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Isolation of Deoxyribonucleic Acid from Mammalian Tissues

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1. DNA has been isolated from different mammalian tissues. The DNA preparations were free from RNA, protein and polysaccharides and have a similar range of sedimentation coefficients (approx. 24s). 2. Protein was removed by a two-stage extraction with a phenol–cresol mixture by using a detergent with 4-aminosalicylate in the first stage and sodium chloride in the second. 3. Polysaccharides remained in solution when DNA was precipitated with 2-butoxyethanol in the presence of 0·5M-sodium chloride and 1·5M-sodium benzoate. 4. Ribosomal RNA was removed by precipitation in the presence of 3M-sodium chloride at 0°, when DNA remained soluble.

The isolation and characterization of DNA from different tissues is important in deciding whether there are variations in the nature of DNA–protein binding in the tissues (Kirby, 1964). The means by which DNA may be released and the yield of DNA are therefore clues in this investigation. Earlier studies with phenol and different salts have proved that hydrophilic salts released little or no DNA, lipophilic salts released DNA to which some protein remained bound, and salts that had lipophilic properties and the ability to form complexes with metals released DNA with minimum amount of protein (Kirby, 1957, 1958, 1959; Frearson & Kirby, 1964).

These results indicate that the chromosomes or nucleus contain a lipoprotein membrane that, if first denatured, renders the subsequent release of DNA difficult. The preferential extraction of rapidly labelled RNA by detergent and phenol at 60° is another example of DNA being trapped by denatured chromosomal protein (Scherrer & Darnell, 1962). The necessity for a complex-forming agent is evidence for some metal (possibly a transition metal) maintaining the chromosomal structure or function.

The phenol–*m*-cresol–8-hydroxyquinoline mixture used for the isolation of RNA (Kirby, 1965) proved to be a more powerful deproteinizing agent than phenol alone, and moreover DNA was separated from RNA without the use of enzymes. As a blender was used the DNA had sedimentation coefficients of 12–16s, and a method has now been devised that will avoid this shearing action. A number of modifications were required as DNA of 12–16s is easily soluble in 3M-sodium acetate, ribosomal RNA remaining insoluble, but DNA of higher molecular weight does not dissolve in this solution so that the separation of DNA from RNA is made more difficult.

The criteria of the quality of the product have been the absence of protein, RNA and polysaccharide from DNA rather than the molecular size of the DNA. The use of enzymes was avoided, because residual ribonuclease in particular may cause difficulties if the DNA is required for studies of hybridization with rapidly labelled RNA from different tissues.

METHODS

Preparation of 12–16s *DNA*. Method 2 of Kirby (1965) was used, except that 6% (w/v) 4-aminosalicylate and 1% (w/v) NaCl were used with an equal volume of phenol–cresol mixture in a blender. DNA, glycogen and transfer RNA were solubilized in cold 3M-sodium acetate buffer, pH 6·0, and the DNA with the glycogen were precipitated with 2-ethoxyethanol (1 vol.). The precipitate, which was centrifuged off (approx. 250*g*), was dissolved immediately in 0·1M-sodium acetate buffer, pH 6·0 (20–30ml. for DNA from three rat livers), and the glycogen centrifuged off at 60000*g* for 90min. at 2°. The supernatant solution was made 0·3M with respect to sodium acetate, and the DNA was precipitated by adding 2-ethoxyethanol (1 vol.). The precipitate was dissolved in 0·1M-sodium acetate buffer, pH 6·0, and dialysed against two or three changes of 0·1M-sodium acetate buffer, pH 6·0, or 5mM-KF.

Preparation of 22–24s *DNA*. The tissue was broken down in a glass vessel with a Teflon pestle with 10ml. of the aqueous mixture/g. of rat liver. This mixture consisted of sodium tri-isopropylnaphthalenesulphonate (1g.) (Kodak Ltd., Kirkby, Liverpool) to which butan-2-ol (6ml.) was added, then 100ml. of water, 6g. of sodium 4-aminosalicylate and 1g. of NaCl. The constituents should be mixed in

this order, the butan-2-ol being present to maintain the solubility of the detergent at 2°. Tissues were removed, dropped into this cold mixture, cut up and then broken down in a Teflon–glass homogenizer before adding an equal volume of the phenol–cresol mixture. Spleen and tumour tissues (Kirby, 1960*a*; O'Sullivan & Kirby, 1964) were broken down in 15–20 ml. of the mixture/g. of tissue.

The two-phase system was shaken gently for 20 min. at 20° and then centrifuged at 12000*g* for 25 min. at 5°. The top phase was carefully removed, made 3% with respect to NaCl and re-extracted (10 min. at 20°) with 0·5 vol. of the phenol–cresol mixture. The phases were again separated by centrifugation (17000*g* for 10 min. at 5°), the upper phase was carefully poured off and made 20% with respect to sodium benzoate, and the DNA was precipitated by the gradual addition of 2-butoxyethanol (distilled over 2,4-diaminophenol hydrochloride at 15 mm. Hg; Kirby, 1960*b*). The viscosity of the solution at first increased and then suddenly fell after the addition of about 0·1 vol. of butoxyethanol. DNA was precipitated as a gel and was centrifuged off at 500–1000*g* for 3 min. at 5°. The gel was dissolved in 0·1 M-sodium acetate buffer, pH 6·0, NaCl (3%, w/v) and sodium benzoate (20%, w/v) were added and the DNA was again precipitated with 2-butoxyethanol (0·15–0·18 vol., as no phenol was present). The dissolution and precipitation was repeated. The final precipitate was washed once with ethanol–water–sodium acetate (75:25:2, v/v/w) to remove butoxyethanol, dissolved in 0·1 M-sodium acetate buffer, pH 6·0, then made 3 M with respect to NaCl, and the solution remained at 0° for 16 hr. Ribosomal RNA precipitated as a gel was centrifuged off after layering the viscous solution over 5 ml. of 6 M-NaBr (17000*g* for 20 min. at 5°). This method gave a good separation of RNA and DNA. [The recovery and properties of ribosomal RNA and rapidly labelled RNA prepared by this method have been described by Parish & Kirby (1966).] The clear supernatant solution (including some NaBr solution) was poured off and DNA was precipitated by adding 2-ethoxyethanol (0·8 vol.); the DNA was removed with a spatula, dissolved in 0·1 M-sodium acetate buffer, pH 6·0, and then dialysed against 0·1 M-sodium acetate buffer, pH 6·0, or 5 mM-KF.

DNA was also separated from ribosomal RNA by extracting the material precipitated by butoxyethanol with a mixture of sodium acetate trihydrate (4 g.), sodium butyrate (12 g.) and water (100 ml.) brought to about pH 7·2 with 20% (v/v) acetic acid. Most of the DNA was soluble in the first extract (20 ml.), but usually two more extractions (with 10 ml. and 5 ml.) were carried out to complete the separation. Ribosomal RNA was centrifuged off each time and DNA was precipitated from the acetate–butyrate mixture, redissolved and dialysed as before.

Preparations were also made in which sodium lauryl sulphate (1%, w/v) (specially purified; British Drug Houses Ltd., Poole, Dorset) was used instead of tri-isopropylnaphthalenesulphonate, or with 0·3 M-sodium acetate buffer, pH 6·0, instead of the 4-aminosalicylate–detergent mixture. Other purifications of DNA after the second extraction with the phenol–cresol mixture were carried out by precipitation with 2-ethoxyethanol and extraction with 2-methoxyethanol–phosphate mixture as described by Kirby (1957). The 2-methoxyethanol and phosphate buffer were cooled to 0° before mixing and DNA was precipitated from the upper phase with 1 vol. of ethoxyethanol before dissolving and dialysing.

Other methods. The melting profiles of DNA dissolved in 5 mM-NaCl–5 mM-sodium acetate buffer, pH 6·0, or in 5 mM-KF were determined with a Unicam SP. 800 spectrophotometer.

The sedimentation profiles of DNA dissolved in 0·1 M-sodium acetate buffer, pH 7·0, were determined in a Spinco model E ultracentrifuge by the method of Shooter & Butler (1956).

Tests for the presence of glycogen, RNA and protein in the DNA were carried out as described previously (Kirby, 1957; Frearson & Kirby, 1964).

RESULTS AND DISCUSSION

The present method has been devised to isolate from mammalian tissues DNA that was free from RNA, protein and polysaccharides. The amount of

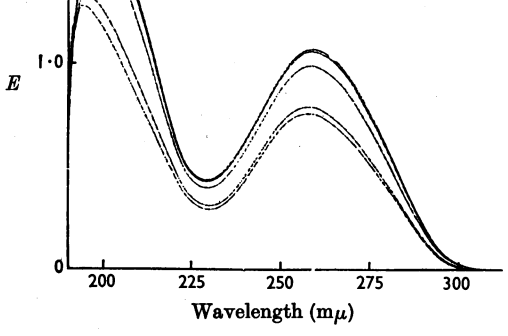

Fig. 1. Thermal denaturation of DNA from rat liver. The solvent was 5 mM-KF and five curves are shown. The spectrum remained unaltered between 24·5° and 60·5°. The remaining spectra in ascending order were measured at 68·5°, 76·0°, 87·0° and 90·0°.

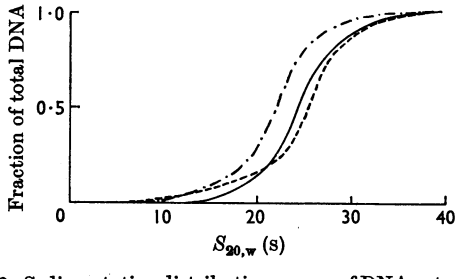

Fig. 2. Sedimentation distribution curves of DNA extracted with tri-isopropylnaphthalenesulphonate and 4-aminosalicylate from rat spleen (——), rat liver (– – – –) and rat sarcoma (—·—·) and dissolved in 0·1 M-sodium acetate buffer, pH 7·0. The sedimentation was carried out in the Spinco model E ultracentrifuge at 50470 rev./min. at 20°.

shearing has been minimized and the use of ribonuclease and 2-methoxyethanol have been avoided. 2-Methoxyethanol (70%, v/v) denatured DNA at a Na^+ concentration of 55mM (Hastings, 1964), as judged by hyperchromicity measurements.

As phenol is not immediately present when the tissue is broken down it is essential to use a detergent to inhibit nuclease activity. Tri-isopropylnaphthalenesulphonate and lauryl sulphate (especially purified) are equally suitable for this purpose, but the former has been generally used as it is a better lytic agent for bacteria (Kirby, Fox-Carter & Guest, 1967) and has produced RNA in good yield with no loss of secondary structure on centrifuging in a gradient of sulpholane (Parish & Kirby, 1966).

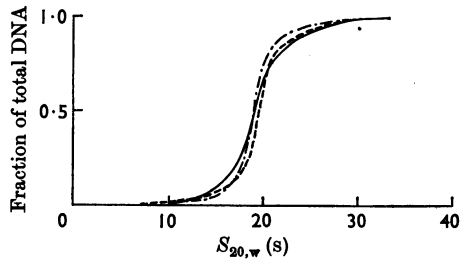

Fig. 3. Sedimentation distribution curves of DNA extracted with 0·3M-sodium acetate buffer, pH6·0, from rat spleen (—·—·), rat liver (——) and hepatoma (-----) and dissolved in 0·1M-sodium acetate buffer, pH7·0. The sedimentation was as in Fig. 2.

2-Butoxyethanol is a suitable solvent for the precipitation of DNA in the presence of sodium chloride and sodium benzoate, the latter salt being necessary as the solvent forms a two-phase system with 0·5M-sodium chloride. Relatively small volumes of the organic solvent are required for the precipitation and DNA is precipitated as a gel under these conditions. Polysaccharides are not easily precipitated at this concentration of butoxyethanol, but there is contamination with ribosomal RNA which can be completely removed from solution by precipitation in the presence of 3M-sodium chloride at 0°. The absence of an increase in E_{260} between 25° and 60° in 5mM-potassium fluoride is an indication of minimal amounts of RNA in a sample of DNA (Fig. 1) (cf. Hastings & Kirby, 1966). Potassium fluoride solutions do not absorb in the range 200–300mμ.

Another effective method is the selective extraction of DNA by a sodium acetate–butyrate mixture. DNA that had been precipitated by butoxyethanol was more readily soluble in this mixture than in sodium acetate alone, and though the same separation is achieved in these cases as with 3M-sodium chloride the mixture has advantages in separation of bacterial nucleic acids.

Table 1. *Yields of DNA extracted and purified by different methods*

DNA was extracted from the tissue (column 1) by the method shown in column 2 and purified as shown in column 3. The yield in column 4 is expressed as mg./100g. of tissue. 4AS (4-aminosalicylate) and methoxyethanol–phosphate are methods described in Kirby (1957). TIPNS–4AS and 0·3M-acetate are methods of preparation described in this paper (tri-isopropylnaphthalenesulphonate–4-aminosalicylate and 0·3M-sodium acetate respectively) and methods of purification (butoxyethanol–3M-NaCl or butoxyethanol–butyrate) are also as described in this paper.

Tissue	Method of extraction	Purification	Yield (mg./100g.)
Rat liver	4AS	Ribonuclease Methoxyethanol–phosphate	200
	TIPNS–4AS	Butoxyethanol–3M-NaCl	100
	TIPNS–4AS	Butoxyethanol–butyrate	120
	0·3M-Acetate	Butoxyethanol–3M-NaCl	100
Rat spleen	4AS	Ribonuclease Methoxyethanol–phosphate	950
	TIPNS–4AS	Butoxyethanol–3M-NaCl	900
	0·3M-Acetate	Butoxyethanol–3M-NaCl	720
Hepatoma	4AS	Ribonuclease Methoxyethanol–phosphate	450
	TIPNS–4AS	Butoxyethanol–3M-NaCl	270
	0·3M-Acetate	Butoxyethanol–3M-NaCl	100
Sarcoma	4AS	Ribonuclease Methoxyethanol–phosphate	435
	TIPNS–4AS	Butoxyethanol–3M-NaCl	340

The absence of detectable protein (less than 0·05%) from the final DNA preparation emphasizes the value of a two-stage extraction, one with detergent and one with sodium chloride in the aqueous phase, and the improved deproteinizing capacity of the phenol–cresol mixture. When 0·3 M-sodium acetate was used in place of detergent and 4-aminosalicylate, DNA with a slightly lower sedimentation coefficient (evidence of nuclease activity) was isolated but with about 0·5% of protein (Figs. 2 and 3). The detergent inhibits nucleases and with the aminosalicylate effects the separation of the protein from the DNA.

The yields of DNA are shown in Table 1.

DNA of low molecular weight dissolves easily in 0·1 M-sodium acetate, but DNA prepared by the detergent method and precipitated with butoxyethanol in the presence of high Na^+ concentration dissolves more slowly. The yield of DNA isolated by the present method varies with tissue compared with that obtained by the original method (Kirby, 1957). The yields of DNA may depend to some extent on the proportion of DNA in the tissue: liver has the smallest amount of DNA/g. of tissue and spleen has the greatest, and these give the smallest and largest yields of DNA by the present method.

However, the important feature is that DNA with a similar range of sedimentation coefficients can be isolated from different normal and tumour tissues and that in all cases it can be freed from protein, polysaccharide and RNA without the use of enzymes. It is possible that much of the shearing that does fragment the DNA takes place during the shaking of the two-phase system, and DNA of higher molecular weight may be obtained by suitable modifications of the systems for extraction.

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