CTYOPLASMIC DNA: SEPARATION INTO TWO COMPONENTS WITH DETERGENTS

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SUMMARY. Homogenates of rat liver in media containing sucrose, Triton X-100 and Mg^{++} were separated by centrifugation into nuclei and cytoplasm. A method is described for separating two types of DNA from the cytoplasmic fraction that closely resemble the DNA obtained from mitochondria and microsomes isolated from liver by conventional cell fractionation procedures.

INTRODUCTION

In previous papers (1,2) it was reported that deoxyribonucleic acid (DNA) was associated not only with mitochondria isolated from rodent liver but also with the microsomal fraction. Both of these types of DNA could be distinguished from nuclear DNA on the basis of [^{3}H]-thymidine incorporation.

In the present report, the separation of two types of DNA corresponding to mitochondrial and microsomal DNA from cytoplasmic extracts of liver homogenates prepared in Triton X-100 containing media is described. These experiments provide additional evidence for the existence of more than one type of cytoplasmic DNA in the liver and also provide a novel means for obtaining them that obviates isolation of mitochondria and microsomes.

MATERIALS AND METHODS

Thymidine-methyl-[^{3}H] (3 - 15 c/m mole) was obtained from Schwarz Bioresearch. Several commercial preparations of Triton X-100 were used with indistinguishable results. Sodium deoxycholate was obtained from Fisher Scientific Co.

Male Sprague Dawley rats (250-300 g.) were injected intraperitoneally with 250 μc [^{3}H]-thymidine and fasted overnight. The livers were then removed and one-half was homogenized in sucrose solution and fractionated by differential centrifugation into nuclei, mitochondria and microsomes as described previously (1). The other half was homogenized with 9 times its weight of

cold 0.25 M sucrose containing 0.5% Triton X-100 and 2 mM Mg^{++} (3). The nuclei were sedimented at 2000 r.p.m. The supernatant fluid (cytoplasm) was then supplemented with sufficient 0.1 M EDTA, 10 per cent Triton X-100, 10 per cent deoxycholate, and NaCl to give final concentrations of 3 mM, 1.0 per cent, 0.6 per cent, and 0.25 M, respectively. The mixtures were kept at 2° overnight and then were centrifuged for 30 min. at 13000 r.p.m. in the Sorvall HB-4 rotor to give a pellet, A. The supernatant fluid was recentrifuged for 90 min. at 45000 r.p.m. in the A-211 rotor of the International B-60 centrifuge to yield a pellet, B, and a final supernatant, C.

Isolation of nucleic acids. Nucleic acids were obtained from the isolated fractions by extraction with sodium dodecyl sulfate (SDS) and phenol (1) and precipitation with ethanol.

For analytical purposes, the DNA containing samples were redissolved in water containing 0.3 N NaOH and the mixtures were incubated for 1 hour at 37° to remove RNA and small molecules (4). Perchloric acid was added to provide a concentration of 0.6 N and the DNA was centrifuged down in the cold at 13000 r.p.m. for 30 min. in the HB-4 rotor. The precipitate was washed once with 0.3 N perchloric acid and then dissolved in 0.1 N NaOH or in 0.3 N perchloric acid by heating for 20 minutes at 90°.

Measurements. DNA concentration was determined by the diphenylamine reaction (5) or was calculated from the ultraviolet absorption of the solutions at 260 mμ. Radioactivity was measured in a Beckman LS-235 liquid scintillation spectrometer using samples dispersed in a Triton X-100-toluene scintillator mixture.

RESULTS AND DISCUSSION

In our earlier work on the isolation of microsomal DNA (1), it was noted that after disruption of the microsomal fraction with deoxycholate (DOC) the microsomal DNA remained sedimentable. It was therefore of interest to determine if mitochondrial DNA behaved in a similar or different manner.

TABLE I

Separation of cytoplasmic DNA from liver homogenates with detergents. The homogenates were fractionated as described in the text.

Isolation medium	Fraction	DNA Content	DNA Specific Activity
		μ mole P per liver	d.p.m./μ mole P
Sucrose	Mitochondria	0.254	90,200
	Microsomes	1.44	34,400
Sucrose-Mg^{++}-triton	Sediment A	0.08	47,900
	Sediment B	0.216	36,800
	Supernatant C	0.208	113,000
	Nuclei	59.4*	19,100
Sucrose	Mitochondria	0.104	167,000
	Microsomes	0.316	58,900
Sucrose-Mg^{++}-triton	Sediment A	0.082	115,000
	Sediment B	0.222	67,500
	Supernatant C	0.266	163,000
	Nuclei	58.1*	18,300

* Recovery of DNA from nuclei was always incomplete because of the difficulty of separating the highly viscous aqueous phase from the phenol phase.

Preliminary experiments showed that the DNA in isolated mitochondria was completely solubilized by 0.3% DOC. Triton X-100 at a concentration of 0.5% solubilized about two-thirds of the DNA in mitochondria and none of the DNA in microsomes.

Since Hymer and Kuff (3) had shown that nuclei could be isolated from homogenates of tissues in Triton X-100 containing media, our findings suggested that it should be possible to separate mitochondrial and microsomal DNA from the cytoplasmic fraction of such homogenates by centrifuging after adding deoxycholate. The results in the Table show that this was the case provided that the detergent concentrations were double those used for the isolated mitochondria and microsomes. The high speed supernatant fraction C, and the sediment B, from the triton homogenate cytoplasm contained about equal amounts of DNA that closely resembled the mitochondrial and microsomal DNA, respectively, in specific activity. The low speed sediment, A, contained smaller amounts of DNA that appeared to be a mixture of the two types, judging from the specific activity.

It should be noted that the separations were not perfect since the specific activity of the B fraction DNA and the microsomal DNA did not agree exactly nor did that of the C fraction DNA and the mitochondrial DNA. Nevertheless, the data indicated that the efficiency of the separations must have been quite high and the fact that isolated mitochondria and microsomes are also subject to some cross contamination (1) must also be considered as a possible explanation for the discrepancies.

Attempts to further characterize the B and C fraction DNA have been limited by the similarity of the physical properties of liver mitochondrial, nuclear, and microsomal DNA. However, a preliminary experiment with Dr. W. R. Kidwell of this Institute indicated that the fractions could be distinguished by their reassociation kinetics; this phenomenon is under further investigation.

Efforts to simplify the detergent procedure have not been successful. Failure to add NaCl, EDTA to chelate the Mg^{++}, or to store the samples overnight at 2° led to inadequate separations of the two types of DNA. Recent experiments have shown that similar separations of cytoplasmic DNA could be achieved with very mild homogenization. Minced liver was dispersed in the Triton X-100 medium containing deoxycholate using several manual strokes of a

loosely fitting (0.3-0.5 mm clearance) Kel-F pestle in a smooth glass test tube; the yields of both types of cytoplasmic DNA were the same as with the regular procedure. This result would appear to effectively dispel the claims of Fromson and Nemer (7), based upon the use of a Dounce homogenizer (clearance 0.01-0.03 mm), that microsomal DNA was an artefact of homogenization.

The use of detergents for the isolation of mitochondrial and microsomal DNA is a new method which avoids the usual cell fractionation normally required for the isolation of these particles. This method should be useful for multiple samples since several homogenates can be processed at the same time more readily than if mitochondria and microsomes had to be isolated from each. The method by which the detergents exert their effect in separating the two types of DNA is not clear, since the effect is not immediate, but requires overnight storage. In the latter respect it resembles the widely used Hirt procedure which employs sodium dodecyl sulfate for separating nuclear from mitochondrial and viral DNA (6). This detergent solubilizes both mitochondrial and microsomal DNA under our conditions.

Disruption of cells in detergent media provides a simple, efficient method for separating nuclei from cytoplasm in a single step and consequently can be expected to produce fewer artifacts than other procedures. Since two types of DNA resembling mitochondrial and microsomal DNA were isolated from the cytoplasmic fraction of such preparations, the earlier findings of DNA associated with the microsomal fraction of rodent liver (1,2) are corroborated. The functional significance of this DNA remains to be established.

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