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Histone F1 from Rat Thymus

SUBFRACTIONATION AND INCORPORATION OF [^{32}P]PHOSPHATE *IN VITRO*

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Four subfractions of histone F1 from rat thymus were obtained by preparative polyacrylamide-gel electrophoresis. These subfractions are closely related in primary structure but show marked differences in radioactivity when derived from rat thymus nuclei labelled *in vitro* with [^{32}P]phosphate.

The capacity of histones to repress transcription and replication of DNA by RNA and DNA polymerases has led many authors to suggest that these proteins are involved in the control of gene expression (Allfrey, Littau & Mirsky, 1963; Bonner & Huang, 1963). Though many comparisons of histones from different sources have failed to reveal widespread specificity (Hnilica & Busch, 1963; Laurence, Simson & Butler, 1963; Hnilica, Taylor & Busch, 1964; Laurence, Phillips & Butler, 1966; Hnilica, 1966; Palau & Butler, 1966), there is a well-established exception in the case of the nucleated erythrocyte (Vendrely, Genty & Coirault, 1965; Vendrely & Picaud, 1968), and recent work indicates more widespread microheterogeneity in certain histones between species and even between different tissues in the same species. Evidence so far reported suggests that this is restricted to the lysine-rich histones (Stellwagen & Cole, 1968; Bustin & Cole, 1968; Buckingham & Stocken, 1969).

The discovery that the structure of a histone may be modified after the synthesis of the primary sequence has led to the suggestion that this may be a means of modulating the repressing function. Modifications by acetylation (Phillips, 1961, 1963), methylation (Murray, 1964), phosphorylation (Ord & Stocken, 1966*a*,*b*), and disulphide-bridge formation (Deakin, Ord & Stocken, 1963; Hilton & Stocken, 1966) have been described and some correlations have been made between the extent of modification and the metabolic activity of chromatin (Pogo, Allfrey & Mirsky, 1966; Pogo, Pogo, Allfrey & Mirsky, 1968). With phosphorylation a relation has been shown between the extent of histone F1 phosphorylation and mitotic index in the tissue of origin (Stevely & Stocken, 1968). In histone F1 from regenerating rat liver, a two-fold increase in phosphate content occurs that just precedes DNA synthesis, suggesting that phosphorylation of histone F1 is essentially involved in the events leading to cell division (Ord & Stocken, 1968; Stevely & Stocken, 1968).

In this study preparative polyacrylamide-gel electrophoresis (Buckingham & Stocken, 1965) was applied to the fractionation of the lysine-rich histones from rat thymus. Subfractions were obtained that differ in specific radioactivity when derived from nuclei labelled *in vitro* with [^{32}P]-phosphate. The subfractions nevertheless yield substantially similar sets of peptides on tryptic digestion, and it is suggested that they possess closely related primary structures.

METHODS

Animals. The strain of Wistar rats from this laboratory was used.

Preparation of nuclei. Nuclei were isolated from thymus glands of rats (120g body wt.) in 0.25M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl buffer, pH7.2, as described by Ord, Raaf, Smit & Stocken (1965).

[^{32}P]*Phosphate uptake into isolated nuclei from thymus gland.* Twelve rats were used for the isolation of nuclei. The final concentration of the nuclear suspension was the equivalent of one thymus gland/ml of medium (0.1875M-sucrose – 20mM-glucose – 23.5mM-NaCl – 8mM-$MgCl_2$ – 25mM-tris–HCl buffer, pH7.4) (see Klouwen & Betel, 1963) containing [^{32}P]orthophosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) (50μCi/ml). Incubation was at 37°C for 20min with gentle shaking under aerobic conditions. Before extraction the nuclei were washed twice with 50ml of isolation medium. Before extraction of the histones the nuclei were washed twice with 5mM-$MgCl_2$–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 10mM-HCl.

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

Phosphorus. 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 omission of the H_2O_2.

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 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 a tank of 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 (about 25min). The buffer (pH6.5) contained 10% (v/v) pyridine and 0.4% (v/v) acetic acid. Descending chromatography in the second dimension was carried out in butan-1-ol–acetic acid–water–pyridine (15:3:12:10, by vol.) until the Orange G component had moved 30cm. The paper was developed with the ninhydrin–cadmium reagent of Heilmann, Barrollier & Watzke (1957) at room temperature.

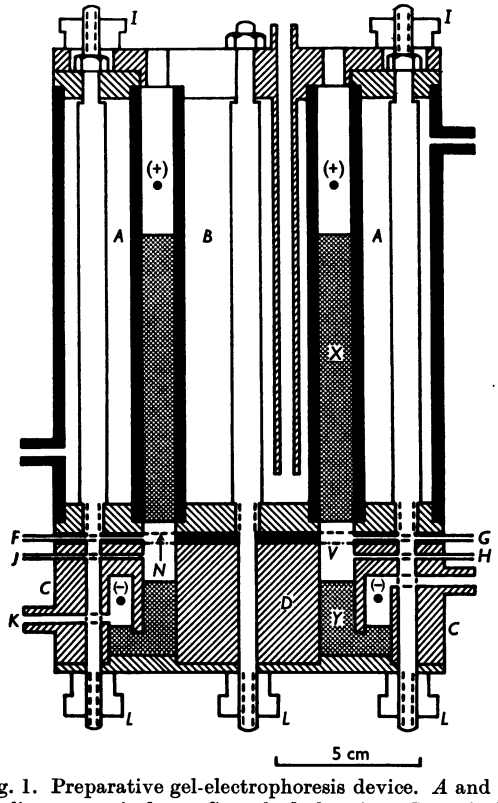

Fig. 1. Preparative gel-electrophoresis device. *A* and *B*, Cooling water jackets; *C*, cathode housing; *D*, cathode-housing central support; *E*, cathode-housing bottom; *F* and *G*, elution-buffer inlets. Three more syringe needles, *F'*, *G'*, and *P* enter the elution chamber. *F'* and *G'* are elution buffer outlets and are at right angles to *F* and *P* is at 45° to *F* and *F'*. *H* and *J*, lower-gel-membrane-cavity access tubes; *K*, cathode-buffer inlet; *L* and *I*, tie-rod thumbnuts; *N*, open-weave nylon cloth; *V*, Visking membrane; *X*, separation gel; *Y*, lower gel. Diagram is to scale except that elution chamber thickness (*N* to *V*) is 1mm.

Polyacrylamide-gel electrophoresis. Analytical gel electrophoresis was performed as described by Reisfeld, Lewis & Williams (1962) at 3mA/tube with gels containing 15% (w/v) (final concentration) of acrylamide. There was negligible retention on top of the gel of any material from histone F1 samples.

The apparatus developed for preparative polyacrylamide-gel electrophoresis is shown in Fig. 1. The separation gel *X* is shown in the annular space between glass-walled cooling chambers *A* and *B* and has a cross-section of 20cm^2. The separators between *N* and *V* are 1mm thick and contain five 22S.W.G. needles, *F*, *F'*, *G*, *G'* and *P*. (See the legend for explanations of *F'*, *G'* and *P*.) In assembly for use the lower gel *Y* is first set, under nitrogen, in parts *C* and *D*. The remaining parts are then assembled, needles *H* and *P* are connected by about 30cm of catheter, and air below *V* and *N* is replaced by elution buffer (56mM-sodium acetate–104mM-acetic acid, pH4.4). *F*, *F'*, *G*, *G'* and *J* are occluded, excess of fluid above *N* is removed and gel solution (200ml) is added. This is simultaneously underlayered with 2M-sucrose (10ml) at 2ml/min, and overlayered with water. When set, elution buffer is pumped in through *F* and *G* at 13ml/h and out at *F'* and *G'*. Elution buffers are circulated through the cathode chamber (elution buffer) and anode chamber [1.56% (w/v) β-alanine–0.4% (v/v) acetic acid, pH4.4] at about 250ml/min, both from reservoirs of 5 litre capacity.

Protein samples (5–50mg) were applied to the top of gel *X* in 1M-sucrose–0–20mM-HCl, in a volume of 3–5ml. The buffer flow around the anode was stopped for 1.5h after application of the sample. A current of 120mA was passed through the apparatus (50–80V) and the effluent was collected in 6ml fractions. Protein was recovered from effluent fractions by adsorption on columns of carboxymethylcellulose (0.6cm×1.0cm high, Whatman CM52) at pH4.5, followed by elution with 50mM-HCl. Recovery of protein was greater than 95%.

Radioactivity measurements. ^{32}P radioactivity was measured by scintillation counting (Beckman Liquid Scintillation Counter CPM200) 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%.

Amino acid analysis. Protein samples were hydrolysed with 6M-HCl for 21 or 72h at 110°C by the method of Moore & Stein (1963). Analyses were conducted on a Locarte Amino Acid Analyser, a single column being used for the acidic, basic and neutral amino acids.

RESULTS

Histone F1 from rat thymus nuclei labelled *in vitro* with [^{32}P]phosphate was fractionated by preparative polyacrylamide-gel electrophoresis. The protein elution profile (Fig. 2) corresponded closely with that expected on the basis of analytical gel electrophoresis of the preparation. In some analytical gels, the band corresponding to sub-fraction F1(2) was clearly visible as a doublet.

This is consistent with the asymmetrical labelling of component F 1(2) that is shown in Fig. 2 and was always observed in fractionations on a preparative scale. It appears that only the more mobile component of the doublet becomes labelled under the conditions described. The subfractions obtained by preparative electrophoresis were subjected to analytical electrophoresis and showed little cross-contamination. Amino acid analyses of these subfractions are given in Table 1. The composition of each subfraction is close to that characteristic of unfractionated histone F 1 preparations. There are nevertheless differences between the subfractions in the contents of some amino acids, which were confirmed by repeated analyses and analyses of independent preparations of subfractions. Differences in contents of threonine, valine [F 1(2) and F 1(3)] and isoleucine [F 1(1a) and F 1(3)] appear significant.

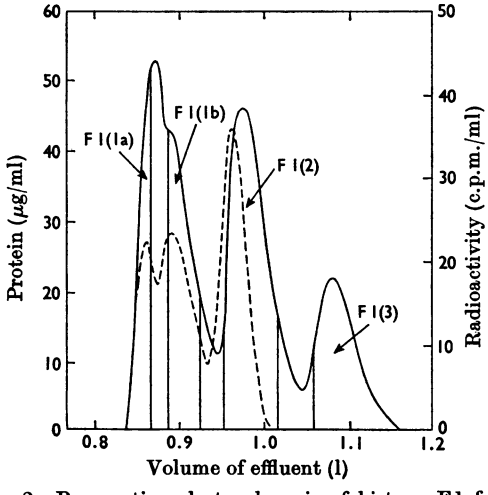

Fig. 2. Preparative electrophoresis of histone F1 from rat thymus, labelled *in vitro* with [^{32}P]phosphate. Electrophoresis was performed as described in the text. Fractions were pooled as shown. ——, Protein; ----, radioactivity.

A further comparison of the subfractions was made by an examination of the peptides produced by digestion with trypsin. Peptides were separated by high-voltage electrophoresis on paper followed by chromatography in the second dimension. About 50 spots could be distinguished in the peptide 'maps', which probably represent 70–80% of the peptides produced by tryptic hydrolysis (Murray, 1964, 1965). The peptide 'maps' from all four subfractions were closely similar, and no differences were observed which were reproducible and could be considered significant. A peptide 'map' obtained from subfraction F 1(2) is shown in Fig. 3.

DISCUSSION

The application, under suitable conditions, of polyacrylamide-gel electrophoresis to histone fractionation has confirmed that the lysine-rich histones are heterogeneous, and suggests that the heterogeneity arises from relatively minor sequence

Table 1. *Amino acid composition of histone* F 1 *subfractions*

The histone F1 subfractions were obtained by preparative polyacrylamide-gel electrophoresis of histone F1 from rat thymus glands as described in the text. The values given are an average of three sets of analyses. Amino acid content is given as mol/100 mol of all amino acids.

Amino acid	Histone subfraction ...	Amino acid composition (mol/100 mol)			
		F1 (1a)	F1 (1b)	F1 (2)	F1 (3)
Lys		27.9	27.4	28.6	28.3
His		0	0	0	0
Arg		1.9	1.8	1.9	1.7
Asp		2.5	2.3	2.1	2.0
Thr		4.5	5.2	6.4	7.8
Ser		7.8	8.8	8.2	7.9
Glu		3.9	3.9	3.6	3.9
Pro		7.9	7.8	8.6	7.5
Gly		6.0	6.9	6.3	8.1
Ala		25.4	23.5	23.7	19.7
Cys		0	0	0	0
Val		5.6	5.3	4.5	6.6
Met		0	0	0	0
Ile		1.4	1.3	1.2	0.9
Leu		4.0	3.9	4.1	4.0
Tyr		0.5	0.4	0.5	0.4
Phe		0.6	0.6	0.5	0.5

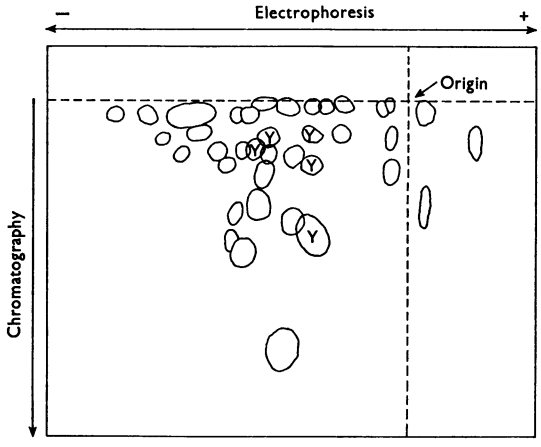

Fig. 3. Peptide 'maps' of tryptic peptides from histone subfraction F1 (2). The histone F1 subfractions were prepared from rat thymus by polyacrylamide-gel electrophoresis and digested with trypsin. These procedures and the separation of peptides are described in the text. Y, Yellow spots.

differences. In addition, the subfractions differ in specific radioactivity when derived from rat thymus nuclei labelled *in vitro* with [^{32}P]phosphate. Although the data indicate that the isolated subfractions differ in primary structure, it is not possible to say that this factor alone is responsible for the resolution effected by gel electrophoresis. Nor is it known how phosphorylation affects electrophoretic mobility. Thus, although the preparation contains at least five components, since histone subfraction F1(2) is a mixture, it is possible that some subfractions may contain, in a different state of phosphorylation, histones present in other subfractions.

Some preliminary experiments have shown, however, that the histone kinase prepared by a modification of the method of Langan (1968) will phosphorylate the mixed subfractions *in vitro* without leading to changes in their electrophoretic pattern (R. H. Buckingham & A. R. Pawse, unpublished work). The histone F1 subfractions became labelled in approximate proportion to their abundance. The incorporation of [^{32}P]phosphate into subfraction F1(3), which contrasted with the pattern from the phosphorylation in isolated nuclei, under these conditions was particularly noteworthy. If subfraction F1(3) obtained in this way is homogeneous in primary structure, these results would suggest that phosphorylation to the extent achieved *in vitro* has little effect on electrophoretic mobility.

In view of the tissue specificity observed in histone F1 subfractions (Buckingham & Stocken, 1969) it would seem that the electrophoretic fractionation of histones labelled with [^{32}P]phosphate may be of value in a study of the metabolic consequences of the phosphorylation of these proteins.

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