Fractionation of Nucleic Acids from *Penicillium chrysogenum* and Associated Ribonucleic Acid Viruses by Selective Exclusion and Retention in Agarose Gels

By S. L. PETROVIĆ, B. D. ŠUMONJA and R. B. VASILJEVIĆ

Department of Biochemistry, Boris Kidrich Institute, P.O. Box 522, Beograd, Yugoslavia

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Double-stranded nucleic acids from a strain of *Penicillium chrysogenum* containing RNA viruses were isolated by agarose-gel filtration, and separated into DNA and double-stranded RNA fractions by agarose-gel chromatography in 2.5M-NaCl. The DNA fraction contained less than 1% alkali-labile polynucleotides, and sedimented homogeneously at 8–10S in alkaline sucrose gradients. In CsCl gradients it tended to band in the density region of 1.66–1.72 g/ml. It had a 'melting' temperature (T_m) of 75°C in 0.015M-NaCl–0.0015M-trisodium citrate, corresponding to 51.5 mol% of G+C. The double-stranded RNA fraction did not contain detectable DNA. It could not band in CsCl up to a density of 1.78 g/ml, and mainly consisted of a 14–15S RNA species with a T_m of 88.5°C in the above solvent, and a G+C content of 49.3 mol%.

Double-stranded polyribonucleotides from viruses associated with *Penicillium* spp. and other fungi (for a review see Border *et al.*, 1972) have received notice as interferon inducers (Lampson *et al.*, 1967; Tytell *et al.*, 1967; Kleinschmidt *et al.*, 1968), viroprophylactics (Sutherland & Bessell, 1969; Edy & Szekely, 1972), possible antitumour agents (Cordell-Stewart & Taylor, 1971) and inhibitors of protein synthesis (Robertson & Mathews, 1973). However, purification of these RNA species from proteins and polysaccharides, and their separation from other types of polynucleotides at either the nucleoprotein stage (Banks *et al.*, 1968, 1971; Wood & Bosarth, 1972), or after isolation of total nucleic acids (Cox *et al.*, 1970), are achieved in a rather elaborate fashion, and could thus be coupled with large losses of intact double-stranded RNA. Moreover, because of the many steps involved, the published isolation and purification procedures do not appear suitable for small-scale preparation and/or analytical characterization of double-helical RNA species. In some cases (e.g. Cox *et al.*, 1970), the separation between native fungal DNA and viral double-stranded RNA, which are isolated together, was not investigated; in this and many other cases, the extent of ultimate contamination of the double-stranded RNA preparations by native DNA is not clear.

Recent findings on the retention behaviour of polynucleotides in agarose gels (Petrović *et al.*, 1971, 1973; Morris *et al.*, 1972) indicated the possibility of improvements in the purification of double-stranded RNA species by agarose chromatography at an increased ionic strength. As shown in the present paper, it was possible to define conditions that result in selective retention or exclusion from the gel of nearly all major types of nucleic acids found in a

Penicillium strain infected with RNA viruses. The separation of native DNA and viral double-stranded RNA achieved by this method appeared to be quantitative.

Experimental

Materials

Radioactive materials. $^{32}\text{P}_i$ (carrier-free; >1000 mCi/mg of P) was supplied as a sterile solution, pH 5–9, by the Department of High Activity, Boris Kidrich Institute. [6- ^{14}C]Orotic acid (28 mCi/mmol) was obtained from the same source.

Chemicals. Sodium dodecyl sulphate (technical grade; Fluka AG, Buchs, Switzerland) was purified by two crystallizations from 95% (v/v) ethanol. Phenol (technical grade; OKI, Zagreb, Yugoslavia) was distilled under atmospheric pressure, saturated with water and protected with 8-oxyquinoline (Kirby, 1968). Sepharose 2B and 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) as hydrated gels ready for use. Kieselguhr (acid-washed) was a product of BDH Chemicals Ltd., Poole, Dorset, U.K. Neutral alumina (AG-7, 100–200 mesh) was supplied by Calbiochem A.-G., Lucerne, Switzerland. All other chemicals were A.R. grade.

Growth and labelling of *Penicillium chrysogenum*. Portions (100 ml) of 15–18 h-old cultures of an industrial strain (possibly similar to strain A.T.C.C. 9480), grown in a corn-steep-liquor-based medium for penicillin production, were given 10 μCi of $^{32}\text{P}_i$ /ml, or 2 μCi of [6- ^{14}C]orotic acid/ml, and shaken for 20–22 h at 24–26°C in an aseptic atmosphere, in 1-litre flasks with cottonwool plugs.

During this period the fungi formed typical globular colonies. This material (wet wt. 21–24 g) was collected on Seitz filters, and washed with 2–3 vol. of 0.15 M NaCl–0.015 M-trisodium citrate (pH 7.5).

Methods

Isolation of total nucleic acids. The washed mycelium was lysed with 100 ml (approx. 5 vol.) of 2.5% (w/v) sodium dodecyl sulphate–2% (w/v) NaCl–0.01 M-Tris–HCl, pH 7.6, by grinding this mixture in a mortar with 2 g of alumina for 10 min at room temperature. The lysate was mixed with 10 ml of water-saturated, 8-oxyquinoline-protected phenol, then shaken, mixed with 5 g of kieselguhr and filtered under suction. The filter cake was washed with 10 ml of the above dodecyl sulphate solution, and the combined filtrates were mixed with $\frac{1}{3}$ vol. of the phenol solution described above (which contained approx. 15% water). This mixture was shaken by hand for 5 min and centrifuged (7 min, 5000g). The resulting aqueous phase was once more treated with phenol as described above, and the second aqueous phase was adjusted to 10% (w/v) NaCl, by dissolving in it the solid salt, and then mixed with 1 vol. of cold 95% (v/v) ethanol (0–4°C). This mixture was left for 2–24 h at 0–4°C, and then the precipitated polysaccharides and nucleic acids were collected by centrifugation. The collected precipitate was washed once with cold (0–4°C) 70% (v/v) ethanol and dissolved in 5 ml of the gel-filtration buffer [0.1 M NaCl–0.1% sodium dodecyl sulphate–0.02 M-Tris–HCl (pH 7.6)–0.001 M-EDTA (sodium salt; pH 7.6)]. The solution was left for 30–60 min at room temperature to allow precipitation of the bulk of polysaccharides, which were then removed by centrifugation (10 min, 7000g).

Isolation of DNA and double-stranded RNA. This was done by gel filtration, by a procedure somewhat similar to that of Cox *et al.* (1970), by using a 2% agarose gel, Sepharose 2B. The clarified solution of total nucleic acids (2–5 ml, up to 40 mg of polynucleotides) was applied to a 200 ml column of the gel packed in the gel-filtration buffer. The filtration was done in the downward direction with a flow rate of 10–20 ml/h. The material emerging from the column in the void-volume zone (DNA and double-helical RNA, plus some particulate polysaccharides) was pooled and precipitated, at 10% (w/v) NaCl in the aqueous phase, with 1 vol. of cold (0–4°C) 95% (w/v) ethanol for 24 h at 0–4°C.

Separation of double-stranded RNA from DNA. The precipitate from the previous stage was collected by centrifugation and dissolved in 2–5 ml of 2.5 M NaCl–0.01 M-Tris–HCl (pH 7.6)–0.001 M-EDTA (sodium salt; pH 7.6) and applied to a 50 ml column of Sepharose 2B, or preferably Sepharose 4B (the more concentrated agarose gel being less prone to shrinking

in contact with double-helical DNA), packed in the same solvent. The chromatography was done with a flow rate of 0.3–0.5 ml/min, and 1.5 column volumes of 2.5 M NaCl–Tris–EDTA were collected. After this, the elution was continued with the gel-filtration buffer described under 'Isolation of total nucleic acids', to recover gel-associated polynucleotides (Petrović *et al.*, 1971), identifiable chiefly as 14–15S double-stranded RNA (see the Results section).

Sucrose-gradient characterization of DNA and RNA. For determination of alkali stability and approximate sedimentation range in the denatured state, native DNA, purified by the chromatography in 2.5 M NaCl described above, was recovered with 67% (v/v) ethanol, and the fibrous precipitate was dissolved in 0.35 M-KOH to give a concentration of 0.3 mg/ml or less, and then digested for 20–24 h at 37°C. Portions of this hydrolysate were applied on top of linear 10–40% (w/v) alkaline sucrose gradients (2.3 ml of either sucrose solution in 0.1 M-NaOH) and centrifuged for 3.5 h at 39000 rev./min and 4°C in an SW 50 rotor of a Beckman Spinco model L ultracentrifuge. The gradients were fractionated and analysed essentially by the method of Martin & Ames (1961).

RNA species were dissolved in the gel-filtration buffer to a concentration of 0.2–0.5 mg/ml, and portions of up to 0.3 ml were layered on linear 10–40% (w/v) sucrose gradients (2.3 ml of each sucrose solution in the gel-filtration buffer), and centrifuged at 20°C, as described above.

CsCl-gradient characterization of nucleic acids. This was done essentially as described by Pettijohn & Hanawalt (1964), except that the samples were not centrifuged to full equilibrium.

Determination of RNA nucleotide composition and thermal-denaturation assays. These were done as described previously (Petrović & Brkić, 1970).

Indole reaction for detection of deoxypolynucleotides. This was performed by the method of Ceriotti (1952).

Results

Filtration through Sepharose 2B of total nucleic acids extracted from *Penicillium* resulted in exclusion of a relatively minor portion of the load from the gel (Fig. 1). This fraction contained, as might be expected (Cox *et al.*, 1970; Falcoff & Falcoff, 1970), chiefly double-stranded polynucleotides. When subjected to gel chromatography in 2.5 M NaCl, this material was resolved into two fractions (Fig. 2). The indole-positive fraction was eluted in the region of the void volume. DNA recovered from this zone by ethanol precipitation displayed a fairly homogeneous sedimentation profile in alkaline sucrose gradients even after exhaustive alkaline digestion; less than 1% of the total radioactivity failed to enter the gradient,

Fig. 1. Separation of $U\text{-}^{32}\text{P}$ -labelled double-stranded *Penicillium* nucleic acids from ribosomal RNA species by gel filtration Sepharose 2B

200ml column of Sepharose 2B was used, equilibrated with 0.1 M-NaCl-0.02 M-Tris-HCl (pH 7.6)-0.001 M-EDTA (sodium salt; pH 7.6)-0.1% sodium dodecyl sulphate, which was also used for elution. For further details, see the Experimental section. $\circ$, E_{260} $\bullet$, ^{32}P radioactivity. Fractions (5ml) were collected. V_0 , Void volume; V_t , total volume; d.s. RNA, double-stranded RNA.

Fig. 2. Separation of *Penicillium* DNA from double-stranded RNA by agarose-gel chromatography in 2.5 M-NaCl

A mixture of $U\text{-}^{32}\text{P}$ -labelled polynucleotides, isolated as in Fig. 1, was chromatographed on a 50ml column of Sepharose 4B in 2.5 M-NaCl-0.01 M-Tris-HCl (pH 7.6)-0.001 M-EDTA (sodium salt; pH 7.6) as described under 'Methods'. $\circ$ — $\circ$, E_{260} ; $\bullet$ — $\bullet$, E_{490} in indole reaction (Ceriotti, 1952); $\bullet$ — $\bullet$, ^{32}P radioactivity. Fractions (5ml) were collected. V_0 , Void volume. The arrow indicates beginning of elution with 0.1 M-NaCl.

indicating only minor contamination with polyribonucleotides (Fig. 3). This is in agreement with our observations on the separation of DNA from high-molecular-weight RNA by agarose chromatography (Petrović *et al.*, 1973).

The fraction retained in the gel at 2.5M-NaCl and recovered with 0.1M-NaCl (Fig. 2) gave an essentially negative indole reaction, and its refiltration through Sepharose 2B at 0.1M-NaCl resulted in a single component, which was eluted with a rather broad

Fig. 3. Sedimentation of an alkaline hydrolysate of $U\text{-}^{32}\text{P}$ -labelled *Penicillium* DNA

DNA was isolated by agarose chromatography as in Fig. 2 and denatured by overnight exposure to 0.35M-KOH. Centrifugation was on 10–40% (w/v) alkaline sucrose gradients, for 3.5 h at 39000 rev./min at 4°C. For further details see under 'Methods'. $\circ$, E_{260} ; $\bullet$, ^{32}P radioactivity. Fractions (approx. 0.16 ml) were collected.

Fig. 4. Sedimentation of $U\text{-}^{32}\text{P}$ -labelled double-stranded RNA from *Penicillium*, purified by agarose chromatography (as in Fig. 2)

Sedimentation positions of 28S and 18S rRNA species from rat liver were determined in the same run by co-sedimentation of unlabelled rat RNA and ^{32}P -labelled double-stranded RNA in separate tubes. Centrifugation was on 10–40% (w/v) sucrose gradients, centrifuged for 3.5 h at 39000 rev./min at 20°C. $\circ$, E_{260} ; $\bullet$, ^{32}P radioactivity. Fractions (approx. 0.16 ml) were collected.

Fig. 5. Behaviour of *Penicillium* nucleic acids in self-generated *CsCl* density gradients

U-³²P-labelled double-stranded RNA (○—○; 0.13 mg), U-³²P-labelled 23S RNA (○—○; 0.10 mg) and U-¹⁴C-labelled DNA (●—●; 0.3 mg), each in 3.0 ml of 0.02 M-Tris-HCl buffer, pH 7.8, were used to dissolve portions of *CsCl* (4.0 g). The resulting solutions were centrifuged for 30 h at 37 500 rev./min in an SW 50 rotor of a Spinco L ultracentrifuge. Δ, Density of *CsCl* at 20°C (from refractometric data). Fractions (approx. 0.15 ml) were collected.

profile in or near the void-volume region. Examination in sucrose gradients indicated the presence of homogeneous 14–15S material (as determined from cosedimentation of rat liver rRNA species) (Fig. 4). Some inhomogeneity was detectable on the faster-sedimenting side of this peak, but it is not clear whether this should be regarded as a faster-sedimenting species of single- and double-stranded polynucleotides, or as an aggregative sedimentation of 14–15S double-helical RNA, analogous to that of native DNA (Burgi & Hershey, 1963); both factors could contribute. Such radioactivity, however, in no case represented more than 15% of the total load. The nucleotide composition measured for our preparations (adenylic acid, 25.6%; uridylic acid, 25.3%; cytidylic acid, 25.0%; guanylic acid, 24.3%) did not greatly deviate from that expected for a uniformly double-helical structure. It was similar to the nucleotide composition reported by Cox *et al.* (1970) for their 'Fraction 1'.

Examination of chromatographic polynucleotide fractions by sedimentation in self-generated *CsCl* gradients in conditions of near-equilibrium (Fig. 5) also excluded any larger cross-contamination of agarose-separated DNA and double-helical RNA.

Less than 5% of the DNA fraction sedimented outside the density region 1.66–1.72 g/ml. The double-stranded RNA fraction largely sedimented to the bottom during 30 h of centrifugation, indicating a slow dehydration by concentrated *CsCl*. The 23S rRNA from *Penicillium* precipitated nearly quantitatively under these conditions (Fig. 5).

The 'melting' profile of double-helical RNA (Fig. 6) was also in good agreement with published data (Cox *et al.*, 1970). The T_m of *Penicillium* DNA isolated by agarose chromatography was 3.5°C higher than that of rat liver DNA (75°C versus 71.5°C in 0.015 M-NaCl–0.0015 M-trisodium citrate), corresponding to G+C content of 51.5 mol% (Mandel & Marmur, 1968). *Penicillium* 23S rRNA gave, as expected (Cox, 1966), a broad, biphasic 'melting' range (Fig. 6). The 'melting' profiles thus clearly distinguished viral double-helical RNA from fungal polynucleotides.

Discussion

The separation of native DNA from viral double-stranded RNA reported here could result from their different abilities to bind and organize water.

Fig. 6. Thermal-denaturation profiles of *Penicillium* nucleic acids in 0.015M-NaCl-0.0015M-trisodium citrate (pH 7.5)

The readings were obtained with an Unicam SP. 500 spectrophotometer, equipped with a two-position water-heated thermoinsulated compartment. ○—○, Rat liver DNA, T_m 71.5°C; ○—○—○, *Penicillium* DNA, T_m 75°C; ●—●, double-stranded viral RNA from *Penicillium*, T_m 88.5°C; ●—●—●, 23S *Penicillium* rRNA.

Hydration of either native or denatured DNA in concentrated salt solutions is known to be considerably higher than that of either single- or double-stranded RNA species (De Kloet & Andrean, 1971); native DNA species support an ice-like intrahelical organization of water (Dahlberg & Rupprecht, 1971), and efficiently compete for water with agarose gels (Novaković *et al.*, 1972). The viral double-stranded RNA species obviously do not share this behaviour, and in fact resemble rRNA species with regard to retention in agarose gels at high ionic strength (Petrović *et al.*, 1973). Their retentional separation from native DNA should be useful in studies on RNA-virus replication (e.g. Zweerink *et al.*, 1972) and in detection and screening of RNA viruses.

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