Phosphate and Thiol Groups in Histone f3 from Rat Liver and Thymus Nuclei

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1. Differences in washing procedures and conditions of dialysis affected the type of histone f3 obtained from rat liver and thymus nuclei. 2. Sulphur-rich preparations of f3 contained phosphate and had SH+SS:phosphorus ratio 1:2. 3. Sulphur-rich f3 from livers of [^{14}C]adenine-labelled rats gave counts indicating a phosphorus:adenine ratio 1:1. 4. Evidence was presented that histones f3 and f1 exist in nuclei as families of proteins, differing with respect to their sulphur or phosphorus content or both. 5. The significance of the different states of phosphorylation and oxidation of histones f1 and f3 was discussed.

Ord & Stocken (1965, 1966*a*) reported the presence of phosphate in histones from rat liver and thymus nuclei. In histone f1 (Johns, 1964) the phosphate was present as serine phosphate and no thiol groups were detected. Phosphate and thiol were both present in the components (extracted from nuclei with 50mN-hydrochloric acid) which run fastest on electrophoresis on acrylamide gel at pH4·0; they were also found in histone f3. Phillips (1965) has reported that f3 contains thiol groups, and Hnilica & Bess (1965) and Kleinsmith, Allfrey & Mirsky (1966) have found phosphate in f3. Stevely & Stocken (1966) and Hilton & Stocken (1966) showed that increasing the number of phosphate groups on f1 or oxidizing the thiol groups on f3 affects the capacities of these histones to reduce the ability of DNA to support RNA synthesis. The possibility that f3 contained both thiol and phosphate groups and that these might be modified in nuclei and so alter the activity of the histone in depressing RNA synthesis, necessitated a closer examination of preparations of f3 to determine the nature of the thiol- and phosphorus-containing component(s).

METHODS

Preparation of nuclei and acid extraction of histones. These were performed as described earlier (Ord & Stocken, 1966*a*).

Differential precipitation of f3. Histone f3 was prepared by method 2 of Johns (1964). f3-1 comprised the proteins which were precipitated by dialysing the acid–ethanol extract for 2–3hr. against 3vol. of ethanol at 0°. f3-2 were the proteins precipitated by further dialysis against 100vol. of ethanol for 17hr. at 0°. In some experiments the precipitate f3-1 was washed twice with 5% (w/v) $HClO_4$ before its solution in 50mN-HCl. After washing with $HClO_4$ the remaining precipitate could be brought into solution by dialysing the suspension in 50mN-HCl overnight against 50vol. of 50mN-HCl.

Protein. This was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), by using acid-extracted protein from thymus nuclei as standard.

Thiol+disulphide. SH and SH+SS groups were estimated by the method of Ellman (1959) as described by Marsh, Ord & Stocken (1964).

Phosphate. The procedure of Berenblum & Chain (1938) was used.

Flavianic acid precipitation. Protein solution (2ml.) containing 2mg. of protein/ml. was made 5M in urea. Flavianic acid (2,4-dinitro-1-naphthol-7-sulphonic acid) (10%, w/v, in water) was added to give a permanent yellow precipitate; 100–150μl. was commonly required. The precipitate was allowed to stand for 30min. at 20°. After centrifugation it was redissolved in 5M-urea–250mN-HCl and dialysed against 250mN-HCl. The supernatant was also dialysed against 250mN-HCl until only traces of flavianic acid remained in the sac.

Analytical electrophoresis. Electrophoresis on acrylamide gel was performed in glycine–acetic acid, pH4·0, at 300v, 4–5mA/tube (McAllister, Wan & Irvin, 1963). Usually the samples to be analysed were made to 5M-urea for an hour before running. If isotopically labelled histones were used, the bands could be cut out, dissolved in 30% (w/v) H_2O_2 and the radioactivity was measured by gas-flow counting (Nuclear–Chicago Corp.) as described by Young & Fulhorst (1965).

Chromatography on Sephadex G-75. This was carried out as described by Ord & Stocken (1966*a*).

Amino acid analyses. These were kindly performed for us by Mrs C. Walker as described by Ord, Raaf, Smit & Stocken (1965).

End-group analysis. A modification of the method of Sanger & Thompson (1953) was used. Loss of DNP-amino acids during hydrolysis was corrected by adding tritiated DNP-amino acids to the ^{14}C-labelled DNP-protein and measuring the ^{3}H as well as the ^{14}C content of the products after hydrolysis and separation by thin-layer chromatography (Randerath, 1963). Acetyl groups were not determined.

We are indebted to Mr R. Buckingham for carrying out these analyses.

Table 1. *Effect of conditions of dialysis on the nature of the proteins precipitated from acid–ethanol extracts of rat thymus and liver nuclei*

Nuclei were isolated and washed as described in the text. They were extracted quickly with a loose homogenizer with ethanol–1·25 N-HCl (4:1, v/v) (Johns, 1964). f3-1 was the proteins precipitated by dialysis for 2 hr. at 0° against 3 vol. of ethanol; f3-2 was the proteins precipitated by further dialysis for 17 hr. at 0° against 50–100 vol. of ethanol. SH+SS: mμmoles/mg. of protein. P: mμg.atoms/mg. of protein.

	Thymus		Liver	
	SH+SS	P	SH+SS	P
2–4 hr. dialysis > 50 vol. of ethanol	—	36·5	51·0	52·7
17 hr. dialysis > 50 vol. of ethanol	14·2	14·7	42·0	33·0
17 hr. dialysis > 50 vol. of ethanol	26·6	22·5	—	—
As above, plus acetone wash	25·5	8·3	—	—
2 hr. dialysis, 3 vol. of ethanol	—	—	—	61·2
As above, > 50 vol. of ethanol	—	—	—	51·1
f3-1	29·3	74·6	53·3	132·9
f3-2	24·7	18·7	38·6	42·3

RESULTS

Earlier work (Ord & Stocken, 1966*a*) had indicated that histone f3 prepared by method 2 of Johns (1964) appeared to contain both phosphate and thiol groups. Comparison of the phosphate content of f3 from rat liver and thymus with other data in our Laboratory on f3 from calf or lamb thymus (J. Hilton & R. Buckingham, unpublished work) showed great differences, the preparations from rat nuclei regularly containing more phosphorus than those from the other sources. Some variations in technique existed between the procedure used to obtain f3 from rat tissues and that used for the larger animals. Smaller amounts of nuclei were available from rat tissues and relatively larger volumes of acid–ethanol were used for the extraction. Shorter times of extraction with a hand homogenizer were also used (1–2 min.). Another important difference was in the washing procedure before acid–ethanol extraction. The rat nuclei were washed with 10 mM-tris–hydrochloric acid, 5 mM-magnesium chloride, pH 7·1, and 1 mN-hydrochloric acid before acid extraction, in contrast to the 0·14 M-sodium chloride and ethanol washes used by Johns (1964).

Examination of the conditions of dialysis also showed differences from the method used with larger-scale preparations. Variations in these conditions (Table 1) markedly affected the properties of the proteins precipitated; prolonged dialysis or dialysis against a large volume of ethanol in the final system reduced the amount of phosphorus found in the precipitate, with less effect, however, on the sulphur content. When the precipitates were examined electrophoretically (Fig. 1) differences were apparent between the material precipitated in 2 hr. against 3 vol. of ethanol (f3-1) and that precipitated by further dialysis for 17 hr. against

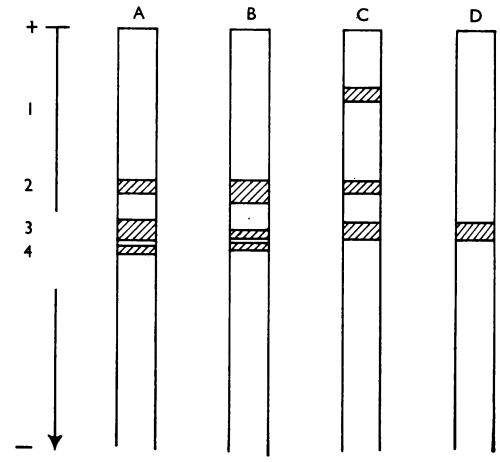

Fig. 1. Electrophoretic patterns obtained from various preparations of histone f3 from rat thymus or liver nuclei. Conditions of electrophoresis are as described in Marsh *et al.* (1964). The proteins to be analysed were made to 5 M in urea for 1 hr. before running and 70–80 μg. was applied/tube. Faint traces of other slow-moving proteins were seen in B and C, especially in preparations from liver. The length of the arrow represents 6 cm. Differences in the hatched areas indicate the intensity of staining in the different bands. A, f3-1 obtained after washing the nuclei with tris and 1 mN-HCl, extracting with acid–ethanol and washing the precipitate which separated after 2 hr. dialysis against 3 vol. of ethanol, with 5% $HClO_4$. B, f3-1 obtained after washing the nuclei with tris and 1 mN-HCl, extracting with acid–ethanol and dialysing the extract for 2 hr. against 3 vol. of ethanol. C, f3-2 obtained after removal of f3-1 above, and dialysing for a further 17 hr. against 50–100 vol. of ethanol. D, Material obtained after flavianate precipitation of f3-2 above. Identical patterns were also shown for f3-2 obtained when two washes with ethanol preceded the extraction with acid–ethanol. Band 1 migrated in the same position as f2a1 and band 2 in the same position as f1.

more than 50vol. of ethanol (f3-2). The main components migrated at similar rates in f3-1 and f3-2 (columns B and C, Fig. 1) but the proportion of the bands changed; in f3-1 more material migrated in band 2 than in f3-2. In preparations of f3-2 there was also more of a very slow-moving component, especially in material obtained from liver nuclei. This slow-moving component migrated in a position identical with that of histone f2a1, which was obtained by precipitation of the supernatant remaining after dialysis, with 3vol. of acteone.

Table 2. *Amino acid composition of histone f2a1 from rat liver and thymus nuclei*

f3-1 and f3-2 were precipitated from the acid–ethanol extract of washed nuclei by dialysis against ethanol as described in the text. The supernatant that remained was treated with 3 vol. of acetone to precipitate f2a1.

	Composition (moles/100 moles)	
	Thymus	Liver
Lys	10·8	11·0
His	2·5	2·8
Arg	12·9	12·6
Asp	5·8	6·0
Thr	6·4	6·1
Ser	2·7	2·9
Glu	7·3	7·5
Pro	1·5	1·6
Gly	15·0	14·4
Ala	7·3	7·2
Val	7·4	7·5
Met	1·1	1·4
Ile	4·8	4·8
Leu	8·3	8·5
Tyr	3·6	3·6
Phe	2·0	2·2
NH_3	7·9	8·6

Comparison of the amino acid composition of the material precipitated with acetone (Table 2) with that reported by Phillips & Johns (1965) for calf thymus f2a1 indicated considerable similarities. In agreement with their data, the preparations of f2a1 from rat liver and thymus had no detectable *N*-terminal amino acid residues and had less than 10 mμmoles of SH + SS or mμg.atoms of phosphorus/mg. of protein.

f3-1 and f3-2 from liver had higher sulphur and phosphorus contents than the proteins obtained from thymus (Table 1) but no differences were detected between the positions of the two main components separating on electrophoresis from the two tissues. Traces of f2a1 could be removed from f3-2 by precipitation with flavianic acid (Table 3). The sulphur- and phosphorus-containing material was precipitated; the protein remaining in solution migrated in electrophoresis in the same position as f2a1 and had no free *N*-terminal amino acids.

Analysis of the *N*-terminal groups of f3-1 and f3-2 (Table 3) showed that only alanine end groups were present. Although f3-1 gave approximately equimolar proportions of SH + SS, phosphorus and end groups (Tables 3 and 4), the apparent molecular weight was about twice that reported for calf thymus f3 (Phillips, 1963). The amino acid analysis (Table 5) clearly indicated that f3-1 from liver and thymus contained a lysine-rich protein which was less evident in f3-2. It was assumed that this lysine-rich contaminant was f1; Dr E. W. Johns (personal communication) suggested that preliminary washing with ethanol would decrease the possibility of removing f1 with f3 in the acid–ethanol extraction. Two washes with ethanol were therefore introduced after the second wash with 1mN-hydrochloric acid.

f3-1 obtained after the ethanol washing was very much reduced in quantity compared with that obtained in the earlier procedure. It also contained

Table 3. *Effect of flavianate precipitation on the proteins of f3-1 and f3-2 from rat thymus and liver nuclei*

f3-1 and f3-2 were precipitated with flavianic acid as described in the text. SH: mμmoles/mg. of protein. P: mμg.atoms/mg. of protein. End groups: mμmoles of alanine/mg. of protein.

	Thymus			Liver		
	SH	P	End group	SH	P	End group
f3-1						
Original material	22·2	24·8, 22·8	36	—	—	25
Flavianate precipitate	30·7	24·8, 22·0	41	—	—	—
Flavianate supernatant	4·7	— 0	—	—	—	—
f3-2						
Original material	19·9, 24·5	10·0, 8·2	67	34·9	18·9	—
Flavianate precipitate	21·6, 29·8	10·4, 7·5	69	50·5	22·4	70
Flavianate supernatant	4·4, 5·2	7·2, 2·9	0	4·7	7·0	2

Table 4. *Thiol + disulphide and phosphorus contents of different types of f3 prepared from rat thymus and liver nuclei*

SH+SS: mμmoles/mg. of protein. P: mμg.atoms/mg. of protein. No. of experiments and range are given in parentheses.

	Thymus		Liver	
	SH+SS	P	SH+SS	P
Acid–ethanol extractions after 1 mN-HCl wash: f3-1	29 (4) (27–29)	36 (4) (28–45)	51, 53	67 (3) (60–79)
f3-2	23 (4) (18–26)	13 (4) (8–19)	39, 42	32 (4) (19–42)
Acid–ethanol extraction after ethanol wash: f3-2	14, 20	14	25, 30	10
f3-1 washed with 5% $HClO_4$	41 (3) (32–48)	83 (3) (79–88)	45	79
f1 obtained from $HClO_4$ wash above	0	5, 5	0	0
f1 obtained by subsequent extraction with 250 mN-HCl	—	103, 105	—	—

Table 5. *Amino acid composition of preparations of f3 from rat thymus and liver nuclei*

	Composition (moles/100 moles)						
	Thymus				Liver		
	Whole f3-1	Flavianate precipitate f3-2	Ethanol washed f3-2	$HClO_4$ washed f3-1	Whole f3-1	Flavianate precipitate f3-2	Ethanol washed f3-2
Lys	18·1	12·4	11·4	14·7	17·3	11·6	11·1
His	1·6	2·7	3·0	2·3	2·0	2·6	2·3
Arg	5·6	9·7	9·8	7·8	6·6	10·1	8·8
Asp	5·7	6·4	5·6	6·6	7·2	5·7	6·4
Thr	6·5	5·5	5·7	5·9	6·7	5·8	5·4
Ser	8·0	6·2	6·2	6·5	8·3	5·9	6·1
Glu	11·7	10·4	10·0	9·5	10·6	10·1	10·5
Pro	7·8	5·2	4·5	5·1	7·8	4·6	4·4
Gly	8·0	8·7	8·5	8·4	9·3	8·5	8·4
Ala	15·7	9·7	11·2	11·1	13·4	11·1	10·7
Val	6·4	6·1	6·1	6·4	6·3	5·8	5·9
Met	1·3	1·4	1·0	0·7	1·7	1·2	1·4
Ile	3·1	4·3	4·1	3·7	3·7	4·2	4·4
Leu	6·9	8·7	8·8	7·5	7·5	8·5	9·0
Tyr	2·0	3·0	2·6	1·7	2·4	2·7	2·8
Phe	2·0	2·2	1·6	2·4	2·5	1·9	2·3
NH_3	8·2	12·6	10·4	—	9·8	10·2	14·0

very much less of the slower of the two main components (Fig. 1). The amino acid composition of the proteins (Table 5) showed a great similarity in composition between the material precipitated with flavianic acid from liver and thymus f3-2 and that obtained as f3-2 after preliminary ethanol washing. As with the flavianate precipitate, less than equimolar proportions of SH + SS and phosphorus were present.

As the ethanol wash before acid extraction gave preparations of f3 which were poor in phosphorus and sulphur, f3-1 was prepared from nuclei that had not been washed with ethanol. The precipitate obtained after 2 hr. dialysis against 3 vol. of ethanol was washed with 5% perchloric acid (Johns, 1964) to remove any f1. After two washes no further f1 was removed. This gave a perchlorate-insoluble residue which had considerably enhanced phosphorus and SH + SS contents (Table 4). On electrophoresis the less-mobile major component was relatively decreased in amount (Fig. 1, column A). Amino acid analysis (Table 5) showed that although some lysine-rich material was probably still present in the preparation examined, the amino

acid composition was appreciably closer to that found in f3-2 than it was for f3-1 from which f1 had not been removed by washing with perchloric acid.

Examination of the absorption spectrum of the protein soluble in perchloric acid and determination of its amino acid composition confirmed that the material removed by the acid was f1. In contrast with f1 prepared directly from thymus nuclei (Ord & Stocken, 1965) the treatment used here gave f1 which contained very little phosphorus (Table 4). It migrated on electrophoresis in exactly the position of the less-mobile major component of f3-1. If procedure 2 of Johns (1964) was continued by extracting the nuclei with 250mN-hydrochloric acid after the acid–ethanol treatment, further f1 could be obtained. This f1 did contain phosphorus and the amount (Table 4) was considerably higher than found previously (Ord & Stocken, 1965; thymus f1 23·4mμg.atoms of phosphorus/mg.).

Our earlier results and those of Hnilica & Bess (1965) had indicated that the phosphorus in f3 might be associated with nucleotide. This was confirmed in liver by isolating f3 from rats which had received 10μc of [8-^{14}C]adenine sulphate/100g. body wt. 90min. previously. f3-1 was obtained after washing with perchlorate; its phosphorus content was 79·3 mμg.atoms/mg. of protein, compared with 31·8 for f3-2. The ratio of [^{14}C]adenine counts was 3:1, suggesting a phosphorus:adenine ratio 1:1 in the proteins isolated. The activity in the material from thymus was too low to allow any conclusions to be drawn. No data are yet available on the nature of the adenine and phosphate binding to f3.

DISCUSSION

The significance of the presence of phosphate in histones from rat liver and thymus was discussed in our previous paper (Ord & Stocken, 1966*a*). With histone f1 from rat thymus serine phosphate could be isolated; with f3 the nature of the phosphate remains to be established. The specific activities of the phosphate in the various histones were considered to be different from and greater than those in DNA and in nuclear ribosomal or residual RNA. The nuclei in our experiments were washed with tris–magnesium chloride, which is known to remove nuclear ribosomes and other proteins (Frenster, Allfrey & Mirsky, 1960). The washing procedure is different from the saline and ethanol washes used by Johns (1964): his wash with ethanol is believed to remove phosphoprotein of the type described by Dr T. A. Langan (personal communication) but since phosphoprotein is separable from nucleoprotein in dilute saline–tris (Dr Langan) it seems probable that our washing procedure also will have lowered the content of the phosphoprotein in the nuclei before extraction of f3. The composition of f3-1 which has been washed with 5% perchloric acid is consistent with this view; although the protein is still heterogeneous by electrophoretic analysis, its amino acid content suggests that the contamination is from the lysine-rich f1 rather than from the phosphoprotein (amino acid analysis from Dr Langan, personal communication).

The amino acid composition of f3-2 shows that the material has a higher lysine content than that reported by Hnilica, Johns & Butler (1962); its *N*-terminal alanine content is also slightly lower than that given by Phillips (1963).

The data here on the composition of different preparations of f3 suggest that this material exists in nuclei as a number of species, which vary with respect to their phosphorus and sulphur contents. f3-2 obtained by extracting the nuclei with acid–ethanol, after preliminary washes with alcohol, had the minimum sulphur content. Its phosphorus was also low, but this may have been due to the prolonged dialysis in the presence of acid–ethanol. The amounts shown in Table 4 suggest that in thymus the minimum SH + SS and phosphorus content may give 1 (SH + SS): 1 phosphorus/mg. of protein and with liver 2 (SH + SS). f3-1, obtained after washing the protein first precipitated by dialysis, with 5% perchloric acid, probably has, for both tissues, 3 (SH + SS): 6 phosphorus. In our hands electrophoretic analysis did not separate the different species of f3 although there were sometimes indications that the main band (D3, Fig. 1) might contain two components. Mr J. Hilton in our Laboratory has separated calf thymus f3 into sulphur-rich and sulphur-poor components on Sephadex G-100 and Dr E. W. Johns has also obtained a sulphur-rich species of f3 (personal communication). Examination of bands 2 and 3 from [^{14}C]*N*-ethylmaleimide-treated nuclei showed that both bands had counts; there is some evidence that the slower of these two bands (2) increases in amount in oxidized material, so that the label may represent thiol groups present in partially oxidized f3.

The data at present available are insufficient to demonstrate whether f3-2 and perchlorate-washed f3-1 are identical proteins, differing only in their thiol and phosphate contents, or whether they represent two different proteins with similar amino acid compositions and electrophoretic mobilities. The suggestion that the thiol and phosphorus contents might be due to a low-molecular-weight nucleotide peptide that becomes associated with certain histones, has been made earlier (Ord & Stocken, 1966*a*; Jellum & Eldjarn, 1965). The fast-moving component present in the 50mM-hydrochloric acid extract from thymus nuclei, which has been purified by preparative electrophoresis and by separation on Sephadex G-75 (Ord

& Stocken, 1966*b*), also showed [^{14}C]adenine uptake and had SH + SS : phosphorus ratio 1 : 2.

f1 also exists in nuclei in varying forms. Stevely & Stocken (1966) have shown that the extent of phosphorylation could be increased *in vitro* and preliminary experiments (W. S. Stevely, M. G. Ord & L. A. Stocken, unpublished work) have shown that f1 prepared from diffuse and dense chromatin differs sharply in phosphate content. The low phosphate content of f1 obtained by washing f3-1 with 5% perchloric acid suggests that acid–ethanol extracts a phosphorus-poor species of f1, the phosphate-rich fraction being removed subsequently with 250mN-hydrochloric acid. The material normally extracted from nuclei with 5% perchloric acid (method 1, Johns, 1964) would therefore represent a mixture of phosphorylated forms.

The importance of these differences in fine composition is indicated from the effects that oxidation of f3 (Hilton & Stocken, 1966) and phosphorylation of f1 (Stevely & Stocken, 1966) have on the capacity of these histones to reduce the ability of DNA to support RNA synthesis. That such differences in composition may occur naturally is shown by the difference in thiol : disulphide ratio of histones from diffuse and dense chromatin (Ord & Stocken, 1966*a*) and their difference in phosphate content (see above). The gross composition and amount of histone in the two types of chromatin does not differ (Frenster, 1965). The effects of acetylation and methylation on the properties of histones has already been noted (Allfrey, Faulkner & Mirsky, 1964). Variation in thiol and phosphate content of f1, f3 and the fast-moving components may now be additional means by which the capacity of histones to decrease RNA synthesis can be influenced. Acetylation, methylation, phosphorylation and oxidation of thiol groups are all simple reactions which may be demonstrated in isolated nuclei. There is therefore the possibility that such reactions can affect the capacity of nucleochromatin to support RNA synthesis. No data are yet available on the specificity of interaction of different species of f1 or f3 with DNA.

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