Studies on Histones

5. THE HISTONES OF THE CROCKER SARCOMA AND SPONTANEOUS MAMMARY TUMOURS OF MICE*

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(Received 24 September 1962)

A number of conflicting reports have appeared on the nature and quantity of histones of tumours as compared with normal tissues. Cruft, Mauritzen & Stedman (1954) reported a lower isoelectric point for these histones, as a result of which they were precipitated at pH 7 on neutralizing the solutions. Davison (1957), however, found that the neutral precipitate was a complex of the histones and an acidic protein, and that when separated from the latter the histones did not appear to be abnormal. Hniliča, Johns & Butler (1962) examined histones derived from a number of normal and leukaemic tissues. The whole histone prepared from these tissues could not be chromatographically resolved into the components under the same conditions as calf-thymus histone and methods were devised to obtain various fractions separately. These did not differ markedly from the corresponding fractions from calf-thymus histone. The same authors reported that the histone from Ehrlich ascites cells was very much degraded.

A comparison of tumour and normal histones is important in view of suggestions (Stedman & Stedman, 1950) that the histones act as gene-regulators controlling cell specificity. The question is, however, not one that can be simply resolved, as differences between different histones may be due to extraneous causes such as contamination with other proteins, which are present in varying amounts in different tissues, or to degradation during preparation by proteolytic enzymes. The tissue on which most fractionation procedures have been carried out, namely calf thymus, is one that readily gives a whole histone only slightly contaminated with other proteins. Methods of extracting certain histone fractions directly from the tissues have also been devised (Johns, Phillips, Simson & Butler, 1960; Hniliča, Johns & Butler, 1962) but it is not known whether they give a quantitative yield or are applicable to all tissues.

To obtain an estimate of the amounts of the different histone fractions present in any tissue, the most direct procedure would be to extract the

whole of the histone present and divide it into the various fractions. Although this can be done with calf thymus, it is much more difficult to achieve with other tissues and the question of extraneous proteins becomes a crucial one. We have therefore made a study of the preparation of histones by various procedures from two tumours. It was found initially that a better product was obtained if the nuclei were first separated intact from the cytoplasmic fraction, and the amount of contamination of the nuclei became an important consideration.

MATERIALS AND METHODS

Tumours. Crocker sarcoma transplants were obtained by growth in CB stock mice and were removed from the animals 11–12 days after implantation and rapidly frozen by pressing between flat blocks of solid CO₂. Spontaneous mouse tumours (from a stock with a high incidence of these) were taken when between 1 and 1½ cm. in diameter and were scooped or poured into a cavity in a block of solid CO₂. After partial freezing had occurred the tissue was rapidly frozen as for the Crocker tumour.

Methods of tissue disintegration. At first the MSE homogenizer (a blender with stainless-steel blades) was used at the maximum speed of 14000 rev./min. for 2 min. both for the original homogenization and for subsequent suspension of the nuclear material. With this method of blending and with HCl–NaCl soln. (see below) as suspension medium, the nuclei are broken during the first homogenization. The insoluble material brought down by centrifuging at 1600g for 15 min. contains DNA and proteins and is termed ‘nucleo-protein’, although it is not certain that all the protein in the pellet is of nuclear origin.

Alternatively, a Potter–Elvehjem homogenizer (Potter & Elvehjem, 1936) with a Teflon pestle (Arthur Thomas Co., Philadelphia, Pa., U.S.A.) was used to produce the homogenate. This was then strained through gauze and centrifuged at 1600g for 15 min. For subsequent resuspensions a homogenizer of the Dounce (1955) type was used. This was a Fluon (Imperial Chemical Industries Ltd.) plunger shaped as a cone joined to a hemisphere, which was moved up and down in the glass tube provided for the Arthur Thomas Co. homogenizer. Close proximity of the plunger to the glass tube is thus limited to a circle and this allowed a much closer fit between the plunger and tube than is possible with the Potter–Elvehjem design.

* Part 4: Johns & Butler (1962b).

In these methods of homogenization, the nuclei are isolated without the extensive fragmentation which occurs from use of the blender. After resuspension the nuclei were sedimented with a centrifugal force of 680g applied for 10 min.

Suspension media. Homogenization and nuclear suspensions were made in media based either on 15% (w/v) sucrose soln. or on 0.9% (w/v) NaCl soln. Sucrose(calcium) contained 2 mM-CaCl₂ for the initial centrifuging and 0.2 mM-CaCl₂ for washing stages, as recommended by Hogeboom, Schneider & Striebich (1952). In one case (EDTA-sucrose) EDTA was added to the medium before homogenization to a concentration of 0.2M and to the resuspension medium to a concentration of 0.02M. Before centrifuging a tenfold excess of CaCl₂ was added to neutralize the EDTA and prevent nuclear clumping.

Of the NaCl media, HCl-NaCl soln. contained 0.5 M-HCl. After three suspensions in this medium the pH of the nucleoprotein suspension was about 4.9; this medium was used only during nuclear fragmentation with the MSE homogenizer. Citrate-NaCl soln. contained 0.01 M-sodium citrate at pH 7.

In the original homogenate and washings an eightfold dilution of the tissue was used at first. In later work with larger amounts of tissue a four- to five-fold dilution was used. Usually three or four washings of the nucleoprotein or nuclei were carried out. To improve the extraction of soluble proteins from the nucleus with the citrate-NaCl soln. method, the final resuspension was carried out by blending with the MSE homogenizer after earlier washings with the Dounce homogenizer.

Methods of histone extraction. 0.25N-H₂SO₄ was used to extract the whole histone from the nuclei or nucleoprotein. It was found that extraction with 0.25N-HCl gave a more contaminated product. The Dounce homogenizer was found to be convenient for dispersing the nuclear material in the acid, the stainless-steel shaft of the plunger being protected by a plastic sleeve. After dispersal, the material was shaken with the acid (about 1 ml. for every g. wet wt. of original tissue) for 1 hr. and then centrifuged at 1600g for 1 hr. A second extraction gave about 5% more histone material, but, except for the experiments in which amounts of histone and DNA were compared, the second extraction was omitted. There was no marked difference in composition between the histones from the first and second extractions.

For extraction of histone fractions the methods of Johns *et al.* (1960), with ethanolic HCl solutions, and of Johns & Butler (1962a), with HClO₄, were used. The arginine-rich (f3) and slightly lysine-rich (f2) fractions were extracted from a nuclear pellet, previously washed with 80% ethanol, by 0.25N-HCl in 80% (v/v) ethanol. The f3 fraction was precipitated by dialysis against 98% ethanol and the f2 fraction was then precipitated with ether after addition of a drop of conc. H₂SO₄ to the supernatant after removing f3. The lysine-rich f1 was either extracted from the whole tissue or from the nuclei by 5% (w/v) HClO₄.

Isolation of histones from solution. The histones were precipitated by adding 100% (w/v) trichloroacetic acid to a histone solution to a final concentration of 20%. The mixture was shaken, rapidly transferred to centrifuge tubes and spun at 1000g for 10 min. The precipitate was drained and washed with acid acetone (1 drop of conc. HCl to 10 ml. of acetone) and then repeatedly with acetone.

The washed precipitate was dried first in air and then *in vacuo*.

Methods of solubilizing nucleoprotein. Nucleoprotein was dissolved either by dissociation in 2M-NaCl or by swelling in water. The insoluble residue was discarded. Salt was removed before swelling by a washing with aq. 50% (v/v) ethanol. As this treatment might itself solubilize some non-nuclear or acidic proteins a preliminary wash with aq. 50% (v/v) ethanol followed by citrate-NaCl soln. was also given. Some experiments were also done with the method of Crampton, Moore & Stein (1955), in which an unwashed nucleoprotein pellet is dissociated in 2M-NaCl soln., centrifuged and the nucleoprotein reprecipitated by dilution with water.

In all cases swelling or dissociation was assisted by blending with the MSE homogenizer for 5 min. The histone was isolated after precipitation of DNA and acidic proteins with N-H₂SO₄ to a final concentration of 0.25N.

Total amino acids and N-terminal groups. These were estimated by chromatography of the dinitrophenyl derivatives (Phillips, 1958; Phillips & Johns, 1959).

Total phosphate. This was estimated by the method of King (1932).

Deoxyribonucleic acid. This was determined by the modification of the Dische reaction given by Burton (1956). To correct for the destruction of deoxyribose in hot HClO₄, a calf-thymus DNA standard of known phosphate content was heated under the same conditions. No difference was observed between the results of extraction at 70° and 90° in N-HClO₄ for 30 min. when the corresponding standard was used for comparison.

Electrophoresis in starch gel. This was carried out at pH 2.3 (Johns, Phillips, Simson & Butler, 1961).

RESULTS

Preparation of histones. Histones were prepared from nucleoprotein fractions obtained from Crocker tumours and spontaneous mouse tumours and also from adult rat liver by a number of methods.

Method A. Blending in hydrochloric acid-sodium chloride solution with the MSE homogenizer and washing the nucleoprotein with the same medium.

Method B. Homogenizing with the Potter and Dounce homogenizers with either B1, sucrose(calcium), or B2, EDTA-sucrose, as media and washing in the same media. Table 1 summarizes the important features of the analyses of these substances and of calf-thymus histone.

It is seen that, compared with calf thymus, the proportion of acidic amino acids is increased in all cases. The highest content of acidic amino acids results from method A, and the closest approach to the calf thymus is obtained by method B2 with spontaneous mouse tumour.

Starch electrophoresis was carried out on these samples and the results are shown in Fig. 1. The general pattern of bands is the same in all cases. Both tumours and rat liver show very similar patterns. There are a number of additional bands

Table 1. *Whole histones prepared from hydrochloric acid-sodium chloride solution and sucrose homogenates*

Values are moles/100 moles of all amino acids found. Method A: blended with HCl-NaCl soln. Method B1: mild homogenization with sucrose(calcium); method B2 = mild homogenization with EDTA-sucrose.

Tissue ...	Crocker tumour		Rat liver		Spontaneous mammary tumour		Calf thymus*
Method ...	A	B1	A	B1	B1	B2	—
Total diacidic amino acids	17.8	16.3	17.9	15.6	15.7	14.6	14.2
Total dibasic amino acids	24.2	24.7	23.3	24.9	24.4	25.1	26.8
Lysine	13.9	14.6	13.2	13.1	13.9	15.2	15.6

* Values for calf thymus in this and subsequent Tables are from the results from this Laboratory summarized by Johns (1962).

Fig. 1. Starch-gel-electrophoresis patterns of some of the histones described.

near the origin in the rat-liver sample prepared by method A. E. W. Johns (unpublished results) has shown that this set of bands corresponds to the acidic proteins of the rat-liver nucleoprotein that do not go into solution during dissociation of the nucleoprotein with 2M-sodium chloride, but which may be extracted with 0.25N-hydrochloric acid from the insoluble residue. The histones prepared by the present methods differ from the calf-thymus histone as previously prepared in some details of the electrophoresis pattern. These differences are

probably due to the greater aggregation of the arginine-rich component of the calf-thymus histone that had been isolated by dialysis and freeze-drying.

The principal *N*-terminal groups present in these histones are given in Table 2.

The proportions of proline and alanine *N*-terminal groups in these preparations are clearly correlated with the proportion of acidic amino acids shown in Table 1. Thus rat-liver histone has the highest proportion of acidic groups and also the lowest content of the two principal *N*-terminal amino acids found in calf-thymus histone. This also suggests that the extra acidic components in histones made by method A are non-histone impurities.

Method C. To show the possible influence of proteolytic enzymes during histone preparations from tumours, a nuclear preparation with the Potter and Dounce homogenizers was carried out in neutral citrate-sodium chloride medium. Phillips & Johns (1959) showed that proteolytic enzymes present in calf-thymus-histone preparations were especially active at pH 7-8. After three washes the nuclei were blended in the MSE homogenizer and the resulting nucleoprotein was extracted with acid. The results are given in Table 3. A feature of the results is the high proportion of the two principal *N*-terminal groups (84-88% of total end groups). This shows either that proteolysis has not occurred in these preparations or that the proteolysed fragments have been lost. Details of the histone yields given below suggest that if a loss is occurring it is not more than 20% of the total histone.

Methods AS and BS. In a modification of method A, the washed nucleoprotein was dissociated with 2M-sodium chloride as described above. The main features of the analysis of this histone (AS) are given in Table 3. The product of the same method of dissociation applied to nuclei (BS) is also illustrated in this Table. Histones AS and BS are characterized by a rather high lysine content, which suggests that the lysine-rich histone is preferentially

extracted. The low value for alanine *N*-terminal groups suggests that the arginine-rich histone was not completely extracted. This may be related to the failure to extract about 8 % of the DNA from the nucleoprotein.

Chromatography of histones. With carboxymethyl-cellulose samples that gave satisfactory results with calf-thymus histone a number of differences were noticed in the behaviour of the tumour histones as compared with calf-thymus histones.

(i) The f2 fraction was more difficult to elute with acid and tended to come off with f3. This was shown by the relatively high amount of the 'f3' fraction, the ratio of arginine to lysine (which was too low) and the starch-gel-electrophoresis pattern. Hniliča *et al.* (1962) observed a similar effect with Ehrlich ascites samples.

(ii) Part of the material, especially from samples prepared by method A, was insoluble in the first buffer at pH 4.2. Analysis showed that this was an acidic protein.

(iii) With method B there was little insoluble protein, but a fraction failed to be adsorbed on the column. This appeared to contain a lysine-rich fraction with high acidic content.

(iv) Part of the lysine-rich f1 trailed and remained behind to be eluted with f2 and f3.

(v) All fractions isolated tended to have an unusually high content of acidic amino acids.

The best results were obtained with a sample from spontaneous mouse tumour prepared by method C. For this sample the proportions of f1:f2:f3 were 16:62:22, as compared with 25:50:25 for calf thymus. The proportions were estimated from the extinction at 280 m μ of the fractions. The low value for f1 may be due to the observed trailing of the fractions. Analysis of the fractions showed that, though the lysine-rich f1 was of satisfactory purity, both f2 and f3 were mixtures of proteins with proline and alanine end groups (Table 4). Both the f2 and f3 fractions reported were recovered by precipitation with 5 % trichloroacetic acid, which does not precipitate the lysine-rich histone present as a result of trailing.

Extraction of histone fractions. The specific extraction procedures were also carried out on the nuclei prepared by method C, which had been found to yield a satisfactory whole histone after the general extraction of the histones. The extraction of f1 with 5 % perchloric acid was done both on the

Table 2. *Principal N-terminal amino acids expressed as moles/100 moles of all N-terminal groups found for samples from Table 1*

Method A: blended with HCl-NaCl soln. Method B1: mild homogenization with sucrose(calcium).

Tissue	...	Crocker tumour	Liver	Spontaneous mammary tumour	Calf thymus
Method	...	B1	A	B1	—
<i>N</i> -Terminal amino acid					
Proline		48	35	46	50
Alanine		30	26	36	35
Proline + alanine		78	61	82	85
Other		22	39	18	15
<i>N</i> -Terminal equiv. wt. ($\times 10^{-4}$)		3.5	3.6	3.8	3.0

Table 3. *Whole histones prepared by sodium chloride controls and salt-dissociation methods*

Method AS: blended with HCl-NaCl soln., nucleoprotein extracted with 2M-NaCl. Method BS: homogenized in sucrose(calcium) and nuclei extracted with 2M-NaCl. Method C: homogenized and then blended with citrate-NaCl soln.

Tissue	...	Total amino acids and <i>N</i> -terminal amino acids (moles/100 moles)				
		Crocker tumour			Spontaneous mammary tumour	Calf thymus
					C	—
Method	...	AS	BS	C		
Amino acids						
Diacidic		15.7	15.0	16.3	14.9	14.2
Dibasic		24.0	24.3	24.2	24.7	26.8
Lysine		16.4	17.0	14.2	13.6	15.6
<i>N</i> -Terminal amino acids						
Proline		53	—	49	53	50
Alanine		27	—	35	35	35
Proline + alanine		80	—	84	88	85
Other		20	—	16	12	15
<i>N</i> -Terminal equiv. wt. ($\times 10^{-4}$)		3.1	—	3.2	3.1	3.0

whole fresh Crocker tumour tissue and on the isolated nuclei. The results (Table 5) show that only extraction of nuclei yields a histone similar to that prepared from calf-thymus tissue by extraction of the whole thymus histone. With ethanolic hydrochloric acid extraction of Crocker nuclei both the f2 and f3 fractions appear to be mixtures of histones, and this is supported by the starch-gel-electrophoresis patterns of these products.

Histone yields. Comparisons were made of the histone isolated from spontaneous mouse tumour in a number of cases by method C with the DNA content of the nucleoprotein pellet from which the histones were isolated. It was found that about 0.9 g. of histone was obtained/g. of DNA in the nucleoprotein. The value for calf thymus in routine preparations is about 1.1–1.2 g. of histone/g. of DNA. Thus the yield is about 80 % of that for calf thymus. A similar yield was obtained for a histone isolated from a nucleoprotein prepared by the

method of Crampton *et al.* (1955), in which there is no washing of the nucleoprotein material.

Further comparisons were made for Crocker tumour by analysis of the nucleoprotein extracted with water as described above. These results are shown in Table 6. The nucleoprotein extracted from rat thymus tissue by the same method is shown for comparison and the molar ratio of arginine + lysine to DNA phosphorus is similar to that obtained by Davison & Butler (1956) for calf thymus. With the tumour, the ratio of total arginine + lysine to DNA phosphorus is greater than for calf thymus, but evidence from the acidic amino acid content of the nucleoprotein shows that all the protein is not histone. An estimate of the histone arginine + lysine was made by assuming that the protein is a mixture of a typical histone (25 % basic groups, 13.5 % acidic groups) and an acidic protein (14 % basic groups, 25 % acidic groups) similar to those found in acid-insoluble residues from nucleopro-

Table 4. *Histone fractions isolated from whole histone of spontaneous mammary-tumour cells by carboxymethylcellulose chromatography*

Amino acid values are given as moles/100 moles of all amino acids found.

Amino acids	f1		f2		f3	
	Tumour	Thymus	Tumour	Thymus	Tumour	Thymus
Diacidic	9.1	8.0	14.9	14.3	17.2	16.9
Alanine	22.2	23.6	11.3	11.1	12.0	13.0
Lysine	24.0	25.9	11.7	13.1	10.3	10.3
Arginine	2.5	2.9	10.2	9.8	10.3	13.2
N-Terminal amino acid						
Proline	37	—	59	70	19	24
Alanine	16	—	26	10	61	69
N-Terminal equiv. wt. ($\times 10^{-4}$)	5.3	—	2.8	2.9	2.4	1.8

Table 5. *Histone fractions isolated by specific extraction from Crocker tumour by perchloric acid and ethanolic hydrochloric acid extraction*

Amino acid values are given as moles/100 moles of all amino acids found. Method C: homogenized and then blended with citrate-NaCl.

Amino acids	f1			f2		f3	
	Tumour		Thymus	Tumour nuclei (method C)	Thymus	Tumour nuclei (method C)	Thymus
	Whole tissue	Nuclei (method C)					
Diacidic	17.7	11.9	9.7	16.8	15.4	15.2	16.2
Alanine	16.3	20.3	22.9	9.6	10.3	12.4	13.7
Lysine	22.3	26.2	29.7	11.0	11.4	10.8	9.0
Arginine	2.8	2.2	2.1	9.5	10.9	10.8	14.0

Table 6. *Histone:deoxyribonucleic acid ratio expressed as moles of lysine + arginine to moles of deoxyribonucleic acid phosphorus*

Tissue	Total (Arg + Lys)/ DNA P	Amino acids (moles/100 moles)		Histone (Arg + Lys)/ DNA P
		Diacidic	Dibasic	
Rat thymus	0.83	13.2	24.0	0.83
Crocker tumour	0.96	17.5	20.8	0.77

teins. The ratio of arginine + lysine to DNA phosphorus estimated for histone is then somewhat less than for thymus histone.

DISCUSSION

To answer the question whether histones from tumours and the normal tissues of similar animals are identical, even with respect to the total amino acid analysis, is difficult. Preparations of the whole histone from tumours, and also from some normal tissues such as liver, contain a greater proportion of acidic amino acids than the histone prepared from calf thymus. The amount of this excess can be reduced by previous isolation of the nuclei and by other procedures which have been described. The histone then obtained approaches that from calf thymus in composition and there is no evidence that would indicate that such differences as remain are not due to remaining contaminating acidic proteins. The separation and characterization of the fractions of tumour histones is complicated by the fact that small proportions of acidic contaminants appear to change the chromatographic behaviour so that the procedures worked out for calf-thymus histone are no longer completely effective. However, when further fractionation steps were used, fractions were obtained which approach in composition the corresponding similar fractions from calf thymus.

No evidence of a large quantitative deficiency of the histone from tumours as compared with normal tissues has been obtained. Even in normal tissues there is an overall deficiency of histone arginine + lysine, as compared with DNA phosphorus of about 20 %. The corresponding deficiency in the Crocker and spontaneous mammary tumours is not much greater.

A solution of this problem requires more effective methods of quantitative separation of exactly characterized compounds than are yet available. At present it can only be concluded that the tumour histones approach in quantity and in composition those from the normal tissues of similar species, at least to the same degree as those from different normal tissues.

SUMMARY

1. Histones have been isolated from a spontaneous and a transplanted tumour by various methods. The preparations differ mainly in the content of acidic protein impurities.

2. The best preparations were obtained by mild homogenization in a medium containing ethylene-

diaminetetra-acetate or sodium citrate; 84–88 % of the *N*-terminal groups of these histones was proline or alanine.

3. Chromatography or specific extraction procedures gave a fairly satisfactory lysine-rich fraction (f1) but only partial separation of the slightly lysine-rich (f2) and arginine-rich (f3) fractions. The normal content of arginine-rich fraction is, however, suggested by the normal content of alanine *N*-terminal groups in the whole histone.

4. These results, together with consideration of electrophoresis patterns and yields, support the suggestion of Hniliča *et al.* (1962) that any differences between tumour and non-tumour histones are too small to be detected by the methods available.

We are indebted to Mr C. Smith for supplying the tumours and to Mr E. W. Johns for carrying out the electrophoresis. This investigation has been supported by grants to the Chester Beatty Research Institute of the Institute of Cancer Research: Royal Cancer Hospital from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Crampton, C. P., Moore, S. & Stein, W. H. (1955). *J. biol. Chem.* **215**, 787.
- Cruft, H. J., Mauritzen, C. M. & Stedman, E. (1954). *Nature, Lond.*, **174**, 580.
- Davison, P. F. (1957). *Biochem. J.* **66**, 708.
- Davison, P. F. & Butler, J. A. V. (1956). *Biochim. biophys. Acta*, **21**, 569.
- Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Hniliča, L., Johns, E. W. & Butler, J. A. V. (1962). *Biochem. J.* **82**, 123.
- Hogeboom, G. H., Schneider, W. C. & Striebig, M. J. (1952). *J. biol. Chem.* **196**, 111.
- Johns, E. W. (1962). Thesis: Royal Institute of Chemistry.
- Johns, E. W. & Butler, J. A. V. (1962*a*). *Biochem. J.* **82**, 15.
- Johns, E. W. & Butler, J. A. V. (1962*b*). *Biochem. J.* **84**, 436.
- Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1960). *Biochem. J.* **77**, 631.
- Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1961). *Biochem. J.* **80**, 189.
- King, E. J. (1932). *Biochem. J.* **26**, 292.
- Phillips, D. M. P. (1958). *Biochem. J.* **68**, 35.
- Phillips, D. M. P. & Johns, E. W. (1959). *Biochem. J.* **72**, 538.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Stedman, E. & Stedman, E. (1950). *Nature, Lond.*, **166**, 780.