide were carried out as described previously (Kirby, 1957; Frearson & Kirby, 1964).

Bacteria were also grown in the presence of 20 μc of L-[^{14}C]leucine (in 1·6 l. of medium), and a portion of DNA that was isolated was degraded with pancreatic deoxyribonuclease (Worthington Biochemical Corp.), before being mixed with the scintillation fluid and the radioactivity estimated by liquid-scintillation spectrometry.

The presence of RNA or single-stranded DNA was examined by studying the effect of temperature on the optical extinction of the DNA dissolved in 5 mM-KF. The experiments were carried out in a Unicam SP. 800 spectrophotometer with a solution that had E_{260} about 0·8 at 20°. The presence of RNA or single-stranded DNA was observed by an increase in extinction before a temperature of 60° was reached (cf. Hastings & Kirby, 1966).

Sedimentation studies on DNA were carried out on a solution in 0·1 M-sodium acetate buffer, pH 7·0, having E_{260} 1·0, in the Spinco model E ultracentrifuge.

The presence of the two characteristic components of ribosomal RNA was checked by centrifugation in a sucrose (5–20%, w/v) gradient in 0·15M-sodium acetate buffer, pH 6·0, or in a sulpholane gradient (Parish & Kirby, 1966).

Transformation by DNA of *B. subtilis* was carried out essentially as described by Anagnostopoulos & Spizizen (1961) and Kelly & Pritchard (1965) except that, before adding the DNA to the competent bacteria, the transforming medium was shaken with Zeo-Karb 225 (10mg./ml. of transforming medium) (containing 2% of divinylbenzene) that had been previously washed with the same medium. Results obtained in this way were more consistent and usually the transformation rate was about twice that found when the ion-exchange resin was absent.

RESULTS

DNA. The DNA was free (less than 0·05%) from protein and polysaccharides, after hydrolysis, paper-chromatographic separation of the products and spraying the papers either with a solution of aniline phthalate to detect sugars or a solution of ninhydrin to detect amino acids (cf. Kirby, 1957; Frearson & Kirby, 1964). No radioactivity above background was detected in DNA from *E. coli* when grown in the presence of [^{14}C]leucine. Any protein contamination must therefore be very small (less than 0·001%). The effect of heating on the optical extinction of DNA free from RNA or single-stranded DNA is shown in Fig. 1, and Fig. 2 shows the result found when RNA or single-stranded DNA was still present. The increases observed in Fig. 2 between 25° and 60° could be eliminated by the action of ribonuclease in a solution of DNA followed by extraction with phenol–cresol mixture and recovery of the DNA.

The sedimentation profiles of DNA from *E. coli* that had been precipitated once and three times with *m*-cresol are shown in Fig. 3.

The transformation rate of DNA from *B. subtilis* for the tryptophan marker was usually 0·3–0·6%, although results were sometimes more variable.

Ribosomal RNA. The ribosomal RNA was characterized by gradient centrifugation with either sucrose or sulpholane (Parish & Kirby, 1966). A typical pattern obtained with ribosomal RNA from *B. subtilis* is shown in Fig. 4. The RNA isolated after labelling for 30sec. had about 10^5 counts/min. ($2{\cdot}65 \times 10^5$ μc)/mg., whereas after labelling for 3–5hr. the RNA had about 10^6counts/min. (9×10^6 μc)/mg., and the radioactivity and

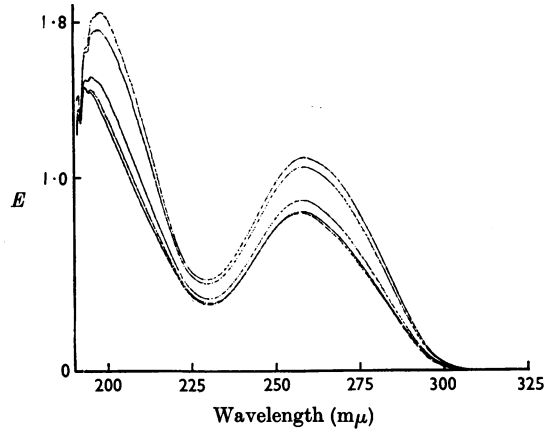

Fig. 1. Thermal denaturation curve of DNA from *E. coli*. The solvent was 5mM-KF and five curves are shown. The lowest curve remained unaltered between 22° and 60°. The next three curves, in ascending order, were measured at 62°, 65° and 70°; the top curve represents the spectrum at 75° and 80°.

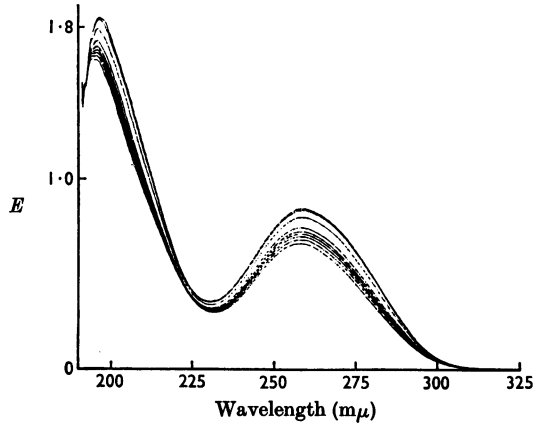

Fig. 2. Thermal denaturation curve of DNA, containing some RNA, from *B. subtilis*. The solvent was 5mM-KF and nine curves are shown in ascending order. The measurements were made at 25°, 35°, 40°, 45°, 50°, 55°, 60°, 65° and 70°.

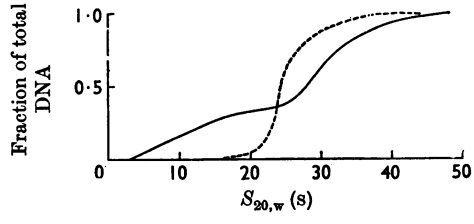

Fig. 3. Sedimentation distribution curves of DNA extracted from *E. coli* and dissolved in 0·1M-sodium acetate buffer, pH 7·0. The sedimentation was carried out in the Spinco model E ultracentrifuge at 50740rev./min. at 20°. ——, DNA that had been precipitated once with *m*-cresol; – – – –, DNA that had been precipitated three times with *m*-cresol.

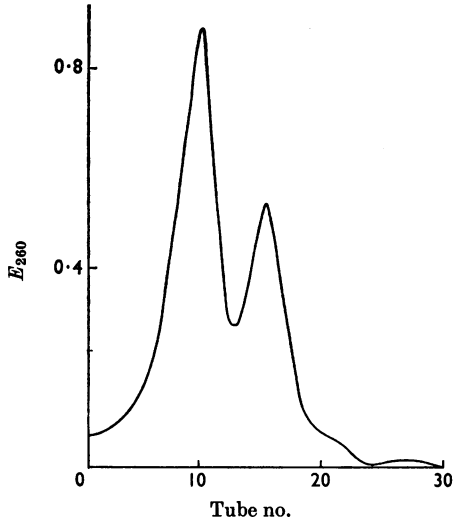

Fig. 4. Centrifugation pattern in sucrose density gradient (5–20% sucrose in 0·15 M-sodium acetate buffer, pH 7·0) of ribosomal RNA from *B. subtilis*. Centrifugation was at 24000 rev./min. for 16 hr. in the MSE TC50 centrifuge at 5° (SW 30 rotor).

extinction profiles were identical after centrifugation in a density gradient of sucrose (5–20%) in 0·15 M-sodium acetate buffer, pH 7·0.

DISCUSSION

Although it is necessary to use lysozyme to break the cell wall of *B. subtilis* the separation of DNA and RNA can be achieved without the use of other enzymes. The reagents were chosen to give the greatest degree of deproteinization and inhibition of nucleases. The phenol–cresol mixture is an improvement on phenol for removal of protein and ribonuclease (Kirby, 1965), and tri-isopropylnaphthalenesulphonate proved to be more effective than dodecyl sulphate for the lysis of *E. coli*, the yield of DNA and the maintenance of good secondary structure of ribosomal RNA. This detergent is also more effective in the isolation of RNA from mammalian tissues (Parish & Kirby, 1966). The second extraction in the presence of sodium chloride is essential for the removal of the protein. DNA is precipitated by *m*-cresol or butoxyethanol in the presence of sodium benzoate with little ribosomal RNA and this has the advantage that practically no polysaccharide is precipitated. Fig. 3 shows that material of low molecular weight is first precipitated with the DNA but on further precipitation this material is removed together with some of very high molecular weight. The final removal of ribosomal RNA proved a difficult step, but was effected by precipitation of the RNA in the presence of 4 M-sodium chloride for the nucleic acids from *E. coli* (Fig. 1). Because of its higher molecular weight, mammalian ribosomal RNA could be precipitated with 3 M-sodium chloride (Kirby & Cook, 1967). Traces of ribosomal RNA remained with DNA from *E. coli* when 3 M-sodium chloride was used for the precipitation, as judged by the increase in E_{260} on heating from 20° to 60° in the presence of 5 mM-potassium fluoride. DNA from *B. subtilis* was never completely separated from RNA even after repeated treatment with 4 M-sodium chloride. This difficulty may be due to the time required for lysozyme to act, since some RNA may be degraded during this period and it is possible that the fragments are difficult to separate (Fig. 2). It should be noted that an increase in extinction at about 220 mμ was always less when RNA was present than when it was absent (Figs. 1 and 2). This has been a regular feature of the thermal denaturation curves carried out in 5 mM-potassium fluoride. The increase in optical extinction found on heating *B. subtilis* DNA to 60° in 5 mM-potassium fluoride is, in itself, no proof that the contaminant is RNA. However, since the effect of ribonuclease treatment is to abolish this increase, it is likely that the contaminant is RNA, in accord with previous experience (Hastings & Kirby, 1966).

The method devised for the separation (sodium acetate–butyrate mixture) is a general method for nucleic acids. However, though mammalian ribosomal RNA is insoluble in a mixture containing 12 g. of sodium butyrate/100 ml., bacterial ribosomal RNA is partly soluble in this mixture and one containing 18 g. of sodium butyrate/100 ml. was necessary to maintain the insolubility of the RNA. We have evidence that some degree of fractionation of RNA may be obtained by partial solution in these mixtures (K. S. Kirby & E. Fox-Carter, unpublished work). The DNA thus prepared is therefore free from other cellular constituents and had a sedimentation range 15–46 s ($S_{20,w}$ 24·2 s). It seems probable that some shearing takes place during the two-phase extraction and subsequent precipitation and dissolution. The transforming activity of DNA from *B. subtilis* is similar to that reported by other authors and hence no impairment of the biological activity is induced by the reagents used.

Butoxyethanol also precipitates ribosomal RNA and very little polysaccharide. Some DNA of low molecular weight is present and is removed by precipitation of the RNA from 4 M-sodium chloride and then washing with 3 M-sodium acetate, or by washing with the acetate–butyrate mixture. This RNA contains the bulk (approx. 90%) of the rapidly labelled RNA after labelling with [^{3}H]-uracil for 30 sec.; a small amount of rapidly labelled

RNA is precipitated with the DNA fraction. The characterization of the RNA by centrifugation in sucrose and sulpholane density gradients has already been reported (Hastings, Parish, Kirby & Klucis, 1965; Parish & Kirby, 1966).

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