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Histone F1

PURIFICATION AND PHOSPHORUS CONTENT

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1. Two minor protein fractions (B and C) were separated from histone F1 by chromatography on DEAE-cellulose. Fraction B was acidic. 2. Uptake of ^{32}P *in vivo* into histone F1 but not into fractions B and C was stimulated by partial hepatectomy. 3. It is suggested that partial hepatectomy causes an increase in the number of histone F1 molecules phosphorylated.

The phosphorus content of histone F1 in regenerating rat liver has been shown to increase about twofold 16–24h after partial hepatectomy, just before DNA synthesis (Ord & Stocken, 1968; Stevely & Stocken, 1968). This is notable in view of the relation between the phosphorus content of histone F1 obtained from a variety of tissues and the ability of this histone to repress replication or transcription of DNA *in vitro* by DNA polymerase and RNA polymerase respectively (Stevely & Stocken, 1966; Ord & Stocken, 1968). The interpretation of these experiments is complicated, however, by the possibility of non-histone contamination of the histone preparations (R. H. Buckingham, unpublished work). Because of the presence in cell nuclei of phosphorylated acidic proteins (Langan, 1967), it is possible that part of the phosphorus content of histone F1 extracts may be due to non-histone contamination. This has been investigated by chromatography of histone F1 on DEAE-cellulose. It was also decided to investigate if the positions of phosphorylation of histone F1 molecules isolated from regenerating liver 22h after partial hepatectomy are similar to those in resting liver. Results consistent with this hypothesis have been obtained from an examination of the radioactive peptides obtained by digestion with trypsin of histone F1 extracts labelled *in vivo* with ^{32}P.

METHODS

Animals. This laboratory's strain of Wistar rats was used. Male rats (180–220g body wt.) were partially hepatectomized by the method of Higgins & Anderson (1931) between 10 and 11 a.m. They were given 10% (w/v) sucrose to drink and food *ad lib.* after the operation. Control rats were sham-operated.

Irradiation. This was provided from a ^{60}Co source, which delivered 100rd/min 87cm from the source. The rats were exposed in individual Perspex cages; the control rats were similarly confined for the same period.

Nuclei. Nuclei were isolated from rat liver as described by Chauveau, Moulé & Rouiller (1956) by using 2.2M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl medium, pH7.2. Before extraction of the histones, the nuclei were washed twice with 10mM-tris–HCl buffer, pH7.2, and twice with 1mM-HCl.

Extraction of histone F1. Washed nuclei were extracted with 5% (w/v) $HClO_4$ to remove histone F1 (method 1 of Johns, 1964). The $HClO_4$ extract was made 20% (w/v) with 100% (w/v) trichloroacetic acid to precipitate the histone, which was redissolved in water and reprecipitated with trichloroacetic acid (20%, w/v). The protein was redissolved in water and dialysed against water or, before chromatography, 50mM-tris–HCl buffer, pH7.2.

Protein determination. This was done by the method of Lowry, Rosebrough, Farr & Randall (1951), with acid-soluble protein from thymus nuclei as standard.

Phosphorus determination. The total phosphorus content of histone preparations was measured by the method of Bartlett (1959), but with 60% (w/v) $HClO_4$ instead of 5M-H_2SO_4, and omitting the H_2O_2.

Radioactivity. [^{32}P]Orthophosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) was given intramuscularly to the rats (100μCi/100g body wt.) 1h before death.

Tryptic digestion. Histone was digested with trypsin (80:1, w/w) for 5h at 37°C in 0.2M-NH_4HCO_3 buffer adjusted to pH8.5 with 5M-NH_3. Chymotrypsin-free trypsin (Worthington TRSF13/14) was a gift from Dr R. E. Offord. The solution was freeze-dried after digestion in preparation for electrophoresis.

Peptide 'maps'. Electrophoresis was conducted on Whatman no. 1 paper in tanks of white spirit or white spirit–pyridine (Michl, 1951). The peptide mixture (150–300μg) was applied to the paper, and electrophoresis was conducted at pH6.5, at 75V/cm, until an Orange G marker spot had moved 10cm (after about 25min). The paper was air-dried before electrophoresis in the second dimension at pH1.9. The buffers were 10% (v/v) pyridine–0.4% (v/v) acetic acid, pH6.5, and 8% (v/v) acetic acid–2% (v/v) formic acid, pH1.9.

Radioautography. Peptide 'maps' were placed in contact with Kodak Kodirex X-ray film for 2–3 weeks.

Column chromatography. A column (0.7cm×5.0cm) of Whatman DE52 DEAE-cellulose was washed with 0.1M-NaOH before equilibration with 50mM-tris–HCl buffer,

pH 7.2. Histone F1 (3–5 mg) was dialysed against the same buffer before application to the column. After elution with 30 ml of this buffer, elution was continued with 0.5 M-NaCl–50 mM-tris–HCl, pH 7.2 (30 ml), and then 0.1 M-NaOH. The elution rate was controlled by pumping at 12 ml/h.

Amino acid analysis. Protein samples were hydrolysed with 6 M-HCl for 21 h at 110°C by the method of Moore & Stein (1963). Analyses were conducted on a Locarte Amino Acid Analyser, with a single column for estimation of acidic, neutral and basic amino acids.

Radioactivity measurements. ^{32}P radioactivity was measured by scintillation counting (Beckman liquid-scintillation counter CPM 200) in a fluid containing 0.8% (w/v) 5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (CIBA Ltd., Duxford, Cambs., U.K.) and 8% (w/v) naphthalene in dioxan. Sufficient counts were reproduced to give an accuracy of ±3%. Counting efficiency was about 99%.

RESULTS

Separation of histone F1 and non-histone protein on DEAE-cellulose. Histone F1 prepared from normal liver and liver 22 h after partial hepatectomy was applied to columns of DEAE-cellulose in 50 mM-tris–HCl buffer, pH 7.2. On elution with this buffer the bulk of the protein was not appreciably retarded by the column and emerged as a single peak (fraction A). A minor adsorbed fraction (2–5%) was then eluted with 0.5 M-NaCl–50 mM-tris–HCl, pH 7.2 (fraction B). A further minor fraction was eluted with 0.1 M-NaOH (fraction C). The phosphorus content of fraction A, from both normal and regenerating liver, was lower than that of the corresponding unfractionated histone F1 preparation (Table 1). The higher content of phosphorus in fraction A from regenerating liver confirmed the observations made on unfractionated histone F1. Insufficient amounts of the minor fractions from chromatography on DEAE-cellulose were obtained to permit accurate phosphorus determinations. The recovery of radioactivity in these minor fractions from histone F1 labelled *in vivo* with ^{32}P showed that they contained about 22% of the radioactivity of unfractionated histone F1 from normal liver and about 11% of that from regenerating liver (Table 1). Incorporation of ^{32}P into fractions B and C, unlike fraction A, was not greatly stimulated by partial hepatectomy.

Amino acid analyses of unfractionated histone F1 and fractions A and B from normal and regenerating liver are shown in Table 2. The acidic nature of fraction B is consistent with the behaviour of this fraction on DEAE-cellulose under the conditions used.

^{32}P-labelled peptides from histone F1. To compare the phosphorylated histones from normal and regenerating rat liver, partially hepatectomized animals were labelled with ^{32}P during the period of rapid increase in phosphate content of histone F1 (21–22 h after partial hepatectomy). A second group of partially hepatectomized rats was similarly treated except that 1000 rd of γ-rays was given immediately before injection with [^{32}P]-orthophosphate. Laparotomized animals were used as controls. Irradiation has been shown (Ord & Stocken, 1967) to inhibit the increase in phosphate content of histone F1 that normally occurs in regenerating liver 18–22 h after partial hepatectomy. Liver histone F1 from each group of rats was digested with trypsin and the peptides were separated by high-voltage electrophoresis on paper. About 45 ninhydrin-positive spots were detected, compared with the value (about 60 peptides) reported by Murray (1964, 1965) (see also Butler, Johns & Phillips, 1968). In a second experiment histone F1 was purified by chromatography on

Table 1. *Phosphorus content, specific radioactivity and recovery of protein fractions obtained by chromatography on DEAE-cellulose of ^{32}P-labelled histone F1 from normal and regenerating rat liver*

The preparation of the histone and the analytical procedures are described in the text. Results from two experiments are shown. Phosphorus content is expressed as ng-atoms of P/mg of protein, and specific radioactivity as c.p.m./mg of protein. Protein recovery is referred to the amount (3–5 mg) applied to the column.

	Phosphorus content		Specific radioactivity		Protein recovery (%)	
Expt. no. ...	1	2	1	2	1	2
Regenerating liver:						
Unfractionated	57	54	4600	3200		
Fraction A	40	43	4400	3140	83.0	80
Fraction B			4750	8400	5.0	2.3
Fraction C			7000	6350	3.9	2.3
Normal liver:						
Unfractionated	28	29	1850	1470		
Fraction A	26	24	1500	1275	84.0	81.0
Fraction B			5100	8250	4.2	2.2
Fraction C			6000	5500	3.3	1.7

Table 2. *Amino acid composition of protein fractions obtained by chromatography on DEAE-cellulose of histone F1 from normal and regenerating liver*

Amino acid content is given as mol/100 mol of all amino acids.

	Unfractionated		Fraction A		Fraction B	
	Normal	Regenerating	Normal	Regenerating	Normal	Regenerating
Lys	28.7	27.2	28.7	27.7	14.7	9.7
His	0.2	0.3	0.1	0.2	3.1	1.6
Arg	2.7	2.6	2.3	2.2	2.2	1.6
Asp	3.1	3.2	2.3	2.6	11.3	10.3
Thr	5.3	5.6	6.1	5.7	3.2	4.5
Ser	7.0	7.6	8.3	7.2	8.1	10.6
Glu	5.0	3.8	3.6	3.9	11.0	10.7
Pro	8.8	9.1	8.6	8.4	6.4	5.4
Gly	6.6	7.4	6.4	7.6	8.7	12.7
Ala	20.2	22.4	22.3	22.7	9.5	14.5
Val	4.4	4.6	5.2	4.7	3.1	4.2
Met	0.3	0.3	0.1	0.1	10.4	7.6
Ile	1.5	0.8	1.1	1.0	2.0	1.9
Leu	3.9	4.0	4.0	4.7	2.8	3.0
Tyr	0.6	0.4	0.4	0.5	0.9	0.3
Phe	0.8	0.8	0.5	0.6	2.8	1.8

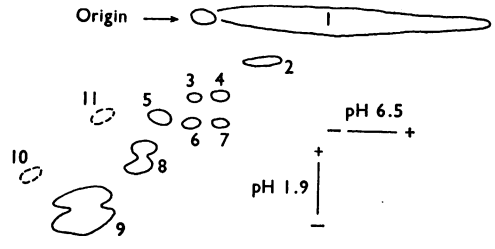

Fig. 1. Radioautograph of tryptic peptides from histone F1, labelled *in vivo* with ^{32}P. Peptides were prepared from regenerating rat liver and separated by two-dimensional paper electrophoresis as described in the text. Spots 10 and 11 belong to 'maps' derived from normal rat liver.

DEAE-cellulose before digestion with trypsin. In this experiment the peptide 'map' was virtually the same.

A radioautograph of a peptide 'map' from unfractionated regenerating liver histone F1 is shown in Fig. 1. Minor differences only were found in radioautographs of peptide 'maps' derived from normal liver and regenerating liver from irradiated animals. Spots 3 and 7 could not be detected in radioautographs from normal liver, though this may have been due to their lower general intensity. Two weak additional spots (10 and 11) were peculiar to these radioautographs. The greater part of the radioactivity was in spots common to all radioautographs. Fractionation of the ^{32}P-labelled histone F1 from all three sources on DEAE-cellulose indicated that the poorly resolved band (spot 1) was derived from the material adsorbed by the ion-exchanger (fraction B). This component was not observed to contribute any other spots to radioautographs made from unfractionated material.

DISCUSSION

Chromatography of histone F1 extracts on DEAE-cellulose has been shown to be effective in removing some minor non-histone proteins from these preparations. With the reservation that amide content was not determined, the minor component eluted with 0.5 M-NaCl–50 mM-tris–HCl, pH 7.2, was acidic in nature, which is consistent with its behaviour on ion-exchange chromatography. The type of DEAE-cellulose employed was found to be important; when Whatman DE50 was used instead of DE52 there was adsorption of up to 30% of the histone F1 applied, due possibly to the participation of minor anionic groups in the material. Johns (1964) has also reported the presence of minor components in histone F1 preparations. These had high contents of the acidic amino acids (or their amides) and were separated from histone F1 by chromatography on CM-cellulose at pH 9.

Part of the phosphate content of extracts containing histone F1 has been shown to be associated with the acidic components, and is readily distinguished by the peptide-'map' radioautographs from that bound to the histone. Changes in the phosphorylation of the acidic component may account for some of the increase in phosphate content in histone F1 extracted from regenerating liver. A substantial increase is nevertheless observed in the

phosphate content of the purified histone preparation (fraction A) obtained by chromatography on DEAE-cellulose. This confirms earlier conclusions (Ord & Stocken, 1968; Stevely & Stocken, 1968) that an increased phosphorylation of histone F1 is involved in the events leading to cell division.

The resolution of phosphorylated tryptic peptides by two-dimensional electrophoresis was possibly incomplete. With this reservation, the similarity between the peptide-'map' radioautographs of material derived from normal liver, regenerating liver and regenerating liver from irradiated animals suggests that the increased phosphorylation of histone F1 during liver regeneration is due to an increase in the number of histone F1 molecules phosphorylated.

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