SEPARATION OF NATIVE AND DENATURED DNA, RNA AND HYBRID ON SODIUM IODIDE GRADIENTS

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1. Introduction

Cs_2SO_4 is the salt most frequently used for buoyant density analyses of DNA–RNA hybrids because DNA, RNA and hybrid all band in Cs_2SO_4 gradients [1]. CsCl can also be used for this purpose although RNA is too dense to band in CsCl gradients. However, neither of these salts is entirely satisfactory since they resolve native and denatured DNA poorly, an important consideration in studies of RNA-driven DNA–RNA hybridisation reactions using labelled DNA [2], in the analysis of which it is necessary to distinguish between RNA–DNA hybrids and reassociated DNA duplexes. The introduction of NaI gradients for DNA [3] prompted us to examine this salt as a substitute for caesium salts although, in a later investigation, De Kloet and Andrean [4] reported that most RNA species are insoluble in solutions of NaI. Experiments reported in this paper show that NaI gradients clearly distinguish between native DNA, denatured DNA, RNA and DNA–RNA hybrid, and that RNA is soluble in NaI solutions so long as care is taken to remove heavy metal ions from the NaI.

2. Materials and methods

[^{3}H] DNA was isolated [5] from the nuclei of LS cells (a substrain of mouse NCTC strain L929 which grows in suspension) which had been grown for 18 hr in the presence of methyl-[^{3}H] thymidine (22.4 mCi/μmole, 1–10 μCi/ml); it was denatured in 0.05 M NaCl by heating at 100° for 10 min. DNA–RNA hybrid was synthesized [6] by Dr. G. Threlfall: heat-denatured [^{3}H] DNA (10 μg) was incubated for 1 hr at 37° in 50 mM Tris-HCl, pH 8, 2.5 mM $MnCl_2$, 1 mM dithiothreitol and 0.1 mM EDTA with 0.8 mM ATP, GTP, CTP and UTP, and RNA polymerase (125 units) prepared from *E. coli* to fraction 6 of the Burgess [7] procedure omitting the phosphocellulose step, in a total volume of 1.0 ml. The hybrid was isolated by chromatography on Sephadex G-50 in 0.05 M NaCl. [^{3}H] RNA was isolated from LS cells grown for 18 hr in the presence of [5-^{3}H] uridine (29.8 mCi/μmole, 1 μCi/ml). Nuclear and cytoplasmic fractions were prepared [8] and RNA extracted from both by shaking with phenol at 20° in the presence of 6-aminosalicylate. The nuclear RNA preparation was digested exhaustively with DNAase I. Both RNA preparations were purified by chromatography on Sephadex G-100 in water; only the excluded peak was collected. *Neurospora crassa* nuclease was prepared as described by Linn and Lehman [9] omitting the phosphocellulose and hydroxyapatite chromatography steps.

NaI (May and Baker Ltd.) was dissolved in 0.1 M Tris-HCl, 0.01 M EDTA, pH 8 (150 g in 90 ml) to give a solution of density 1.86 g/cm^3. Na_2SO_3 (10 mg/100 ml) was added to prevent oxidation of the NaI and the solution was passed through a Millipore filter, then a bed of Chelex-100 resin (10 ml) at about 1 ml/min; Na_2SO_3 (10 mg/100 ml) was again added to the solution. CsCl (BDH Ltd.) was dissolved in the same buffer (150 g in 100 ml) to give a solution of density 1.8 g/cm^3, filtered and passed through Chelex-100 resin as for the NaI solution. These stock solutions were stored at room temperature. The labelled nucleic acids, together with 5 μg of carrier mouse-embryo DNA (native and denatured) were mixed with stock

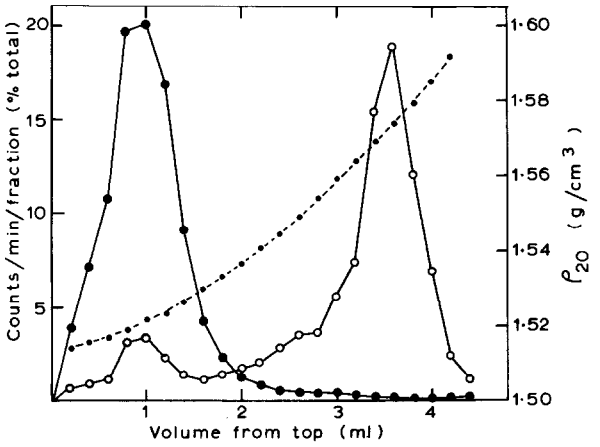

Fig. 1. Isopycnic banding of native (●–●–●) and denatured (○–○–○) LS cell [^{3}H] DNA (20,000 cpm) in NaI gradients (ρ_i 1.546 g/cm^3) centrifuged at 45,000 rpm for 66 hr at 20°; (●--●--●) shows the density gradient.

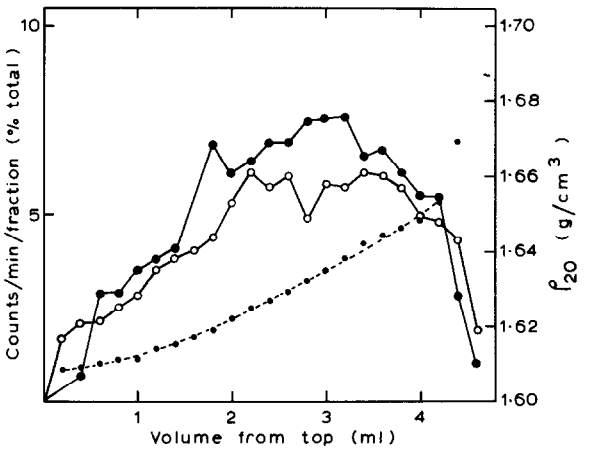

Fig. 2. Isopycnic banding of [^{3}H] RNA from nuclei (●–●–●; 25,000 cpm) and cytoplasm (○–○–○; 30,000 cpm) of LS cells in NaI gradients (ρ_i 1.630 g/cm^3) centrifuged at 45,000 rpm for 65 hr at 20°; (●--●--●) shows the density gradient.

NaI or CsCl and sufficient buffer was added to adjust the density of each mixture to that required as measured by its refractive index. The solution (4.6 ml) was placed in a 10 ml polypropylene tube, overlaid with paraffin, and centrifuged at 45,000 rpm and 20° in the 10 × 10 titanium fixed-angle rotor of the MSE Superspeed 65 Mark II centrifuge for 63–66 hr. The rotor was allowed to come to rest without braking, the gradients were unloaded by upwards displacement with saturated NaI or CsCl solution, respectively, and 0.2 ml fractions were collected. The refractive index of each fraction, from which its density was calculated, was measured; 0.1 ml of each fraction was counted after mixing it with 1 ml of water and 10 ml of Triton X-100 toluene-based scintillator. For NaI solutions it was necessary to add 1 drop of mercaptoethanol to each vial to prevent formation of free iodine, a very effective quenching agent.

3. Results

The density gradient generated in a NaI solution centrifuged in a fixed-angle rotor is much shallower than that in equivalent CsCl or Cs_2SO_4 solutions under the same conditions. For example, the density gradient formed at 45,000 rpm in a NaI solution of initial density (ρ_i) 1.545 g/cm^3 was 1.514 to 1.584 g/cm^3 while that in a CsCl solution (ρ_i 1.750 g/cm^3) was 1.702 to 1.820 g/cm^3 and that in a Cs_2SO_4 solution (ρ_i 1.540 g/cm^3) was 1.430 to 1.670 g/cm^3.

Although NaI gradients are shallow, both denatured and native LS cell DNA of molecular weight 1–2 × 10^6 daltons band sharply in them (fig. 1). The separation between native and denatured DNA is much greater, in terms of gradient volume, than in CsCl (or Cs_2SO_4) gradients, partly because of the shallow gradient but mainly because there is a much greater difference between the buoyant densities of native and denatured DNA (table 1). This large difference is not due to iodination of the denatured DNA since denatured DNA recovered from a NaI gradient (and centrifuged through Sephadex G-25 [10] to remove NaI) banded in CsCl with the same buoyant density as denatured DNA which had not been dissolved in NaI. Also, it is unlikely to be caused by the formation of heavy metal salts of denatured DNA, as can occur in CsCl [11], since the NaI solutions had been passed through Chelex-100 resin.

As in CsCl and Cs_2SO_4, in NaI the buoyant density of RNA is much greater than that of DNA (table 1). Unfractionated RNA does not band sharply in NaI, but is rather heterogeneous with respect to buoyant density (fig. 2). However, this is not due to insolubility of RNA in NaI solutions as previously reported by De Kloet and Andrean [4]; although RNA does pre-

Table 1
Buoyant densities of LS-cell nucleic acids in sodium iodide and caesium chloride.

	Sodium iodide			Caesium chloride
	Buoyant density (g/cm^3)	Range (g/cm^3)	No. of measurements	Buoyant density (g/cm^3)
Native DNA	1.522	1.519–1.523	4	1.700
Denatured DNA	1.574	1.571–1.577	5	1.715
RNA	1.63	1.62 –1.65	4	> 1.9
DNA–RNA hybrid	1.540	1.536–1.542	5	1.775

For these measurements the initial density of the sodium iodide was adjusted so that the nucleic acid banded at, or near, the centre of the gradient; centrifugation conditions were as described in Materials and methods.

cipitate from untreated NaI solutions, even in the presence of 0.01 M EDTA, it is quite soluble in NaI solutions which have been passed through Chelex-100 resin (table 2).

Synthetic DNA–RNA hybrids band sharply in NaI gradients (fig. 3), in a position intermediate between those of native and denatured DNA (table 1). In contrast, in both CsCl and Cs_2SO_4, the buoyant density of DNA–RNA hybrid is intermediate between those of denatured DNA and RNA. Incubation of this hybrid with *N. crassa* nuclease, which removes the short 'windows' of single-stranded DNA present in this hybrid [12], reduces its mean buoyant density only slightly. However, it does cause significant broadening of the band (fig. 3), reflecting the reduction in the mean molecular weight of the hybrid from 400,000 to 50,000 daltons (Birnie, Hell and Threlfall, unpublished).

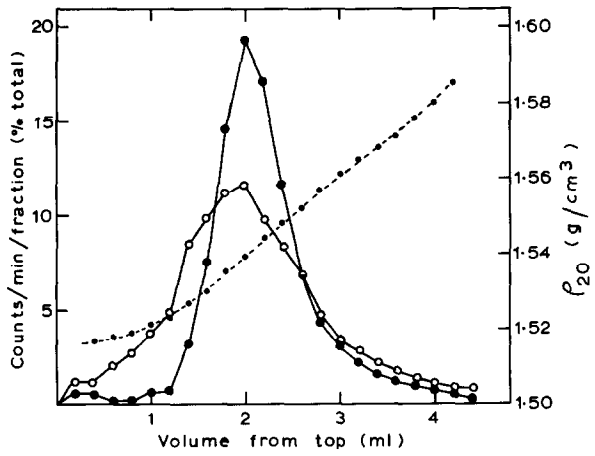

Fig. 3. Isopycnic banding of synthetic DNA–RNA hybrid before (●–●–●; 15,000 cpm) and after (○–○–○; 10,000 cpm) treatment with *N. crassa* nuclease in NaI gradients (ρ_i 1.545 g/cm^3) centrifuged at 45,000 rpm for 63 hr at 20°; (●--●--●) shows the density gradient. The nuclease-treated hybrid was first centrifuged through Sephadex G-25 in 0.05 M NaCl [10] to remove small oligodeoxynucleotide fragments.

4. Discussion

There are two striking differences between density gradients of NaI and those of CsCl and Cs_2SO_4. These are, first, the much greater effect that denaturation has on the buoyant density of DNA and, second, the relatively small increase in buoyant density which occurs when one strand of the DNA duplex is replaced with an RNA strand. De Kloet and Andrean [4] have reported that the buoyant density of double-stranded RNA in NaI is much less than that of single-stranded RNA. Thus, although it has been shown that the buoyant density of a DNA in NaI gradients is dependent on its GC content [3,4], it appears that the buoyant density of both DNA and RNA is more dependent on secondary than on primary structure. The reason for this is unknown. In the case of DNA, it is not due to iodination of denatured strands, nor to formation of heavy metal salts. A possible explanation is that one (or more) of the ring nitrogen atoms involved in base-pairing in the duplex becomes available to form an

Table 2
Solubility of RNA in sodium iodide solution.

Solvent for NaI	Treatment of NaI solution	RNA precipitated in 4 hr (%)	RNA precipitated in 24 hr (%)
Water	None	48	73
Tris-EDTA	None	24	38
Water	Chelex-100	0	1

LS-cell nuclear [^{3}H]RNA (0.2 μg, 4000 cpm) in 0.5 ml of water was mixed with 1.5 ml of NaI solution (ρ_i 1.86 g/cm^3). The mixtures were incubated at 20° for the times indicated then centrifuged at 11,000 rpm (Sorvall HB-4 rotor) for 20 min; two 0.5 ml samples were taken from the supernatant solution for liquid scintillation counting.

iodide salt with a concomitant increase in density of the denatured strand. This could also occur in, for example, CsCl gradients, but would have a much less noticeable effect because of the low density of the chloride ion compared to that of the iodide ion.

Another observation of possible significance is the heterogeneity of RNA in NaI gradients, also noted by De Kloet and Andrean [4]. This may well be due to variations both in base composition and secondary structure and suggests the possibility of fractionating RNA on some basis other than size and shape of the molecule.

Although NaI cannot replace CsCl or Cs_2SO_4 as a gradient material for all measurements of the buoyant density of nucleic acids, it has a number of practical advantages in some applications. This is particularly so in studies of DNA–RNA hybridisation. In CsCl and Cs_2SO_4 gradients unpaired stretches of DNA reduce the buoyant density of a hybrid while unpaired stretches of RNA increase it. Thus an annealed hybrid which appears to be a well-matched duplex by virtue of its buoyant density in these salts could in fact consist of a few duplexed regions with very long stretches of unpaired DNA and RNA. In contrast, 'tails' of unpaired DNA and RNA both increase the buoyant density of hybrids in NaI gradients so facilitating the detection of extensive mismatching in a hybrid. Also, in similar studies of the reannealing of denatured DNA, in which the quality of the duplex formed must be assessed, the very large separation between native and denatured DNA in NaI gradients is extremely useful.

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