High Total Histone/Deoxyribonucleic Acid Ratios for Rat Liver Nuclei

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The ratios of total histone to DNA for rat liver nuclei isolated by four methods as well as for calf liver nuclei isolated by one method were determined by obtaining the ratios of the total areas of the electrophoretic histone peaks for the liver nuclei to the corresponding total area given by a known amount of standard calf thymus histone. Ratios of total histone to DNA of approx. 2 for rat liver nuclei isolated at pH 3.8 or 5.8 and for calf liver nuclei isolated at pH 3.8 were confirmed twice by the above procedure and also by direct measurement, by the method of Lowry *et al.* (1951), of histone extracted in 0.2M-H₂SO₄. The histones of calf thymus, calf liver and rat liver were characterized by their amino acid compositions and by polyacrylamide-gel electrophoresis.

Previous work from this laboratory has indicated that rat liver nuclei isolated either at pH 5.8 or 3.8 contain at least twice as much histone as DNA on a dry-weight basis (Umaña *et al.*, 1964; Chanda & Dounce, 1971a). On the other hand, when nuclei are isolated at pH 3.1 or in the presence of CaCl₂ or MgCl₂ without pH control, the ratio of histone to DNA drops to 1.0–1.2 (Dounce *et al.*, 1966a; Dounce & Ickowicz, 1969), although the electrophoretic patterns of histone extracted from nuclei isolated at pH 3.1 or in the presence of CaCl₂ or MgCl₂ are nearly indistinguishable from those of rat liver nuclei isolated at pH 5.8 or 3.8 (Chanda & Dounce, 1971b).

The histone/DNA ratio of calf thymus nuclei is generally accepted as approx. 1.0, and this ratio has also been found in our laboratory (Dounce *et al.*, 1966a). Therefore calf thymus histone has been used as a standard in the work to be reported here. The new evidence for total histone/DNA ratios of approx. 2 for liver cells is based in part on determining the amount of total histone present from the total areas of electrophoretic histone profiles measured with a Du Pont curve analyser.

The histones isolated from liver and thymus have been characterized by amino acid compositions and also by polyacrylamide-gel electrophoresis.

Materials and Methods

Isolation of nuclei

Sprague–Dawley rats weighing 180–200 g were used for isolation of liver nuclei at pH 5.8, 3.8 and 3.1 and without pH control in the presence of 5mM-CaCl₂ (Dounce *et al.*, 1966a; Dounce & Ickowicz, 1969). Calf liver nuclei were isolated at pH 3.8 from the liver of freshly slaughtered calves. Calf thymus nuclei were isolated from fresh calf thymus at pH 3.8 as previously

reported (Dounce *et al.*, 1966a). In all cases 0.44M-sucrose was used as suspending medium for the isolation of nuclei, except for the Chauveau step, where 2.2M-sucrose was used (Chauveau *et al.*, 1956). All preparations were checked for purity by use of the phase-contrast microscope. In the past electron microscopy and Coulter-counter distribution curves as well as the amount of DNA per nucleus (Chanda & Dounce, 1971a) have also been used in investigating the purity of the nuclei. It is judged that all preparations of nuclei described in the present paper are of comparable degrees of purity.

Extraction of histones and DNA from nuclei

Histone and DNA were extracted from all types of nuclei as described previously, 0.1M-HCl and 0.2M-H₂SO₄ being used to extract histone from the liver nuclei and 0.2M-HCl for the thymus nuclei (Dounce *et al.*, 1966a). In all cases the nuclei were extracted three times with 0.9% NaCl at pH 5.8 to remove soluble proteins before extraction of the histone fraction.

Determination of DNA

DNA was measured by the Schneider–Dische technique, by using the residues obtained after removal of globulin and histone fractions, as described previously (Monty & Dounce, 1959). Lipid was first removed from the residue by extraction with ethanol–ether (3:1, v/v) and then with chloroform–methanol (1:1, v/v). The residue after lipid extraction was washed twice with 10% (w/v) trichloroacetic acid solution at room temperature and DNA was then extracted in 5% trichloroacetic acid solution by heating to 95°C for 15 min. After removal of protein

by centrifugation, portions of the supernatants were used for DNA determination by the Dische colour reaction as described previously (Dounce & Umaña, 1962).

Determination of histones

Histones isolated from calf thymus and rat liver nuclei were measured both by the dry-weight method as described previously (Chanda & Dounce, 1971c) and by measuring total areas of the electrophoretic histone peaks as described below. Histones were also determined by the method of Lowry *et al.* (1951). Bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was taken as a standard, since this gives a colour per dry weight that is practically the same as that given by histone.

Polyacrylamide-gel electrophoresis of histones and determination of histone/DNA ratios

Known quantities of histone in HCl extracts from known weights of the different types of nuclei, whose dry weights had previously been determined from samples, were applied after dilution in 1 M-sucrose to polyacrylamide gels for gel electrophoresis. The volume of sample was 0.05 ml in all cases. Polyacrylamide-disc-gel electrophoresis was then carried out by the method of Reisfeld *et al.* (1962) and Clarke (1964), as modified by Panyim & Chalkley (1969). After electrophoresis the gels were stained and scanned with a Gilford instrument and the histone areas corresponding to the sums of the individual bands correlating with F1, F3, F2b + F2(a)2, and F2(a)1 histones (which comprise all but a small percentage of the total histone area) were estimated by means of a Du Pont curve analyser. In this work the area of a standard calf thymus histone sample containing 66 µg of histone/0.03 ml (the amount used in electrophoresis) was arbitrarily taken as 6.6. This makes the area 100 for a sample containing 1000 µg of histone. The areas corresponding to the total histone from rat liver nuclei isolated at pH 3.8, pH 5.8, and in the presence of CaCl₂ without pH adjustment, and from calf liver nuclei at pH 3.8, were then determined relative to the area of histone from the standard histone sample.

The following equations apply to this situation:

$$\frac{W_{HT}}{A_{HT}} = K, \frac{66}{6.6} = K, \text{ or } K = 10 \quad (1)$$

where W_{HT} = amount (µg) of calf thymus histone used for electrophoresis, and A_{HT} = the arbitrary area (6.6) corresponding to 66 µg of calf thymus histone.

$$W_{HL} = K \times {}_{HT}A_{HL} = 10 \times {}_{HT}A_{HL} \quad (2)$$

where W_{HL} = total amount (µg) of liver histone and

${}_{HT}A_{HL}$ = total electrophoretic area for liver histone expressed as percentage of total area for the calf thymus histone standard.

$$R_{HL} = 10({}_{HT}A_{HL}/W'_{HL}) \quad (3)$$

where R_{HL} is the calculated dry-weight ratio of liver histone to DNA, and W'_{HL} is the amount of DNA (µg dry wt.) corresponding to the amount of liver histone used in electrophoresis.

The relationship between histone weight and area of electrophoresis peak was also determined by measuring the absolute areas of electrophoresis peaks for calf thymus nuclei and rat liver nuclei, isolated in the presence of CaCl₂ without pH control, by means of a planimeter. The following equations were used:

$$W_{HT}/A_{HT} = C_{HT} \text{ and } W_{HL}/A_{HL} = C_{RL}$$

where W_{HT} and W_{HL} are the dry weights of calf thymus and rat liver histones used for electrophoresis, A_{HT} and A_{HL} are the corresponding absolute electrophoretic areas measured by planimetry and C is a constant.

Amino acid analysis

Histones isolated from calf thymus, calf liver and rat liver nuclei under different conditions were analysed for amino acids by the technique of Moore & Stein (1954), by using a Beckman model 120B amino acid analyser.

Results

Polyacrylamide-gel electrophoresis of histones isolated from calf thymus, calf liver and rat liver nuclei

Plate 1A shows a typical polyacrylamide-gel electrophoretic picture of calf thymus and calf liver histones from nuclei isolated at pH 3.8. It also includes rat liver histones isolated at pH 5.8, 3.8, 3.1 and in the presence of CaCl₂ without pH control. Microdensitometric tracings of the electrophoretic pattern of the total histones isolated from thymus and liver are shown in Fig. 1(a-f). After electrophoresis the gels were scanned densitometrically with a Gilford instrument and the curves for scanning were analysed by means of a Du Pont curve resolver to obtain approximate percentages of the histone components corresponding to the electrophoretic bands (Table 1). Histones for the above experiments were extracted with either 0.1 M- or 0.2 M-HCl (see the Methods and Materials section).

Though there are quantitative differences in patterns (Table 1) all the histones were resolved into four major bands, i.e. bands 4, 6, 7 and 8 (Plate 1A). Lysine-rich histone fraction F1 (band 4) was strongest in calf thymus histones (29% of the total; see Table 1). The ratios of areas of band 4 to band 8, i.e. the ratios of lysine-rich histone F1 to arginine-rich

Disc-gel electrophoresis of total histones of calf thymus, calf liver and rat liver nuclei

Histones were extracted from liver with 0.1 M-HCl and from thymus with 0.2 M-HCl. Further details are given in the Methods and Materials and Results sections. (a) Calf thymus (nuclei isolated at pH 3.8); (b) calf liver (nuclei isolated at pH 3.8); (c) rat liver (nuclei isolated at pH 5.8); (d) rat liver (nuclei isolated at pH 3.8); (e) rat liver (nuclei isolated at pH 3.1); (f) rat liver (nuclei isolated in 5 mM-CaCl₂-0.44 M-sucrose without pH adjustment).

Disc-gel-electrophoresis of histones extracted from rat liver nuclei with 0.2 M-H₂SO₄

Details are given in the Results section. (1) Nuclei isolated in 5 mM-CaCl₂-0.44 M-sucrose without pH adjustment. (2) Nuclei isolated at pH 3.8. (3) Nuclei isolated at pH 5.8.

histone F2(a)1 were high in calf thymus histones (2.32) and lowest in histones extracted from rat liver nuclei isolated at pH3.1. In the latter case the ratio

was 1.19. The corresponding ratios for histones of rat liver nuclei isolated at pH3.8, 5.8 and in the presence of CaCl_2 without pH adjustment were 1.32, 1.28 and

Fig. 1. Scanning profile of disc-gel-electrophoretic bands of total histones extracted from calf thymus, calf liver and rat liver nuclei under various conditions

Further details are given in the Methods and Materials and Results sections. See also Plate 1A. (a) Calf thymus (nuclei isolated at pH3.8); (b) calf liver (nuclei isolated at pH3.8); (c) rat liver (nuclei isolated at pH5.8); (d) rat liver (nuclei isolated at pH3.8); (e) rat liver (nuclei isolated at pH3.1); (f) rat liver (nuclei isolated in 5mM- CaCl_2 -0.44M-sucrose without pH adjustment).

Table 1. *Quantitative measurement of areas under the densitometric tracings (% of total area)*

Percentages of total histone area corresponding to the various electrophoretically separated histone components for calf thymus, calf liver and rat liver nuclei isolated under various conditions are shown. The areas of photo-metric scans of the bands were estimated by means of the Du Pont curve analyser (see the text for further details). Histones were extracted with 0.1 M-HCl from liver nuclei and with 0.2M-HCl from thymus nuclei (Dounce *et al.*, 1966a; Mackay *et al.*, 1968).

Source of nuclei ...	Histone (% of total)					
	Rat liver				Calf thymus	Calf liver
	pH 5.8	pH 3.8	pH 3.1	5 mM-CaCl ₂	pH 3.8	pH 3.8
Preparation conditions ...						
Band no.						
4 (F1)	20.5	20.5	15.8	19.5	29.0	21.5
6 (F3)	16.0	20.4	18.0	16.0	17.0	19.0
7 F2b+F2(a)2	34.0	31.5	37.0	37.0	36.0	36.5
8 F2(a)1	16.0	15.5	16.8	15.0	12.5	10.5
Others	11.5	12.1	13.2	12.5	5.5	12.5
Ratio 4 (F1): 8 [F2(a)1]	1.28	1.32	1.19	1.56	2.32	2.04

1.56 respectively. The major bands observed are designated in terms of the nomenclature of Johns (1964).

Ratios of histone to DNA obtained from total areas of the electrophoretic histone peaks

The ratios of total histone to DNA of calf thymus, calf liver and rat liver nuclei isolated under different conditions are listed in Table 2. The amount of histone in each case was calculated from the ratios of the total areas of the electrophoretic histone peaks for the liver nuclei to the corresponding total area given by a known amount of standard calf thymus histones (see the Methods and Materials section). From this calculation, the ratios of histone to DNA for rat liver nuclei isolated at pH 3.8 and 5.8 were 1.81 and 2.00 respectively. On the other hand, these ratios were 1.06 and 1.14 respectively for rat liver nuclei isolated at pH 3.1 and in the presence of CaCl₂ without pH adjustment. The histone/DNA ratio for calf liver nuclei isolated at pH 3.8 was 1.98.

Relationship between dry weight of total histone and total electrophoretic histone area

The ratios between histone weight and electrophoretic area for calf thymus and for rat liver histones extracted from nuclei in the presence of CaCl₂ without pH control were also determined by measuring the absolute areas of the electrophoretic peaks by means of a planimeter. The area for the standard calf thymus histone (equivalent to 66 µg of histone) was 599 mm², and that corresponding to the electrophoresis of 91 µg of histone from rat liver nuclei isolated in the presence of CaCl₂ without pH adjust-

ment was 840 mm². The ratios C_{HT} and C_{RL} (see the Methods and Materials section) were found by this method to be 0.110 for calf thymus nuclei and 0.108 for the rat liver nuclei isolated in the presence of CaCl₂ without pH adjustment.

Histones extracted from rat liver nuclei with 0.2M-H₂SO₄

Plate 1B represents polyacrylamide-gel electrophoretic pictures of histones extracted with 0.2M-H₂SO₄ from rat liver nuclei isolated at pH 3.8, 5.8, and in 5 mM CaCl₂-0.44M-sucrose without pH adjustment. Histone/DNA ratios, measured by the dry-weight method and by area analyses, were about 2 for nuclei isolated at pH 5.8 and 3.8 and 1 for nuclei isolated in the presence of CaCl₂ (Table 3). Similar results were obtained when histones were measured by the method of Lowry *et al.* (1951) (see Table 3).

Quantitative amino acid analyses

Table 4 contains amino acid analyses of histones of calf thymus, calf liver and rat liver nuclei isolated under different conditions; the ratios of arginine+lysine+histidine to the acidic amino acids (aspartic acid+glutamic acid) for the total histones of calf thymus, calf liver and rat liver nuclei were similar. Values of this ratio for histones from rat liver nuclei isolated under different conditions are also quite comparable. The values varied from 1.70 for rat liver nuclei histone isolated at pH 3.8 to 1.86 for calf thymus nuclear histones. The lysine/arginine ratios were 1.67 for calf thymus and 1.43 and 1.46 for rat liver nuclei isolated at pH 3.8 and 5.8 respectively. The cor-

Table 2. *Histone/DNA ratios for rat liver, calf liver and calf thymus nuclei isolated under different conditions*

Histone values obtained from electrophoretic areas of histone peaks as well as by dry-weight determinations are shown. Areas are expressed as percentages of the total histone area of the standard calf thymus histone. Histones were extracted from liver nuclei with 0.1 M-HCl and from thymus nuclei with 0.2 M-HCl. DNA was determined by the diphenylamine colorimetric reaction (Chanda & Dounce, 1971c). See the text for definitions of abbreviations used.

Source of nuclei	Histone (μ g dry wt.) used for electrophoresis	DNA from nuclei equivalent to μ g of histone in nuclei used for electrophoresis	Total area under histone curve ($A_T \times A_{HL}$)	Total histone ($K \times H_T \times A_{HL}$) (μ g)	Ratio total histone/DNA	
					Based on area determination	Based on dry-wt. determination
Calf thymus (pH 3.8)	66.0	60.0	6.60	66.0	1.10	1.10
Calf liver (pH 3.8)	28.0	13.3	2.64	26.4	1.98	2.10
Rat liver (pH 5.8)	60.0	28.6	5.72	57.2	2.00	2.09
Rat liver (pH 3.8)	52.5	25.5	4.62	46.2	1.81	1.92
Rat liver (pH 3.1)	62.5	57.0	6.17	61.7	1.06	1.10
Rat liver (5mM-CaCl ₂)	91.0	76.0	8.71	87.1	1.14	1.20

responding values for histones isolated from rat liver nuclei at pH 3.1 and in the presence of CaCl₂ without pH adjustment are 1.45 and 1.49 respectively.

Discussion

In setting up the equations for the determination of histone/DNA ratios from area analyses, the basic assumption has been made that the relationship between true weight of total histone and total electrophoretic histone area is the same or very nearly the same for all kinds of histone dealt with. Since the ratios of peak areas are practically identical for all types of liver histone studied, the basic assumption must hold among these classes of histones. There are small differences among peak ratios for calf thymus and rat liver histones, but these are too small to call into question the basic assumption made above unless the histone weight/area ratios vary greatly from one peak to another, and even then the assumption would still be approximately correct. It does not seem possible that the species difference between calf and rat could cause an appreciable deviation from the basic assumption.

The ratios of total histone to DNA based on the calculations outlined above are listed in Table 1, which shows that the ratios for rat liver nuclei that have been claimed previously by us (Umaña *et al.*, 1964; Dounce *et al.*, 1966a) are approximately verified.

To check experimentally the argument that the relationship between histone weight and electrophoretic area should be nearly the same for calf thymus histone and rat liver histones, we have also determined absolute rather than relative areas of electrophoretic peaks, by means of a planimeter, for calf thymus nuclei and rat liver nuclei isolated in the presence of CaCl₂ without pH control. These histones are the purest of any of the histone samples studied by us and total histones extracted from nuclei isolated by these two methods have been generally accepted as relatively pure. The ratio of histone dry weight to the corresponding electrophoretic area was approximately the same for nuclei from calf thymus and rat liver isolated by the CaCl₂ method (0.110 and 0.108 respectively).

This corroborates the assumption made above that the weight/area constants for calf thymus histone and rat liver histone must differ only slightly if at all. The histones of liver nuclei isolated under different conditions were characterized both by polyacrylamide-gel electrophoresis and by amino acid analyses. In all cases, the gel picture as well as the amino acid compositions showed patterns typical of histones (Plate 1A, Table 4). Although the ratios of histone to DNA for rat liver nuclei isolated at pH 3.8 and 5.8 are approximately twice the corresponding ratios for rat liver nuclei isolated at pH 3.1 and in the presence of

Table 3. *Histone/DNA ratios for rat liver nuclei*

Histone values were determined from electrophoretic areas corresponding to total histone and expressed as percentages of the total histone area for the standard calf thymus histone, as described for Tables 1 and 2. Histone values were also obtained from protein and dry-weight determinations. Histone was extracted from nuclei with 0.2M-H₂SO₄.

Conditions of isolation of nuclei	Histone/DNA ratio		
	Based on protein determination (Lowry <i>et al.</i> , 1951)	Based on area determination	Based on dry-wt. determination
CaCl ₂	1.10	0.98	1.10
pH 3.8	1.99	1.96	1.98
pH 5.8	1.88	1.90	1.92

Table 4. *Amino acid composition of total histone of rat liver, calf thymus and calf liver nuclei isolated under different conditions*

Values for amino acids are expressed as μmol of amino acid/100 μmol of total amino acids recovered from the column. Histones were extracted in all cases with 0.2M-H₂SO₄ and amino acids were determined by the method of Moore & Stein (1954).

Amino acid	Calf thymus (pH 3.8)	Calf liver (pH 3.8)	Rat liver (pH 3.8)	Rat liver (pH 5.8)	Rat liver (pH 3.1)	Rat liver (5 mM-CaCl ₂)
Lysine	14.6	15.0	12.6	13.5	13.8	13.4
Histidine	2.0	2.2	1.9	2.1	2.3	2.2
Arginine	8.7	9.2	8.8	9.1	9.5	9.0
Aspartic acid	4.9	4.9	5.6	5.1	5.1	5.5
Threonine	3.7	5.1	5.4	5.1	3.6	5.2
Serine	4.2	4.0	5.2	5.4	3.8	5.4
Glutamic acid	8.7	8.8	8.1	8.5	8.8	8.3
Proline	6.0	5.5	4.5	4.8	5.0	5.0
Glycine	9.2	9.1	10.8	11.1	9.8	10.8
Alanine	15.2	14.7	11.9	12.2	13.7	12.4
Valine	6.1	6.0	6.0	5.8	6.3	6.0
Methionine	0.2	0.2	0.9	0.7	0.22	0.4
Isoleucine	3.9	4.0	3.9	3.7	4.7	4.0
Leucine	9.0	7.4	8.1	8.3	9.3	8.5
Tyrosine	1.8	1.9	2.4	2.5	2.1	2.2
Phenylalanine	1.6	1.6	1.9	2.1	1.6	2.0
Lysine/arginine	1.67	1.63	1.43	1.46	1.45	1.49
Lys + Arg + His	1.86	1.92	1.70	1.81	1.84	1.78
Asp + Glu						

CaCl₂, there is practically no difference in the amino acid composition (Tables 2 and 3). The qualitative and quantitative electrophoretic patterns are also comparable.

It has been observed that when histones are extracted from nuclei or from chromatin with 0.2M-H₂SO₄ they are accompanied by very little non-

histone proteins (Hearst & Botchan, 1970). In our laboratory when histones were extracted either with 0.1M-HCl or with 0.2M-H₂SO₄ from rat liver nuclei isolated at pH 3.8 or 5.8, the ratios of histones to DNA measured by the dry-weight method or by area analyses as well as by the method of Lowry *et al.* (1951) were about 2 (Tables 2 and 3).

The question may be raised whether the relatively high histone/DNA ratios reported in the present paper for liver nuclei isolated at pH 3.8 and 5.8 (about 2) might be caused by loss of DNA through deoxyribonuclease action during the isolation procedure. We have the following comments to make about this possibility.

(1) Analyses of nuclei isolated at pH 3.8 and pH 5.8 previously published from this laboratory (Chanda & Dounce, 1971a) showed values of DNA per nucleus in agreement with values taken from the literature for nuclei prepared in the presence of CaCl_2 . In this connexion it can also be stated that according to our results diploid and polyploid nuclei have the same ratios of histone to DNA, and hence changes in the ratios of diploid to polyploid nuclei should not change the overall ratio of histone to DNA.

(2) We have in the past encountered a situation where DNA is partially depolymerized in nuclei isolated in water instead of sucrose at pH 5.8–6.0. In spite of this partial depolymerization of the DNA (Dounce *et al.*, 1957; Monty & Dounce, 1959) the percentage of DNA in the nuclei and the ratios of the principal protein fractions to DNA are similar to those found for nuclei isolated in 0.44M-sucrose at pH 5.8–6.0. Evidently DNA must be rather heavily depolymerized before it is lost from nuclei isolated at pH 5.8. This statement should hold even more strongly for nuclei isolated at pH 3.8, where the condensation of chromatin is greater than in the nuclei isolated at pH 5.8–6.0. DNA apparently would have to be extensively degraded to escape the histone with which it is complexed in cases where chromatin is condensed, as in the nuclei isolated at pH 5.8 and especially at pH 3.8.

(3) We have a rather sensitive test for the action of deoxyribonuclease on DNA, which is concerned with gel formation by isolated nuclei or DNA-residual protein complex isolated from previously isolated nuclei (Dounce *et al.*, 1957, 1966b). A relatively small nuclease action will render the nuclei or the DNA-residual protein complex non-gellable at pH 8–10, or in the presence of sodium dodecyl sulphate at lower pH values. Nuclei isolated in sucrose at pH 3.8 and 5.8 and DNA-residual protein complex from these nuclei form good gels, which are stable under the conditions previously stated by us to be criteria for demonstrating that the gels have not been partially degraded (Dounce *et al.*, 1957; Mackay *et al.*, 1968). Further details of the formation and properties of nuclear gels are given by Volkman & Dounce (1972) and Dounce *et al.* (1972). The nuclei isolated at pH 3.8 in sucrose form particularly stable gels.

The only way to avoid the conclusion that the DNA of nuclei isolated in 0.44M-sucrose at pH 5.8 or 3.8 has not been subjected to appreciable deoxyribonuclease action would be to assume that the nuclease had severely depolymerized half the DNA of the

nuclei, leaving the remainder untouched. We know for the nuclei isolated at pH 5.8 that deoxyribonuclease does not act in this way, since if it does act (Dounce *et al.*, 1957; also see above) we do not find half the DNA untouched, but rather find all of it depolymerized to some extent. This depolymerization is apparent from a failure of the DNA to form long fibres and by the necessity of adding more than 0.5 vol. of ethanol to precipitate it from sodium dodecyl sulphate solution at 1M-NaCl concentration.

(4) We have been unable to find a deoxyribonuclease that has a pH optimum near pH 3.8 in mammalian tissue. Considering the stability of DNA to non-enzymic hydrolysis in the cold at this pH value, this makes it hard to understand how depolymerization of DNA could occur at all in nuclei prepared in sucrose at pH 3.8. Moreover there is so much citrate used in the isolation of liver nuclei at pH 3.8 that any deoxyribonuclease requiring Mg^{2+} for inactivation would not be active. Even the action of lysosomal deoxyribonuclease is blocked in the mitochondrial-lysosomal fraction isolated from homogenates used in isolating liver nuclei at pH 5.8.

(5) The use of a pH as low as 3.8 makes it almost or absolutely impossible to lose DNA unless of very low molecular weight, because of the high degree of insolubility of DNA-histone complex at this pH. A pH of 3.8 confers great stability of DNA in cell nuclei during the isolation procedure. Thus even if one raises the question of loss of DNA from the nuclei isolated at pH 5.8, it appears that a loss of DNA from those isolated at pH 3.8 can be ruled out. The analytical results show that nuclei isolated at pH 5.8 do not differ appreciably in ratios of the various protein fractions, including the histone fraction, to DNA.

Some of the possible implications of the presence of an extra amount of histone in liver nuclei isolated at pH 3.8 or 5.8 or the loss of approximately half the histone complement when liver nuclei are isolated at pH 3.1 or in the presence of CaCl_2 without pH adjustment have been discussed previously (Dounce & Ickowicz, 1969; Chanda & Dounce, 1971c; Dounce *et al.*, 1972). We cannot say at the present time how the extra complement of histone is bound in liver nuclei when it occurs, but the observation of Phillips (1968) and Paul & More (1972) that calf thymus chromatin can bind an extra complement of histone when suspended in a solution of free histone may have bearing on this point. The carboxyl groups of the histones already present in the chromatin were thought by Phillips (1968) to be involved in the binding of the extra histone. It is possible that the extra histone complement observed by us in liver nuclei may be bound in the same way. It is also possible, however, that the extra 'loosely bound' histone could be bound to the residual protein of chromatin, which is acidic in nature. In any case we now are forced to recognize the presence of a second histone

complement in liver cell nuclei, which we originally referred to as 'loosely bound histone' (Dounce *et al.*, 1966a; Chanda & Dounce, 1971b).

The distinction between the terms 'loosely bound histone' and 'easily extractable histone' (Chanda & Dounce, 1971c) is purely operational. It is possible to extract about half the histone complement from nuclei isolated at pH 3.8 in 0.44M-sucrose by quickly lowering the pH (in the cold-room) to 2.7–2.9. The histone thus extracted consists mostly of fractions F1 and F2(a)2 (Chanda & Dounce, 1971c). We have recently found that it is possible, although more difficult, to do the same thing with nuclei isolated at pH 5.8 in sucrose in the presence of 0.2mM-lead acetate (R. Ickowicz & A. L. Dounce, unpublished results; see Dounce & Ickowicz, 1970). 'Easily extractable histone' is the term applied to the histone extracted from nuclei isolated at pH 3.8 and 5.8 as described above.

We think that an effect of using CaCl_2 -sucrose solution in isolating liver nuclei without pH control is also to cause loss of about half the histone of the nuclei, but in this case the histone that is extracted and lost during the isolation procedure must have the same composition as whole histone, since disc-gel-electrophoresis patterns and amino acid analysis of nuclei isolated in the presence of CaCl_2 or at pH 3.8 or pH 5.8 are practically indistinguishable. If the histone extracted from nuclei isolated with CaCl_2 -0.44M-sucrose were not of the same composition as that remaining in the nuclei, the latter (on extraction with 0.1M-HCl or 0.2M- H_2SO_4) would not be expected to show the same electrophoretic pattern as those of the whole histone of nuclei isolated at pH 3.8 or 5.8, which according to our claim show ratios of histone to DNA about twice that of the nuclei isolated with CaCl_2 .

The histone that we claim is lost during the isolation of nuclei with CaCl_2 has been termed 'loosely bound histone', which must also have the same composition as that of whole histone extracted from nuclei isolated at pH 3.8 or 5.8.

If nuclei isolated at pH 5.8 are extracted with 5mM- CaCl_2 mainly lysine-rich histone dissolves. Thus the effect of CaCl_2 on isolated nuclei is different from its effect in the homogenate, with regard to extraction of histones.

Nuclei isolated from rat liver at pH 3.1 in 0.44M-sucrose solution are similar to nuclei isolated in CaCl_2 -sucrose solution, since the nuclei isolated at pH 3.1 have a histone/DNA ratio of about 1 and therefore must have lost about half their complement of histone. However, there is a relatively small but noticeable difference between the nuclei isolated at pH 3.1 and those isolated in CaCl_2 -sucrose solution, since the disc-gel-electrophoresis pattern of histone from the former nuclei differs appreciably from that of whole histone from the latter nuclei and

from nuclei isolated at pH 3.8 and 5.8 (the major difference is loss of some of the lysine-rich F1 fraction from the nuclei isolated at pH 3.1; see Table 1). Nevertheless, as stated above, the composition of the histone from the nuclei isolated at pH 3.1 is still quite similar to that of nuclei isolated with CaCl_2 or at pH 3.8 and 5.8, and hence it can be said that the effect of suddenly lowering the pH of nuclei isolated at pH 3.8 or 5.8 to pH 1.7–2.9 is distinctly different from that of lowering the pH of the homogenate to approximately the same value (3.1.)

It cannot yet be stated why the effects of low pH or CaCl_2 or both on rat liver nuclei differ, depending on whether they are imposed on already isolated nuclei or nuclei suspended in the original liver homogenate. These questions have been discussed previously (Chanda & Dounce, 1971c; Dounce *et al.*, 1972).

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