Metabolic Properties of Histones from Rat Liver and Thymus Gland

By MARGERY G. ORD AND L. A. STOCKEN

Department of Biochemistry, University of Oxford

(Received 20 September 1965)

1. The incorporation of ^{14}C -labelled amino acids into acid-extractable proteins from rat-liver and -thymus nuclei confirmed the existence of a protein component with a higher uptake than that into the major histone components. 2. This rapidly labelled component appeared to contain the thiol groups detectable in the acid extracts. 3. Histone f1 contained 1 mol. of serine phosphate/mol. of mol.wt. of 42000–43000. 4. Phosphate was present in other components of the 50mm-hydrochloric acid extract from liver and thymus nuclei, and was probably associated with the thiol-containing component. 5. The difference in amino acid uptake into the histones of diffuse and dense chromatin was confirmed. Dense chromatin was found to have a higher proportion of disulphide than did diffuse chromatin.

Examination of the histones that could be separated by preparative electrophoresis on polyacrylamide gel showed that when rat-thymus nuclei were incubated with ^{14}C -labelled amino acids there was a marked difference in rate of uptake of the amino acids into the different proteins, one component (6) having an especially high uptake (Ord, Raaf, Smit & Stocken, 1965). In the electrophoretic procedure used, 2–3 days were required for the separation of the different components, so that proteolysis could have occurred, and only 5mg. of material could be examined. To confirm the existence of a basic protein into which amino acids were incorporated rapidly, and to obtain sufficient material for the nature of the component to be investigated, it was decided to use other methods for the separation of the histones and also to see whether the component was found in histones from nuclei of another tissue. A comparison was also made of the pattern of amino acid and $[^{32}\text{P}]$ -phosphate uptake into thymus histones *in vivo* and *in vitro*.

METHODS

Preparation of nuclei. Nuclei were isolated from liver and thymus glands of rats (120g. body wt.) in 0.25M-sucrose–5mm-MgCl₂–5mm-tris-HCl, pH 7.2, as described by Ord *et al.* (1965). The final concentration of the nuclear suspension was the equivalent of one thymus gland or liver/ml. of suspending medium.

Isolation of histones. Nuclei from a suspension in 10–12ml. were washed twice with 20ml. of 0.01M-tris-HCl–5mm-MgCl₂, pH 7.1 (Frenster, Allfrey & Mirsky, 1960), to remove nuclear ribosomes. The sediment was then washed two or three times with 20ml. of 1mm-HCl to remove low-molecular-weight nucleotides and was extracted with 5ml. of 50mm-HCl (Marsh, Ord & Stocken, 1964). A second

extract with 20mm-HCl was rejected and the residue re-extracted with 5ml. of 250mm-HCl. The extracts in 50mm- or 250mm-HCl were clarified by centrifuging for 20–30 min. at 100000g (35000 rev./min. in a Spinco model L centrifuge, rotor SW 39).

Preparation of diffuse and dense chromatin. These were obtained from rat-thymus nuclei by the procedure of Frenster, Allfrey & Mirsky (1963). After preparing the nuclei in the usual way they were washed in tris-MgCl₂ and allowed to stand for 10 min. at 0° with 0.25M-sucrose. The preparation was then treated ultrasonically for 1 min. at 1.5A and 20000 cyc./sec. in an MSE Sonicator. The material was centrifuged for 10 min. in a Servall centrifuge at 1000g to bring down the dense chromatin. The material that sedimented after a further 30 min. at 3000g was discarded and diffuse chromatin was sedimented after 60 min. at 80000g (35000 rev./min. in a Spinco model L centrifuge, rotor 40). After two or three washes with 1mm-HCl the histones were extracted from dense and diffuse chromatin in the normal way.

Separation of the histones. (a) Chemical methods. (1) In some experiments nuclei that had been washed with tris-HCl-MgCl₂ and with 1mm-HCl were extracted with 5% (v/v) HClO₄ (method 1 of Johns, 1964). The histone f1 was usually precipitated from such an extract by the addition of 100% (w/v) trichloroacetic acid to give a final concentration of 20%. The nuclear residue was further extracted with 50mm- and 250mm-HCl as described above, the extracts were made 1.25N with respect to HCl, 4 vol. of ethanol was added and the solutions were dialysed overnight against ethanol at 0°. The precipitates that formed in the dialysis sacs were separated by centrifugation and redissolved in 50mm- or 250mm-HCl. Then 3–5 vol. of acetone was added to the ethanolic supernatants and the resulting precipitates were also dissolved in 50mm- or 250mm-HCl.

(2) In other experiments the nuclei were extracted with 50mm- and 250mm-HCl in the usual way and these extracts made to 1.25N with respect to HCl. After the addition of 4 vol. of ethanol the solutions were dialysed against ethanol

Fig. 1. Separation of histones from a 50mm-HCl extract from rat thymus nuclei on Sephadex G-75. A 16–20mg. sample of protein in 4–5ml. was applied to a column (130cm. $\times$ 2.5cm. diam.) of Sephadex G-75. Fractions (5ml.) were collected at a flow rate of 15ml./hr. with 20mm-HCl as eluent. The protein content was measured on 0.1ml. from each tube by the method of Lowry *et al.* (1951). The peak numbers marked on the Figure correspond to those in Table 1.

Fig. 2. Separation of histones from 250mm-HCl extracts of liver or thymus nuclei on Sephadex G-75. A 16–20mg. sample of protein in 4–5ml. was applied to a column (130cm. $\times$ 2.5cm. diam.) of Sephadex G-75. Fractions (5ml.) were collected at a flow rate of 15ml./hr. with 20mm-HCl as eluent. The protein content was measured on 0.1ml. from each tube by the method of Lowry *et al.* (1951). (No differences were observed between the elution patterns of the extracts from liver and thymus.) The peak numbers marked on the Figure correspond to those in Table 1.

as in (1), and the ethanol- and the acetone-insoluble materials separated.

(3) Histones f3, f2a and f2b were obtained from thymus nuclei by method 2 of Johns (1964).

Table 1. *Histones detected by analytical electrophoresis in the peaks eluted from Sephadex G-75*

The main peaks were numbered as they were eluted from the Sephadex. Peak 1 was defined as the first big peak from the 50mm-HCl extract which contained component 9; peak 7 was the peak from the 50mm-HCl extract which contained only components 1–3. All the peaks were not found in every run and sometimes clearly separated minor peaks were present. Components in parentheses were present in trace amounts only. The components were identified by the position to which they moved after analytical electrophoresis under standard conditions. As far as could be ascertained the same component might be present in a number of different peaks and many were found in both the 50mm- and 250mm-HCl extracts.

50mm-HCl extract		250mm-HCl extract	
Peak	Components	Peak	Components
0	4, 5, 6, 7, 8	A ₁	4, 5, 6, 7, 8, (10), (12)
1	4, 5, (6), 7, 8, 9	A ₂	(6), 7, 8, (10), 12
2	4, 5, (6), 7, 8, 9	B	1–3, 4, 5, 7, 8, 10, 11, 12
3	(1–3), (4, 5), 7, 8, (9)	C	4, 5, 7, 8, 10, 12
(–)	(1–3), 4, 5, (6), (7, 8, 9)	D	4, 5, 7, 8, 10, (12)
4	1–3, 4, 5, (7, 8, 9)	E	4, 5, 7, 8, 10
5	1–3, 4, 5		
6 ₁	1–3, 4, 5, 7, 8		
(–)	1–3, 4, 5		
6 ₂	1–3, (4, 5)		
7	1–3		

(b) Exclusion chromatography. Some separation of the components in the 50mm- and 250mm-HCl extracts from rat-liver and -thymus nuclei was obtained by chromatography on Sephadex G-75 (Cruft, 1961; Johnson, Driedger & Marko, 1964). A column 130cm. $\times$ 2.5cm. diam. was used. The proteins were eluted with 20mm-HCl; 5ml. fractions were collected at a flow rate of 15ml./hr. Figs. 1 and 2 show the peaks obtained from a 50mm-HCl extract from thymus nuclei and from the 250mm-HCl extract of both liver and thymus. The components detected in the different peaks after analytical electrophoresis are shown in Table 1.

Analytical electrophoresis on polyacrylamide gel. The histones present in the different fractions obtained by the chemical procedures or by chromatography were analysed by electrophoresis on polyacrylamide gel (McAllister, Wan & Irwin, 1963). Fig. 3 shows the components that have so far been distinguished, and their probable identification with the histones from calf thymus described by Johns (1964). The numbering of the bands agrees with that of the components detected by preparative electrophoresis (Ord *et al.* 1965). Where components 1–3 could not be distinguished they are referred to as fast-moving components, f_{mc}. About 75–80 μ g. of proteins was applied to the gels and the electrophoresis was run for 2.5 hr. at 300v and 3–4 mA/tube at pH 4.1 (37mm-glycine-acetic acid buffer) at 20°.

Uptake of labelled precursors. A nuclear suspension (10–12ml.) was incubated for 45 min. at 37° with 100 μ C of ¹⁴C-labelled protein hydrolysate (The Radiochemical

Centre, Amersham, Bucks.) (specific activity 600 $\mu\text{C}/\text{mg.}$) and 0.8 ml. of 0.9% NaCl containing 100 mg. of glucose/100 ml. At the end of the incubation the nuclei were washed five times with medium containing casein hydrolysate (1%) before extraction of the histones in the usual way.

In experiments on incorporation *in vivo* the ^{14}C -labelled amino acids, [^{14}C]adenine sulphate (28.9 mc/m-mole; The Radiochemical Centre) or inorganic [^{32}P]phosphate were injected intramuscularly (15 $\mu\text{C}/100\text{g.}$ body wt., 5 $\mu\text{C}/100\text{g.}$ and 50 $\mu\text{C}/100\text{g.}$ respectively) and the rats killed after 30–120 min.

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The procedure was standardized by using dialysed HCl extracts from nuclei that were then dried *in vacuo* over P_2O_5 .

Thiol and disulphide groups. These were measured by the method of Ellman (1959) as described by Marsh *et al.* (1964). 1- ^{14}C -labelled *N*-ethylmaleimide (8 $\mu\text{C}/\text{mg.}$; Schwartz Bio-Research Inc., Mount Vernon, N.Y., U.S.A.) was used to label the thiol groups present in a 50 mm-HCl extract from thymus nuclei. The extract was brought to pH 4.5 and a 50% excess of *N*-ethylmaleimide added. After standing at 20° for 6 hr. the solution was dialysed overnight at 0° against 20 mm-HCl.

Phosphate determinations. Inorganic phosphate was measured by the method of Berenblum & Chain (1938). A final volume of 2 ml. of 2-methylpropan-1-ol-ethanol was

used. The methods for acid-labile phosphates, total phosphate and for nucleic acid phosphorus were as described by Ord & Stocken (1961).

Serine phosphate. An 8 mg. sample of histone f1 was hydrolysed for 20 min. in 6N-HCl at 100° as described by Schwartz & Lipmann (1961). After removal of the HCl *in vacuo* over KOH the hydrolysed material was dissolved in water and applied to a column (20 cm. $\times$ 0.4 cm. diam.) of Bio-Rad AG 50W (X8; 200–400 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The column was eluted with water and 1 ml. fractions were collected. The fractions containing phosphate were freeze-dried and examined by ascending paper chromatography with propan-2-ol–10N-HCl–water (65:16.6:18.4, by vol.) (Fink, Cline & Fink, 1963). Serine phosphate and threonine phosphate hardly separate in this solvent (R_F 0.53 and 0.55 respectively). The area containing the phosphate was eluted with water, and the eluate freeze-dried and hydrolysed in 6N-HCl at 110° for 17 hr. (Crestfield, Moore & Stein, 1963). The HCl was removed under vacuum at 40° and the hydrolysed material from histone f1 and from the serine phosphate marker was examined by ascending paper chromatography in 2-methylpropan-2-ol–butan-2-one–water–aq. NH_3 (sp.gr. 0.88) (4:3:2:1, by vol.) (Fink *et al.* 1963), with serine and threonine as markers (R_F 0.38 and 0.54 respectively).

Ribose. Ribose was determined by the method of Dische & Schwartz (1937).

Deoxyriboside. This was measured as described by Burton (1956).

Radioactivity measurements. The ^{14}C radioactivity was determined by gas-flow or scintillation counting (Nuclear-Chicago Corp. Tritomat; Isotope Developments Ltd., Beenhams, Berks.). (The efficiency of the scintillation counter was 34%; that of the gas-flow counter was about 2%. Sufficient counts were recorded to give an error of less than 2%.) The ^{32}P radioactivity was measured in a liquid M6 Geiger-Müller tube or 2 ml. of the sample was placed within a glass tube surrounded by 5 ml. of scintillator and the activity measured in the Tritomat. The ^{32}P content of the bands separated by electrophoresis on polyacrylamide gel was determined by gas-flow counting as described by Marsh *et al.* (1964).

RESULTS

Chemical separation of histones. In confirmation of previous findings (Ord *et al.* 1965) our results (Table 2) show that after incubation with ^{14}C -labelled amino acids *in vitro* the histones extracted from thymus nuclei with 50 mm-hydrochloric acid were of higher specific activity than those removed by 250 mm-hydrochloric acid. The 50 mm-hydrochloric acid extract was characterized by the presence of component 9 (histone f1 of Johns, 1964) and the relative absence of components 10 + 12 (histone f3); the 250 mm-hydrochloric acid extract normally contained no histone f1 but fairly large amounts of histones f3, f2a (components 7 + 8) and f2b (components 4 + 5) were present in both extracts. These four types of histone made up the greater part of the proteins extracted but components 1–3, which moved ahead of histone f2 on electrophoresis at pH 4.1, were also present and were relatively

Fig. 3. Bands obtained after electrophoresis of 50 mm- and 250 mm-HCl extracts of rat-liver and -thymus nuclei on polyacrylamide gel. A 70–80 $\mu\text{g.}$ sample of protein was applied to the gel (6 cm. $\times$ 0.4 cm. diam.). Electrophoresis was performed for 2½ hr. at 3 mA/tube and 300 v in glycine-acetic acid buffer, pH 4.1. The gels were stained with Naphthalene Black and destained electrophoretically. Bands 1–9 were commonly found in the 50 mm-HCl extracts and bands 4–8 and 10–12 in the 250 mm-HCl extracts. Band 10 probably corresponds to histone f3 of Johns (1964), band 9 to histone f1, and bands 4, 5, 7 and 8 to the components of histone f2.

Table 2. *Specific activity of different histones from rat-thymus nuclei obtained after chemical separation of the proteins*

Nuclei were isolated from rat thymus gland and incubated with ^{14}C -labelled protein hydrolysate for 45 min. at 37° . After washing the nuclei with medium containing casein hydrolysate (1%) the histones were extracted and precipitated as described in the text. Specific activity is expressed as counts/min./ μg . of protein. Components in parentheses were present in trace amounts only.

	Method 1		Method 2	
	Probable components	Specific activity	Probable components	Specific activity
50mm-HCl extract	—	—	1-9	66.9
250mm-HCl extract	—	—	4, 5, 7, 8, 10, 12	19.5
5% HClO_4 extract	9	6.8	—	—
	1-3, 7, 8, 9	15.5	—	—
50mm-HCl extract, ethanol ppt.	4, 5, (9), 10, 12	17.6	1-3, 4, 5, 6, 9, (10, 12)	81.6
		25.2*		48.4*
50mm-HCl extract, acetone ppt.	7, 8, 10, 12	5.4	1-3, 7, 8, (10, 12)	35.7
				19.8*
250mm-HCl extract, ethanol ppt.	4, 5, 10, 12	15.7	4, 5, 7, 8, 10, 12	41.5
		29.0*		25.9*
250mm-HCl extract, acetone ppt.	7, 8, 10, 12	7.5	7, 8, 10, 12	12.8
		16.5*		11.3*

* These fractions were prepared from a batch of nuclei of which half was treated by method 1 and half by method 2.

Table 3. *Thiol contents of different histones from rat-thymus nuclei*

The nuclei were isolated and the histones extracted and precipitated as described in the text. The total thiol content was measured by the method of Ellman (1959) after reduction of any disulphide to thiol by borohydride. The total thiol content is given as μmoles of thiol plus disulphide/mg. of protein. Components in parentheses were present in trace amounts only.

Fractionation method	Components present	Total thiol content
Method 2		
50mm-HCl extract, ethanol ppt.	1-3, 4, 5, 6, (7, 8), 9	37.0, 27.2
50mm-HCl extract, acetone ppt.	1-3, (4, 5), 7, 8	3.3, 8.6
250mm-HCl extract, ethanol ppt.	4, 5, (7, 8), 10, 12	36.0, 30.9
250mm-HCl extract, acetone ppt.	(4, 5), 7, 8, (10, 12)	7.2, 8.8
Original 50mm-HCl extract	1-9	37.0, 23.8
Original 250mm-HCl extract	1-5, 7, 8, 10, 12	33.0, 10.3
Method 3: f3	10, 12	30.0
f1	9	0
f2b	4, 5	8.0

concentrated in the mixture of histones extracted with 50mm-hydrochloric acid. The histone patterns obtained after electrophoresis, from nuclei before and after incubation at 37° , did not show any significant differences, suggesting that proteolysis during incubation did not account for the presence of relatively large amounts of components 1-3.

Precipitation with ethanol or acetone of the mixtures of histones already extracted with hydrochloric acid did not give clear-cut separation of the different components, although extraction of the nuclei with 5% perchloric acid usually gave fairly clean histone f1 (Johns, 1964). The specific activities of the different mixtures (Table 2), however, show that, in agreement with Laurence & Butler (1965), incorporation of amino acids into

component 9 (histone f1) was relatively slow, as was that into components 10+12 (histone f3) and 7+8. Incorporation into components 4+5 was more rapid. When nuclei were extracted with 5% perchloric acid before further extraction with 50mm- and 250mm-hydrochloric acid much less clear separation between histones f1 and f3 was obtained and the amounts of components 1-3 appeared to be decreased, although this may have resulted from the presence of relatively large amounts of histones f1 and f3 in the extracts that were analysed by electrophoresis. It was also found that the material of very high specific activity was not extracted from nuclei if they were first treated with 5% perchloric acid (Table 2). This material, with high specific activity, was precipitated from

a normal 50 mM-hydrochloric acid extract by method 2, and may correspond to band 6 on electrophoresis.

As previous results (Ord *et al.* 1965) had suggested that the fast-moving components on electrophoresis might be the thiol-containing histones (Marsh *et al.* 1964), the thiol plus disulphide contents of the different components obtained by precipitation were determined (Table 3). In agreement with the findings of Butler and his colleagues (Laurence & Butler, 1965; Phillips, 1965), histone f1 had no detectable thiol or disulphide groups, histones f2a and f2b contained some thiol plus disulphide groups and histone f3 was relatively sulphur-rich. The fairly large thiol content associated with the 50 mM-hydrochloric acid extract appeared to be due to the presence of either component 6 or one or more of the fast-moving components.

Separation of the histones on Sephadex G-75. As the precipitation methods did not allow unequivocal separation of the different histones, an attempt was made to separate them by chromatography on Sephadex G-75 (Cruft, 1961; Johnson *et al.* 1964). Usually all the protein applied to the columns could be recovered and fairly good reproducibility was obtained between the different runs except that band 6, which in some experiments (Fig. 1) was in the first peak (0) off the column, was also obtained in a shoulder (2) immediately after the peak (1) containing a mixture of histones in which f1 predominated. As reported by Johnson *et al.* (1964) separation on Sephadex did not yield peaks containing single histones, although it was more satisfactory for the low-molecular-weight fast-moving components.

The pattern of incorporation of amino acids into histones extracted with 50 mM-hydrochloric acid (Table 4) confirmed that obtained after differential precipitation. The presence of component 6 was again associated with material of high specific activity and uptake into histone f1 was low. When

sufficient protein was available histone f1 could be separated from contaminants also present in the first large peak off the column by its solubility in 5% perchloric acid (Johns, 1964). Such treatment confirmed the low specific activity of this protein.

The specific activities of the histones after uptake of amino acids *in vivo* followed a very similar pattern to that found after uptake *in vitro*, and the distribution of specific activities between the histones of liver nuclei *in vivo* was very similar to that found in the histones from thymus (Table 4). The similarity of our results *in vivo* and *in vitro* suggest that the data are not invalidated by the observations of Sangster & Poort (1965) on the unsuitability of algal-protein hydrolysate as a source of ^{14}C -labelled amino acids for studies on incorporation *in vitro*. Qualitatively similar histones were extracted from the liver nuclei with 50 mM- and 250 mM-hydrochloric acid to those found from thymus but liver nuclei appeared to contain relatively more of components 7 + 8. This may have been due to the different method of preparation of the nuclei, but the electrophoretic behaviour of the histones extracted from thymus nuclei that had been prepared in 2.2 M-sucrose medium was not significantly different from those obtained from nuclei made in 0.25 M-sucrose medium.

When the histones from a 50 mM-hydrochloric acid extract of thymus nuclei were labelled *in vitro* with ^{14}C -labelled *N*-ethylmaleimide the insignificant thiol content of histone f1 was substantiated (Table 4) and a fast-moving component and band 6 were found to be the thiol-rich components.

Incorporation of amino acids into the histones of the 250 mM-hydrochloric acid extracts was also similar for liver and thymus *in vivo* (Table 5). The two small peaks of high-molecular-weight material eluted first from the columns were much more radioactive than the major peaks B and C. Electro-

Table 4. *Specific activities of different histones from rat-thymus and -liver nuclei obtained after separation of the histones from 50 mM-hydrochloric acid extracts on Sephadex G-75*

The nuclei were isolated and the histones extracted and separated on Sephadex columns as described in the text. Expt. 1: specific activities of different peaks after thymus nuclei had been incubated with ^{14}C -labelled amino acids. Expt. 2: peaks from thymus nuclei after labelled amino acids had been injected into rats 1 hr. previously. Expt. 3: as Expt. 2, but with liver nuclei. Expts. 4 and 5: peaks from 50 mM-HCl extracts from thymus nuclei, labelled *in vitro* with ^{14}C -labelled *N*-ethylmaleimide. The numbering of the peaks corresponds to that in Fig. 1, and the components present are shown in Table 1. Specific activities are expressed as counts/100 sec./ μg . of protein.

Expt. no.	Specific activity											
	Peak...	0	1	2	3	—	4	5	6 ₁	—	6 ₂	7
1	—	—	27.5	21.5	8.7	20.9	—	1.25	—	1.03	—	—
2	—	—	0.81	5.65	2.38	—	—	0.86	—	0.81	—	0.96
3	—	—	0.35	1.18	0.46	—	0.69	—	—	—	—	—
4	—	—	4.5	19.4	—	—	—	—	7.5	—	7.4	—
5	50	50	4.2	—	23.4	—	34.8	—	16.9	—	38.4	20.5

Table 5. *Specific activities of different histones from rat-thymus and -liver nuclei obtained after separation of the histones from 250mM-hydrochloric acid extracts on Sephadex G-75*

The nuclei were isolated and the histones extracted and separated on Sephadex columns as described in the text. Expt. 1: specific activity after thymus nuclei had been incubated *in vitro* with ^{14}C -labelled amino acids. Expt. 2: peaks from thymus nuclei after labelled amino acids had been injected into rats 1 hr. earlier. Expt. 3: as Expt. 2, but with liver nuclei. The numbering of the peaks corresponds to that in Fig. 2, and the components present are shown in Table 1. Specific activities are expressed as counts/100sec./ μg . of protein.

Expt. no.	Specific activity						
	Peak ...	A ₁	A ₂	B	C	D	E
1	—	—	—	24.1	27.3	—	—
2	1.71	1.71	1.47	0.69	0.69	0.99	0.86
3	1.71	1.71	3.12	0.33	0.27	—	—

Table 6. *Amino acid uptake and thiol plus disulphide contents of diffuse and dense chromatin obtained from rat-thymus nuclei*

Diffuse and dense chromatin were prepared as described by Frenster *et al.* (1963) and the histones extracted therefrom in the usual way. Conditions for amino acid uptake are described in the text. Thiol and total thiol plus disulphide contents are expressed as $\text{m}\mu\text{moles/mg.}$ of protein and ^{14}C uptake is expressed as $10^{-3} \times \text{counts/min./mg.}$ of protein.

	Dense chromatin			Diffuse chromatin		
	Histone fl	50mM-HCl extract	250mM-HCl extract	Histone fl	50mM-HCl extract	250mM-HCl extract
Amino acid uptake	12.0	20.2	9.4	37.0	38.7	26.0
SH	—	8.3*, 10.8†	11.5*, 28.0†	—	20.4*, 31.4†	15.4*, 29.0†
SH+S.S	—	15.6*, 21.2†	25.0*, 31.6†	—	26.9*, 31.4†	23.4*, 29.0†

* Expt. 1. † Expt. 2.

phoretic analysis of the protein of peaks A₁ and A₂ indicated the presence of traces of component 6.

Diffuse and dense chromatin. Frenster *et al.* (1963) described the greater rate of incorporation of a number of precursors into the RNA and protein of diffuse (active) chromatin as compared with dense (repressed) chromatin. Frenster (1965) has also established that the amount and type of histone obtained from the two chromatins are very similar. Our results on amino acid incorporation into the two types of chromatin (Table 6) and the pattern obtained after electrophoresis of the histones confirm Frenster's (1965) observations. When the thiol contents of the histones were examined (Table 6), however, the 50mM-hydrochloric acid extract from dense chromatin contained relatively more disulphide than the extract from diffuse chromatin. There may also have been rather less total sulphur. The total amounts of thiol in the 250mM-hydrochloric acid extracts did not differ. Dense chromatin is obtained much earlier in the preparation than diffuse chromatin, and histone extraction and thiol estimations were performed immediately so that preferential spontaneous oxidation of the thiol groups from dense chromatin seems unlikely. There was some indication that dense chromatin contained relatively more histone fl than did active chromatin; this is substantiated by the

observations of Littau, Burdick, Allfrey & Mirsky (1965) and explains the lower total sulphur content in the 50mM-hydrochloric acid extract from dense chromatin as histone fl does not contain thiol or disulphide groups.

Incorporation of [^{32}P]phosphate into 50mM- and 250mM-hydrochloric acid extracts of nuclei. Examination of the spectrum of peak 0 (Table 1) showed that the protein(s) present absorbed in the ultra-violet with a broad shoulder at 255–280 μ (Fig. 4). As the extinction in the region/mg. of protein appeared to be considerably greater than for histone fl, which also does not show a prominent tyrosine peak, it seemed possible that nucleotide might be associated with component 6 either as a contaminant or bound to the protein.

To test this hypothesis [^{32}P]phosphate was injected intramuscularly 30–120 min. before death and the nuclei from liver and thymus were washed an additional time with 1mM-hydrochloric acid to ensure removal of low-molecular-weight phosphate compounds. The radioactivity of all the washes was followed: ^{32}P was detected in both the 50mM- and 250mM-hydrochloric acid extracts from both sources of nuclei (Table 7). The radioactivity was not decreased by dialysis overnight against 50mM- or 250mM-hydrochloric acid, and when 230 μg . of protein from a 50mM-hydrochloric acid extract

from thymus nuclei was analysed electrophoretically 13% of the counts remained at the origin, 27% was in the band associated with histone f1 and 53% was distributed over the region of histone f2 (components 4+5+7+8). The mean phos-

phorus contents of the 50mm- and 250mm-hydrochloric acid extracts were 26.0 (19.9–31.4) and 15.8 (8.4–22.6) $\mu\text{g}.\text{atoms}/\text{mg}.$ of protein respectively (five determinations). Histone f1 was also prepared (method 1 of Johns, 1964); on electrophoresis only very small traces of other proteins were detectable. The specific activity of ^{32}P -labelled histone f1 and its ^{31}P content were unchanged by dialysis. It contained 23.4 (20.4–27.8) $\mu\text{g}.\text{atoms}$ of P/mg. of protein (five determinations).

Because of the high radioactivity of many of the phosphorylated derivatives in nuclei, the extent of ^{32}P incorporation into a number of phosphates was followed over a 2 hr. period. Previous experiments (Ord & Stocken, 1961) had shown the presence of inorganic phosphate which was bound to thymus nuclei, but which could be removed with cold dilute acid. N-Perchloric acid was used and it was noted that a small amount of phosphorus in this extract was associated with a soluble protein which was precipitated by phosphomolybdate in the procedure of Berenblum & Chain (1938) for determining inorganic phosphate. It is now believed that the protein precipitated under these conditions was histone f1.

The time-course of ^{32}P uptake into bound inorganic phosphate, acid-labile phosphate, RNA and DNA suggested (Table 7) that the radioactivities of histone f1 and the 50mm-hydrochloric acid extract were unlikely to be due to contamination from the types of nucleic acid phosphorus examined. The distribution of counts through the preliminary washes showed that ^{32}P radioactivity was removed with tris-magnesium chloride and

Fig. 4. Absorption spectrum of histones obtained from a 50mm-HCl extract of rat-thymus nuclei. Histone f1 (component 9) was obtained by method 1 of Johns (1964) and histone f2a by method 2. Peak 0 was obtained after separation of a 50mm-HCl extract of rat-thymus nuclei on Sephadex G-75. The spectra were examined in the range 320–240 $\text{m}\mu$ in a Beckman DK-2A spectrophotometer. The protein concentrations were: histone f1 (A), 1.30 mg./ml.; histone f2a (B), 1.94 mg./ml.; peak 0 (C), 0.24 mg./ml.

Table 7. *Specific activity of phosphorylated compounds from rat-thymus nuclei*

[^{32}P]Phosphate was injected intramuscularly (50 $\mu\text{C}/100\text{g}.$ body wt.) 30–120 min. before death. Nuclei were isolated and extracted as described by Marsh *et al.* (1964). Histone f1 was prepared by the method of Johns (1964). The specific activities of bound inorganic phosphate and acid-labile phosphate were recalculated from the data of Ord & Stocken (1961). Ribosomal RNA was the RNA extracted from the nuclei with 0.01 M-tris-HCl–5 mM-MgCl₂, pH 7.1 (Frenster *et al.* 1960), and residual RNA was RNA remaining in the nuclei when ribosomal RNA, acid-extractable proteins and lipids had been removed. Specific activities are expressed as counts/100 sec./ $\mu\text{g}.$ of phosphorus.

Expt. no. (Ord & Stocken, 1961)	Time after injection (min.).....	Specific activity		
		30	60	120
{	Bound inorganic P	990	648	512
	Acid-labile P	610	521	501
1	Ribosomal RNA P	2	3	3
1	Residual RNA P	7	26	31
1	DNA P	2	9	15
1	Histone f1	46	56	73
2	Histone f1	—	223	195
3, 4, 5*	Histone f1	29	188	79
3, 4, 5*	50mm-HCl extract	6	65	64
3, 4, 5*	250mm-HCl extract	14	15	36

* In Expts. 3–5, each of the time-periods represents a different experiment, but histones f1 and the 50mm- and 250mm-HCl extracts were all prepared from the same batch of nuclei.

that the washes with 1mM-hydrochloric acid took out progressively less and less activity; 5% perchloric acid of 50mM-hydrochloric acid markedly increased the amount of ³²P extracted but the results after dialysis appeared to exclude contamination of the proteins by low-molecular-weight compounds.

At 60min. after injection the uptake of ³²P into the histones of the 50mM-hydrochloric acid extract considerably exceeded that into the histones of the 250mM-hydrochloric acid extract (Table 7), and this is supported by the radioactivities of the proteins after separation on Sephadex G-75 (Table 8). In the second experiment with thymus component 6 was found by electrophoresis to have separated in peak 0 just ahead of the first big peak, and had a lower specific activity/ μ g. of P than the other components of the 50mM-hydrochloric acid extract, although its activity/mg. of protein was higher. The behaviour of the 50mM-hydrochloric acid extract from liver nuclei was similar to that from thymus although the activity/ μ g. of P was higher throughout.

Nature of the phosphate groups. In examining the nature of the phosphate group in histone f1 and in the whole 50mM-hydrochloric acid extract the possibilities considered were that the phosphate, either as inorganic phosphate or as nucleotide phosphate, was covalently bound to the proteins, or that there was a particularly stable salt formed between the proteins and the ester phosphate. Because of the small amounts of material available [¹⁴C]adenine uptake *in vivo* was investigated to see whether adenine was present in any of the fractions. Histone f1 and the 50mM- and 250mM-hydrochloric acid extracts were prepared in the usual way from liver and thymus nuclei 2hr. after injection. Uptake into extracts from liver nuclei was much higher than into those from thymus (thymus: 50mM-hydrochloric acid extract, 2.5 counts/min./mg. of protein; 250mM-hydrochloric acid extract,

$\pm$; liver: 50mM-hydrochloric acid extract 15.5; 250mM-hydrochloric acid, 4.7). No counts were detected in histone f1 from either source.

The absence of nucleotide from histone f1 allowed the possibility that the phosphate might be present as serine phosphate or threonine phosphate. ³²P-labelled histone f1 was prepared and hydrolysed for 20min. in 6N-hydrochloric acid at 100° (Schwartz & Lipmann, 1961). The phosphate was not liberated as inorganic phosphate but remained as organophosphate. After chromatography on Bio-Rad AG 50W (see the Methods section), ninhydrin-positive material was not detected in the first two fractions, which contained the ³²P. Ascending paper chromatography of the phosphorus-containing material showed that the ³²P migrated in the same position as an authentic sample of serine phosphate. The ³²P-containing area and that of the authentic sample of serine phosphate were eluted, hydrolysed overnight in 6N-hydrochloric acid at 110° and rechromatographed. The material obtained from histone f1 behaved identically with that from authentic serine phosphate. No sign of threonine was detected.

The [¹⁴C]adenine uptake into the 50mM- and 250mM-hydrochloric acid extracts suggested that their phosphorus contents might be due to contamination from nucleotide material other than that examined above. Treatment of the 50mM-hydrochloric acid extract with ribonuclease or with phenol (Kirby, 1956) failed to decrease the phosphorus content nor was it removed by passing the proteins through a column of Dowex 1 (Cl⁻ form). When the phosphorus contents of histones prepared from thymus nuclei after extraction with 50mM-hydrochloric acid and dialysis against ethanol were compared with those obtained by method 2 of Johns (1964), ³¹P and ³²P were detected mainly in the precipitate from the 50mM-hydrochloric acid extract dialysed against ethanol and in histones f1 and f3. Histones f2a and f2b did not contain

Table 8. [³²P]Phosphate uptake by proteins extracted from liver and thymus nuclei with 50mM- and 250mM-hydrochloric acid

[³²P]Phosphate was given to the rats 60min. before death. The nuclei were washed and the proteins extracted and separated on Sephadex columns as described in the text. The components in the peaks were identified by electrophoresis on polyacrylamide gel, and the peaks numbered as in Figs. 1 and 2. ³²P radioactivity is expressed as counts/10³sec. per mg. of protein or per μ g. of phosphorus.

		50 mM-HCl extract						250 mM-HCl extract		
	Peak.....	1	2	3	5	6	7	B	C	D
³² P uptake/ μ g. of P	Liver	2970	—	5290	2640	—	—	—	—	—
	Thymus: 1	1875	1350	1270	—	1370	—	—	—	—
	2	1882	447	—	1242	1140	2115	97	75	149
³² P uptake/mg. of protein	Liver	2860	—	927	—	—	—	—	—	—
	Thymus: 1	1376	1760	1226	—	240	—	—	—	—
	2	1350	2440	—	1115	584	1349	237	111	795

phosphorus. Since extraction of nuclei with 50mm-hydrochloric acid or higher acid strength appeared to remove a phosphorus compound in either method, it seemed that the phosphorus component(s) other than histone f1 might be extracted in either procedure and become associated with the other proteins present in the extracts.

A strong similarity was observed between the behaviour of this phosphorus compound, the component into which rapid uptake of amino acids occurred and the thiol-rich material examined above. This was confirmed in an experiment in which the histones from thymus nuclei were labelled with ^{14}C -labelled amino acids and ^{32}P -phosphate *in vivo* and the phosphorus and thiol contents of the 50mm-hydrochloric acid extract were followed (Table 9). Both the thiol groups and the phosphorus appeared to be associated with material extracted in 50mm-hydrochloric acid that was precipitated by dialysis against ethanol, was not removed by dialysis against hydrochloric acid and was not histone f1. The amounts of phosphorus and of thiol plus disulphide were equimolar and were also associated with protein having an increased uptake of amino acids. It was not possible from the electrophoretic analyses to define precisely the component with the appropriate properties. Component 6 did not separate in any of these analyses.

The separation of the ^{14}C adenine-labelled extract from thymus nuclei on Sephadex G-75 referred to above gave a small peak 0 containing ^{14}C adenine radioactivity and ^{31}P , which was eluted before the first big peak. When this peak 0 was analysed for ribose by the orcinol method

(Dische & Schwartz, 1937) a brown colour was produced, masking any possible ribose content, and probably being attributable to glucose derived from oligosaccharide. Oligosaccharide was also detected in peak 7, the only other material to give low, but unequivocal, ^{14}C adenine radioactivity. Thymus nuclei are known to contain glycogen (Ord & Stocken, 1962). It seems possible therefore that the thiol- and phosphorus-containing compound extracted from nuclei with 50mm-hydrochloric acid becomes associated with oligosaccharide or low-molecular-weight basic protein or both and distributes itself on electrophoresis or on Sephadex according to the properties of its associated molecule.

Hydrolysis of peak 0 in 6N-hydrochloric acid overnight at 110° and chromatography of the hydrolysed material in the butan-2-one solvent showed the presence of some material absorbing in the ultraviolet and five spots reacting with ninhydrin. Three of these have been tentatively identified as threonine, serine and glutamic acid or aspartic acid.

DISCUSSION

The rates of uptake of amino acids into the main histones extracted from liver and thymus nuclei confirm observations of Laurence & Butler (1965) that there is little difference in the rapidity of synthesis of these proteins, which are renewed at a detectable rate. The greater incorporation of amino acids into the histones extractable from diffuse chromatin compared with those from dense chromatin also confirms observations by Frenster *et al.* (1963). The same amount of histone is found

Table 9. *Phosphorus and thiol contents of proteins from a 50mm-hydrochloric acid extract from thymus nuclei*

Rats were given $25\mu\text{C}$ of ^{32}P phosphate and $17.7\mu\text{C}$ of ^{14}C -labelled amino acids/120 g. body wt. 60 min. before death. The 50mm-HCl extract was obtained in the usual way. It was then dialysed against 100 vol. of ethanol and the precipitate redissolved in 50mm-HCl and made 0.74N (5%) with respect to HClO_4 . The resulting precipitate was dialysed against two changes of 10 vol. of 50mm-HCl. ^{31}P is expressed as $\text{m}\mu\text{g. atoms/mg. of protein}$; SH (or SH+S.S) is expressed as $\text{m}\mu\text{moles/mg. of protein}$. ^{32}P radioactivity is expressed as $\text{counts}/10^3\text{sec./mg. of protein}$, determined by liquid counting; ^{14}C radioactivity is expressed as $\text{counts}/10^3\text{sec./mg. of protein}$, determined by scintillation counting.

	^{32}P	^{14}C	SH	^{31}P	Components present
Original 50mm-HCl extract	215	9 590	25.9	—	1-9
Ppt. after dialysis against ethanol	371	11 340	30.7	—	—
Material not pptd. after dialysis against ethanol	84	8 320	—	—	4, 5, 7, 8, (9)
Ethanol-insoluble material pptd. in 0.74N- HClO_4	329	12 440	27.0 37.6*	36.8	2, 3, 4, 5, 9
Ethanol-insoluble material not pptd. by 0.74N- HClO_4	246	8 700	0	23.4†	2, (3), (7, 8), 9

* SH+S.S.

† From previous determinations in this paper.

in these two types of chromatin (Frenster, 1965) and our results support the view that the histones present in these two kinds of chromatin are qualitatively identical, although relatively more histone f1 may be located in dense chromatin. The greater proportion of disulphide in dense chromatin could contribute to the cross-linking of lysine-rich histones between double helices of DNA suggested by Littau *et al.* (1965) and indicates that alterations in thiol/disulphide balance may be linked with alterations in the rate of synthesis of associated components.

Unfortunately, we are still unable to define precisely the nature of the thiol compound. The present results suggest that, although the material is extracted together with basic protein, of the histones obtained by the second procedure of Johns (1964) only histone f3 contains thiol groups, and it may be that this sulphur is only present because it is extractable from nuclei under the same conditions as histone f3 and becomes linked with it. If nuclei are extracted with 50mM-hydrochloric acid histone f3 is not removed but a considerable proportion of the thiol is, and now becomes associated with the basic proteins of this extract.

The presence of small amounts of phosphorus in the acid extracts was unexpected and it is difficult to eliminate entirely the possibility that it may be due to fragments of messenger RNA which either are, or become, attached to histone. The linkage of the phosphorus with the protein is not random and phosphorus is not present in histone f2a or f2b. The parallel properties of the phosphorus and thiol components and of the material with higher uptake of amino acids are striking and lend support to the view that nuclei possess a fairly rapidly synthesized thiol- and nucleotide-containing peptide. Such nucleotide peptides containing cysteine have been described in liver (Steinberg, Vaughan, Sherman & O'Dell, 1960; Chang & Wilken, 1965). Separation of histones on Sephadex is not clear-cut, but the data on adenine and amino acid uptake and thiol and phosphorus contents of the different peaks are consistent with the presence of a thiol- and phosphate-containing compound in association with oligosaccharide of mol.wt. at least 40 000 and with proteins 1-3, which move ahead of histone f2 on electrophoresis. Until its properties are substantiated it is only possible to speculate on the significance of such a compound in nuclear metabolism.

The presence of phosphate on one of the serine residues of histone f1 is more clearly established.

Frenster (1965) has discussed the possible functions of nuclear phosphoproteins in binding histones and so preventing repression of RNA synthesis. It would be surprising if one phosphate group in addition to the other carboxylic acid residues in histone f1 affected its capacity to bind nucleic acids but the presence of phosphorylated protein in acid extracts from nuclei may be a complication for other work on nuclear metabolism.

We thank Professor J. A. V. Butler and his colleagues for manuscripts before their publication and Mr T. Bridgwater for technical assistance.

REFERENCES

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Burton, K. (1956). *Biochem. J.* **62**, 315.
 Chang, S. H. & Wilken, D. R. (1965). *J. biol. Chem.* **240**, 3136.
 Crestfield, A. M., Moore, S. & Stein, W. H. (1963). *J. biol. Chem.* **238**, 622.
 Cruft, H. J. (1961). *Biochim. biophys. Acta*, **54**, 611.
 Dische, Z. & Schwartz, K. (1937). *Mikrochim. Acta*, **2**, 13.
 Ellman, C. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.
 Fink, K., Cline, R. E. & Fink, R. M. (1963). *Analyt. Chem.* **35**, 389.
 Frenster, J. H. (1965). *Nature, Lond.*, **206**, 680.
 Frenster, J. H., Allfrey, V. G. & Mirsky, A. E. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 432.
 Frenster, J. H., Allfrey, V. G. & Mirsky, A. E. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 1026.
 Johns, E. W. (1964). *Biochem. J.* **92**, 55.
 Johnson, L. D., Driedger, A. & Marko, A. M. (1964). *Canad. J. Biochem.* **42**, 795.
 Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
 Laurence, D. J. R. & Butler, J. A. V. (1965). *Biochem. J.* **96**, 53.
 Littau, V. C., Burdick, C. J., Allfrey, V. G. & Mirsky, A. E. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 1204.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 McAllister, M. C., Wan, Y. C. & Irwin, J. L. (1963). *Analyt. Biochem.* **5**, 321.
 Marsh, W. H., Ord, M. G. & Stocken, L. A. (1964). *Biochem. J.* **93**, 539.
 Ord, M. G., Raaf, J. H., Smit, J. A. & Stocken, L. A. (1965). *Biochem. J.* **95**, 321.
 Ord, M. G. & Stocken, L. A. (1961). *Biochem. J.* **81**, 1.
 Ord, M. G. & Stocken, L. A. (1962). *Biochem. J.* **84**, 593.
 Phillips, D. M. P. (1965). *Biochem. J.* **97**, 669.
 Sangster, A. N. & Poort, C. (1965). *Biochem. biophys. Res. Commun.* **20**, 218.
 Schwartz, J. H. & Lipmann, F. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1996.
 Steinberg, D., Vaughan, M., Sherman, F. G. & O'Dell, B. (1960). *Biochim. biophys. Acta*, **40**, 225.