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The Dissociation of Chicken Erythrocyte Deoxyribonucleoprotein and some Properties of its Partial Nucleoproteins

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Histones were completely dissociated from their native complex with DNA in 2.0 M-sodium chloride. Histone fractions IIb, V and I were dissociated in 1.2 M-sodium chloride, fractions V and I in 0.7 M-sodium chloride and fraction I in 0.45 M-sodium chloride. Repeated extraction of partial dRNP (deoxyribonucleoprotein) preparations with sodium chloride of the same concentration as that from which they were prepared resulted in release of histones that previously had remained associated with the DNA of the complex. Gradual removal of histones from dRNP was paralleled by an improvement in solubility, a decrease in wavelength of the u.v.-absorption minimum, and a fall in sedimentation coefficient of the remaining partial dRNP. X-ray diffraction patterns of partial dRNP preparations showed that removal of histone fractions I and V from dRNP did not destroy the supercoil structure of the dRNP, but further removal of histones did. Infrared spectra of partial dRNP preparations showed that in native dRNP histone fraction I was present in the form of extended, isolated polypeptide chains, and that the other histone fractions probably contain a helical component that lies roughly parallel to the polynucleotide chains in the double helix and an extended polypeptide component that is more nearly parallel to the DNA helix axis. An analysis of the sedimentation of partial dRNP preparations on sucrose gradients showed that native dRNP consists of DNA molecules each complexed with histone fractions of all types.

The heterogeneity of histones and the dissociation of these proteins from their native complex with DNA at high salt concentrations are now very well known. It is equally well known that in solutions of low ionic strength these oppositely charged macromolecules will reunite, usually with the formation of an insoluble, gelatinous mass. Analytical studies of the dissociation and reassociation behaviour indicate, as one might expect, that the reassociated product differs from the native complex (Crampton, Lipshitz & Chargaff, 1954; Crampton, 1957; Fredericq, 1963). These observations led to the concept that a specific and ordered interaction may exist between different histones and DNA in native dRNP,‡ but this specific, native, organization may well not be reformed on reassociation of the components at low ionic strength, for this would be expected to be random. More recent studies with reassociated dRNP preparations suggest, however, that the reassociation may not be altogether random (Skalka, Fowler & Hurwitz, 1966; Leng & Felsenfeld, 1966; Bekhor, Kung & Bonner, 1969; Gilmour & Paul, 1969) but one still feels that a return to the precise native organization or configuration is improbable. It may therefore be preferable that studies of the structure and function of dRNP be based on stepwise dissociation of different histone fractions from the native complex, rather than on reassociated complexes. There are now several reports of analytical and physical

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‡ Abbreviation: dRNP, deoxyribonucleoprotein.

studies of native dRNP and some of its dissociation products, but correlation is not always easy because the preparations studied often had different histories. The work described here was an attempt to organize a comparative study of several aspects of a series of partial dRNP fractions on identical preparations. Chicken erythrocyte dRNP was selected for study because it has an additional histone fraction that appears to be unique to nucleated erythrocytes (Neelin, 1964; Hnilica, 1964; Murray, Vidali & Neelin, 1968) and hence offers the possibility of providing an additional step in the preparation of a series of partial dRNP fractions compared with a corresponding series of partial dRNP fractions from calf thymus nuclei, for example. Although acid extraction at low temperature (Murray, 1964*a*, 1969) provides a means for generation of a series of partial dRNP fractions (because steps of histone removal are clearly defined) dissociation as a function of sodium chloride concentration was used instead in this series of experiments: in retrospect this was perhaps an unfortunate choice technically.

The presence of the additional histone fraction (fraction V; Murray *et al.* 1968) in chicken erythrocyte dRNP necessitated an analytical study of the effect of sodium chloride concentration on histone dissociation, to which the work on thymus and liver dRNP (Giannoni & Peacocke, 1963; Hindley, 1964; Georgiev, Ananieva & Kozlov, 1966; Ohlenbusch, Olivera, Tuan & Davidson, 1967) could provide only a rough guide. This was done spectrophotometrically, and provided a basis for preparative experiments in which the principal histone fractions were dissociated from dRNP in discrete steps at a given salt concentration and separated from the remaining partial dRNP by ultracentrifugation. The dissociated histones were examined by zone electrophoresis in starch gel and their amino acid composition was determined.

The pellets of partial dRNP fractions obtained by ultracentrifugation were used for X-ray diffraction and spectroscopic investigations of the effect of progressive histone removal on the structure of native dRNP. X-ray diffraction analyses of partial dRNP fractions could show whether certain or all of the histone fractions are involved in maintenance of the supercoiled structure proposed for native dRNP (Pardon, Wilkins & Richards, 1967). Studies of i.r. dichroism exhibited by dRNP fractions could, in an analogous way, provide information about the conformation of the histone fractions in association with DNA, and the orientation of the proteins with respect to the axis of the DNA double helix (Bradbury, Price, Wilkinson & Zubay, 1962*b*). Solutions of the partial dRNP pellets in sodium chloride of the same concentration as that used for their preparation were used in further dissociation experiments and in studies, by sedimentation through sucrose gradients, of the distribution of histone fractions along the polynucleotide chains (Murray, 1969). The solutions were also used for u.v.-absorption measurements, solubility studies, analytical ultracentrifugation and light-scattering measurements.

The results of these studies were reported at meetings of the European Federation of Biochemical Societies, Warsaw (Murray, 1966*a*), the British Biophysical Society, Portsmouth, 1966, and the International Symposium on Biophysics, Madras (Bradbury, Crane-Robinson, Rattle & Stephens, 1967).

EXPERIMENTAL

Nuclei and dRNP preparations. Pooled blood was obtained from a production-line slaughter of chickens through the generosity of Messrs J. Sainsbury and Co. Ltd., Bury St Edmunds, Suffolk, U.K. Erythrocytes and saline-washed dRNP were prepared from the blood as described by Murray *et al.* (1968). All operations were carried out at 4°C. After ten washes in 0.14M-NaCl by repeated homogenization and centrifugation the final product was a creamy, somewhat fibrous material. [Electron microscopy of osmium-stained preparations showed a progressive change during washing with 0.14M-NaCl from an open structure reminiscent of euchromatin (Frenster, Allfrey & Mirsky, 1963) to a compact fibrous material.] This was used as a suspension in 0.14M-NaCl in all the salt dissociation experiments. The 'concentration' of this suspension was about 0.1–0.2ml of dRNP pellet/10ml of saline, and it is very desirable not to exceed this concentration, otherwise solutions after dissociation at the higher NaCl concentrations become very viscous.

Partial dRNP preparations, (i) Analytical experiments. Suspensions of saline-washed dRNP in 0.14M-NaCl were adjusted to the desired concentration of NaCl in a total volume of 5ml, by the dropwise addition of the calculated volume of 2M- or 4M-NaCl. The concentration range covered was 0.2–2.0M-NaCl. After vigorous agitation for about 12h the samples were centrifuged at 39000rev./min for 12h at about 5°C (Spinco 40.3rotor), and the extinctions of the supernatant solutions were measured at 230, 259 and 275nm. The pellets of partial dRNP were used for X-ray-diffraction and spectroscopic studies.

(ii) Preparative-scale experiments. These were based on the analytical results. In the first series of experiments, suspensions of dRNP were dispersed in 0.45M-, 0.8M-, 1.2M- and 2.0M-NaCl, but in the later series of experiments the dRNP suspensions were dispersed in 0.45M-, 0.7M-, 1.2M- and 2.0M-NaCl. The preparations were made from suspensions of dRNP in 0.14M-NaCl as in the analytical experiments, but with larger volumes, usually about 300ml (final volume). The preparations were stirred overnight and centrifuged at 39000rev./min for 12h at about 5°C (Spinco 40.3rotor); sometimes a preparation was stirred for 36h before centrifugation. The supernatant solutions (s_1) were analysed spectrophotometrically and protein was then recovered from them. Pellets of partial dRNP (p_1) were used as such for X-ray diffraction and

spectroscopy, and were dispersed in NaCl of concentration equal to that used in their preparation for further dissociation studies, spectrophotometric analysis, solubility observations, sedimentation and light-scattering measurements, and sucrose-density-gradient centrifugation. On completion of partial dRNP preparations, samples in sterile glassware were packed in ice for transport to other laboratories, which they usually reached in a few hours.

Preparation and analysis of histones. Histones were recovered from the supernatant solutions after ultracentrifugation by dialysis against water followed by rotary evaporation, further dialysis against water and freeze-drying.

Samples for zone electrophoresis were dissolved in 0.01M-sodium acetate, pH4.1 (0.1–0.3mg in 0.05ml) and placed in slots in starch gels made in the same buffer containing 4M-urea.

Samples for amino acid analysis were dissolved in 6M-HCl (1–2mg in 0.1–0.2ml) and heated at 105°C in evacuated sealed tubes for 22h. The hydrolysates were dried *in vacuo* and analysed with an automatic aminoacid analyser (Spackman, Stein & Moore, 1958; Beckman/Spinco 120B). No corrections were made for hydrolytic losses of any of the amino acids and analyses for ϵ-*N*-methyl lysine, which is present in some histones (Murray, 1964*b*), were not carried out.

Solubility and further dissociation of partial dRNP preparations. Partial dRNP preparations (pellets, p_1, after ultracentrifugation) were dispersed (usually with difficulty, particularly with preparations obtained in the lower NaCl concentrations) in NaCl of concentration equal to that from which they had been made. This was effected by gentle shaking with ballotini for 12h followed by centrifugation for 10min at 10000rev./min (MSE HS18 centrifuge, 8×50 rotor). The clear solutions were then centrifuged for 18h at 39000rev./min (Spinco 40.3 rotor) to give a second partial dRNP pellet (p_2) and supernatant solution (s_2) which was analysed spectrophotometrically and retained for protein analysis. This cycle of operations was repeated to give a third partial dRNP pellet (p_3) and supernatant solution (s_3); this third pellet dissolved readily in NaCl solution of appropriate concentration. Samples of the partial dRNP pellets and their solutions were retained at each stage. Proteins were recovered from the supernatant solutions from the ultracentrifuge steps and their electrophoretic behaviour and, where possible, amino acid composition, were ascertained as described in the preceding section.

Sedimentation measurements. Partial dRNP solutions were dialysed overnight against NaCl of molarity equal to that used in their preparation. This solution was then diluted with the diffusate to give four or five solutions whose E_{260} was 0.15–1.00. These solutions were centrifuged in a Spinco model E ultracentrifuge equipped with an absorption optical system. Light of wavelength 260nm was selected with a monochromator attachment, and the image was recorded on Kodak CF8 film at 2 or 4min intervals. The light-absorption of the film was measured with a Joyce–Loebl model Mk. IIIC microdensitometer, and sedimentation coefficients from the resulting traces were calculated as described by Schumaker & Schachman (1957). All measurements were made at 20°C, so that extrapolation of the sedimentation coefficients to zero concentration gave the quantity s^0_{20}. To correct this parameter for the effect of viscosity and density of the solvent (Svedberg & Pedersen, 1940), the partial specific volume of the solute is required. From the s^0_{20} values and other observations, the partial dRNP solutions $2p_1$ and $3p_1$ resembled nucleohistone solutions and a partial, specific volume of 0.68ml/g was assumed (Zubay & Doty, 1959). Other partial dRNP solutions more nearly resembled DNA solutions and a partial specific volume of 0.56ml/g was assumed for the solute (Hearst, 1962).

Light-scattering measurements. Partial dRNP solutions were dialysed against 0.45M-NaCl. The solvent was clarified from dust by the method of Bernardi (1964) and partial dRNP solutions were clarified by centrifugation at 30000rev./min (Spinco model L ultracentrifuge) for 30min. The intensity of light scattered from solvent and from solutions was measured at angles between 30 and 90° to the incident beam with a Sofica model 42000 light-scattering photometer. The results were plotted by the method of Zimm (1948) and molecular weights of the solutes obtained by extrapolation of the curves to zero concentration and zero angle.

Infrared spectroscopy. Spectra were recorded on two spectrometers, a Grubb–Parsons grating spectrometer, the Spectromaster, and a Grubb–Parsons S3A monochromator with NaCl prism which had been converted into a double-beam-in-time micro-spectrometer by coupling the monochromator to a moving-sample stage and reflecting microscope (Bradbury & Ford, 1966). With the micro-spectrometer, small areas (approx. 1mm×0.1mm) of oriented films of nucleoprotein may be examined. Dichroic effects in the orientated films were observed with a selenium film transmission polarizer (Elliott, Ambrose & Temple, 1948), placed between the Nernst source and the reflecting microscope.

Orientated films of partial dRNP preparations were produced by unidirectional stroking out of the gelatinous pellets (from ultracentrifugation) with a horn spatula on a BaF_2 plate until the films were dry. The orientated films were examined in a polarizing microscope and uniform areas of film having high birefringence were selected for dichroic studies in the micro-spectrometer. Films were then mounted in a deuteration cell designed for use with the reflecting microscope (Bradbury, Price & Wilkinson, 1962*a*). Spectra of both orientated and unorientated films were recorded at room humidity, which was maintained at 50% relative humidity. To obtain the deuteration rate of the films, the wavelength scan was stopped at the position of maximum absorption in the N–H stretching bands (3300cm^{-1}) and saturated KCl in 99.9% D_2O was pipetted into the dry deuteration cell, which was then sealed with Apiezon grease. These operations were carried out without disturbing the position of the cell on the specimen stage of the reflecting microscope. The fall in intensity of the bands at 3300cm^{-1} was then recorded as a function of time with a recorder chart speed of 1in/min. After 1h the complete spectrum was recorded and this was repeated at regular intervals. Plots of optical density (3300cm^{-1}) against time then gave rates of deuteration of the partial dRNP preparations.

Measurement of the protein content of partial dRNP. Since different portions of a dRNP gel may have slightly different protein/DNA ratios it is essential to determine the protein content of the film actually under examination. This ratio was calculated from the measured extinctions

of i.r. bands characteristic of the protein and DNA components of the dRNP specimen. The absorption bands used were the amide II band at 1540 cm^{-1}, which lies in a spectral region of low extinction by DNA, and the DNA phosphate band at 1240 cm^{-1} (the PO_2^- antisymmetrical stretching vibration), which lies in a region of low extinction by histones (Fig. 2). Although cross-absorption in these bands is low, there is in the nucleoprotein spectrum, however, some background absorption due to protein in the region of the phosphate band. Corrections for these background absorptions were obtained from spectra of films of pure histone and pure DNA of several thicknesses. Average values of the ratios $\left[\frac{E_{1540\,cm^{-1}}}{E_{1240\,cm^{-1}}}\right]_{protein}$ and $\left[\frac{E_{1240\,cm^{-1}}}{E_{1540\,cm^{-1}}}\right]_{DNA}$ were determined from these films and used to correct the observed extinctions of the partial dRNP samples at 1240 and 1540 cm^{-1}. The final value of $\left[\frac{E_{1540\,cm^{-1}}}{E_{1240\,cm^{-1}}}\right]_{partial\ dRNP}$ was related to composition by calibration with a native dRNP gel of known protein/DNA ratio. Measurements made on different parts of the same uniform partial dRNP film gave values for the protein content, which agreed to within 2%. It has been assumed in this method that the extinction coefficients of the amide bands are independent of conformation of the histones; Bendit (1966) showed that this is so for the different conformations of keratin within an experimental error of about 10%. Thus, to this extent, there is an uncertainty in the determination of the histone content of partial dRNP.

X-ray-diffraction studies (*cf. Pardon* et al. 1967). X-ray-diffraction studies were carried out on our partial dRNP samples by Dr B. M. Richards and Dr J. F. Pardon of the Biophysics Department and Medical Research Council Biophysics Unit, King's College, Drury Lane, London W.C.2, U.K. Earlier samples were sent to them in the form of pellets and later ones in the form of fibres prepared (within 24h) from the partial dRNP pellets by the method described for DNA fibres (Langridge, Wilson, Hooper, Wilkins & Hamilton, 1960). The fibres (diam. 100–200 μm) were mounted on phosphor-bronze stretchers to keep them under tension in the X-ray camera. Specimens were exposed in cameras having pin-hole collimation (Langridge *et al.* 1960). Exposures varied with both specimen and camera; a typical time was about 65h.

Sucrose-density-gradient sedimentation. Linear gradients (4.4ml) of sucrose were made in 5ml cellulose nitrate centrifuge tubes with equal volumes of 5% and 20% (w/v) sucrose solutions in 0.7M-, 1.2M- and 2.0M-NaCl; the gradients were made with a mixing chamber as described by Britten & Roberts (1960). Partial dRNP samples (0.2ml) were carefully layered on top of a gradient made in their own concentration of NaCl. These operations were carried out in the cold-room and the centrifuge (Spinco model L) was run at about 5°C. After centrifugation at 28000rev./min for 7h (Spinco SW39 rotor), the tubes were carefully removed and 2-drop fractions were collected in sterile tubes. Fractions were diluted with 0.25ml of water and the E_{259} was measured. When mixtures of dRNP samples were to be analysed all the samples were first dialysed against several changes of NaCl solution of the lowest concentration of any sample to be included in the mixture; the dRNP samples were then mixed and layered on the sucrose gradients.

RESULTS AND DISCUSSION

Several independent preparations of dRNP were used and the studies made on each preparation are listed in Table 1.

The dissociation of protein from dRNP as a function of sodium chloride concentration is represented in Fig. 1 as plots of the E_{230} of the supernatant solution after ultracentrifugation against the concentration of sodium chloride in the solution. Since the extinction coefficients (at any wavelength) of the various histone fractions vary appreciably (Murray *et al.* 1968) these curves have little quantitative meaning, but they show that the dissociation is a rather gradual process and served to indicate the most useful sodium chloride concentrations for preparative experiments. Accordingly, preparative dissociations were made in the first series of experiments at 0.45M-, 0.8M-, 1.2M- and 2.0M-sodium chloride and, in the light of this experience,

Table 1. *Experiments on partial dRNP preparations from different independent preparations of native dRNP*

Experiment carried out	dRNP preparation used			
	A	B	C	D
Histone dissociation at various NaCl concentrations	+	+	+	
Amino acid analysis of dissociated histones		+	+	
Electrophoresis of dissociated histones in starch gel	+	+	+	
Further dissociation of partial dRNP	+	+		
Sedimention analysis of partial dRNP	+		+	+
Light-scattering measurements	+			
U.v.-absorption spectra	+	+		+
X-ray diffraction of partial dRNP pellets	+	+	+	
I.r. spectroscopy of partial dRNP pellets	+	+	+	
Sedimentation of partial dRNP preparations through sucrose gradients		+		+

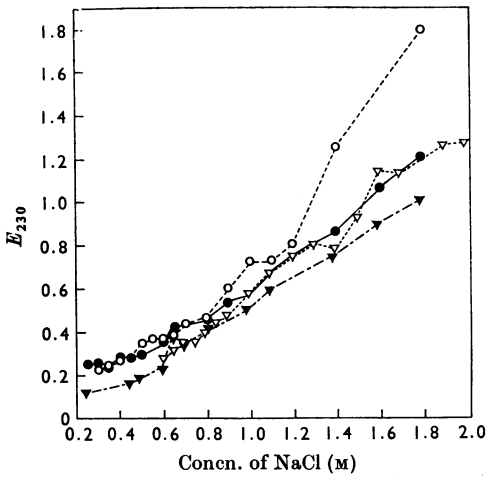

Fig. 1. Release of protein from dRNP at various NaCl concentrations. Extracts were shaken for 12h at 4°C, ultracentrifuged and the E_{230} of the supernatant solutions was measured. Curves 1 (○) and 2 (●) were obtained with dRNP preparations A and B and curves 3 (▽) and 4 (▼) represent two experiments with dRNP preparation C.

at 0.45M-, 0.7M-, 1.2M- and 2.0M-sodium chloride in subsequent series of experiments. Preparations made in 0.45M-sodium chloride were described by the prefix 1, those made in 0.7M- or 0.8M-sodium chloride by the prefix 2, those made in 1.2M-sodium chloride by the prefix 3, and those made in 2.0M-sodium chloride by the prefix 4. Subscripts 1, 2 and 3 after the partial dRNP pellets (p) or supernatant solutions (s) from ultracentrifugation steps denote first, second, and third cycles of extraction or dissociation in a given concentration of sodium chloride (Table 2).

The amino acid composition of the protein recovered from the supernatants (s_1) in a series of dissociation experiments is given in Table 3 and the behaviour of the proteins on zone electrophoresis in starch gels is shown in Plate 1. These results show the anticipated progression in complexity of the dissociated protein, the amino acid analyses being especially useful because they show that although the protein first dissociated (0.45M-sodium chloride) is almost certainly histone fraction I, a considerable quantity of other protein, presumably non-histone or 'acidic' protein, is also present. [The nomenclature used for histone fractions is that of Murray *et al.* (1968)] This conclusion [which may be drawn also from analyses published by Wilhelm & Champagne (1969) for protein dissociated from dRNP in 0.3–0.5M-sodium chloride] is reflected in the presence of histidine and methionine, the relatively high proportion of aromatic amino acids and

Table 2. *Summary of partial dRNP samples made in each series, from the same preparation of native dRNP*

Partial dRNP no.	Native dRNP dispersed in NaCl	Ultracentrifuge →	Supernatant + Pellet (dispersed in NaCl)	Ultracentrifuge →	Supernatant + Pellet (dispersed in NaCl)	Ultracentrifuge →	Supernatant + Pellet
1	0.45M	$1s_1$	$1p_1$ (dRNP $1p_1$)	$1s_2$	$1p_2$ (dRNP $1p_2$)	$1s_3$	$1p_3$ (dRNP $1p_3$)
2	0.7 or 0.8M	$2s_1$	$2p_1$ (dRNP $2p_1$)	$2s_2$	$2p_2$ (dRNP $2p_2$)	$2s_3$	$2p_3$ (dRNP $2p_3$)
3	1.2M	$3s_1$	$3p_1$ (dRNP $3p_1$)	$3s_2$	$3p_2$ (dRNP $3p_2$)	$3s_3$	$3p_3$ (dRNP $3p_3$)
4	2.0M	$4s_1$	$4p_1$ (dRNP $4p_1$)	$4s_2$	$4p_2$ (dRNP $4p_2$)	$4s_3$	$4p_3$ (dRNP $4p_3$)

Table 3. *Amino acid composition of histones dissociated from dRNP at various* NaCl *concentrations*

Amounts of amino acids are expressed as mol/100 mol of total recovered amino acids; no corrections were applied for hydrolytic losses of any amino acids.

dRNP preparation used ...	B			C			
Concn. of NaCl	0.45 M	0.45 M*	0.7 M	0.45 M	0.7 M	1.2 M	2.0 M
Amino acid							
Lys	22.9	21.4	21.9	22.8	20.2	16.1	14.2
His	1.4	0.8	0.1	1.2	1.7	2.3	1.9
Arg	2.9	4.1	8.0	3.5	6.0	6.1	8.0
Asp	3.5	3.9	2.8	4.5	3.6	4.8	4.9
Thr	4.2	4.4	3.7	4.2	3.8	4.5	5.4
Ser	7.1	8.3	10.0	6.5	9.6	7.7	6.4
Glu	5.7	5.7	5.0	6.9	5.8	7.3	8.3
Pro	9.2	8.8	8.0	8.1	8.4	6.3	5.3
Gly	8.1	7.2	6.2	7.2	6.8	7.6	8.4
Ala	22.6	21.7	18.4	20.3	19.6	16.1	13.5
Val	4.8	4.7	4.4	4.9	4.8	5.7	6.0
Met	0.3	0.3	0.3	0.3	0.5	0.6	0.9
Ile	1.5	1.8	2.6	2.0	2.5	4.1	4.6
Leu	5.1	4.8	4.9	5.3	4.8	7.2	7.9
Tyr	0	1.2	2.2	1.1	1.3	2.3	2.7
Phe	0.8	0.9	1.5	1.3	1.1	1.3	1.8
Arg/Lys molar ratio	0.13	0.19	0.40	0.15	0.30	0.38	0.56

* This extract was stirred for 36 h before ultracentrifugation. All other extracts were stirred for 12 h before ultracentrifugation.

isoleucine, and the relatively low proportion of lysine and alanine. The difference in behaviour on zone electrophoresis and in amino acid composition of proteins dissociated at higher sodium chloride concentrations indicate that histone fractions I and V are dissociated in 0.7 M-sodium chloride, histone fraction IIb is also dissociated in 1.2 M-sodium chloride, and histone dissociation is completed with the release of histone fractions III and IV in 2.0 M-sodium chloride. This progession, which was observed in part by Wilhelm & Champagne (1969) in a somewhat similar study, resembles the stepwise removal of histone fractions from dRNP attending a gradual increase in H^+ concentration (Murray *et al.* 1968).

The initial pellets of partial dRNP (p_1) obtained in all the dissociation experiments (whether preparative or analytical) always comprised two phases, a lower, creamy, opaque layer, and an upper, clear, glassy layer. Both layers were sampled for all the analytical and physical experiments but no differences between the layers were observed. For physicochemical experiments, dissolution of the dRNP pellets (which were essentially insoluble in 0.8 mM-sodium chloride or 1 mM-EDTA, pH 7.0) was attempted in sodium chloride of the same concentration as that from which they had been sedimented. This proved distinctly difficult and after prolonged gentle resuspension these second extracts were clarified and again ultracentrifuged, and the protein from the supernatant solution (s_2) was examined by zone electrophoresis. The results (series B, Plate 1) show, surprisingly, that histone fractions that previously had remained associated with DNA in the partial dRNP in 0.7 M- or 1.2 M-sodium chloride were now dissociated at this same sodium chloride concentration. Pellets (p_2) from the second extracts, or dissociations, dissolved more readily than before (though still with a little difficulty) in sodium chloride solutions of the same concentration, and after a third cycle of extraction and sedimentation the pellets (p_3) dissolved quite readily. This progressive ease of dissolution, at a

EXPLANATION OF PLATE I

Zone electrophoresis in starch gels of histones dissociated from dRNP in 0.45 M-, 0.7 M- (or 0.8 M-), 1.2 M- and 2.0 M-NaCl. The gels contained 15 g of starch/100 ml of 0.01 M-acetate buffer, pH 4.1, containing 4 M-urea and were stained with Amido Black 10B. The behaviour of these preparations may be compared with that described in earlier studies of histone preparations and isolated fractions (Rasmussen, Murray & Luck, 1962; Murray, 1964*b*; Neelin, 1964).

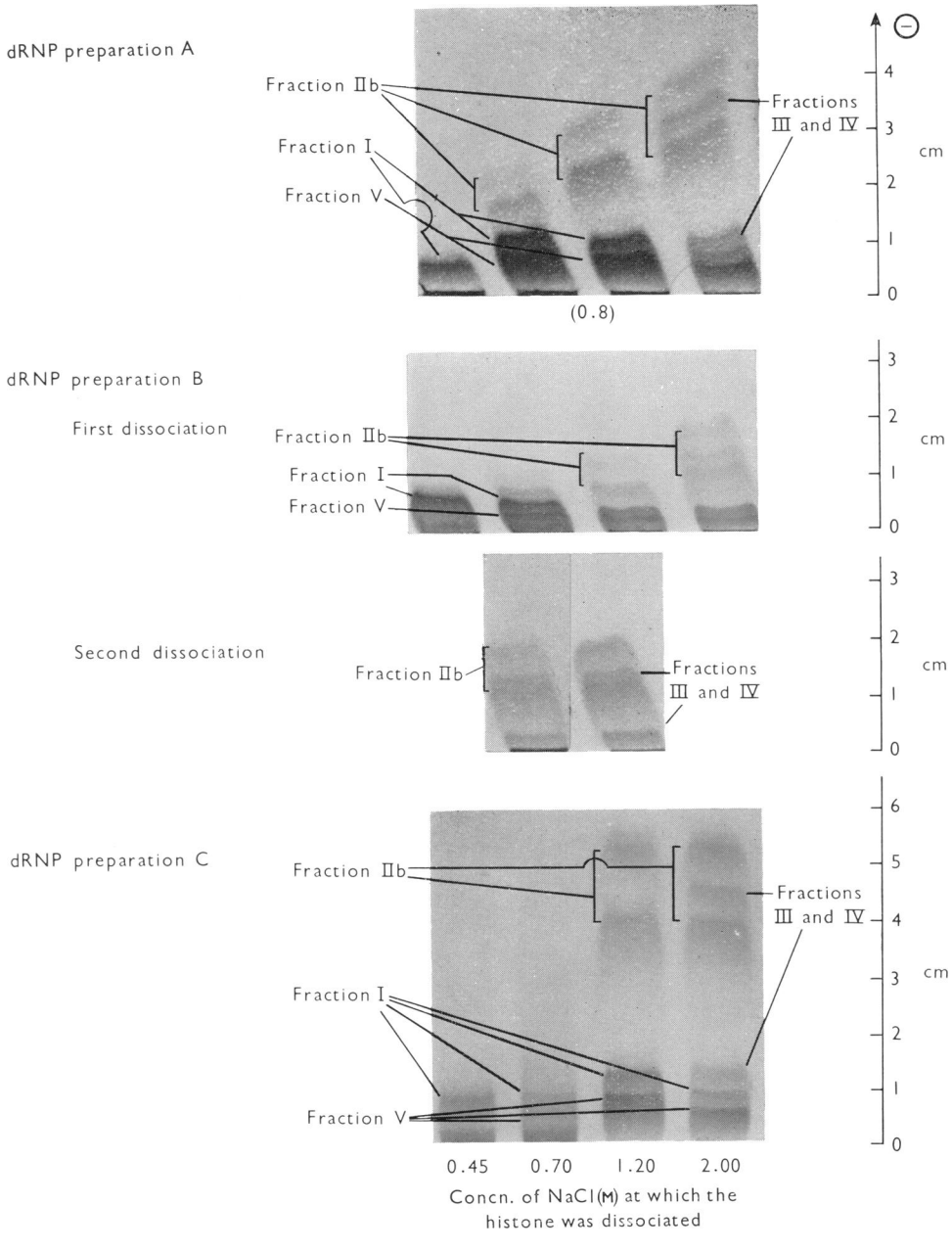
dRNP preparation A
Fraction IIb
Fraction I
Fraction V
Fractions III and IV
⊖
4
3
2
1
0
cm
(0.8)
dRNP preparation B
First dissociation
Fraction IIb
Fraction I
Fraction V
3
2
1
0
cm
Second dissociation
Fraction IIb
Fractions III and IV
3
2
1
0
cm
dRNP preparation C
Fraction IIb
Fractions III and IV
Fraction I
Fraction V
6
5
4
3
2
1
0
cm
0.45
0.70
1.20
2.00
Concn. of NaCl(M) at which the histone was dissociated

Table 4. *U.v.-absorption characteristics of supernatants and redissolved dRNP pellets at various stages of dissociation in* NaCl

dRNP preparation used	Sample no.	Concn. of NaCl (M)	Supernatant s_1 230	Supernatant s_1 259	Supernatant s_1 277	Pellet p_1 230	Pellet p_1 259	Pellet p_1 277	Pellet p_1 $\lambda_{min.}$	Supernatant s_2 230	Supernatant s_2 259	Supernatant s_2 277	Pellet p_2 230	Pellet p_2 259	Pellet p_2 277	Pellet p_2 $\lambda_{min.}$	Supernatant s_3 230	Supernatant s_3 259	Supernatant s_3 277	Pellet p_3 230	Pellet p_3 259	Pellet p_3 277	Pellet p_3 $\lambda_{min.}$
Wavelength (nm)	..	..																					
A	1	0.45	0.302	0.088	0.073																		
	2	0.80	0.552	0.065	0.072	2.24	4.0	2.5		0.167	0.072	0.055	0.422	0.921	0.561	234	0.054	0.045	0.022	0.338	0.772	0.466	
	3	1.20	0.840	0.088	0.106	1.63	2.88	1.8		0.234	0.089	0.072	0.338	0.747	0.447	232	0.051	0.055	0.024	0.309	0.711	0.428	
	4	2.00	1.75	0.147	0.212	0.966	1.98	1.23		0.173	0.078	0.061											
B	1	0.45	0.315	0.133	0.111	0.100	0.230	0.174															
	2	0.70	0.482	0.118	0.102	3.30	5.49	3.48	235	0.337	0.110	0.088	2.16	4.32	2.72	233	0.129	0.047	0.037	1.40	3.33	2.10	230
	3	1.20	0.980	0.240	0.175	2.69	5.18	3.06	233	0.356	0.112	0.085	2.28	5.12	3.02	231.5	0.103	0.072	0.038	1.24	2.89	1.55	230
	4	2.00	1.41	0.195	0.201	1.85	3.86	2.41	231.5	0.175	0.103	0.072	1.42	2.97	1.85	231	0.042	0.051	0.030	0.84	1.81	1.12	230

fixed sodium chloride concentration, of the partial dRNP pellet resulted from gradual removal of histones and led to changes in the physical properties of the partial dRNPs which might be anticipated. The series of preparations from repeated extracts of dRNP are summarized in Table 2, and the u.v.-absorption characteristics of some of them are given in Table 4; the progressive fall in $\lambda_{min.}$ (seen most clearly in the partial dRNP solutions of series B) from 235nm to 230nm parallels removal of protein. Although partial dRNP pellets from a sequence of repeated dissolutions or extractions dissolved increasingly readily in their characteristic molarity of sodium chloride, they did not dissolve in dilute sodium chloride (i.e. about 0.5M) until protein dissociation was essentially complete (at the p_3 stage); similarly, dilution with water of salt solutions of any of the partial dRNP p_1 preparations, or of partial dRNP preparation $2p_2$ gave a gelatinous precipitate of nucleoprotein. Salt solutions of partial dRNP preparations $3p_2$ or $4p_2$, or any of the p_3 preparations, however, gave no such precipitate on dilution with water.

Sedimentation coefficients of several partial dRNP samples and molecular weights obtained from light-scattering measurements on one series of p_3 preparations are given in Table 5. Since sedimentation coefficient is related to molecular weight and frictional coefficient, variation in $s^0_{20,w}$ values may reflect changes in molecular weight or molecular size, or both. However, the fall in $s^0_{20,w}$ in the series $2p_1$, $3p_1$, $4p_1$ (preparations A and D) is consistent with other evidence indicating a dissociation of histones and a resulting fall in molecular weight with increasing concentration of sodium chloride. Similar lowering of $s^0_{20,w}$ values of dRNP in solvents of high ionic strength was observed by Giannoni & Peacocke (1963). Table 5 also shows that successive equilibrations in sodium chloride of a given ionic strength, for example in the series $2p_1$, $2p_2$, $2p_3$ (preparations A and D), result in progressive dissociation of histones and that after three equilibrations the $s^0_{20,w}$ value has reached a constant minimum value which is about the same for all sodium chloride concentrations and is, presumably, that of DNA. Light-scattering measurements on dRNP preparations $2p_3$, $3p_3$ and $4p_3$ give a weight-average molecular weight of about 3×10^6 which, again, is presumed to be that of the DNA component of the dRNP. The separations in sucrose-density-gradient experiments are in good agreement with predictions from the $s^0_{20,w}$ values of the partial dRNP solutions.

Pellets of partial dRNP from ultracentrifugation in both analytical and preparative-scale experiments were used for X-ray diffraction and i.r. spectroscopy. The X-ray diffraction work on our samples was done by Dr B. M. Richards and Dr

Table 5. *Sedimentation coefficient and molecular weight (from light-scattering) of partial dRNP preparations*

	dRNP preparation used			
	A		C	D
Partial dRNP sample	$s^0_{20,w}$ (S)	$10^{-6} \times$ Mol.wt.	$s^0_{20,w}$ (S)	$s^0_{20,w}$ (S)
$2p_1$	54.0 ± 3.0		50.4 ± 2.0	32.0 ± 2.0
$2p_2$	19.5 ± 1.0			24.0 ± 2.0
$2p_3$	18.0 ± 1.0	2.8		21.0 ± 1.0
$3p_1$			32.8 ± 2.0	24.0 ± 1.0
$3p_2$	22.4 ± 1.0			23.0 ± 2.5
$3p_3$	19.4 ± 0.5	2.9		18.0 ± 1.0
$4p_1$	24.0 ± 1.0		19.2 ± 1.0	19.0 ± 1.5
$4p_2$	20.0 ± 1.0			25.0 ± 3.0
$4p_3$	16.8 ± 0.4	2.4		20.0 ± 2.0

Table 6. *Analysis of X-ray-diffraction patterns of partial dRNP samples*

Pellets of partial dRNP from analytical-scale dissociation experiments were used. Results are expressed as the presence (+) or absence (0) of the low-angle X-ray-diffraction rings at 22, 27, 35 and 55 Å which are characteristic of dRNP in its native conformation.

	dRNP preparation used		
Concn. of NaCl used for partial dRNP preparation (M)	A	B	C
0.35		+	
0.40			+
0.45	+	+	
0.50		+	
0.6		+	
0.7			+
0.8	+		
0.9		+	
1.0			
1.1		0	
1.2	+		0
1.3			
1.4			
1.5			
1.6		0	
1.7			
1.8			
1.9			
2.0			0

J. F. Pardon, and the following, with Table 6, is a summary of their results. As noted already, no significant differences were observed between the different layers of partial dRNP pellets. Diffraction patterns from freshly prepared fibre specimens of native dRNP from both chick erythrocyte and calf thymus contain, in addition to unoriented reflexions from DNA, a characteristic set of low-angle rings (corresponding to spacings of 22, 27, 35, 55, and 110 Å). These are attributed to diffraction by a supercoil of the DNA–histone complex having a pitch of 120 Å and diameter of 100 Å, and are therefore called the supercoil rings (Pardon *et al.* 1967). In fibre specimens that had lost the supercoil configuration, the DNA molecules showed better orientation than in native dRNP; this is expected with release of the DNA molecules from a supercoiled form (Pardon *et al.* 1967). The results obtained with the present series of partial dRNP fibres are recorded in Table 6, as the presence or absence of the supercoil rings in the diffraction patterns obtained and pertain to samples from three independent dRNP preparations. (In the entire series of partial dRNP samples from two other independent dRNP preparations no supercoil rings were found, the reason for which is unknown.) In these three series of preparations (A, B and C), supercoil rings were observed in diffraction patterns from all partial dRNP samples prepared from 0.35 M- to 0.9 M-sodium chloride. Three partial dRNP samples from 1.1 M- or 1.2 M-sodium chloride gave mixed results, two having lost the supercoil characteristics and one having retained them. No supercoil rings were found in diffraction patterns from the partial dRNP samples prepared at sodium chloride concentrations above 1.2 M. Since histone fractions I and V are dissociated from dRNP in 0.7 M-sodium chloride and the supercoil characteristics persist in partial dRNP prepared from 0.9 M-sodium chloride, these two histone fractions would not appear to be critical for the existence of the supercoil conformation. In 1.2 M-sodium chloride histone fraction IIb is dissociated, but fractions III and IV are not. Partial dRNP prepared from sodium chloride at concentrations above 1.2 M has lost the supercoil conformation; thus the presence of these histone

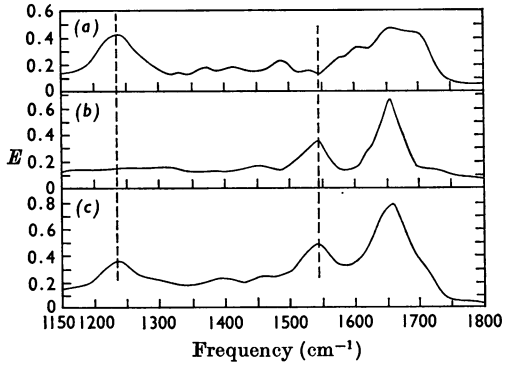

Fig. 2. I.r. spectra of films of (*a*) DNA, (*b*) unfractionated histone and (*c*) native dRNP. The broken lines show the regions used for measurements of the intensity of protein and DNA bands.

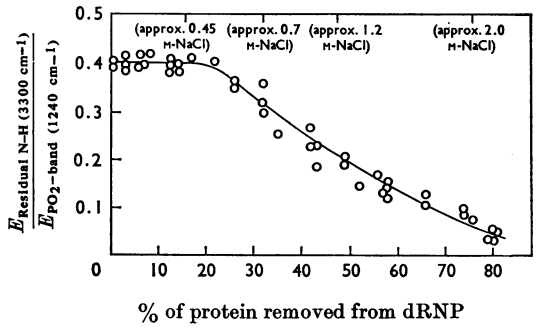

Fig. 3. Effect of protein removal from dRNP on the proportion of slowly deuterated protein in the remaining partial dRNP.

fractions would appear necessary for the supercoil structure of dRNP preparations, with the presence of histone fraction IIb (which dissociates in 1.2M-sodium chloride) perhaps being the limiting factor.

Spectroscopic studies showed that histone fraction I, which contains 9mol % of proline, has very little α-helix content in sodium chloride solutions. The other histone fractions have a greater tendency to adopt an α-helical configuration and this increases with increasing ionic strength in aqueous solutions (Bradbury, Crane-Robinson, Phillips, Johns & Murray, 1965; Bradbury, Crane-Robinson, Goldman, Rattle & Stephens, 1967). I.r. spectroscopy (Fig. 2), combined with deuterium exchange, of partial dRNP films was used here to study the conformational behaviour of histones in their native complex. Native dRNP has a protein component that is slowly deuterated. This slow exchange of hydrogen for deuterium has been attributed to shielding of amide N-H protons that are involved in an α-helical form (Elliott & Hanby, 1958). The residual intensity of the absorption band due to the amide N–H stretching vibration ($3300\,cm^{-1}$) after 2h exposure of the sample to D_2O vapour gives a measure of the slowly exchanging component. (Variations in film thickness of the different partial dRNP samples were accommodated by measurement of the ratio of the intensity of this band to that of the DNA phosphate absorption band at $1240\,cm^{-1}$.) If the slowly exchanging protons were equally distributed among the various protein fractions of native dRNP, then a plot of the residual $E_{3300\,cm^{-1}}/E_{1240\,cm^{-1}}$ ratio against the amount of protein removed from the dRNP would give a monotonically decreasing curve. However, Fig. 3 shows that removal of the first 20–25% of the protein does not change the amount of slowly deuterated protein remaining in the partial dRNP. Thus, the

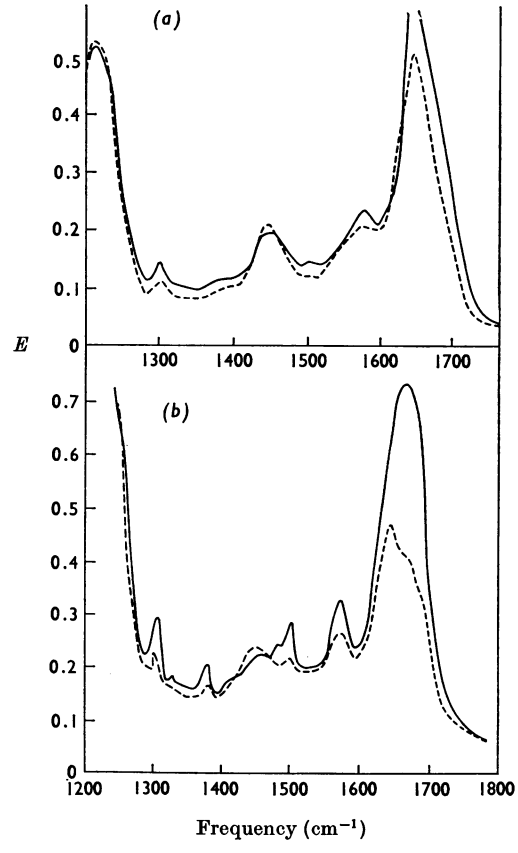

Fig. 4. Dichroic i.r. spectra of oriented films of (*a*) partial dRNP preparation $2p_1$ (prepared from 0.7M-NaCl) and (*b*) partial dRNP preparation $3p_1$ (prepared from 1.2M-NaCl). ----, Electric vector parallel, and ——, electric vector perpendicular, to the direction of orientation of the film.

labile amide protons in the protein most readily removed (i.e. dissociated first) from native dRNP are not shielded in any way. Further, since this protein has a high lysine content (Table 3) the ϵ-amino protons must also exchange rapidly with deuterium. Of the total histone in chicken erythrocyte dRNP about 11% (w/w) is histone fraction I and about 15% (w/w) is histone fraction V (Murray *et al.* 1968; P. J. Greenaway & K. Murray, unpublished work). These two fractions are the only histones dissociated from dRNP in sodium chloride up to 0.7M, and only fraction I is dissociated in 0.45M-sodium chloride, but the amino acid composition of histone fraction I prepared in this way shows that it contains other (non-histone) protein. A rough estimate based on the histidine and methionine content and the depressed content of lysine and alanine in the preparations from 0.45M-sodium chloride extracts, and the composition of some non-histone nuclear protein (fraction R; Murray, 1966*b*), suggests that about two-thirds of the protein dissociated from native dRNP in 0.45M-sodium chloride is histone fraction I. It therefore seems reasonable to conclude that at least most of the protein possessing rapidly exchangeable amide protons is histone fraction I, and this is present in native dRNP in the form of isolated extended polypeptide chains similar to those found in nucleoprotamine (Feughelman *et al.* 1955; Bradbury *et al.* 1962*a*). The other histone fractions contain, in their native complex with DNA, slowly exchangeable amide protons attributable to shielding in some other conformation such as α-helix (since it is known that native dRNP does not contain protein in the β-form; Bradbury *et al.* 1962*b*). This conformational difference between histone fraction I and the other fractions may indicate that the various histone fractions have differing roles in the native dRNP complex.

Polarized i.r. spectra were obtained from the partial dRNP preparations to study the orientation of histones with respect to DNA in the dRNP complex. Dichroic spectra of series of partial dRNP preparations after deuteration showed that the residual N-H stretching band at 3300 cm^{-1} alway exhibited a very slight parallel dichroism. This low dichroism (compared with that of DNA of the same samples) indicates that if shielding of the resistant amide protons results from helix formation, then the axes of the polypeptide helices must either be distributed randomly or lie at an angle close to 54° to the DNA axis (Bradbury *et al.* 1962*b*). The

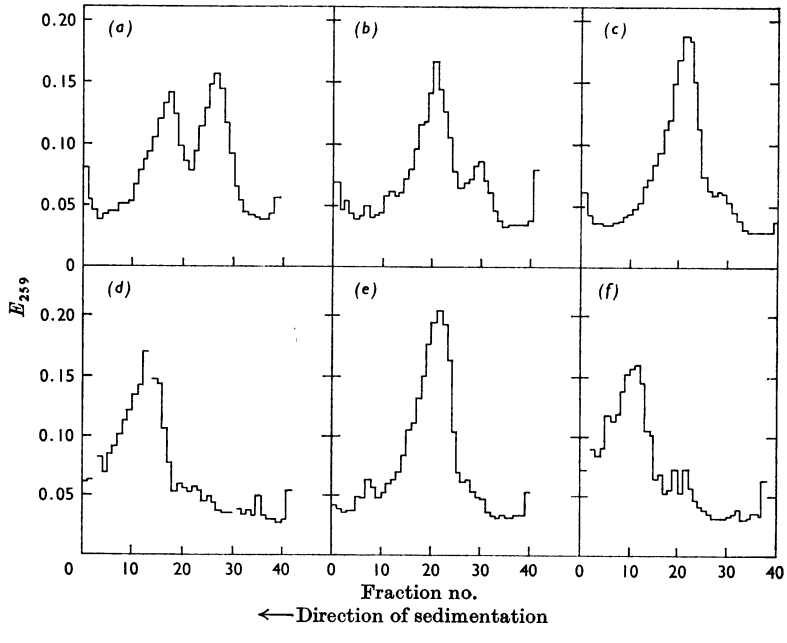

Fig. 5. Sedimentation of mixtures in various proportions of partial dRNP preparations $2p_1$ and $4p_3$ through 5–20% (w/v) sucrose density gradients in 0.7M-NaCl (in the analysis shown in the upper left the mixture was of dRNP preparations $2p_1$ and $2p_3$). The proportions, given as the percentage of each component in terms of E_{259} units, are (*a*) $2p_1$ 62%, $2p_3$ 38%; (*b*) $2p_1$ 84%, $4p_3$ 16%; (*c*) $2p_1$ 92%, $4p_3$ 8%; (*d*) $2p_1$ 95%, $4p_3$ 5%; (*e*) $2p_1$ 97%, $4p_3$ 3%; (*f*) $2p_1$ 98%, $4p_3$ 2%.

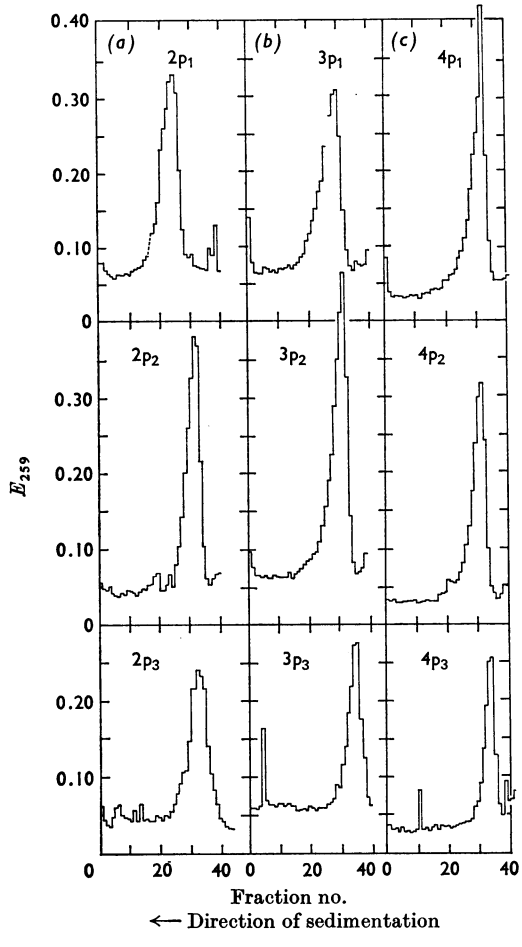

Fig. 6. Sedimentation of the partial dRNP samples (from dRNP preparation B) through 5–20% (w/v) sucrose density gradients in: (*a*) 0.7 M-NaCl; (*b*) 1.2 M-NaCl; (*c*) 2.0 M-NaCl.

latter interpretation would allow the alignment of helical segments of histones with the polynucleotide strands of the DNA which make an angle of 54° with the axis of its double helix. An amide II band at 1452 cm^{-1} appeared almost immediately on exposure of partial dRNP films to D_2O vapour, showing that the histones remaining in the complex contain a rapidly deuterated amide component as well as a helical component. This band results from a C–N stretching vibration in the –C–N– group and

D

exhibits a parallel dichroism that becomes more pronounced as protein is progressively removed from the dRNP. Fig. 4 shows the dichroic spectra of partial dRNP preparations $2p_1$ and $3p_1$. The rapidly exchanging amide component to which this band may be assigned is from a non-shielded, non-helical conformation and the parallel dichroism of the band indicates that the axis of this polypeptide chain lies more nearly parallel to the axis of the DNA molecule than perpendicular to it. These results suggest, therefore, that in a partial dRNP, such as that which has lost histone fractions I and V, the remaining histones contain both helical components that are essentially parallel to the polynucleotide chains, and extended polypeptide components that are more nearly parallel to the DNA axis. Here, it is notable that the amino acid sequence of histone fractions III (or f2a; DeLange, Fambrough, Smith & Bonner, 1969) and IIb_2 (or f2b; Iwai, Ishikawa & Hayashi, 1970) and a high-resolution n.m.r. study of their conformation (Boublik, Bradbury & Crane-Robinson, 1970; M. Boublik, E. M. Bradbury, C. Crane-Robinson & E. W. Johns, unpublished work) suggest that, after dissociation from DNA, these histones may comprise a globular portion and a similar proportion of extended polypeptide chain.

One further aspect of dRNP structure studied was the distribution of the different histone fractions amongst the population of native dRNP molecules. The partial dRNP preparations (except dRNP 1p, of which insufficient material was available) were sedimented through sucrose gradients, because this method was shown to permit the detection of about 5% of DNA in a dRNP sample (Fig. 5). All the preparations sedimented as single components with a characteristic velocity (Fig. 6), and when mixtures of two or three partial dRNP fractions were analysed in the same way, the components of the mixtures retained their individual sedimentation behaviour (Fig. 7). The various partial dRNP fractions therefore represent discrete populations of molecules (see also Table 5). This means that in native dRNP, all the DNA molecules (or at least fragments of molecular weight of about 3×10^6; Table 5) are complexed with histone fractions of all types (otherwise free DNA would appear in a partial dRNP at some step of histone removal). This conclusion is in accord with an earlier analysis of partial dRNP preparations obtained from calf thymus dRNP by titration with acid at low temperature (Murray, 1969). The possibility of any appreciable redistribution of histones between different partial dRNP molecules appears unlikely because the partial dRNP fractions retained their identity when sedimented as mixtures (Fig. 7), but it is not precluded by this analysis.

We are indebted to Dr J. F. Pardon and Dr B. M. Richards for their X-ray diffraction analysis of our samples, and to the Science Research Council (E. M. B., C. C. R. and R. M. S.) and the Medical Research Council (A. J. H. and A. R. P.) for financial support.

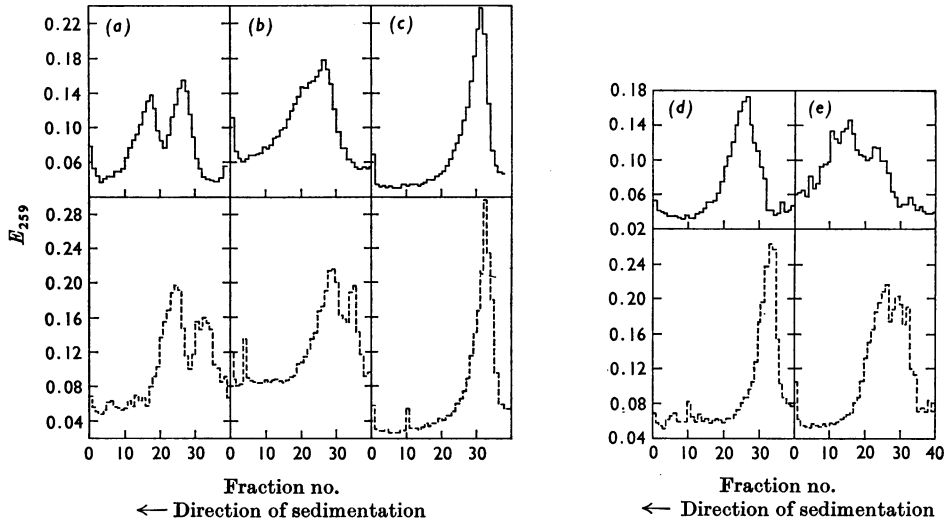

Fig. 7. Sedimentation of various mixtures of partial dRNP preparations through 5–20% (w/v) sucrose density gradients in NaCl. The figure in broken lines beneath each experimental result was obtained by simple addition of profiles shown in Fig. 6 for the components of each mixture. (*a*) 0.7M-NaCl, $2p_1$ 62%, $4p_3$ 38%; (*b*) 1.2M-NaCl, $3p_1$ 64%, $3p_3$ 36%; (*c*) 2.0M-NaCl, $4p_1$ 68%, $4p_3$ 32%; (*d*) 0.7M-NaCl, $2p_3$ 65%, $4p_3$ 35%; (*e*) 0.7M-NaCl, $2p_1$ 38%, $3p_1$ 35%; $4p_1$ 27%.

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