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The properties of native and denatured DNA in buoyant rubidium trichloroacetate at neutral pH

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ABSTRACT

Aqueous RbTCA is generally suitable as a buoyant solvent for both native and denatured DNA at neutral pH and room temperature. Native PM-2 DNA II, for example, is buoyant at 3.29 M salt, 25°C; whereas the denatured strands band together at 4.52 M. Two properties of the solvent make this system uniquely useful for separations based upon the extent of secondary structure. First, the melting transition temperature for chemically unaltered DNA is depressed to room temperature or below. Second, the buoyant density increase accompanying denaturation is extraordinarily large, 174 mg/ml for PM-2 DNA II. This value is three times that found in aqueous NaI and ten times that for CsCl. The properties of the RbTCA buoyant solvent presented here include the compositional and buoyant density gradients and the buoyant density dependence upon base composition. The DNA remains chemically unaltered after exposure to RbTCA as shown by the absence of strand scissions for closed circular DNA and by the unimpaired biological activity in transformation assays. Intact virion DNA may be isolated by direct banding of whole virions in RbTCA gradients without prior phenol extraction. Strongly complexed or covalently bound proteins may be detected by their association with the buoyant polymer in the denaturing density gradient.

INTRODUCTION

A number of aqueous inorganic salt solutions (including Cs_2SO_4, KBr, NaI, RbBr, RbCl, $Cs(CH_3CO_2)$, $Cs(CO_2H)$, CsBr and CsI) are used as binary solvents for the buoyant banding of, in particular the nucleic acids. CsCl is presently the best characterized of the density gradient salts. For a description of this technique and a survey of specific experimental applications, general reviews are available (1, 2). The choice of a density gradient salt is governed primarily by the accessible solution density range. Other desirable characteristics include chemical inertness and stability; a relatively low viscosity to reduce centrifugation time; solubility of the macrospecies; and transparency in the wavelength region of interest. Neutral aqueous CsCl is most useful for the buoyant banding of native and of heat-denatured DNAs but has too low a density range for RNA. Aqueous Cs_2SO_4 often causes precipitate formation with high molecular weight

single-stranded RNAs (3). Mixed Cs_2SO_4-DMSO gradients have been used for banding RNA (4) but this technique, involving a four component system, is complex and the results difficult to reproduce, the apparent RNA buoyant density being a strong function of the band position in the tube. More recently an alternative mixed salt gradient, CsCl-guanidinium chloride, has been used to band DNA, DNA-RNA hybrids, and RNA as resolved components in a single preparative gradient (5). Although guanidinium chloride is a denaturant, nucleic acids band in the native form in this mixed salt system.

A neutral, chemically inert binary solvent able to form a denaturing density gradient avoids the complexities of the mixed solvent systems. Alkaline CsCl, a commonly used denaturing buoyant medium, is not suitable for the banding of DNA with included ribonucleotides, nor of RNA. There is a small but measurable rate of strand scission for DNA due to depurination in alkaline CsCl (6). In addition, this system is four-component in nature and involves the formation of a partially titrated DNA. Aqueous sodium and potassium TFA* have been used (7) to study the hydration of native and denatured T7 DNA. However, T7 DNA at buoyant salt densities in KTFA denatures at 53°C, a temperature too high to be useful for routine applications in the ultracentrifuge.

The effects of a number of denaturing salts, all 1:1 electrolytes, on the stability of the native DNA duplex structure have been examined (8), and only the alkali salts of TFA^-, ClO_4^-, and TCA^- were found capable of displacing the melting transition of DNA to the vicinity of room temperature. In this paper and those to follow we document the use of a series of highly potent denaturing salts, the neutral alkali metal salts of trichloroacetic acid, to form density gradients suitable for the isolation, purification, and characterization of native and denatured DNA and RNA and of certain nucleoproteins at neutral pH and room temperature.

MATERIALS AND METHODS

A. Preparation of Salts. RbTCA was prepared by titration of Rb_2CO_3 with HTCA to a pH of 7.0. The solution was flushed with N_2 during the titration. The solution was treated with heat-activated charcoal overnight at room temperature then filtered through a 0.22 μ Millipore filter and evaporated to dryness at room temperature in a Büchi rotary vacuum evaporator. The resulting white, needle-shaped crystals were crushed to a powder and dried to constant weight under vacuum over P_2O_5 at room temperature. The anhydrous salt is stable indefinitely if stored under desiccation at room

temperature. The results of a chemical analysis of the RbTCA sample used in this investigation (Galbraith Laboratories, Knoxville, Tennessee) are shown in Table I.

TABLE I: Chemical Analysis of Anhydrous RbTCA.

Element or Group	Calculated % (by weight)	Determined %
C	9.7	9.39
H	0.0	0.08
Cl	42.9	42.6
Rb	34.5	34.18
Na	0.0	0.006
K	0.0	0.11
Li	0.0	< 0.001
Cs	0.0	0.0176
Carbonate	0.0	0.37

All salt solutions were prepared immediately before use and were buffered with 0.03 M Tris and 0.0025 M EDTA at pH 8.0. Freshly prepared solutions normally exhibited a slight turbidity, which was readily removed by filtration through a 0.22 μ Millipore filter. The TCA anion rapidly decomposes in solution at temperatures near 80°C. The rate of this decomposition is imperceptible at room temperature. A 4.0 molar solution of RbTCA typically exhibits an A_{280} of 0.5 and is opaque at 260 nm. The saturation concentration is approximately 5 M at 25°. Nucleic acids were therefore detected by their absorbance at 280 nm, where the DNA molar absorbance is reduced by 48% for a DNA of the base composition of PM-2, 41.5% (G+C), compared to that at 260 nm (9).

B. Properties of Aqueous RbTCA Solutions. Solution densities, ρ_{25}, were determined with a precision of ± 0.001 g/ml by weighting 500 μℓ aliquots in a calibrated pipette at approximately 25°C. Refractive indices, n^D_{25}, were measured at 25° with a thermostated Zeiss refractometer. The resulting data was fit to a first degree polynomial by least squares, with the results

$$\rho_{25} = -7.7805 + 6.5869\ n^D_{25} \quad (1a)$$

$$\rho_{25} = 1.0045 + 0.1448\ c_{25} \quad (1b)$$

where c_{25} is the solution molarity at 25°C. These values are in good agreement with a previous report of the dependence of density upon molarity for aqueous RbTCA solutions (10).

C. Preparation of DNAs. Bacterial virus PM-2 was grown in the Pseudomonad bacterium BAL-31 and the DNA isolated as described by Salditt et al. (11).

Tritium labelled PM-2 DNA was prepared similarly, except that the bacteria were grown in modified BAL synthetic media (12) containing 1/4 the normal amount of yeast extract. The bacteria were infected with a phage multiplicity of 10 at a turbidity of 0.2-0.4 OD_{650}. Ten minutes post infection, 0.25 mC of [^{3}H]thymidine/liter of culture were added followed by a second equal addition 10 minutes later. The specific activity of the [^{3}H]PM-2 DNA thus prepared was 2.6 x 10^4 cpm/µg.

PM-2 DNA II was prepared from DNA I by irradiation with a 25 watt tungsten lamp at a distance of 6.5 cm in the presence of 0.2 moles EtdBr per mole of nucleotide. The solution was irradiated 1-2 hrs. at room temperature until 50% of the molecules were converted to DNA II, as monitored by agarose gel electrophoresis in the presence of 0.5 µg/ml EtdBr (13). The two circular DNA forms were separated by banding in CsCl/EtdBr. Alkaline sedimentation velocity analysis of this DNA showed that 50% of the molecules contained only one strand scission.

Lysates were prepared from bacterial virus T7 (14), and T7 DNA was isolated by PEG precipitation of the virus followed by CsCl equilibrium banding and phenol extraction (15). The DNA exhibited an absorbance ratio, A_{260}/A_{280}, of 1.86 and sedimented as a single band in neutral CsCl. Ad2 DNA, prepared by pronase treatment of phenol-extracted virion DNA followed by a second phenol extraction, was a gift from Dr. Carl Anderson. λ DNA (λh80CI857St68) was received as a gift from Dr. Susan Mickel, [^{3}H]mouse mitochondrial DNA from Dr. David Clayton, and ColE1 plasmid DNA from Dr. Martin Freundlich. [^{3}H]Ad2 DNA and [^{32}P]Ad2 virus were generously provided by Dr. Richard Roberts, [^{35}S]Ad2 virus by Dr. Carl Anderson, and [^{14}C]Poly d(AT) by Dr. Joseph Kates. M. luteus DNA was purchased from Sigma Chemical Co. and was phenol extracted prior to use.

D. Analytical Ultracentrifugation. A Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner, multiplex accessory, rapid return assembly and mirror optics was used. Charcoal-filled Epon centerpieces, 12 mm, were used for buoyant density experiments. Sedimentation equilibrium was reached within 36-96 hrs., depending upon the initial salt molarity and temperature. A DNA concentration of 0.1-0.5 µg/DNA per band was used. The salt redistribution at equilibrium was measured using the Schlieren optical system (16). Metallographic plates developed with Kodak D19 were measured on a Nikon comparator at 10 X magnification.

E. Preparative Ultracentrifugation. Step gradients of 3 ml total volume were prepared by sequentially layering five solutions differing in density

by 60 mg/ml into polyallomer tubes. The DNA sample was then directly mixed with the layer of greatest density in order to band in the denaturing density range. To band in the native density range, the DNA sample was layered as a thin lamellum on top of the preformed gradient. The tubes were filled to 4.5 ml with an inert mineral oil and centrifuged in the Beckman SW65 rotor at 40-45 Krpm, 25°C for 36 hrs. If DNA is directly mixed with a density layer close to the expected equilibrium buoyant layer, centrifugation times may be reduced to less than 24 hrs. The resulting density gradient is steep enough so that both native and denaturing density ranges are accessible in the same tube. The tubes were fractionated by dripping from the bottom onto filter paper. Radioactively labelled polymer was TCA-precipitated and detected by liquid scintillation counting. Rubidium contains a natural isotope, $^{37}Rb_{88}$, of 27.8% relative abundance which decays with the emission of beta particles. Although the isotope half life is long (5×10^{11} yrs.) and therefore the specific activity is very low, a detectable increase in the background was obtained unless the filters were carefully washed.

F. Transformation. ColE1 plasmid DNA was incubated in RbTCA and the effect on the transformation frequency of E. coli C was investigated by the procedure described by Goebel and Bonewald (17). A portion of the plasmid DNA was pretreated by incubation in a 3.5 M RbTCA solution at room temperature for 3 days, followed by dialysis. Parallel DNA samples, incubated and control, were added to a $CaCl_2$-treated bacterial culture and aliquots of the culture were plated on L-plates. Transformants were detected by the appearance of plaques in a sparse lawn of colicin sensitive bacteria (W 3310) overlaid above $CHCl_3$-killed, transformed colonies.

G. Chemicals. EtdBr, Tris and EDTA were purchased from the Sigma Chemical Company. CsCl was obtained from the Harshaw Chemical Co., Rb_2CO_3 was obtained as optical density gradient grade from ICN, and reagent grade HTCA from Baker. Reagent grade isopropanol was purchased from Mallinckrodt.

RESULTS

A. Neutral Buoyant Banding of Native and Denatured DNAs. Native PM-2 DNA II is buoyant in RbTCA (25°) at 3.29 M, whereas the denatured DNA bands at 4.52 M. This increase in molarity corresponds to a buoyant density increment of 174 mg/ml and is more than tenfold greater than that obtained with heat-denatured DNA in CsCl (18). A similar buoyant separation between native and denatured Ad2 DNAs in a preparative RbTCA equilibrium gradient is presented in Figure 1. [^{3}H] Ad2 DNA was denatured by addition to a 4.7 M

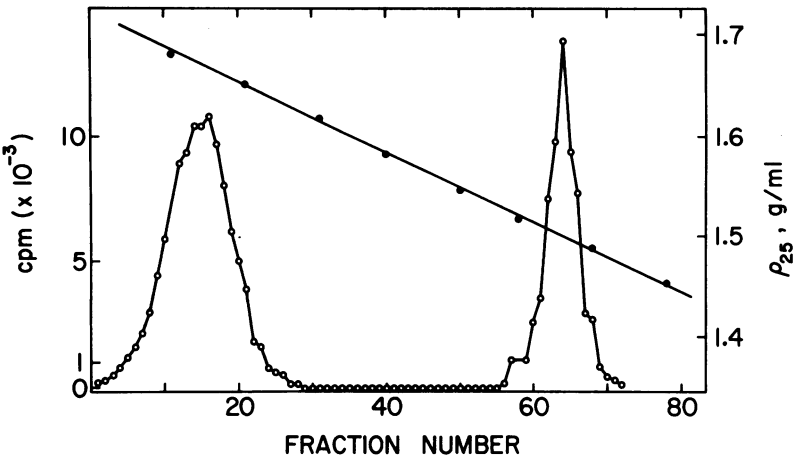

Figure 1. Buoyant banding of [^{3}H] Ad2 DNAs, native and denatured, in a RbTCA preparative gradient. A 10 µℓ aliquot of a [^{3}H] Ad2 DNA solution was mixed with 0.6 ml of a RbTCA solution at 1.68 g/ml to form the bottom layer of the step gradient. Four additional RbTCA solutions, each of 0.6 ml volume and differing in density by 0.06 g/ml were sequentially layered into a 5 ml polyallomer tube. A lamellum of 10 µℓ of [^{3}H] Ad2 DNA solution was layered on the top of the gradient and the tube topped with a light mineral oil. Centrifugation was performed in a Beckman SW65 rotor at 45 Krpm, 20°C for 36 hrs. The tube was fractionated by dripping 5 drop fractions from the tube bottom onto Whatman 3 MM filter paper. Approximately every 10th fraction was collected in a vial for refractive index determination. The filters were treated with 10% cold TCA, washed with 95% ethanol, dried, and counted in a liquid scintillation cocktail.

RbTCA solution at room temperature and added to a step gradient prepared as described above. Denatured Ad2 DNA is buoyant at 4.67 M RbTCA (1.675 g/ml), whereas native Ad2 DNA bands at 3.41 M (1.498 g/ml). The band of denatured DNA is broader than that of the native species, as expected.

B. Practical Buoyant Density of Native and Denatured PM-2 DNA II. The practical buoyant density, θ, is the density at one atmosphere of a solution in which the polymer bands at the root-mean-square (rms) position of a 1.1 cm solution column in a sector shaped cell at 44 Krpm and 25°C (19). In RbTCA solutions there are two nonoverlapping buoyant density ranges useful for banding of native and for denatured DNAs. The practical buoyant densities of both native and denatured PM-2 DNA II were determined by banding in a series of salt solutions differing in density by approximately 0.010 g/ml and interpolating to the rms position (20). In Figure 2 the radial difference between band center (r_o) and the rms location (r_e) is shown as a function of the initial solution density or salt molarity for both the

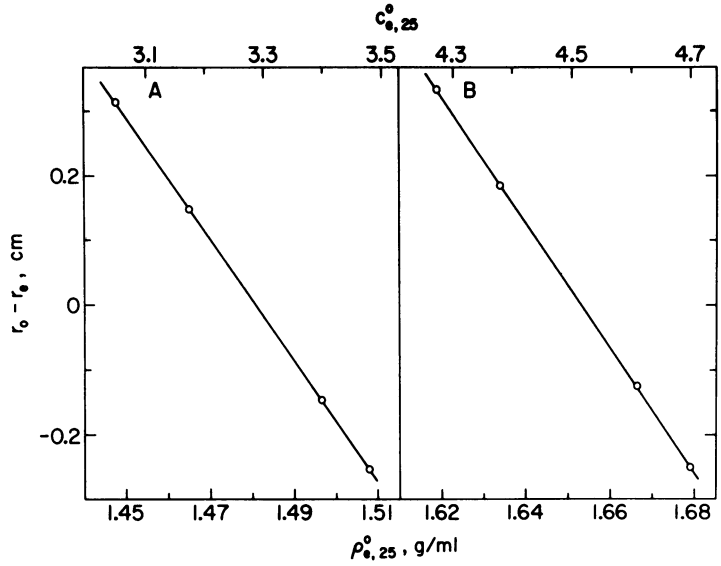

Figure 2. Determination of the practical buoyant density of PM-2 DNA II. The radial distance between band center (r_o) and the rms position (r_e) was plotted as a function of the initial solution density measured at atmospheric pressure and 25° (lower abscissa) or of the initial solution molarity (upper abscissa) for the native (A) or the denaturing (B) density range. All experiments employed 0.5 µg of DNA in a sector shaped cell at 44 Krpm, 25°C, in 0.38 ml (1.1 cm column).

native and the denaturing density ranges. The practical buoyant density of native PM-2 DNA II, determined from the interpolated density at $r_o = r_e$, is $1.480_8 \pm 0.001$ g/ml and that of denatured PM-2 II is $1.653_3 \pm 0.001$ g/ml.

C. The Compositional Density Gradient. The equilibrium density gradient due to the redistribution of salt was determined with the Schlieren optical system (16). The compositional density gradient is

$$\frac{d\rho^{\circ}}{dr} = \frac{1}{\beta^{\circ}} \omega^2 r \qquad (2)$$

where ρ° is the solution density measured at atmospheric pressure and β°, a proportionality constant, is a function of temperature and of the activity, molecular weight, and partial specific volume of the solute (component 2) at solution density ρ°. (In general a superscript ° refers to a quantity measured or calculated at one atmosphere pressure.) The values of β° obtained at three different solution densities are listed in Table II.

D. Pressure Dependence of Buoyant Density. The buoyant density gradient, $\frac{d\theta}{dr}$, is the sum of the compositional density gradient, $\frac{d\rho^{\circ}}{dr}$, and a polymer-specific compressional gradient, $\psi\rho^{\circ 2}_{o}\omega^2 r$ (21).

$$\frac{d\theta}{dr} = (1/\beta^{\circ} + \psi\rho^{\circ 2}_{o})\omega^2 r \qquad (3)$$

TABLE II: Determination of the Compositional Density Gradient in Aqueous RbTCA.[a]

ρ° (g/ml)	$\beta^\circ \times 10^9$ (cgs)	$(d\rho/dr)^\circ$ (g/ml)[b]
1.473	1.326	0.107
1.519	1.325	0.106
1.634	1.380	0.101

[a]These experiments employed single sector cells equipped with a lower -1°, flat, or +1° and an upper flat window and filled with 0.6 ml salt solution of increasing density over 40 µℓ of FC43. Centrifugation was at 44 Krpm, 25°C, for 24 hrs.
[b]Calculated for 44 Krpm at r = 6.607 cm.

Here ψ is the pressure coefficient (1) and ρ_o° the solution density at band center (denoted by subscript $_o$). Both PM-2 DNAs I and II were banded together in five cells containing increasing volumes of a RbTCA solution. The pressure dependence of the buoyant density (ψ) was then determined from a plot of the density difference between band center and the rms location ($\rho_o^\circ - \rho_e^\circ$) versus the change in pressure above atmospheric pressure (P_o). Figure 3 shows the variation of density at band center with an increase in pressure for PM-2 DNAs I and II. The values of ψ calculated from these data are presented in Table III.

TABLE III: Determination of the Buoyant Density Gradient for RbTCA in Aqueous RbTCA.

DNA	Slope (g/ml/atm)	Intercept (g/ml)	$1/\overline{v}_{s,o}$ (ml/g)	ψ (atm^{-1})
PM-2 II	$-3.86 \pm .50 \times 10^{-5}$	-4.17×10^{-3}	1.484_5	2.60×10^{-5}
PM-2 I	$-4.17 \pm .54 \times 10^{-5}$	8.74×10^{-3}	1.497_3	2.78×10^{-5}

As indicated in Table III, the buoyant density of DNA I is greater than that of DNA II in RbTCA. This increase in buoyant density may be attributed to a denaturation of approximately 10% of the base pairs at 25° in an early melting transition. The buoyant banding of closed circular DNAs in RbTCA is described in greater detail in a separate report (22). The range of uncertainty in the values obtained for ψ encompass both values which are, therefore, indistinguishable for the totally duplex and partially denatured DNAs.

The buoyant density gradient may also be determined by banding the DNA in two salt solutions differing in density such that the resulting

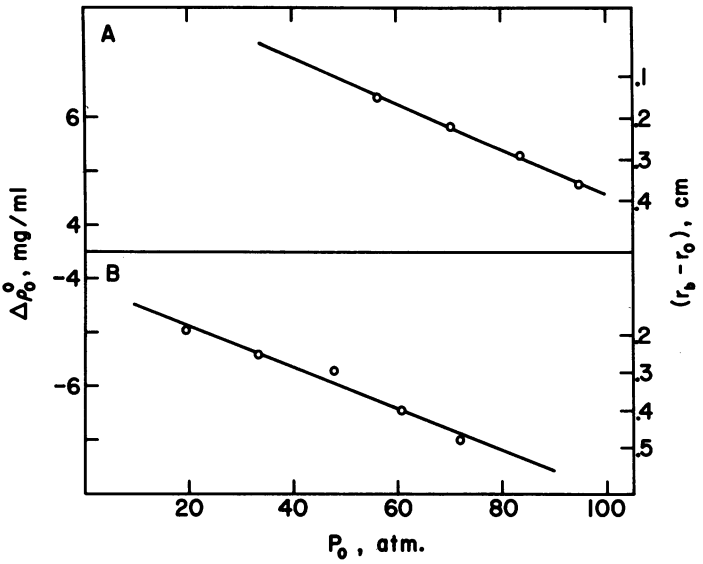

Figure 3. Dependence of the buoyant density of closed (A) and nicked (B) circular PM-2 DNAs upon solution pressure at band center, P_o. The difference in density between each band center and the rms coordinate, r_e, at atmospheric pressure was calculated from the compositional density gradient and is shown in the left coordinate. The shift in band radial location from the bottom meniscus, r_b, is displayed on the right ordinate. PM-2 DNAs I and II were cobanded at 44 Krpm, 25°C in five cells containing increasing volumes of aqueous RbTCA at an initial solution density of 1.489 g/ml. The solid line is the best least squares fit to the data and the coefficients are given in Table III.

bands bracket the rms position (23). The buoyant density gradient is then given by Eq. 4, provided that the solution column heights are identical.

$$\frac{d\theta}{dr} = 1/\beta_B \omega^2 \bar{r} = \frac{\rho_{e,2} - \rho_{e,1}}{r_{o,1} - r_{o,2}} \qquad (4)$$

Here $\rho_{e,i}$ is the initial solution density and $r_{o,i}$ the radial band location in cell i, and $1/\beta_B = (1/\beta^\circ + \psi\rho_o^{\circ 2})$. The banding of PM-2 DNA II in both reference and sample sectors in solutions of equal volumes and densities differing by about 0.03 g/ml is shown in Figure 4. The native density range (average solution density 1.481 g/ml) is shown in panel A and the denatured density range (average solution density 1.650 g/ml) is shown in panel B. Table IV lists the density gradient measured and presents a comparison between this gradient and that calculated by adding together contributions from the compositional and compressional density gradients.

TABLE IV: Buoyant Density Gradient in RbTCA

ρ_{25} (g/ml)	Measured[a] $1/\beta_B \times 10^{-10}$ (cgs)	Calculated $1/\beta_B \times 10^{-10}$ (cgs)	$\frac{d\theta}{dr}$ (g/ml)[b]
1.48	7.93	8.10	0.114
1.66	7.76	7.96	0.112

[a] Average of two trial values
[b] Using the calculated value of $1/\beta_B$ at 44 Krpm and r = 6.607 cm.

The ratio of band widths, denatured/native, is given by (24)

$$\frac{\sigma_d}{\sigma_n} = \left(\frac{M_n r_{o,n} \rho_{o,d} \left(\frac{d\rho}{dr}\right)_{o,n}}{M_d r_{o,d} \rho_{o,n} \left(\frac{d\rho}{dr}\right)_{o,d}} \right)^{1/2} \quad (5)$$

where σ is half the band width at 60.6% of the maximum band height for the denatured, d, or native, n, species and $\left(\frac{d\rho}{dr}\right)$ is the effective density gradient (25). Assuming that the ratio of the compositional density gradients for the two respective density ranges may be used to estimate the ratio of the effective density gradients and setting $M_n = 2M_d$, we calculate $\sigma_d/\sigma_n = 1.53$. From the bands shown in Figure 4 the average measured ratio σ_d/σ_n is 1.55.

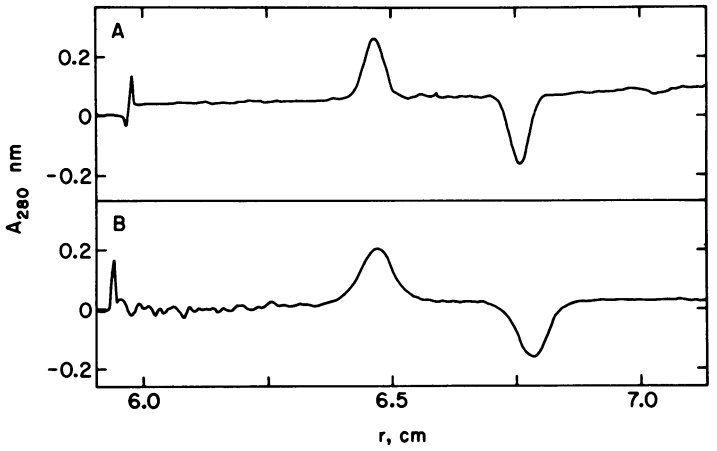

Figure 4. Determination of the buoyant density gradient for the native (A) and denaturing (B) density ranges. Analytical scans taken at 280 nm show PM-2 DNA II in both sample and reference cell sectors. The initial solution densities differ between the two sectors by 31.6 mg/ml in (A) and 32.2 mg/ml in (B). In (A) $\overline{\rho_e^\circ}$ = 1.481 g/ml and in (B) $\overline{\rho_e^\circ}$ = 1.650 g/ml. The solution column heights are identical for both reference and sample sectors and the resultant bands bracket the rms position.

E. Buoyant Density Dependence upon Base Composition for Native and Denatured DNAs. A direct comparison of the resolution between two native DNA species in RbTCA and in CsCl is presented in Figure 5. Ad2 DNA (25 x 10^6 daltons, 54% G+C) and PM-2 DNA II (6.5 x 10^6 daltons, 41.5% G+C) were cobanded in aqueous RbTCA of initial density 1.495 g/ml and in aqueous neutral CsCl of initial density 1.703 g/ml at 44 Krpm and 25°C. Panel A presents the results in CsCl and Panel B those in RbTCA. The buoyant separations in RbTCA are comparable to those obtained in CsCl, although the base compositional dependence of the buoyant density is greater

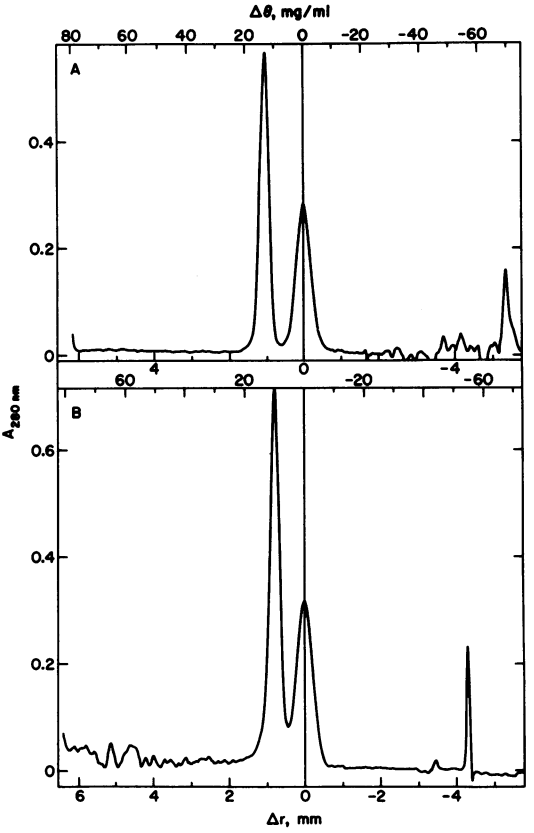

Figure 5. Buoyant banding of PM-2 II and Ad2 DNAs in CsCl of initial solution density 1.695 g/ml (A) and in RbTCA of initial solution density 1.495 g/ml (B) at 44 Krpm and 25°C. The radial distance measured from the PM-2 DNA II band center is shown on the lower abscissa, and the buoyant density increment from PM-2 DNA II is shown on the upper abscissa. The PM-2 DNA II band center was located at 6.531 cm in CsCl and at 6.476 cm in RbTCA. The CsCl buoyant density gradient was calculated from the known β° and ψ values (1). The scans were obtained with a Hewlett-Packard 7004B x-y recorder, and the centrifugal field increases from right to left.

in the latter salt solution. It is apparent that the usefulness of aqueous RbTCA lies in the resolution of native from denatured species, not in the fractionation of native species as a function of base composition.

The variation of buoyant density with mole percent (G+C) is shown in Figure 6 for a series of both native and denatured DNAs. For CsCl, $d\theta/d\%(G+C)$ = 1.06 mg/ml per %(G+C) (26) and is the same for native and alkali-denatured DNAs (18). The buoyant density shift for heat-denatured DNA in CsCl is a function of base composition, presumably due to the maintenance or reformation of secondary structure. In RbTCA two different linear equations were obtained for the two density ranges, employing a least-squares fit to the data of Figure 6.

$$\theta_n = 1.4591 + (6.45 \pm 0.47) \times 10^{-4} \ (\%G+C) \quad (6a)$$

$$\theta_d = 1.6179 + (9.82 \pm 0.60) \times 10^{-4} \ (\%G+C) \quad (6b)$$

The uncertainty in the slope represents the 95% confidence level, as determined with the Student t distribution. The buoyant density shift for the denaturation transition is therefore a function of base composition and is given by

$$\theta_d - \theta_n = 0.159 + 3.36 \times 10^{-4} \ (\%G+C) \quad (6c)$$

In a series of preliminary experiments we also examined [^{3}H]mouse LA9 mitochondrial DNA in a neutral preparative RbTCA buoyant density gradient and found a buoyant density difference between separated strands of 15 mg/ml, with the denser strand banding at 1.667 g/ml and the lighter strand at 1.652 g/ml. These buoyant densities were determined relative to a marker, denatured T7 DNA at 1.667 g/ml. In alkaline CsCl the buoyant density difference between strands is 31 mg/ml (27).

F. Nucleoprotein Complexes. In addition to their effects on nucleic acid structure, chaotropic salts bring about the salting in of proteins at reduced temperatures (28). A complex between a protein and a nucleic acid, the integrity of which depends upon the latter component, is expected to be disrupted by concentrated RbTCA. A precedent for this effect is found in the case of aqueous solutions of 5.0 M $NaClO_4$ to release DNA from the bacterial viruses T7, λ, T4, and B3 (29). At least three exceptions to this general behavior are expected: proteins which are protected from denaturation by association with nucleic acid, those which remain bound in spite of denaturation, and those which are bound covalently to the nucleic acid.

In a first series of experiments we have compared the buoyant profiles

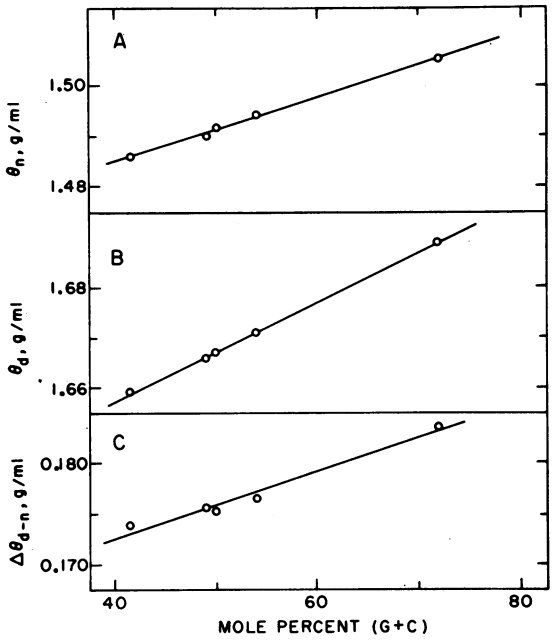

Figure 6. Dependence of the buoyant density in RbTCA upon base composition for the native (A) and the denaturing (B) density range, and the increase in buoyant density upon denaturation (C), plotted as a function of base composition. The DNAs used and their respective base compositions are PM-2 II, 41.5% (39); λh80CI857St68, 49%, calculated from the measured buoyant density in CsCl of 1.7025 g/ml using PM-2 DNA II as a marker at 1.6942 g/ml (measured in our laboratory and in good agreement with a reported value of 1.693 (39)) and using 1.06 mg/ml per %(G+C) in CsCl (26); T7 DNA, 50% (40); Ad2, 54%, calculated from the CsCl buoyant density 1.7077 g/ml using M. luteus, 72% (40); the solid line represents the best least squares fit to the data.

in RbTCA of T7 DNA both following phenol extraction and in the absence of any prior treatment. The complete removal of viral proteins at the buoyant RbTCA salt concentration is demonstrated by the constancy of buoyant density for T7 DNA banded directly from whole virions or following phenol extraction using PM-2 DNA II as a marker. The measured buoyant density shift in these cases between T7 DNA and the marker DNA was 0.004_7 and 0.005_3 g/ml, respectively.

In contrast to the case with T7 virions, covalently bound proteins remain associated with nucleic acids banded in neutral TCA gradients. Lee et al. (30), in an application of the system reported here, have recently examined phenol-extracted polio RNA in a preparative CsTCA density gradient. They detected a compound, presumably a protein, which

remains associated with the RNA recovered from the CsTCA gradient. This compound can be labelled with [^{3}H]-amino acids, is susceptible to pronase digestion, and does not comigrate with viral capsid proteins in polyacrylamide gel analysis. It is possible to isolate polio RNA with associated protein directly by banding in CsTCA, eliminating the necessity for a prior phenol extraction.

In a second set of experiments we examined Ad2 DNA bands isolated by placing whole virions directly into a preparative RbTCA gradient. Ad2 DNA released from virions in 4 M guanidinium chloride has a protein bound to the termini as evidenced by the detection of circular DNA forms by electron microscopy (31). We have examined Ad2 DNA in the electron microscope by direct aqueous spreading of fractions from a RbTCA buoyant banding of Ad2 virions. No circular Ad2 DNA forms were detected by this technique. A buoyant density difference of approximately 1.5 mg/ml has been reported (32) between Ad2 DNA banded as whole virions or a phenol extracted, pronase treated DNA in a CsCl gradient containing 4 M guanidinium chloride. We have confirmed this finding by banding both [^{3}H] Ad2 DNA and [^{32}P] Ad2 virions in RbTCA. In the denaturing density range the double labels are separated by one fraction, corresponding to a density difference of approximately 1.8 mg/ml, with DNA introduced as virions banding as the lighter of the two species. In a separate experiment we determined that the density of denatured proteins from [^{35}S] Ad2 virions is approximately 1.56 g/ml at a pH of 8.5. A buoyant density difference of 1.8 mg/ml corresponds to approximately 100,000 daltons protein associated per DNA strand. A more complete investigation of the buoyant behavior of proteins and nucleoproteins will be reported elsewhere.

G. Integrity of the DNA. Exposure of DNA to RbTCA solutions does not introduce backbone chain scissions, as shown by the maintenance of the integrity of PM-2 DNA I during centrifugation in a 3.3 M RbTCA solution over temperature ranges of 15-35°C for one week. We have also examined the maintenance of biological activity in a transformation experiment. Plasmid ColE1 DNA was incubated in a 3.5 M RbTCA solution for one week then dialyzed to remove salts. Both incubated and control DNAs were used to transform *E. coli* C. Although the transformation frequencies were uniformly low, in three separate experiments no significant differences were detected between salt incubated and untreated DNA samples (Table V). Under no conditions have we seen indications of precipitate formation, for either native or denatured DNA.

TABLE V: Transformation of E. coli C with ColE1 Plasmid DNA following Incubation in RbTCA.

Experiment No.	Transformants	Incubation Conditions
1	5	Treated
	7	Untreated
2	4	Treated
	5	Untreated
3	4	Treated
	6	Untreated

DISCUSSION

On a comparative basis, the buoyant density difference between native and denatured DNAs of approximately the same base composition as PM-2 is 16 mg/ml in CsCl (18), 22 mg/ml in Cs_2SO_4 (2), 52 mg/ml in NaI (33) and 58 mg/ml in alkaline CsCl (18). In RbTCA, denatured PM-2 DNA II is 173.8 mg/ml denser than native PM-2 DNA II. This increase is 10.9 times as large as the comparable buoyant density shift in CsCl and 7.9 times the value in Cs_2SO_4. In both CsCl and Cs_2SO_4 heat-denatured, cooled DNA undoubtedly regains a large amount of secondary structure, a phenomenon which appears to be minimal in concentrated solutions of RbTCA. Heat-denatured T7 DNA banded in KTFA of solution density 1.48 g/ml may regain up to 60% base pairing (7). Even if this extent of secondary structure were regained by heat-denatured DNAs in CsCl or Cs_2SO_4, the potential buoyant density shift for the complete denaturation would still be much lower than that found in RbTCA. The actual structure of the heat-denatured species in CsCl or Cs_2SO_4 is a function of temperature, reannealing time, and DNA concentration. In contrast to its behavior in these nondenaturing solvents, PM-2 DNA II is totally denatured in RbTCA at 4.4 M and 25°C as determined by a separate spectrophotometric melting experiment. The midpoint of the melting transition for PM-2 DNA II in 4.44 M RbTCA is -0.1°C and the transition breadth is about 5.5°C. A more detailed report of the melting behavior of DNAs in aqueous trichloroacetate solutions will be presented elsewhere (22).

The magnitude of the buoyant density increase upon denaturation in RbTCA most likely arises from a large dimunition in the preferential hydration as the DNA helix-coil transition proceeds. The preferential hydration, Γ, is normally expressed as moles water per mole nucleotide or, on a weight basis as Γ', the grams water per gram neutral, dry DNA (20).

This latter quantity may be calculated from the measured buoyant density and eq. 7.

$$\frac{1}{\bar{v}_{s,o}} = \frac{1 + \Gamma'}{\bar{v}_3 + \Gamma'\bar{v}_1} \quad (7)$$

Here $\bar{v}_{s,o}$ is the partial specific volume of the solvated neutral polymer as determined from the buoyant density at band center, $\bar{v}_3$ the partial specific volume of anhydrous neutral DNA, and $\bar{v}_1$ the partial specific volume of water (taken to be unity). The result of this analysis shown in Table VI for several density gradient salts. The value of $\bar{v}_3$ for RbDNA was taken to be 0.516 ml/g (34). The shift in buoyant density in RbTCA which accompanies denaturation can be understood as a substantial diminution in preferential hydration for the denatured coil compared to the native duplex. On a

TABLE VI: Preferential Hydration of Buoyant Neutral DNA

Salt	Γ_n	Γ_d	Γ'_n	Γ'_d	$\Delta\Gamma_{n-d}$	$\Delta\Gamma'_{n-d}$
CsCl[a]	7.09	6.67	0.288	0.272	0.42	0.017
Cs_2SO_4[b]	18.97	17.47	0.774	0.713	1.50	0.061
KTFA[c]	6.9	5.3	0.358	0.275	1.6	0.083
RbTCA	10.51	4.77	0.481	0.218	5.74	0.263

[a]For a 40% (G+C) heat-denatured DNA estimated from values in Vinograd et al. (18).
[b]For a 43.5% (G+C) DNA from Szybalski and Szybalski (2).
[c]For a 50% (G+C) DNA from data of Tunis and Hearst (7).

molar basis the relative preferential association of water with the native polymer is about 14 times greater in RbTCA than in CsCl and about 3.7 times greater in RbTCA than in KTFA or Cs_2SO_4. The preferential hydration of a native, neutral DNA in concentrated, nonchaotropic aqueous salt solutions is predominantly determined by the water activity, a_1, and is relatively insensitive to the nature of the anion present (35). The variation of Γ_n thus reflects a difference in water activity in these salt solutions. We estimate the water activity, a_1, in RbTCA solutions of densities 1.480 and 1.650 g/ml to lie in the ranges $0.85 < a_1 < 0.90$ and $0.60 < a_1 < 0.65$, based upon the plot of Γ as a function of a_1 (7).

Alternatively, the region of preferential hydration surrounding the neutral polymer may be envisioned as a preferential exclusion of salt from immediate contact with the DNA. A preferential exclusion parameter, Γ_2, may be calculated provided that the partial specific volume of the neutral anhydrous salt at the appropriate solution density is available. The

results of this analysis are shown in Table VII. The relative exclusion of salt from the vicinity of the polymer is much greater for the native than for the denatured DNA in RbTCA, by a factor of 4.4 compared to CsCl and by a factor of 8.5 compared to Cs_2SO_4. A definitive explanation of the much greater exclusion of RbTCA from the duplex structure compared to the coil is not yet available. The activity coefficient of the free pyrimidine and purine bases is, however, reduced in chaotropic salt solutions compared to water (36). The change in activity coefficients was detected as a relative increase in the solubility of the free base in the salt solution. It was suggested (36) that the solubility increase results from direct binding between the free base and solute ions. Such

TABLE VII: Preferential Exclusion of Salt from Buoyant Neutral DNA

Salt	θ_n (g/ml)[a]	θ_d (g/ml)[b]	$\theta_d-\theta_n$ (mg/ml)[b]	$\bar{v}_{2,n}$ (ml/g)[c]	$\bar{v}_{2,d}$ (ml/g)[c]
CsCl	1.6942	1.7102	15	0.276	0.277
Cs_2SO_4	1.424	1.446	22	0.209	0.211
RbTCA	1.4855	1.6593	173.8	0.432	0.454

Salt	$\Gamma'_{2,n}$	$\Gamma'_{2,d}$	$\Delta\Gamma'_2$	$\Gamma_{2,n}$	$\Gamma_{2,d}$	$\Delta\Gamma_2$
CsCl	-0.377	-0.367	0.010	-0.987	-0.962	0.025
Cs_2SO_4	-0.467	-0.457	0.010	-0.569	-0.557	0.012
RbTCA	-0.652	-0.583	0.069	-1.035	-0.925	0.110

[a] Buoyant density for 41.5% (G+C) DNA in CsCl and RbTCA and for 40% (G+C) DNA in Cs_2SO_4.
[b] Buoyant density for heat-denatured DNA in CsCl and Cs_2SO_4.
[c] Determined by the chord method from published density and concentration data for CsCl (37), for $CsSO_4$ (38) and for RbTCA from this report. The subscripts n and d refer to the salt concentrations at which native and denatured DNA, respectively are buoyant.

an interaction would be expected to reduce the sphere of preferential hydration for the coil with bases exposed to the solvent environment. At the present time a direct interaction of this type appears consistent with the relatively large value of $\Delta\Gamma_2$ for RbTCA (Table VII).

The very large difference in hydration between native and denatured DNA in RbTCA density gradients resulting in a very large buoyant density

difference is generally useful for the separation of nucleic acids based on degree of secondary structure. For example, closed circular DNA undergoes an early melting transition at temperatures well below those required to reach the midpoint of the melting transition for the cognate nicked species. In place of a large temperature increase, solutions of denaturing salts can be used to lower T_m to near room temperature. In RbTCA, virion PM-2 DNA I is denser and is physically resolved from nicked PM-2 DNA II due to an early melting transition involving the denaturation of about 10% of the base pairs. Other DNAs containing single-stranded regions, such as mitochondrial D loop DNA, are expected to be readily resolved from totally duplex DNA in RbTCA gradients. Hybrid molecules with single-stranded regions could be separated from duplex structures and the extent of single-strandedness calculated from the buoyant density shifts.

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*Abbreviations used are: TCA, trichloroacetate; TFA, trifluoroacetate, DNA I, covalently closed circular duplex DNA; DNA II, nicked circular duplex DNA; Ad2 DNA, Adenovirus type 2 DNA; ColE1 DNA, bacterial plasmid colicinogenic factor E1 DNA; EDTA, disodium salt of ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EtdBr, ethidium bromide; DMSO, dimethylsulfoxide.

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