THE HISTONES OF CALF THYMUS DEOXYRIBONUCLEOPROTEIN

I. PREPARATION AND HOMOGENEITY

by

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INTRODUCTION

Since the researches of KOSSEL many investigations have been made of the basic proteins extracted by acids from cell nuclei and various nucleoprotein preparations. Although histones obtained from different tissues nearly all show a general similarity of amino-acid composition, STEDMAN AND STEDMAN[1] and EADIE AND LEAF[2] have pointed out that the analyses reported by different authors for histones from the same tissues show variations greater than those which could be attributed to the experimental errors of the analytical procedures. Indeed, DALY, MIRSKY AND RIS[3], and HARPER AND MORRIS[4] found that different histone samples extracted from the same tissue, by a uniform procedure, still showed quite marked differences in composition.

These variations may be due to real differences in the nature of the histone in a given tissue, when taken at different times, or from different individuals of the same species; to contamination or breakdown of the histone during the extraction procedures; or, as has been suggested by the above-mentioned authors, to slight differences in the experimental procedures which result in the extraction of different fractions from a whole family of closely similar histones, which, it becomes necessary to assume, are present in the cell nucleus.

The aim of these experiments was to examine the homogeneity of histones extracted from calf thymus nucleoprotein by a variety of procedures. In theory it would be preferable to isolate the cell nuclei before extracting first the nucleoprotein and then the histone, but in practice the isolation of nuclei on a large scale is difficult, and instead we isolated the nucleoprotein directly from the tissue homogenate, and obtained the basic proteins from this. It is also probable that the nucleus contains proteins other than those present in the nucleoprotein.

Nucleoproteins prepared by different methods have been reported to differ considerably and do not necessarily contain the same proteins in the same proportions. Thus, KHOUVINE AND BARON[5] found that the histones, extracted from nucleoproteins, prepared by two different methods from rat epithelioma, differed in composition. The nucleoprotein prepared by the MIRSKY AND POLLISTER[6] method (extraction with concentrated saline followed by dilution) has been dissociated and reconstituted. It may therefore be contaminated with foreign proteins, which are not removed in the prelimi-

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nary washing with dilute salt and which are soluble in the concentrated salt solution. Histones may also be displaced by other proteins in this process.

It seems preferable to use a water extraction of the nucleoprotein, such as has been described by STERN AND DAVIS[7], ORGEL AND STERN[8], GAJDUSEK[9] and STEINER[10]. The method used by us is a slight modification of that of STERN, GOLDSTEIN AND ALBAUM[11]. It offers the great advantage of speed and simplicity.

The nitrogen to phosphorus (N/P) ratios of nucleoproteins prepared by different workers differ very considerably and are themselves evidence of loss or gain of protein. Preparations by the method of MIRSKY AND POLLISTER usually have N/P = *ca.* 4.0 (*e.g.* FRICK[12]). Water extracted preparations vary far more. STERN, GOLDSTEIN AND ALBAUM[11] obtained samples with N/P = 3.4–3.8; but they analysed samples by ORGEL having N/P as high as 7.5. The significance of these variations is not known. Our product has an N/P ratio of 4.3–5.5 and often contained a small amount of fibrous protein. Notwithstanding this the histones prepared from different samples had similar electrophoretic patterns.

The fraction of protein extractable by acids also varies considerably, but it is not known if this fraction is connected with the N/P ratio of the original nucleoprotein. STEDMAN AND STEDMAN[13] regarded this inability to extract the whole of the protein from nucleoprotein as due to simple physical occlusion of the histone by the precipitated DNA, although they were able to extract all the histones from isolated nuclei. HAMER[14] has reported only 50% of thymus nucleoprotein to be soluble in acid, but nevertheless there was a striking similarity of the amino acid composition of both soluble and insoluble fractions. We have found that practically the whole of the protein can be extracted from dilute nucleoprotein solutions with $N/2$ H_2SO_4, but not with $N/10$ sulphuric acid (see below).

Since it was thought that the acids normally used for extraction might themselves cause some degradation, we attempted, in the first place, an alternative procedure in which the isolated nucleoprotein was dissolved in strong salt solutions, which dissociate the protein from the nucleic acid (DNA). By the addition of ethanol or acetone the nucleic acid was then precipitated, leaving the proteins in solution. It was found, however, that this procedure yielded products which were electrophoretically more complex than those obtained by acid extractions. It is believed that this was due to the degradation of the histones in neutral saline, by the action of cathepsins present in the nucleoprotein. The existence of these intra-nuclear cathepsins has been demonstrated by MAVER, GRECO, LØVTRUP AND DALTON[15]. Attempts were made to inactivate the cathepsins and this resulted in an improvement of the product, but the best preparations were no more homogeneous, and often less so, than those obtained by acid extraction.

EXPERIMENTAL

Methods

The sedimentation, diffusion and electrophoresis measurements were carried out in the apparatus and by the methods described by BUTLER, GILBERT AND JAMES[21]. Nitrogen was determined by micro-Kjehldahl, and phosphorus by the method of MARTLAND AND ROBISON[22].

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Preparation of thymus nucleoprotein

All operations were performed in a refrigerated room with the temperature maintained below 5° C.

600 g of clean calf thymus freshly obtained from the slaughter-house was frozen over solid carbon dioxide, minced, and homogenised in the Waring Blendor with 2 litres 0.9% sodium chloride solution. The homogenate was centrifuged 45 min at 2200 r.p.m. on the International Refrigerated Centrifuge at 5° C. The supernate, which contained some floating fatty debris, was discarded, and the sediment was resuspended in 2 litres 0.9% saline. The centrifugation was repeated, and the supernate again discarded. The upper half of the precipitate was removed from the more fibrous and matted lower layer, and resuspended in 1 litre of 0.9% saline. The centrifugation was repeated and all but the lowest layer of the precipitate was resuspended in 1 litre of 0.9% saline. This suspension was centrifuged 30 min at 2200 r.p.m., the whole precipitate was resuspended and the washing process was repeated until the supernatant liquid was clear.

Extraction of histones in neutral salt solutions

The nucleoprotein was dissolved in 10% sodium chloride solution in which the DNA and protein are largely dissociated. The solution was vigorously stirred and the DNA was precipitated by the slow addition of acetone or ethanol to a final concentration of 30%. Some protein was carried down with the DNA as was indicated by the N/P ratio of the precipitate which was usually between 2.2 and 2.7. By redissolving and reprecipitating the nucleic acid, the protein contamination on the nucleic acid was reduced until the final N/P ratio of the precipitate reached 1.9. Pure DNA has an N/P ratio of 1.7, but since the nucleic acid of these preparations was shown by the method of SCHMIDT AND THANNHAUSER[16] to contain up to 8% pentosenucleic acid, and since the procedure described would not be expected to remove the protein bound to the RNA, it was considered that all the protein originally bound to the DNA was obtained in solution by this technique.

If the nucleic acid was precipitated from solutions of nucleoprotein in 5% to 7% sodium chloride, the protein contamination was much larger, and the precipitate would not easily redissolve. This would suggest that the protein – DNA dissociation is incomplete in solutions of sodium chloride below a concentration of 10%.

From the protein solution so obtained, precipitates slowly formed during the subsequent operations which could not be redissolved by any mild reagents. These precipitates, which had N/P ratios between 12 and 20 represented about one third of the protein nitrogen in the solutions. This fraction was not extensively investigated, although by simple paper chromatography on buffered one-dimensional strips (MCFARREN[17]), no large qualitative difference of amino-acid content was detected between the soluble and insoluble proteins. Since the phosphorus content of the precipitate was by no means negligible it is thought that the precipitates possibly represented synthetic nucleoprotein complexes which were denatured by the precipitant. Alternatively the precipitates may represent a particularly easily denatured fraction of the proteins present, carrying down the last traces of the nucleic acid. This precipitate might thus correspond to the insoluble "non-histone protein" which HAMER[14] has analysed. The small differences in amino-acid composition which HAMER reported would not be easily detectable by the analytical procedure adopted.

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The protein which remained in solution was isolated by salting-out with ammonium sulphate, or alternatively the precipitant and salt were removed from the protein solution by dialysis. When the protein solutions were freeze-dried, a white powder was obtained, which dissolved in water easily, although a small insoluble residue was present in most of the preparations.

The first preparations made in this way showed very complex electrophoretic diagrams (Fig. 1a, b). From the rate of spreading of the peaks it appeared that the molecular weight of the material was low. Dialysis studies showed that a considerable proportion of the material of these preparations passed through a Visking membrane, and could be detected by the absorption at 270 mμ. It appeared from this that cathepsins in the nucleoprotein were causing proteolysis. MAVER AND GRECO[18] have studied such cathepsins and reported that their optimum activity was shown at pH 5, while they were almost inactive above pH 7. Accordingly the solutions were buffered at pH 9 during dialysis, and the electrophoretic complexity of the protein finally obtained was markedly reduced (Fig. 1c). FRUTON AND BERGMANN[19], in studies on intracellular cathepsins, reported that some of these enzymes were activated by thiol groups. The inclusion of ethyl iodoacetate, as an enzyme inhibitor, in the isolation media also appeared to improve the product (Fig. 1d). In the final procedure that was adopted ethyl iodoacetate was added to the medium, and the whole preparation, including the dialysis, was carried out as rapidly as possible. Although not all such preparations were satisfactory, the best yielded a protein of similar characteristics to that obtained by the acid extraction (Fig. 1e).

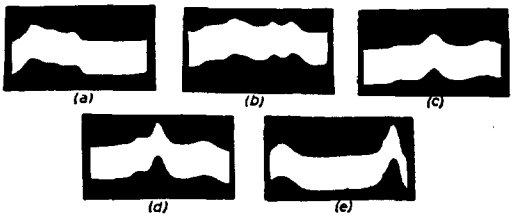

Fig. 1. Electrophoresis of histones in Miller and Golder's buffers, 0.2 ionic strength.

a. Descending boundary 110 min 12.5 mA. pH 6.7
b. Descending boundary 165 min 10 mA. pH 5.5
c. Ascending boundary 104 min 17 mA. pH 9
d. Descending boundary 112 min 17 mA. pH 7
e. Ascending boundary 146 min 18.5 mA. pH 7

Extraction of histones by acid

To compare with the histones prepared as described above, a number of different acid extraction procedures were used.

a. *Sulphuric acid.* The nucleoprotein in aqueous solution was vigorously stirred while *N* sulphuric acid was added to bring the final concentration of acid to *N*/10. After standing some hours the solution was centrifuged. The histone was precipitated from the supernate by the addition of ethanol or isopropanol. The histone was redissolved in water and either dialysed and then freeze-dried, or reprecipitated then redissolved and freeze-dried. By treatment of the residue from the first extraction with more acid, further but small amounts of histone could be obtained.

b. *Hydrochloric acid.* The histone was extracted with *N*/5 HCl, and precipitated from solution by a large excess of acetone (STEDMAN AND STEDMAN[13]). The histone was redissolved in water, dialysed and freeze-dried.

c. *Phosphoric acid.* The histone was extracted with *N*/10 H_3PO_4. From the supernatant solution the protein was precipitated by a large excess of acetone, sometimes

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with the addition of ammonium hydroxide to aid the precipitation. The protein was redissolved in water, dialysed and freeze-dried.

d. *Citric acid.* The nucleoprotein was dissolved in 10% sodium chloride solution and citric acid solution was stirred in to a final concentration of 1%. The DNA precipitated, together with a considerable proportion of the protein. The histone was precipitated from the supernatant by the addition of acetone. The histone was dialysed and freeze-dried.

All of the freeze-dried proteins prepared by these acid techniques were white powders freely soluble in water and buffer solutions except between pH 10 and 12.

Fraction of protein extracted by acids

The fraction of protein remaining with the precipitated nucleic acid after the acid extractions, which was determined by estimating the N/P ratio of the precipitate, varies considerably. In view of SIEDMAN AND STEDMAN's[13] suggestion that the unextracted protein was simply occluded by the precipitated nucleic acid, we thought that a greater amount of protein should be extracted from a more dilute solution of the nucleoprotein. This is borne out by the following figures for single extractions with sulphuric acid, but it is also found that the fraction of protein extracted increases with the concentration of the acid. The figures reached on repeated extraction are given in brackets.

TABLE I

Concentration of sulphuric acid	*N/P in residue Nucleoprotein soln.* (× 1)	*N/P in residue Nucleoprotein diluted* × 5
0	4.2	4.2
0.1	3.25 (3.0)	3.1 (2.3)
0.2	2.9 (2.5)	2.75 (2.2)
0.5	2.3 (2.0)	2.2 (2.0)

Homogeneity of acid-extracted histones

In the electrophoresis of the acid-extracted histones very little separation is observed in short periods of time, but after prolonged electrophoresis (90 min at 17 m.amps.) two sharp peaks are observed in the ascending limb (Fig. 2a), and similar but much more diffuse peaks in the descending limb. Similar pictures of other acid preparations are shown in Fig. 2b and 2c. The electrophoretic pattern in the cases examined did not depend on concentration of acid used for extraction. The peaks are too close to permit an estimate of their relative sizes, but the slower component gives the higher peak. An essentially similar behaviour is observed at all pH's between 4 and 9. The mobility (measured before separation of two peaks) as a function of the pH is shown in Fig. 3, from which the isoelectric point of the histone is apparently at about pH 11.

Corresponding ultracentrifuge pictures of these preparations are also shown in Fig. 2. In the initial stages of these runs, a small rapidly sedimenting component is also observed (Fig. 2d), which rapidly disappears from view. A similar observation has been reported by HAMER[20], who suggested it was caused by a small proportion of denatured protein.

Diffusion and sedimentation runs were also carried out with some of the extracted histones in order to get an approximate estimate of the molecular weights. Notwith-

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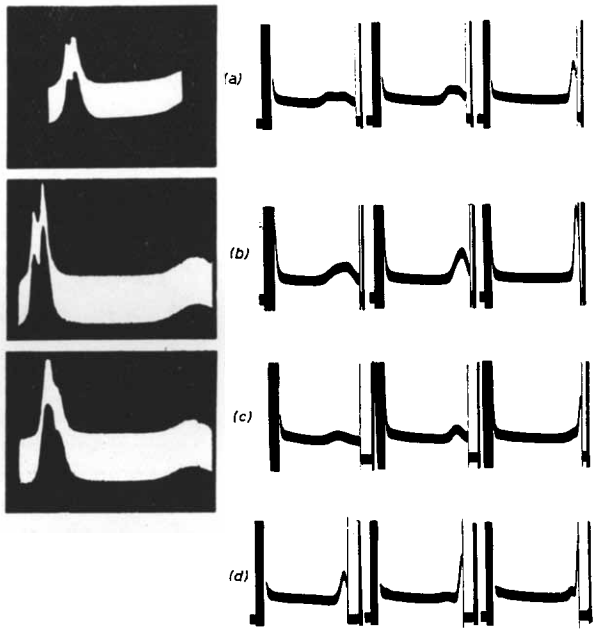

Fig. 2. Electrophoretic and ultracentrifuge pictures of acid extracted histones.

a. Sulphuric acid preparation. Electrophoresis: ascending boundary, 146 min at 16.5 mA, pH 4.38. Ultracentrifugation in 0.2 *M* NaCl. Time intervals, from right to left 0, 65, 115 min.
b. Citric acid preparation. Electrophoresis: ascending boundary, 140 min at 17 mA, pH 4.5. Ultracentrifugation in 0.2 *M* NaCl. Time intervals, from right to left, 0, 52, 118 min.
c. Sulphuric acid preparation, enriched in one component. Electrophoresis: ascending boundary, 146 min at 16.5 mA, pH 4.5. Ultracentrifugation in 0.2 *M* NaCl. Time intervals from right to left, 0, 64, 130 min.
d. Citric acid preparation. Ultracentrifugation in 0.2 *M* NaCl only. Time intervals from right to left, 0, 12, 42 min.

standing the electrophoretic inhomogeneity the diffusing boundaries were approximately Gaussian and are not capable of being analysed into more than one diffusion coefficient.

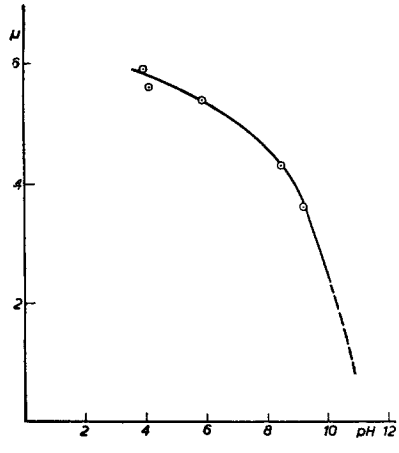

Fig. 3. The mobility (μ in cm^2/sec/volt · 10^{-5}) of sulphuric acid extracted histone SH as a function of pH.

On account of the known inhomogeneity, the results should however be accepted with some reserve. The acid-extracted histones 32/H and SH (Table II) both appear to contain a high molecular weight component with molecular weight 50,000–65,000. 32/H shows two boundaries in the ultracentrifuge (Fig. 2a), the slower of which (using the same diffusion constant) corresponds to a much lower molecular weight. The preparation SH was more nearly homogeneous and had only one apparent sedimentating boundary. The variation of S and D for similar mol. wts. must be due to the varying shape of the histone molecule in the different preparations. Some observations are also shown of a salt-extracted histone SA/D which was known from its electrophoretic pattern to be very heterogeneous. The molecular weight, as might have been expected, is comparatively low.

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TABLE II

Sample	*Concentration*	$D \cdot 10^7$	*D (mean)*	*S*	*M*
32/H	1.0	4.6	4.9	2.6	51,000
	0.5	5.0		0.9	17,000
	0.25	5.3			
SH	1.0	2.45	2.4	1.6	64,000
	0.5	2.20			
	0.25	2.60			
SA/D	0.5	8.9	7.6	1.7	21,000
	0.25	6.3			

DISCUSSION

In this work it has been found that practically all the protein present in the isolated nucleoprotein is removable by salt dissociation, but when isolated from these solutions, after precipitation of the nucleic acid, with an organic reagent, it is found to be complex. A considerable part of it becomes insoluble during dialysis; the remainder is electrophoretically very complex. Since this complexity can be reduced by various means, such as rapid handling and thiol enzyme inhibitors, it has been ascribed a consequence of the action of proteolytic enzymes (cathepsins) present in the isolated nucleoprotein. The insoluble protein which cannot be brought into solution by any mild treatment, may be a denatured material. Another possibility, however, is that it is a result of the synthetic action of the proteolytic enzymes. It has been shown by TAUBER[23] that chymotrypsin, acting on a peptic digest of egg albumin, gives rise to insoluble material of a protein nature, owing probably to its ability to act as a transaminating agent, as a result of which peptide links are formed between the main peptide chains. It may well be that the insoluble material formed in these experiments is the result of a similar process.

It is clear from our experiments that the acid extraction is a superior experimental technique, probably owing to the inactivation of cathepsins in an acid medium. The product is found to be comparatively simple—only two main components being observed both by electrophoresis and in the ultracentrifuge.

It is obvious that the proportion of these components varies somewhat in the different extractions—*e.g.* the preparation SH appears to consist almost entirely of the heavier component. If the components differ in chemical composition we should have a possible explanation of the varying chemical analyses reported. STEDMAN AND STEDMAN[13] have reported the fractionation of their calf thymus histone into two fractions of different composition. We have not undertaken any quantitative amino-acid estimations at this stage as it was considered that analyses of mixtures is not fruitful and we had alternative methods of testing the homogeneity of the preparations, but attempts to achieve a complete separation are being made and a study of the composition of the isolated substances will then be valuable.

Our experiments are in agreement with the main conclusions of several other investigators of nucleoproteins from this and other tissues which have appeared during the writing of this paper. Thus, KHOUVINE, GREGOIRE AND ZOLTA[24] found in a histone extracted by acid from cancerous tissue and necrotic tissue (rat epithelioma) two sharp

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peaks of slightly different mobilities. GREGOIRE, GREGOIRE AND REYNAUD[25], like us, have found that salt-extracted histone gives on dialysis an insoluble fraction, but unlike us they find that it redissolves on prolonged dialysis. They find that solutions of this component are homogeneous but the soluble portion is complex.

It therefore appears that so far as can be judged from electrophoresis and sedimentation, the histone of thymus nucleoprotein is not highly complex, and the hypothesis of a family of related substances showing a continuous range of physical properties is not supported by this work. The varied products of earlier investigators can be accounted for if the extraction procedures used gave varied proportions of the two main histones. It must also be remembered that although acid extraction may stop proteolysis, in many cases an opportunity for some proteolysis occurs in the intact or minced tissue before the acid is introduced.

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SUMMARY

Physico-chemical studies have been made of histones extracted from calf thymus nucleoprotein by various techniques. Those extracted at neutral pH's were found to be considerably degraded, probably by cathepsins, unless precautions were taken. The best preparations obtained by either acid extraction or by a neutral salt technique were very similar and showed, by electrophoresis and ultracentrifugation, two components, of which the proportions differed considerably in the different preparations. The sedimentation and diffusion constants of different preparations show a marked variation.

RÉSUMÉ

Les histones extraits des nucléoprotéines du thymus de veau par diverses techniques ont été étudiées du point de vue physico-chimique. Les histones extraits à pH neutres sont considérablement dégradées, probablement par des cathepsines, à moins que des précautions soient prises. Les meilleures préparations obtenues, soit par extraction acide, soit par la technique aux sels neutres, sont très comparables et présentent, à l'électrophorèse et à l'ultracentrifugation, deux constituants, dont les proportions diffèrent énormément d'une préparation à l'autre. Les constantes de diffusion et de sédimentation varient fortement selon les préparations.

ZUSAMMENFASSUNG

Physikalisch-chemische Untersuchungen wurden mit den aus dem Thymusnucleoprotein des Kalbes mit verschiedenen Methoden extrahierten Histonen unternommen. Die bei neutralen pH extrahierten Histone werden — wie gefunden wurde — wahrscheinlich mit Kathepsin beträchtlich abgebaut, wenn nicht Vorsichtsmassregeln getroffen werden. Die besten entweder durch Extraktion mit Säure oder einer Neutralsalzmethode erhaltenen Präparate waren sehr ähnlich und bestanden nach elektrophoretischen Untersuchungen und Untersuchungen mit der Ultrazentrifuge aus zwei Komponenten, deren Anteile in den verschiedenen Präparaten beträchtlich von einander abwichen. Die Sedimentations- und Diffusionskonstanten der verschiedenen Präparate zeigten eine bemerkenswerte Schwankung.

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