The Histones of some Human Tissues

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1. Histones were examined from five human tissues, namely thymus, liver, placenta, bronchial tumour and peripheral leucocytes. 2. The four main histone fractions [F1, F2(b), F2(a), and F3] were isolated and characterized. 3. The amino acid analyses, N-terminal group analyses and electrophoretic patterns were very similar to those of the corresponding fractions of calf thymus. 4. The yields of fractions F1 and F2(b) were high in human thymus, in human bronchial tumour and in some preparations of normal human leucocytes. 5. It is concluded that the pattern of nuclear histones found in lower forms of life was conserved during the evolutionary process leading to man.

Histones are basic proteins associated with DNA in the nuclei of many cells. It has been suggested (Stedman & Stedman, 1951) that these proteins could control gene expression, but the expected cell specificity of histones has not materialized. For example, little difference has been found between the histones of several tissues of a number of species (Crampton, Moore & Stein, 1957; Lindsay, 1964; Neidle & Waelsch, 1964; Fambrough & Bonner, 1966; Palau & Butler, 1966; Cozcolluela & Subirana, 1968) or between the histones of tumour and normal cells (Hnilica, Johns & Butler, 1962; Laurence, Simson & Butler, 1963; Laurence & Butler, 1965; Hnilica, 1966; Laurence, Phillips & Butler, 1966). Histone specificity has been described only in specialized cells, e.g. wheat germ (Johns & Butler, 1962), nucleated erythrocytes (Neelin, Callahan, Lamb & Murray, 1964; Hnilica & Edwards, 1967), yeast cells (Tonino & Rozijn, 1966), silkworm glands (Yoshida, Yokotsuka & Shimura, 1966) and meiotic plant cells (Sheridan & Stern, 1967). In addition, DNA in certain spermatozoa is associated with the low-molecular-weight protamines, which are rich in arginine (Felix, 1960).

Apart from early investigations by Cruft, Mauritzen & Stedman (1957), Hirschbein & Khourine (1957) and Hirschbein (1960), little work has been done on the histones of human cells. The present report describes an attempt to determine whether the general pattern of histone proteins seen in lower animals (e.g. calf) was conserved during the process of evolution that led to man. Advantage was taken of the improved methods now available for the preparation and fractionation of the

histones of calf thymus tissue (Rasmussen, Murray & Luck, 1962; Johns, 1964). These procedures were applied to a number of human tissues and cells, including thymus and liver, the organs usually studied in other animals, and also several specialized tissues. For example, the placenta is a tissue that develops during adult life and is subsequently rejected at parturition. Peripheral leucocytes contain the granulocytes, which are mature end cells of limited lifespan, and immature forms of such cells are found in blood during leukaemia. In addition, some human tumours have the advantage of being primary, in contrast with the repeatedly transplanted forms frequently studied in animals.

Preliminary reports of this work have been published (MacGillivray, 1966; MacGillivray, Monjardino & Moss, 1967).

EXPERIMENTAL AND RESULTS

Tissues. Calf and human tissues were obtained at operations and were placed on solid CO₂ immediately after excision. They were then stored at -20° for several days before use. Leucocytes were obtained from fresh normal blood and were freed of contaminating erythrocytes by two treatments with an osmotic-shock procedure described by MacGillivray & Monjardino (1968). The cells were then stored at -20° overnight.

Preparation of nucleoprotein. All procedures were carried out at 4°. Human tissue (40–200 g.) was homogenized in 0.25 M-sucrose–3 mM-CaCl₂ in a glass-Teflon homogenizer. After filtration through muslin the homogenates were centrifuged at 600g for 10 min. The pellets were then washed three times in 0.25 M-sucrose–3 mM-CaCl₂ by suspension and centrifugation at 600g for 10 min. The final pellets were suspended in 2.2 M-sucrose and centrifuged at 40 000g for 1 hr. (Chaveau, Moulé & Rouiller, 1956).

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The 40000g pellets were homogenized briefly in 0.14M-NaCl-1mM-CaCl₂-Sodium phosphate buffer, pH7. After centrifugation at 600g for 10min. the extraction was repeated. The final pellet was then blended in 0.14M-NaCl-10mM-trisodium citrate by using an MSE homogenizer at full speed for 2min. After centrifugation at 1100g for 15min. the extraction was repeated but with blending at half speed for 30sec. The homogenate was centrifuged at 1100g for 15min. and the pellet of nucleoprotein retained.

Human leucocytes (20-40g.) were suspended in 5 parts (v/w) of 4% (v/v) acetic acid and stirred vigorously for 4hr. as described by MacGillivray & Monjardino (1968). After filtration through nylon gauze, the suspension was centrifuged at 600g for 10min. The pellet was extracted with approximately 10vol. of aq. 88% (v/v) ethanol containing 0.01N-HCl (Pogo, Allfrey & Mirsky, 1966) by blending in an MSE homogenizer at half speed for 30sec. After centrifugation at 1000g for 10min. this extraction was repeated. The final white pellet of nucleoprotein was retained.

Preparation of whole histone. All procedures, apart from the drying of precipitates, were carried out at 4°.

Calf thymus whole histone was prepared by the method of Johns, Phillips, Simson & Butler (1961). Whole histone was prepared from human tissues as follows. Samples of nucleoprotein from human tissues and leucocytes were suspended by homogenization in 10-20 parts (v/w) of 0.25N-HCl. After being stirred overnight, the suspension was centrifuged at 1000g for 10min. The supernatant was retained and the pellet stirred for 2hr. with 5-10 parts (v/w) of 0.25N-HCl. After centrifugation at 1000g for 10min. the supernatant was retained and the extraction repeated. The three supernatants were combined and were clarified by filtration through a grade 4 sintered-glass funnel. The extract was sedimented by the slow addition with stirring of 10vol. of acetone. The precipitate was collected by centrifugation, washed twice in acetone and dried under vacuum.

Whole histone from human foetal thymus was prepared by a method based on that of Steele & Busch (1963). Soluble nuclear proteins were extracted from a 40000g pellet of nuclei by washing it with 0.14M-NaCl-0.1M-tris buffer, pH7.6. The residue was homogenized in 2M-NaCl and stirred overnight. After centrifugation at 25000g for 1hr. the supernatant was retained and the pellet extracted twice more in the same way for 2hr. Then 5N-HCl was added to the pooled 2M-NaCl extracts so that the final concentration of HCl was 0.25N. After being stirred overnight the suspension was centrifuged at 1000g for 10min. The supernatant was retained, and the pellet was suspended in 0.25N-HCl and stirred for 2hr. After centrifugation at 1000g for 10min. the supernatant was retained, and the extraction was repeated. The three supernatants were pooled and clarified by filtration through a grade 4 sintered-glass funnel. The acid extract was then dialysed against 1mN-HCl and freeze-dried.

Preparation of histone fractions. Attempts to fractionate, for example, leucocyte whole histone by chromatography on CM-cellulose at pH4.2 (Johns, Phillips, Simson & Butler, 1960) or on Amberlite IRC-50 at pH6.8 (Rasmussen *et al.* 1962) failed. This may have been due to the presence of contaminating acidic proteins or to the high content of proteolytic enzymes that leucocytes possess (Cline, 1965; Haschen & Krug, 1966). Therefore histone fractions were

prepared from human nucleoproteins by the methods of Johns (1964). The volumes of solvents (0.75N-HClO₄, acid-ethanol and 0.25N-HCl) required for the extraction of the human histone fractions were calculated on the assumption that preparations of purified nuclei were equivalent to calf thymus tissue on a wet weight basis. Preliminary experiments with human liver and placenta showed that method 2 (Johns, 1964) yielded preparations of fraction F1 that gave amino acid analyses suggesting contamination by other proteins. Therefore method 1 was used with solid tissues, with the following modification for the separation of fractions F2(a) and F3. After precipitation of fractions F2(a) and F3 with 5vol. of acetone and 0.3ml. of conc. HCl, the precipitate was recovered by centrifugation, washed twice with acetone and dried. The weighed precipitate was then extracted with approximately 0.2ml. of ethanol-1.25N-HCl (4:1, v/v)/mg. The insoluble material was removed by centrifugation, dried and weighed. The protein concentration of the ethanol-1.25N-HCl extract was then adjusted to 2-3mg./ml., and fractions F2(a) and F3 were separated by dialysis against ethanol as described by Johns (1964).

Method 2 was found to be more suitable for the isolation of leucocyte histone fractions. The following modifications were introduced. The ethanol-1.25N-HCl extract containing fractions F2(a) and F3 was precipitated with 5vol. of acetone and the fractions were obtained as described in method 1. The residue remaining after the extraction of fractions F2(a) and F3 was then extracted with 0.25N-HCl as described by Johns (1964), and the acid extract was precipitated with 5vol. of acetone. After being washed with acetone and dried, the precipitate was extracted with approximately 0.2ml. of 0.25N-HCl/mg. The insoluble material was removed by centrifugation, dried and weighed. After the protein concentration had been adjusted to 2-3mg./ml., the extract was brought to room temperature and fraction F1 was precipitated with 3vol. of acetone. Fraction F1 was then removed by centrifugation and fraction F2(b) was recovered from the supernatant by the addition of another 2vol. of acetone.

The yields of the four fractions from the human tissues are given in Table 1.

Analyses. Total amino acid analyses were carried out with a Technicon AutoAnalyzer apparatus. The samples were hydrolysed under vacuum in 6N-HCl at 110° for 70hr. The N-terminal amino acids were determined in samples (5-15mg.) by the method of Phillips (1958), except that the DNP-amino acids were separated by two-dimensional chromatography on thin layers of cellulose. The results are shown in Table 1.

Electrophoresis. Preparations of human whole histones and histone fractions were compared with similar preparations from calf thymus (Johns *et al.* 1961; Johns, 1964) by disc electrophoresis by the methods of Shepherd & Gurley (1966) and Mauritzen, Starbuck, Saroja, Taylor & Busch (1967). Some preparations were also characterized by starch-gel electrophoresis (Johns *et al.* 1961).

Electrophoresis in acrylamide or starch gels showed that whole-histone preparations from human tissues contained components with electrophoretic mobilities similar to those of the main fractions of calf thymus, namely fractions F1, F2(a)1, F2(a)2, F2(b) and F3. In the electrophoretic system of Shepherd & Gurley (1966) all fractions F1 showed one major band with several minor bands of higher mobility.

Table 1. Amino acid analyses of histone fractions from human tissues

The amino acid contents are expressed as moles/100 moles of all amino acids found. No corrections have been made for hydrolytic losses. The yield of each fraction is given as a percentage of the total weight of all fractions. N.D., Not detected; t, trace.

Human tissue	Amino acid composition (moles/100 moles)														
	Thymus					Liver					Placenta				
	F1	F2(b)	F2(a)	F3		F1	F2(b)	F2(a)	F3		F1	F2(b)	F2(a)	F3	
Histone fraction ...															
Amino acid															
Asp	3.3	5.4	5.8	4.7		3.2	5.7	7.2	5.0		3.0	6.2	5.6	4.4	
Thr	5.2	5.3	4.7	6.2		4.7	5.4	5.1	5.7		4.7	10.1	5.2	5.8	
Ser	6.0	6.8	3.0	3.4		7.1	7.0	3.5	3.6		6.7	5.9	3.1	3.9	
Glu	5.3	9.2	8.9	12.2		5.1	9.3	9.4	12.3		5.4	7.3	8.4	10.4	
Pro	8.1	4.9	1.7	2.8		6.6	4.0	2.1	4.1		9.5	5.4	0.3	2.5	
Gly	7.7	7.6	13.0	7.1		7.7	7.4	11.5	7.1		10.6	10.2	13.0	7.2	
Ala	23.7	11.8	11.1	14.1		23.7	11.2	9.8	12.7		23.3	8.4	9.5	13.6	
Val	4.7	6.6	7.0	5.0		5.1	6.5	7.1	5.2		5.4	7.4	7.7	5.0	
Cys	t	t	0.1	0.4		t	0.2	0.6	0.9		t	t	0.1	0.5	
Met	t	1.1	0.5	0.8		t	1.2	1.0	1.1		t	1.2	1.0	1.1	
Ile	0.9	4.7	4.9	4.9		1.4	5.1	4.7	4.8		1.3	3.7	5.5	5.0	
Leu	4.2	7.0	10.7	10.2		4.6	7.5	9.6	9.5		4.7	5.1	9.7	10.3	
Tyr	0.5	3.1	2.7	2.0		0.6	3.0	2.8	2.3		0.4	2.6	3.2	2.4	
Phe	0.6	1.5	1.5	2.4		0.6	1.6	1.9	2.6		0.7	1.3	2.2	2.5	
Lys	27.3	13.9	9.5	9.6		26.7	13.5	10.1	9.2		22.5	16.1	11.2	10.1	
His	0.2	2.5	2.5	1.9		0.2	2.6	2.2	1.8		0.2	2.2	2.2	1.7	
Arg	2.2	8.2	12.4	12.3		2.6	8.7	11.4	12.2		2.3	6.9	11.6	13.5	
N-Terminal amino acids (μmoles/g. of protein)															
Pro	9.8	63.6	N.D.	N.D.		N.D.	33.6	N.D.	N.D.		8.2	57.8	—	N.D.	
Ala	1.3	8.3	+	+++*		3.3	5.2	1.8	+++*		2.2	4.1	—	33.2	
Gly	1.1	0.3	N.D.	N.D.		2.5	0.1	0.3	N.D.		1.3	1.6	—	N.D.	
Others	1.6	3.0	N.D.	N.D.		0.4	0.8	1.1	+		1.7	1.4	—	N.D.	
Yield (%)															
33	52	10	4	25	33	29	14	25	19	37	19	48	31	12	8

* Qualitative determination. The relative amounts of the amino acids are given by + signs.

The number and apparent concentration of these minor components varied among the human fractions. Fractions F2(b) showed one major and at least five minor components. Fractions F2(a) were found to consist of a number of major components, namely a band of aggregated material at the origin, the F2(a)1 fraction of intermediate mobility and up to three components of high mobility, which represented fraction F2(a)2. The number and intensity of staining of these fast-moving components varied among the human preparations. However, no such differences could be detected after disc electrophoresis of calf thymus and human fractions F2(a) in the presence of urea as described by Mauritzen *et al.* (1967). Fractions F3 were found to consist of a band of aggregated material at the origin, two major bands of intermediate and high mobilities and several minor components. Electrophoresis in starch gel at pH 2.3 (Johns *et al.* 1961) also did not reveal any major distinctions between human and calf thymus histone fractions.

DISCUSSION

In the present investigation the histones of five types of human cells were examined by analysis of whole histones and of the four main histone fractions. Electrophoresis of these preparations showed that each human tissue contained a pattern of histones similar to that found in calf thymus tissue. The similarity of the histones found in the human tissues studied was confirmed by the total amino acid analyses of the histone fractions. In addition these analyses were very similar to those found in calf thymus (Johns, 1964) and in a number of tissues of other species (Laurence & Butler, 1965; Laurence *et al.* 1966; Palau & Butler, 1966; Cozcolluela & Subirana, 1968). The *N*-terminal amino acids of the respective histone fractions were also very similar. For example, no major *N*-terminal group was found in fractions F1 and F2(a). The *N*-terminal groups of such fractions are blocked in other species (Fambrough & Bonner, 1966; Laurence *et al.* 1966; Palau & Butler, 1966) and this probably represents the presence of *N*-acetyl groups (Phillips, 1963). The major *N*-terminal groups of fractions F2(b) and F3 were found to be proline and alanine respectively, their yields being comparable with those found in the same fractions in other species (Johns, 1964; Laurence *et al.* 1966; Palau & Butler, 1966).

The yields of the histone fractions varied in the human tissues studied. High contents of the lysine-rich histones were found in foetal thymus, bronchial tumour and leucocytes. These results may have been caused by the isolation procedures used, but electrophoretic and amino acid analyses of a whole histone preparation tended to confirm the high concentrations of fractions F1 and F2(b) in human thymus. This pattern was also indicated in other lymphoid cells after analysis of whole histone from

a sample of infected human tonsil tissue (prepared by Dr M. Greaves). The yield of fraction F1 was found to vary considerably among samples of normal human leucocytes. This could have reflected differing proportions of each cell type present. Alternatively, fraction F1 could be lost during the isolation of leucocyte nuclei by the acetic acid procedure described. Electrophoretic examination of the initial 600g supernatant always revealed that small amounts of fraction F1 had been extracted from the nuclei. However, the presence of numerous proteolytic enzymes in leucocytes (Cline, 1965; Haschen & Krug, 1966) could also affect the final yield of histone fractions. In general, there is little information concerning the contents of histone fractions in different tissues (Hnilica, Edwards & Hey, 1966; Johns, 1967; Fambrough, Fujimura & Bonner, 1968) and hence the significance of the present results is unknown.

No evidence was found in the present study for histone specificity in any of the human tissues studied. This means that our preliminary evidence that granulocytes contained a specific group of histones (MacGillivray *et al.* 1967) was not confirmed. Additional investigations showed that these results were due to artifacts produced during the histone-fractionation procedures used. In addition other experiments carried out in this Laboratory showed that the pattern of histones described in normal leucocytes was also found in the immature cell types seen in peripheral blood during chronic lymphatic and chronic myeloid leukaemia.

The same group of histones thus appears to have been conserved during evolution, since the present investigation shows that the histones of human cells are very similar to those found in other mammals (Hnilica *et al.* 1962; Johns, 1964; Laurence & Butler, 1965) and in forms of life lower down the evolutionary scale (Palau & Butler, 1966; Cozcolluela & Subirana, 1968). Final proof that the histones of human tissues are exactly identical with those of tissues from other species can only come from knowledge of the amino acid sequence of each protein. The evidence thus far obtained from peptide analyses (Hnilica, 1966; Laurence *et al.* 1966) and from partial sequences (Smith, 1968) suggests that the primary structure of a histone does not vary greatly from one tissue to another.

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