On the Complexity of Calf Thymus Histone*

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This paper reports upon two problems pertinent to histone chemistry: (a) the possible role of enzymatic and chemical degradation during isolation of the nuclei, extraction of the histone, and subsequent fractionation of the latter; and (b) the amino acid composition of each of the resulting fractions. Three preparations were compared. The first was derived by sulfuric acid extraction of isolated, washed nuclei, the second by direct acid extraction of the acid-homogenized gland, and the third by preliminary heating of the frozen, powdered thymus in guanidinium chloride solution and subsequent acid extraction of DNP isolated therefrom. Column chromatography established that in all three preparations comparable fractions were eluted identically, and the detailed characteristics of the several chromatographic peaks were closely comparable from preparation to preparation. These findings, supported by analytical starch-gel electrophoresis and the amino acid composition data, lead to the conclusion that under the conditions of these studies proteolytic or chemical degradation is not appreciable and that the fractions derived are not artifacts. The complexity of thymus histone is indicated by the multiplicity of fractions and the probability that each of the fractions is still heterogeneous.

It has been demonstrated by diverse methods (fractional extraction, precipitation, ultracentrifugation, and electrophoresis) that histone preparations from various tissues comprise a number of proteins (Cruft *et al.*, 1957; Luck *et al.*, 1956, and references cited therein). Ion-exchange chromatography permitted the recognition and preparation of several fractions (Crampton *et al.*, 1955; Davison, 1957; Moore, 1959; Luck *et al.*, 1958), and, more recently, starch-gel electrophoresis experiments have shown the histones to be highly heterogeneous (Neelin and Connell, 1959; Neelin and Neelin, 1960). The extent to which the observed heterogeneity of histone is a feature of native histone (*i.e.*, histone as it occurs within the cell nucleus) is an important and urgent question (Butler *et al.*, 1954; Moore, 1959). The methods of preparation and fractionation are therefore of great importance, for the possibility of concomitant degradation, both chemical and enzymatic, must be minimized.

The object of the present study was, firstly, to establish whether various fractions were authentic histone components or artifacts arising during their derivation, and, secondly, to investigate in some detail the properties and composition of the histone fractions obtained by chromatography. For these purposes chromatographic fractionations were carried out on histone obtained by three different procedures, each designed to minimize enzymatic degradation. The chromatograms were compared and the isolated fractions from each preparation were examined by starch-gel electrophoresis and with respect to their amino acid composition.

The results of these experiments suggest that the various chromatographic fractions of thymus histone are native proteins and not artifacts arising from enzymatic degradation. That none of these fractions is homogeneous furnishes additional evidence of the extreme complexity of native calf thymus histone.

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Experimental

Histone Preparations

Preparation A.—Isolated, washed nuclei from calf thymus glands[1] were extracted repeatedly with sulfuric acid at pH 1.5, and the histone was precipitated with ethanol (yield, 1.3 g per 100 g of thymus gland) (Satake *et al.*, 1960). This furnished the material designated as histone sulfate$_{1.5}$ in the reference cited. Many such preparations have been studied.

Preparation B.—The residue from the extractions at pH 1.5 was further extracted with sulfuric acid at pH 0.7, and the histone was precipitated with ethanol (yield 1.0 g per 100 g of thymus). This furnished the material previously designated as histone sulfate$_{0.7}$ (Satake *et al.*, 1960). Again, many such preparations have been investigated.

Preparation C.—Frozen calf thymus gland[1] (100 g) was ground to a fine powder with solid carbon dioxide and immediately added to a boiling solution (105°) of 3 M guanidinium chloride (1500 ml). The addition was carried out gradually to maintain active boiling, which was continued for 5 minutes after the complete addition of thymus gland. The solution was strained through four thicknesses of cheesecloth and poured into cold, distilled water (30 liters), and the precipitated deoxyribonucleoprotein was allowed to settle. All subsequent operations were carried out in the cold (2°). The supernatant solution was discarded and the deoxy-

[1] Thymus glands were collected at a slaughter house in South San Francisco. Through the kind cooperation of Swift & Company, the glands were given to us as promptly as possible—within 5 to 15 minutes, we believe, of the death of the animal. They were at once cooled in crushed ice or frozen in solid CO_2. Usually, one preparation of histone was initiated immediately on arrival at the University (1 hour), later preparations being derived from the frozen glands stored at −18°.

ribonucleoprotein was washed four times with 0.14 M sodium chloride solution (2000 ml in all) by homogenization in a Waring blendor for 5 minutes, followed by centrifugation (2000 rpm for 20 minutes; International Centrifuge). Histone was prepared from washed deoxyribonucleoprotein in the manner used for preparation A, and the ethanol-precipitated material was then dissolved in water (200 ml), dialyzed overnight against water, and lyophilized (yield 1.63 g).

PREPARATION D.—This differed from preparation A only in that whole thymus gland (100 g), instead of washed nuclei, was twice extracted at pH 1.5 with sulfuric acid (with 200-ml portions). The acid extracts, even after centrifugation at about 8000 rpm (Servall, type SS-1) for 30 minutes, were quite turbid. The yield of ethanol-precipitated material was 4.6 g.

Chromatography of Histone on Amberlite IRC-50 with Guanidinium Chloride Solution as Eluent.—Fractionation of the four preparations of histone thus obtained was carried out on Amberlite IRC-50 (250 mesh) with a gradient concentration of guanidinium chloride by the procedure described previously (Satake *et al.*, 1960). Histone fractions Iaa, Ia, Ib, Ix, IIaa, IIa, IIb, and III + IV were isolated.[2] Histone fraction IIc, which usually is found only as a small shoulder on the descending limb of fraction IIb, was not further studied in the present work.

Chromatography of Histone on Amberlite IRC-50 with Barium Acetate Solution as Eluent.—For reasons that will become apparent later in this report our standard reference preparation (histone preparation A) was also fractionated by elution chromatography from a column of Amberlite IRC-50 with a barium acetate gradient. Apart from the height of the column, the procedure was essentially as described by Crampton *et al.* (1955) in their preparative scale experiments with histones. When the effluent volume totaled 1670 ml, the column was washed briefly with water (75 ml) and, to remove that residual fraction which is known to resist elution with barium acetate, elution was continued with a gradient concentration of guanidinium chloride. The mixing vessel initially contained 8% guanidinium chloride solution (1000 ml) buffered at pH 6.8 with 0.1 M sodium phosphate, and was supplied with 13% guanidinium chloride solution from a constant-pressure reservoir. At a total effluent volume of 3080 ml, elution was concluded with 40% guanidinium chloride solution (600 ml).

Recovery of Histone Fractions.—Most of the salt was removed from the appropriately combined eluate fractions by dialysis overnight at 2° against a large volume of water which was renewed at least once. During dialysis, both the outer solution and the contents of the dialysis bags were agitated continuously. Each histone fraction was then concentrated by rotary evaporation under reduced pressure (at a temperature not exceeding 30°) to a small volume and again dialyzed during a second night to complete the removal of guanidinium chloride. The dialyzed solutions were clarified by centrifugation and then lyophilized.

Preparation of Histone Fractions Ia_s, Ib_s, Ia_g, and Ib_g by Subfractionation of Ia and Ib.—Eluate fractions Ia and Ib were each diluted with water to a guanidinium chloride concentration below 8%. These solutions were filtered through columns (8 × 2 cm diameter) of Amberlite IRC-50 resin (250 mesh) which had been washed previously with a large amount of water. The effluents from the columns were examined turbidimetrically with trichloracetic acid (Luck *et al.*, 1958) to ensure that the columns had not been overloaded. The histones were eluted stepwise with 0.2 N sulfuric acid, followed by 40% guanidinium chloride solution to remove a fraction which was not elutable with acid. The histone eluted with sulfuric acid (Ia_s and Ib_s) was precipitated with ethanol and, after washing with ethanol, was dried *in vacuo*. The histone eluted by guanidinium chloride (Ia_g and Ib_g) solution was recovered by lyophilization after dialysis.

Amino Acid Analysis of Histone Fractions.—A small amount of each histone fraction (4–5 mg) was dissolved in "constant boiling" hydrochloric acid (1 ml), and portions of the solution (0.400 ml) were transferred to each of two Pyrex test tubes (100 × 12 mm). The tubes were evacuated, sealed, and heated at 110°. In each case, one tube was heated for 22 hours and the other for 70 hours. The hydrolysates were evaporated to dryness *in vacuo* over phosphorus pentoxide and sodium hydroxide pellets; the residue was dissolved in water (0.2 ml) and again evaporated to dryness. Amino acid analyses were carried out with a Beckman/Spinco Automatic Amino Acid Analyzer (Model 120).

Starch-Gel Electrophoresis

(A) ANALYTICAL EXPERIMENTS.—Starch-gels were prepared as described by Smithies (1955). The composition of the gels was 15.6 g starch (Connaught Medical Research Laboratories) per 100 ml 0.03 M acetate buffer solution (4 M in urea; pH 4.1), and the same buffer solution (without urea) was used for a bridge electrolyte. Eleven samples of histone preparations and their chromatographic fractions (0.3 to 1.0 mg each, dissolved in 0.05 ml of the buffer solution) were examined simultaneously in one gel and the gels were stained with Amido-Schwartz 10B after electrophoresis (apparatus by Murray, 1962).

(B) PREPARATIVE EXPERIMENTS.—The composition of the gels was 15.6 g starch per 100 ml 0.056 M acetate buffer solution (4 M urea, pH 4.1); 0.07 M acetate buffer solution (pH 4.1) was used for bridge solutions. The chromatographic fraction IIb (8 mg) of histone preparation A was dissolved in 0.07 M acetate buffer solution (0.25 ml), and separation of the two major components, indicated by analytical starch-gel electrophoresis, was effected with a continuous elution electrophoresis apparatus

[2] The following designations are used: I and II to fractions derived from histone preparation A by chromatography on Amberlite IRC-50 columns with a barium acetate elution gradient; Iaa, Ix, Ia, Ib, IIaa, IIa, IIb, IIc, III, and IV to chromatographic fractions derived from histone preparations A, B, C, or D by use of a guanidinium chloride elution gradient; I asc., I desc., II asc., and II desc. to the fore and rear parts, respectively, of the chromatographic peaks corresponding to fractions I and II as defined above; "fraction A" and "fraction B" to the fractions so designated in the papers by Crampton *et al.*

(Murray, 1962). Appropriate eluate fractions containing components 1 and 2, respectively, were combined and the solutions so obtained from three experiments were passed through columns (8 × 1 cm diameter) of Amberlite IRC-50 resin (250 mesh). After the columns were washed thoroughly with water, elution of the histone fractions was effected with 20% guanidinium chloride solution, and 1-ml eluate fractions were collected. Histone-containing fractions (detected by absorption at 277 mμ) from each column were combined, dialyzed overnight, and lyophilized to furnish components 1 and 2 of histone fraction IIb. The combined yield was about 3 mg of component 1 and 7 mg of component 2.

Results

Chromatographic Fractionations of Histone Preparations on Amberlite IRC-50.—Histone preparations A, B, C, and D, chromatographed on Amberlite IRC-50 with guanidinium chloride solution as the eluent, yielded the chromatographic patterns presented in Figure 1. The chromatogram illustrated by Figure 2 was obtained when histone preparation A was chromatographed with barium acetate solution as the eluent. Replacement of the barium acetate solution by guanidinium chloride solution (buffered with sodium phosphate), after a brief interval of washing with water, resulted in the development of a slight opalescence in subsequent eluate fractions. For this reason, measurement of the optical density at 277 mμ could no longer be used for protein assay.

Amino Acid Composition of Histone Fractions.—The amino acid composition of histone fractions is expressed as moles of the amino acids per hundred moles of total amino acids recovered (moles %). Serine and threonine are partially degraded during hydrolysis, and values for these amino acids were corrected by substituting values obtained from 22- and 70-hour hydrolysates in an equation based on the assumption that first-order kinetics applies to the degradation processes (Hirs *et al.*, 1954). That this modification does not necessarily provide a true correction is fully appreciated, but the values so obtained are nearer to the original (zero hours) than are those observed. No loss of tyrosine has been observed (*cf.* Crampton *et al.*, 1957), and values for this amino acid are therefore uncorrected. In none of the hydrolysates was any indication of the presence of tryptophan or its degradation products found (Spackman *et al.*, 1958). Ammonia was excluded in calculations of the moles % amino acid compositions. The amino acid data reported are, generally, averages of the determinations on 22- and 70-hour hydrolysates.

The amino acid composition of the principal histone fractions obtained from preparations A, B, and C are presented in Table I.

A comparison of the amino acid composition of histone fractions Ia, Ib, and IIb from histone preparations A, C, and D is shown in Figure 3. The corresponding data for histone fractions I and II obtained from histone preparation A by development of the chromatogram with barium acetate solution instead of guanidinium chloride solution

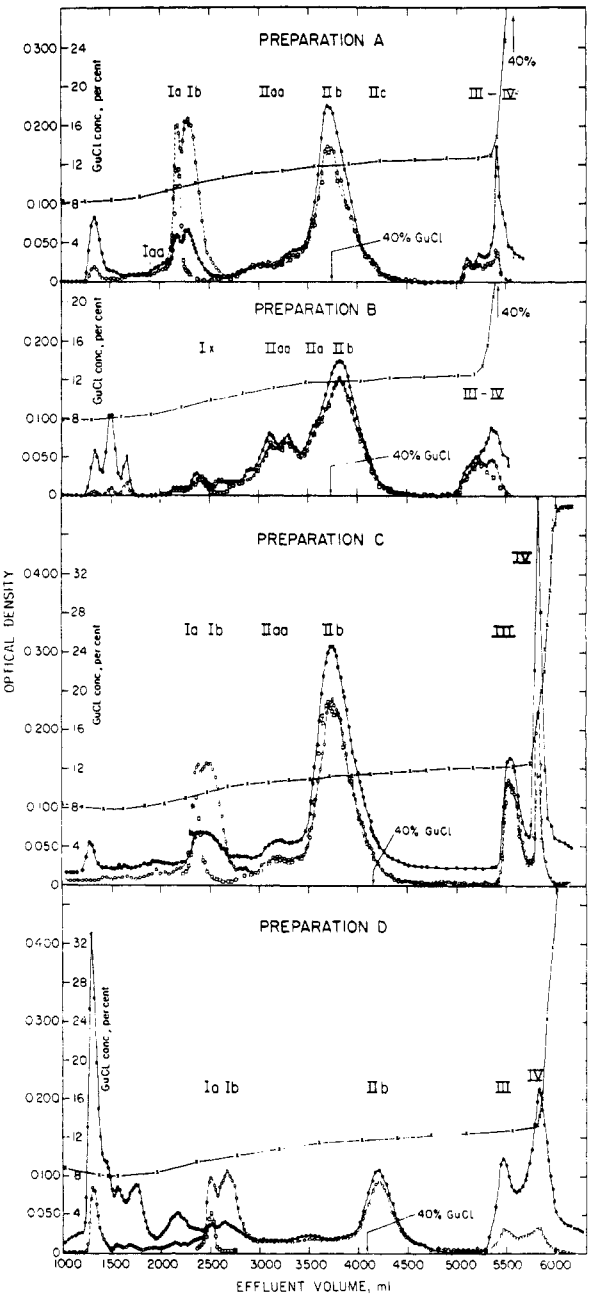


FIG. 1.—Chromatographic fractionation of calf thymus histone preparations on Amberlite IRC-50 columns (55 × 7.5 cm diameter) with a gradient concentration of guanidinium chloride (GuCl). The quantity of histone samples applied to the column was: preparation A, 700 mg; preparation B, 570 mg; preparation C, 790 mg; preparation D, soluble part of 750 mg. Protein concentration of the effluent fractions was determined (a) by the optical density at 277 mμ, ●—●; (b) by the optical density at 400 mμ of the turbid solutions which resulted when 0.1-ml effluent samples were mixed with trichloroacetic acid (1.1 M) in a total volume of 3.0 ml, ○—○; (c) by the optical density at 400 mμ of the turbidity produced in 0.8 M trichloroacetic acid, □—□. Concentration of guanidinium chloride in the effluent, as determined by refractometry, is indicated by X—X.

are also included in this diagram; the ascending part of fraction I is placed with fractions Ia, the descending part of fraction I is placed with frac-

TABLE I

THE AMINO ACID COMPOSITION OF CHROMATOGRAPHIC HISTONE FRACTIONS OBTAINED FROM PREPARATIONS A, B, AND C

	Eluent Solution: Guanidinium Chloride Solution										Barium Acetate Solution			
	Histone Preparation: A	A	A	B	B	B	A	A	B	C	C	A	A	A	A
	Chromatographic Fraction:						IIb[a]	III + IV[a]	III + IV[a]	III	IV	I acs.	I desc.	II asc.	II desc.
Amino Acid	Iaa[a]	Ia[a]	Ib[a]	Ix	IIaa	IIa									
Lysine	13.8	25.3	26.2	13.8	10.3	9.6	13.5	9.7	9.2	9.4	9.0	27.9	28.4	12.9	12.7
Histidine	2.2	0.4	0.2	2.2	1.9	1.9	2.8	1.9	1.6	1.6	1.6	0	0	2.7	2.7
Arginine	8.2	3.0	2.6	6.3	11.3	11.6	7.9	11.9	11.6	12.9	12.8	1.8	1.4	8.3	8.5
Aspartic acid	4.5	2.5	2.5	5.3	4.9	4.8	5.6	5.0	5.0	4.4	4.5	2.0	1.8	5.4	5.5
Threonine	6.2	5.8	5.4	6.5	7.1	6.8	5.2	6.7	6.9	7.4	7.4	6.0	5.8	5.3	5.2
Serine	6.6	6.4	6.5	10.0	5.0	4.6	7.0	4.6	4.8	4.1	4.1	6.2	6.7	6.7	7.2
Glutamic acid	8.9	4.5	4.3	9.7	9.1	9.6	8.7	10.4	9.9	9.9	10.6	3.5	3.4	8.9	8.8
Proline	5.5	8.6	9.1	4.4	3.4	3.7	4.7	4.2	3.9	3.8	4.2	9.2	9.4	4.6	4.5
Glycine	8.8	6.7	7.3	7.1	10.1	9.8	8.2	8.6	9.4	8.8	7.9	6.6	7.0	8.5	8.5
Alanine	14.7	24.0	24.2	11.9	10.3	11.2	11.5	11.6	11.5	11.8	12.3	25.1	25.9	11.9	11.5
Valine	5.0	4.9	4.1	6.8	6.8	6.5	6.7	5.9	5.1	5.8	5.6	5.3	4.6	6.6	6.6
Methionine	0.6	0.1	0.1	1.3	1.2	1.2	0.8	1.3	1.4	1.2	1.2	0	0	0.8	0.8
Isoleucine	3.8	1.3	1.2	4.7	5.4	5.4	4.5	5.3	5.3	5.4	5.4	1.0	0.9	4.8	4.8
Leucine	8.2	5.3	5.0	6.0	8.0	8.7	8.6	8.9	8.5	8.7	8.9	4.8	4.2	8.7	8.6
Tyrosine	1.6	0.7	0.7	2.7	2.9	2.8	3.0	2.2	2.8	2.4	2.3	0.5	0.3	3.0	3.1
Phenylalanine	1.6	0.6	0.6	1.8	2.2	2.3	1.3	2.5	2.6	2.5	2.7	0.5	0.5	1.3	1.3

[a] These values are based on analyses of 22-hour hydrolysates. The serine and threonine values have been corrected for decomposition by means of factors derived from analysis data in which both 22- and 70-hour hydrolyses were carried out.

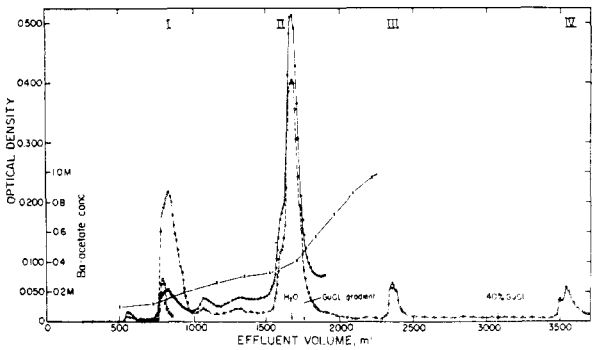


FIG. 2.—Chromatography of histone preparation A (400 mg) on Amberlite IRC-50 column (55 × 4.5 cm diameter) with a gradient concentration of barium acetate, followed at 1760 ml by a gradient concentration of guanidinium chloride. The symbols (●, ○, and □) have the same significance as in Figure 1. Concentration of barium acetate in the effluent, as determined by refractometry, is indicated by X—X.

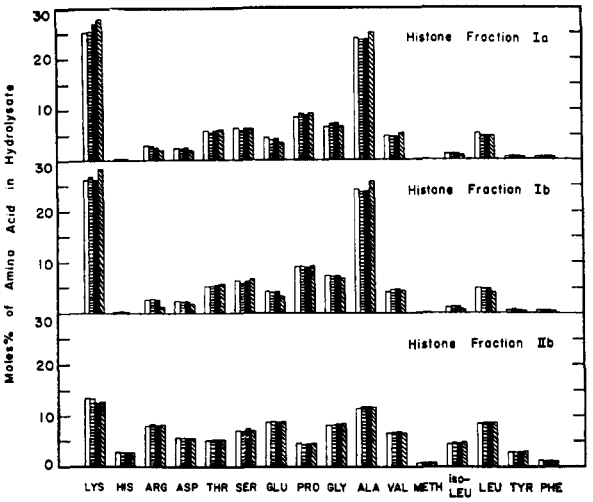


FIG. 3.—Comparison of the amino acid composition of corresponding chromatographic histone fractions from different preparations. Blank bars, fractions from preparation A; horizontally striped bars, fractions from preparation C; solid bars, fractions from preparation D (all three obtained by elution with guanidinium chloride solution); diagonally striped bars, fractions from preparation A (obtained by elution with barium acetate solution).

tions Ib, and fraction II is placed with fractions IIb.

The analytical data for histone subfractions Ia_s, Ib_s, Ia_g, and Ib_g are set forth in Table II.

TABLE II

COMPARISON OF THE AMINO ACID COMPOSITION OF HISTONE FRACTIONS Ia AND Ib, AND THEIR SUBFRACTIONS DERIVED BY DIFFERENTIAL ELUTION FROM AMBERLITE IRC-50

Amino Acid	Fraction: Ia[a]	Ia_s[a]	Ia_g[a]	Ib[a]	Ib_s[a]	Ib_g[a]
Lysine	25.3	27.5	27.0	26.2	28.6	24.3
Histidine	0.4	0	0.3	0.2	0	0.4
Arginine	3.0	2.0	2.5	2.6	1.6	3.1
Aspartic acid	2.5	2.0	2.3	2.5	2.0	3.4
Threonine	5.8	6.1	5.9	5.4	5.4	4.9
Serine	6.4	6.7	6.7	6.5	6.4	6.3
Glutamic acid	4.5	3.3	3.5	4.3	3.6	4.4
Proline	8.6	9.5	8.9	9.1	11.2	10.2
Glycine	6.7	6.1	6.5	7.3	6.6	7.9
Alanine	24.0	25.3	23.2	24.2	24.7	24.5
Valine	4.9	5.6	6.0	4.1	4.3	3.7
Methionine	0.1	0	0.3	0.1	0	0.2
Isoleucine	1.3	0.9	1.1	1.2	0.8	0.9
Leucine	5.3	4.3	4.7	5.0	4.1	5.0
Tyrosine	0.7	0.3	0.5	0.7	0.4	0.8
Phenylalanine	0.6	0.4	0.6	0.6	0.4	0.7

[a] See footnote of Table I for explanation.

Table III shows the amino acid composition of the two subfractions (IIb_1 and IIb_2) of histone fraction IIb which were obtained by preparative starch-gel electrophoresis.

The hydrolysates of the histone fractions IIaa, IIa, III + IV, III, and IV all contained a small amount of an unidentified compound which emerged from the short column (15 cm) of the amino acid analyzer immediately after lysine (*cf.* Crampton *et al.*, 1957, p. 369). None of the other fractions contained this compound.[3]

Starch-Gel Electrophoresis of Histone Fractions.—The results of analytical starch-gel electrophoresis experiments with the various histone preparations

[3] The peak due to the unknown compound was observed in the hydrolysate of fraction IIb from preparation B, presumably because of contamination with fraction IIa (and IIaa).

TABLE III

AMINO ACID COMPOSITION OF THE TWO COMPONENTS (IIb_1 AND IIb_2) OBTAINED BY PREPARATIVE STARCH-GEL ELECTROPHORESIS FROM HISTONE FRACTION IIb

Amino Acid	Fraction IIb[a]	IIb_1[a]	IIb_2[a]
Lysine	13.5	11.5	15.9
Histidine	2.8	2.8	2.4
Arginine	7.9	8.8	6.7
Aspartic acid	5.6	6.1	4.9
Threonine	5.2	4.5	6.2
Serine	7.0	5.6	10.0
Glutamic acid	8.7	9.2	8.2
Proline	4.7	4.5	4.8
Glycine	8.2	10.0	6.0
Alanine	11.5	12.1	10.4
Valine	6.7	6.4	7.2
Methionine	0.8	0.2	1.6
Isoleucine	4.5	4.6	5.0
Leucine	8.6	10.2	5.0
Tyrosine	3.0	2.3	4.4
Phenylalanine	1.3	1.2	1.6

[a] See footnote of Table I for explanation.

and chromatographic fractions are shown in the photographs of the stained gels. Histone preparations A, B, C, and D are compared in Figure 4; the behavior of the various chromatographic fractions obtained from histone preparations A and B by development of the chromatogram with guanidinium chloride solution is illustrated in Figure 5; corresponding chromatographic fractions from histone preparations C and D are compared in Figure 6 with the chromatographic fractions obtained from preparation A by development of the chromatogram with barium acetate solution.

The presence of two major components in histone fractions IIb is demonstrated by analytical starch-gel electrophoresis experiments (Figures 5 and 6). Figure 7 illustrates the separation of these two components of fraction IIb (from preparation A) by a continuous-elution method of preparative starch-gel electrophoresis.

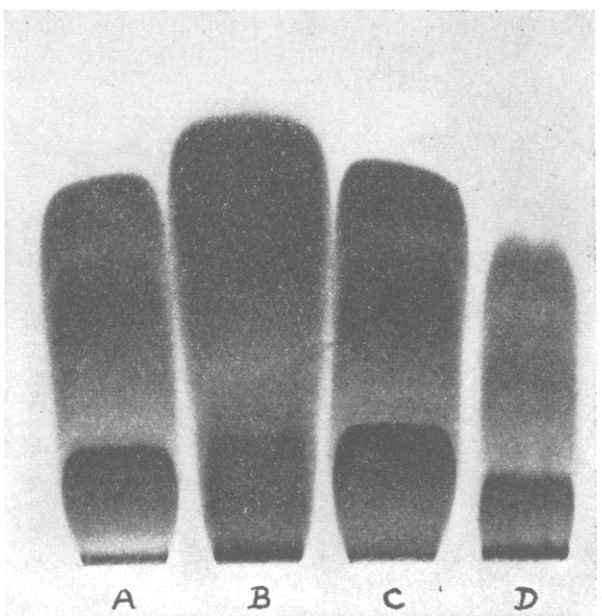


FIG. 4.—Comparative starch-gel electrophoresis of histone preparations A, B, C, and D. A 1-mg sample was used in each case, but a considerable portion of preparation D was insoluble, and the relatively low migration rate of the components of this preparation is a consequence of the lower concentration of the solution applied to the starch-gel.

DISCUSSION

Since histones are susceptible to enzymatic cleavage (Butler *et al.*, 1954; Moore, 1959), the separation of several histone fractions raises doubts concerning their authenticity. Crampton *et al.* (1957) have pointed out that histones are small proteins possessing a rather open, unfolded structure which is not stabilized by disulfide bonds; consequently they are particularly prone to enzymatic attack. Trypsin, for example, rapidly degrades histones that have not been pretreated with denaturing agents. The usual precautions of working rapidly in the cold may thus be insufficient to prevent some enzymatic degradation of histones during their preparation. Most of the work on histone fractionation has been carried out on histone extracted from washed nuclei or solutions of deoxyribonucleoprotein by treatment with dilute mineral acid or with salt and ethanol. Preparations obtained by each method have been shown to be chromatographically heterogeneous (Crampton *et al.*, 1957; Satake *et al.*, 1960), and preparations involving the use of sodium chloride at approximately neutral pH have been found to be electrophoretically more heterogeneous than preparations extracted with acid, probably due to catheptic degradation (Butler *et al.*, 1954). Prolonged dialysis, which was employed at some stage in most preparations, also enhances the possibility of enzymatic degradation (Crampton *et al.*, 1957). More direct extraction procedures have been employed in order to reduce the possibility of enzymatic degradation. Extraction of whole thymus gland with trichloracetic acid was used by Westenbrink (Moore, 1959), and thymus gland has been extracted with dilute sulfuric acid (Neelin and Neelin, 1960). In the latter case, the protein extracted was shown by zone electrophoresis in starch-gel to be comparable in heterogeneity to a preparation obtained by acid extraction of washed nuclei, which implies that the heterogeneity of histone observed in other preparations can be due only in part to enzymatic degradation.

In the work now described, three different procedures, all designed to minimize enzymatic reactions during the extraction process, were used to prepare histone. In the first method thymus gland was repeatedly homogenized and centrifuged in ethylene glycol to furnish nuclei from which histone preparations A and B were obtained by successive extraction with sulfuric acid at pH 1.5 and pH 0.7 respectively; together they constitute "whole histone." The second extraction method was based on thermal inactivation of proteolytic enzymes: finely ground thymus gland was very rapidly heated by dropping into boiling 3 M guanidinium chloride solution. Guanidinium chloride solution at this concentration has been shown to denature bovine serum albumin (Kolthoff *et al.*, 1959); it may therefore help to minimize enzymatic processes during the time taken for the thymus tissue to reach the temperature of the solution. Histones do not appear to be denatured by guanidinium chloride solution

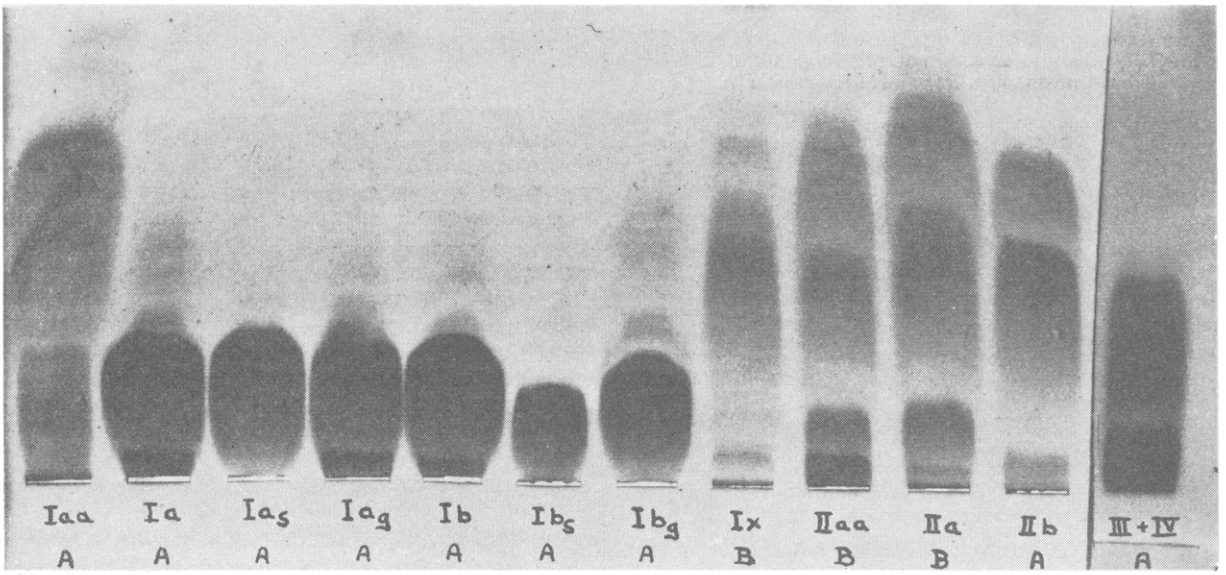
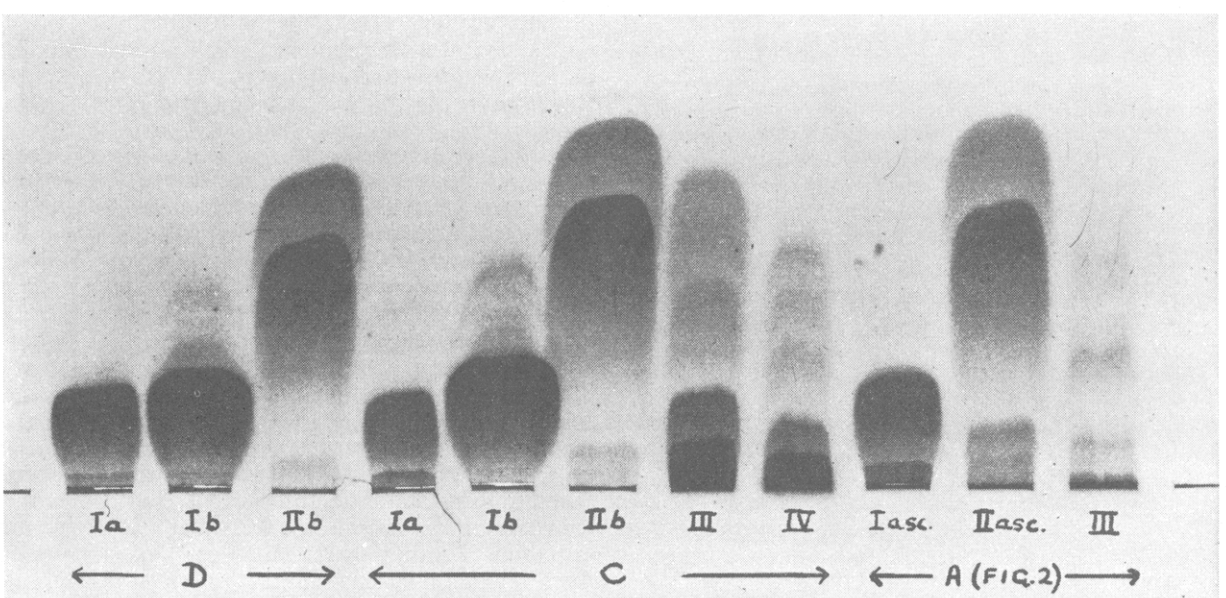


FIG. 5.—The behavior on starch-gel electrophoresis of histone fractions obtained from preparations A and B by chromatography with guanidinium chloride solution. The letter beneath the histone fraction numbers indicates the preparation from which the fraction was derived.

(Luck *et al.*, 1956), probably because of their simple structure. After the heat treatment, deoxyribonucleoprotein (precipitated on dilution to a guanidinium chloride molarity of 0.14) was washed with cold sodium chloride solution and histone was extracted with sulfuric acid at pH 1.5 (preparation C). Direct homogenization of whole thymus gland with sulfuric acid was used in the third extraction method with the intention of minimizing enzymatic reactions by reducing the number of steps, and hence the time involved, and by exposing the tissue to very low pH immediately, rather than after some preliminary treatment. Preparation D was obtained in this way.

It must be emphasized that preparations A and B are obtained from successive extracts, at differing pH, of the same material. Preparation A represents only about 60% of the whole histone, and preparations C and D, also extracted at pH 1.5, likewise may not be regarded as whole histone. It is apparent from Figure 1 that preparation B differs from preparation A in that it does not contain histone fractions Ia and Ib, but contains relatively large proportions of fractions IIaa and IIa. A whole histone preparation will thus contain higher proportions of fractions IIaa and IIa than will preparations A, C, and D. Furthermore, since fractions IIaa and IIa are eluted from Amberlite IRC-50 with an overlap of fraction IIb, it follows that fraction IIb obtained from preparation A (or



FIGS. 6.—The behavior on starch-gel electrophoresis of histone fractions obtained from preparations C and D by chromatography with guanidinium chloride solution, and from preparation A by chromatography with barium acetate solution. The letter beneath the histone fraction numbers indicates the preparation from which the fraction was derived.

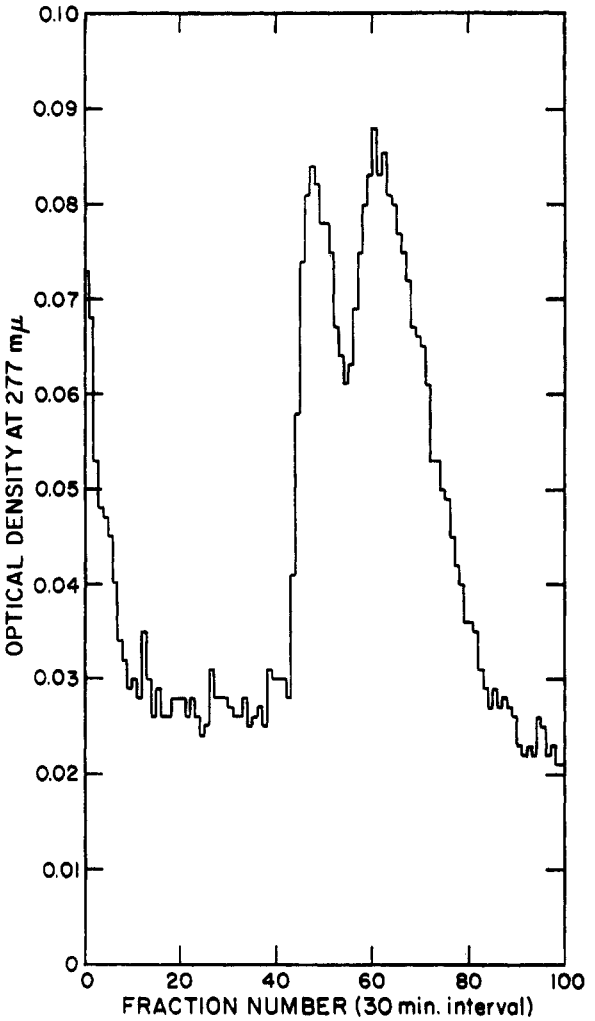


FIG. 7.—Separation of components 1 and 2 of histone fraction IIb by preparative starch-gel electrophoresis.

preparations C or D) will be less susceptible to contamination with fractions IIaa and IIa than will fraction IIb obtained from either whole histone or preparation B. For this reason preparations A, C, and D were used in preference to whole histone extracts in comparative studies of the products of the three preparative methods.

The results strongly suggest that none of the major histone fractions, *i.e.*, Ia, Ib, IIb, III, and IV, is an artifact resulting from enzymatic degradation. The similarity of the chromatograms of histone preparations A, C, and D[4] (Fig. 1) is one important indication to this effect. Another is to be found in the similarity of preparations A, C, and D as illustrated by analytical starch-gel electrophoresis.[5]

A third indication resides in the similarity in both amino acid composition (Fig. 3)[6] and behavior on starch-gel electrophoresis (Fig. 6) of the corresponding histone fractions from preparations A, C, and D. A comparison of histone fractions III and IV in the case of preparations A and C shows that even this residual material—known to be very heterogeneous—is closely comparable for these two preparations (except for arginine, aspartic acid, and serine). This type of comparison could not be extended, unfortunately, to the minor fractions, which are obtainable only from preparations extracted at pH 0.7. Such preparations are not suitable for comparison in this type of study, since most of these minor fractions are known to be heavily contaminated by overlap of neighboring fractions.

Quantitative differences will have been noted in the distribution of the histone fractions in the chromatograms of histone preparations A, C, and D. Precise equivalence of the relative proportions of whole tissue or deoxyribonucleoprotein to volume of extracting solution in the three dissimilar methods was virtually impossible to attain, and the actual pH of the three extracts differed slightly. Since preparations A, C, and D are essentially partial extracts, relatively small changes in these factors may contribute appreciably toward the quantitative differences observed between the three chromatograms. In addition to changes consequent upon slight alterations in extraction conditions, it is possible that the relatively high proportion of histone fraction IIb in preparation C may arise in part because the extraction was made from recombined rather than native deoxyribonucleoprotein (Moore, 1959). Preparation D contained a considerable quantity of insoluble material, and the exact quantity of protein applied to the chromatography column was not known. The relatively large amount of material eluted from the column in the breakthrough peak in the case of preparation D is to be expected because the extract was made from whole thymus tissue and not from isolated nuclei or deoxyribonucleoprotein, and would therefore contain some nonbasic protein.

An incipient resolution into fractions Ia and Ib of the first major histone component eluted from the chromatography column by guanidinium chloride solution is observed with the three preparations, A, C, and D (Fig. 1). These two fractions are particularly rich in lysine and alanine. Crampton *et al.* (1955) chromatographed histone preparations on Amberlite IRC-50 with barium acetate solution and obtained fractions A and B, which were eluted as single, reasonably symmetrical peaks. Our fractions Ia and Ib are similar to this fraction A in amino acid composition except that they contain more than a trace amount of histidine and methionine (Table IV), and fraction IIb is virtually identical in amino acid composition with their fraction B, as is shown in Table V. In later experiments

[4] Note, however, the shift in elution position of fraction IIb in preparation D; this we are inclined to attribute to the reduction in load on the column.

[5] The lower migration rate of the components of preparation D compared with those of preparations A and C was due to the lower ionic strength of the more dilute protein solution; the relatively large amounts of the faster-moving components and the very slowly moving material (near the origin) are a result of extraction from whole thymus tissue rather than isolated nuclei or deoxyribonucleoprotein.

[6] Note also the close similarity in amino acid composition of fraction II (as obtained by chromatography of our preparation A with barium acetate solution), fraction B of Crampton *et al.* (1955), and fraction IIb from preparations A, C, and D—again strongly suggesting that none of them had suffered, or was the result of, enzymatic degradation.

TABLE IV

COMPARISON OF THE AMINO ACID COMPOSITION OF HISTONE FRACTIONS I AND A[a]

Amino Acid	Fraction Ia[b]	Ib[b]	I asc.	I desc.	A(1955)[c]	A(1957)[d]
Lysine	26.1	26.4	27.9	28.4	26.2	28.2
Histidine	0.3	0.3	0	0	0[e]	0
Arginine	2.6	2.7	1.8	1.4	2.3	1.7
Aspartic acid	2.5	2.4	2.0	1.8	2.1	2.0
Threonine	5.8	5.5	6.0	5.8	5.7	5.7
Serine	6.7	6.2	6.2	6.7	7.0	6.4
Glutamic acid	4.3	4.1	3.5	3.4	3.7	3.2
Proline	8.6	9.1	9.2	9.4	9.3	9.4
Glycine	6.9	7.3	6.6	7.0	7.0	6.7
Alanine	23.7	23.8	25.1	25.9	24.4	26.0
Valine	5.3	4.6	5.3	4.6	5.3	4.8
Methionine	0.1	0.1	0	0	0[e]	0
Isoleucine	1.2	1.3	1.0	0.9	1.1	0.9
Leucine	4.8	4.8	4.8	4.2	4.7	4.1
Tyrosine	0.6	0.7	0.5	0.3	0.7	0.5
Phenylalanine	0.7	0.7	0.5	0.5	0.6	0.5

[a] Calculated from the data published by Crampton *et al.* (1955 and 1957). The two fractions A are distinguished by the date in parentheses at the head of the column of amino acid values. [b] Average of values obtained for fractions from histone preparations A, C, and D. [c] Calculated from the values listed in Table II of paper by Crampton *et al.* (1955). [d] Calculated from the average values of the chromatographic preparations of fraction A listed in Table II of paper by Crampton *et al.* (1957). [e] Although listed here as zero, traces of these amino acids were apparently present in some preparations (Crampton *et al.*, 1957).

(Crampton *et al.*, 1957), these workers found that replicate experiments on different batches of histone furnished in some cases chromatograms which differed from the earlier ones in that a new, fast-moving component was eluted from the column before fraction A, the fraction A peak became divided into two overlapping peaks, and the size of the peak due to fraction B diminished as the other changes progressed.

These changes were attributed to decomposition, probably enzymatic, during the rather lengthy preparative procedure, which included an overnight extraction of histone, followed by dialysis for several days. When a much more rapid method was utilized for histone preparation, the chromatographic abnormalities were obviated and the amino acid composition of fractions A and B were somewhat different from those of earlier fractions. Fraction A contained more lysine and alanine, and was devoid of histidine and methionine, while fraction B contained more arginine and less lysine. These changes were explained by the assumption that in the earlier, time-consuming preparations fraction B was enzymatically degraded and some of the products of this fraction were eluted from the chromatography column in the same region as fraction A, thus contributing the traces of histidine and methionine found in some of the earlier fractions A. This process could also have been responsible for the lower arginine and higher lysine contents of the earlier fractions B.

Two lines of evidence show that a similar enzymatic degradation of, for instance, histone fraction IIb cannot be invoked to explain the separations of histone fractions Ia and Ib in the experiments in which guanidinium chloride solution was used to develop the chromatogram. Firstly, histone preparations A, C, and D, in which it is improbable that the same enzymatic reactions would persist, all furnish fraction Ia and Ib when chromatographed with guanidinium chloride solution, and Figure 3 shows that fractions Ia and Ib from the three different histone preparations have very similar amino acid compositions. Secondly, when a sample of preparation A was chromatographed on Amberlite IRC-50 with barium acetate solution, fraction I was obtained as a single peak (Fig. 2). However, its ascending (fraction I asc.) and descending (fraction I desc.) parts (Table I) contained neither histidine nor methionine; in this respect they resembled the histone fraction A of Crampton *et al.* (1957) rather than fractions Ia and Ib. Further fractionation and composition studies on fractions Ia and Ib revealed some important properties that require emphasis. Although they have a similar amino acid composition and are also similar in their behavior on starch-gel electrophoresis (Fig. 5 and 6), they differ not only in their chromatographic behavior (*i. e.*, separation on elution from the Amberlite IRC-50 column with guanidinium chloride solution), but also in their reaction with trichloracetic acid. Histone fraction Ia produces a turbidity in 0.8 M trichloracetic acid, while fraction Ib does not do so; fraction I asc. and I desc. (obtained by elution with barium acetate solution) exhibit this same difference.[7] Another difference between fractions

TABLE V

COMPARISON OF THE AMINO ACID COMPOSITION OF HISTONE FRACTIONS IIb, II, AND B[a]

Amino Acid	Fraction IIb[b]	IIb[c]	II[d]	B(1955)	B(1957)[e]	B(1957)
Lysine	13.0	11.1	12.8	13.2	12.1	11.7
Histidine	2.7	2.3	2.7	2.5	2.3	2.1
Arginine	8.1	10.4	8.4	7.6	10.2	10.8
Aspartic acid	5.5	5.0	5.5	5.5	5.2	5.1
Threonine	5.2	6.2	5.3	5.1	5.7	6.1
Serine	7.2	5.5	6.9	7.3	5.3	5.1
Glutamic acid	8.8	9.1	8.9	8.6	8.7	8.6
Proline	4.5	4.0	4.6	4.7	4.0	3.7
Glycine	8.2	9.2	8.5	8.4	9.1	9.7
Alanine	11.7	11.3	11.7	11.8	11.1	11.7
Valine	6.7	6.4	6.6	6.7	6.8	6.1
Methionine	0.8	1.0	0.8	0.9	1.0	1.0
Isoleucine	4.6	5.0	4.8	4.6	5.0	4.9
Leucine	8.7	8.7	8.7	8.5	8.7	8.8
Tyrosine	3.0	3.0	3.0	3.2	3.0	2.8
Phenylalanine	1.2	1.8	1.3	1.4	1.8	1.8

[a] Calculated from the data published by Crampton *et al.*, 1955 and 1957. The fractions B are distinguished by the date in parentheses at the head of the column of amino acid values. [b] Average of values obtained for fractions from histone preparations A, C, and D. [c] Fraction obtained from preparation B. [d] Average of values obtained for ascending and descending parts of fraction II derived from preparation A by chromatography with barium acetate solution. [e] Calculated from the average values of the six chromatographic preparations of fraction B listed in Table III by Crampton *et al.* (1957). [f] Calculated from the values for preparation 25 (direct extraction) listed in Table III by Crampton *et al.* (1957).

[7] Examination of Figure 2 shows that the fraction I peak is slightly asymmetrical, and it is possible that a more gradual change in concentration of the barium acetate solution used for elution in the chromatographic separation might have permitted at least partial resolution of two components corresponding with histone fractions Ia and Ib. It may also be noted that a separation of two histone components (f1A and f1B), each of which had a somewhat similar amino acid composition to that of histone fractions Ia and Ib, was achieved by stepwise elution of a histone preparation from carboxymethyl cellulose (Johns *et al.*, 1960).

Ia and Ib was demonstrated by Satake *et al.* (1960) in studies of the arginine peptides derived by enzymatic digestion of these fractions; Ia contained a very much higher proportion of serylarginine than did Ib.

Histone fractions Ia and Ib were both further resolved by differential elution from Amberlite IRC-50 with dilute sulfuric acid followed by 40% guanidinium chloride solution to furnish histone fractions Ia_s, Ib_s, and Ia_g, Ib_g respectively. Fractions Ia_s and Ib_s now resemble histone fraction I (obtained by elution with barium acetate solution) and the histone fraction A of Crampton *et al.* (1957) in that they contain neither histidine nor methionine (Table II); Fractions Ia_g and Ib_g contain both of these amino acids. Fractions Ia_s and Ib_s have a slightly lower arginine content than fractions Ia, Ib, Ia_g, and Ib_g, and the arginine content of fractions I asc. and I desc. is also lower than that of these four fractions. Further small but significant differences are apparent between histone fractions Ia and Ib, for the arginine content of fraction Ia_s is higher than that of fraction Ib_s and the valine content of fractions Ia, Ia_s, and Ia_g is higher than that of Ib, Ib_s, and Ib_g respectively (Table I). Corresponding differences are found between histone fractions I asc. and I desc. (obtained by chromatography with barium acetate solution).

All of this information is consistent with the contamination of fractions Ia and Ib by a small quantity of a histone fraction, such as fraction IIb, which contains histidine and methionine and a larger proportion of arginine, but a lower proportion of lysine and alanine, than fractions Ia and Ib. The histone fractions Iaa and Ix (Fig. 1) resemble fraction IIb in amino acid composition and overlapped fractions Ia and Ib, when chromatograms were developed with guanidinium chloride solution. There is no reason to believe that fractions Iaa and Ix are derived from fraction IIb by enzymatic degradation during preparation, for their presence in all three preparations, A, C, and D, is indicated by the amino acid composition of fractions Ia and Ib obtained from these preparations even though it may not be too obvious from the chromatograms shown in Figure 1. Since fractions Iaa and Ix (and also fractions IIaa, IIa, IIb, III, and IV) are not eluted from Amberlite IRC-50 by dilute sulfuric acid, the differential elution procedure removes contaminants (fractions Iaa and Ix) from fractions Ia_s and Ib_s, but concentrates them in fractions Ia_g and Ib_g. Supporting evidence of this is furnished by starch-gel electrophoresis experiments. Figure 5 shows that the minor components of fractions Ia and Ib (which are detected only when the gel is overloaded, as in the gel shown in the photograph) are absent from fractions Ia_s and Ib_s, but more obviously present in fractions Ia_g and Ib_g than in fractions Ia and Ib. Although fraction I (obtained by chromatography with barium acetate solution) does not contain fractions Iaa and Ix (since it is free of histidine and methionine), starch-gel electrophoresis experiments show (Fig. 6) that it is heterogeneous and contains minor components which are absent from fractions Ia_s and Ib_s (Fig. 5).

The chromatographic behavior of the histone fractions I may now be summarized. Development of the chromatogram with barium acetate solution furnished a fraction I which was free from contamination with such histone fractions as Iaa and Ix, but which was not resolved into fractions Ia and Ib. Resolution (if only partial) of histone fractions Ia and Ib was achieved by development of the chromatogram with guanidinium chloride solution, but contamination with other histone fractions (*e.g.* Iaa and Ix) then occurred. Differential elution of fractions Ia and Ib from Amberlite IRC-50 with sulfuric acid yielded preparations (Ia_s and Ib_s) which were free from the contaminating fractions (Iaa and Ix) and which appeared, from starch-gel electrophoresis experiments, to be less heterogeneous than any of the other fractions I.

In contrast with the histone fractions I, there was no discernible difference in either chromatographic behavior or amino acid composition between the histone fractions IIb and II obtained by development of chromatograms with guanidinium chloride and barium acetate solutions respectively. These fractions have virtually the same amino acid composition as the histone fraction B reported on by Crampton *et al.* in 1955, but their arginine content is lower and their lysine content is slightly higher than the fractions B described in 1957 by the same workers using rapid extraction procedures (see Table V). Crampton *et al.* (1957) noticed that the front half of their chromatographic fraction B peak had a higher arginine but lower lysine and histidine contents than the rear half of the peak. The admixture in fraction B (1957) of fractions such as our fraction IIaa or IIa would account for the differences in amino acid composition of the fractions B described by Crampton *et al.* in 1955 and 1957. We suggest that these differences are attributable to changes in the extraction procedure and to loss of resolution in the 1957 chromatography procedure due to the use of a shorter columr (10 cm) and a steeper barium ion concentration gradient, rather than to enzymatic degradation of the earlier histone preparation.

We do not wish to imply that enzymatic degradation may not be a hazard in histone preparation. Several investigators, mentioned earlier, have shown that under certain conditions degradation does indeed take place. Two years ago, in our own studies, we deliberately stored a sedimented paste of washed thymus nuclei at 2° to 3° for 1 week. The subsequently isolated histone when fractionated chromatographically behaved quite atypically and showed anomalies in the principal peaks that were strongly suggestive of degradative changes.

In two other preparations we included diisopropyl phosphorofluoridate (0.005 M) and *p*-chloromercuribenzoate (0.0001 M), respectively, as proteolytic enzyme inhibitors in the media used for washing the nuclei. The resulting histone preparations did not differ chromatographically from our usual preparations. Unfortunately the nuclei in this experiment were not permitted to incubate with the inhibitors under conditions that ordinarily would have provoked degradation (one week at 2° to 3°).

A second objective in this investigation has been to determine the amino acid composition of each of the isolated histone fractions, not only because of the bearing of such data on the question of enzymatic degradation but because of their pertinence to the problem of histone heterogeneity. We have already pointed out that each of the fractions Ia and Ib is heterogeneous. An inspection of Table II shows that there are significant differences in the composition of the subfractions despite the deceptive similarity in composition of the parent Ia and Ib fractions. Histone fractions IIaa and IIa contain much more arginine[8] and less lysine and histidine than does fraction IIb (Table I) and are probable contaminants of fraction IIb. Chromatoceams developed with guanidinium chloride show lgrarly that fractions IIaa and IIa were much more abundant in histone preparation B than in preparation A, evidently because of their low extractability at pH 1.5 and high extractability at pH 0.7; fractions IIb[9] from preparation B was consequently much more heavily contaminated with fraction IIa (and, perhaps, to a lesser extent with fraction IIaa) than was fraction IIb from preparation A. This is also apparent from the amino acid composition of the two fractions IIb (Table V), as well as from starch-gel electrophoresis experiments (not shown here), which demonstrated that fraction IIb from preparation B consistently contained much more of the very slowly moving components (appreciably more than fraction II asc., as shown in Figure 6) than did fraction IIb from preparation A; this would be expected if fraction IIaa or IIa (Fig. 5) were present. Chromatography of preparation A with barium acetate solution under conditions closely resembling those used by Crampton *et al.* (1955) furnished a histone fraction II which was almost identical in amino acid composition with fraction IIb obtained from preparation A, C, or D by chromatography with guanidinium chloride solution (see Fig. 3). In its behavior on starch-gel electrophoresis, fraction II (ascending part of the chromatographic peak) differed from the various fractions IIb in that it exhibited the presence of more minor, slowly moving components (Fig. 6), but the same two major components were found in all cases. Somewhat similar behavior on starch-gel electrophoresis was also observed by Johns *et al.* (1961) with a histone fraction (F2) obtained in an asymmetrical peak (f2) by chromatography on carboxymethyl cellulose (Johns *et al.*, 1960); this fraction also had an amino acid composition resembling that of the fractions II, IIb, and B discussed here.

A small amount of an unidentified compound, which was eluted from the chromatography column immediately after lysine, was found in the hydrolysates of fractions IIaa, IIa, III, and IV, but not in the hydrolysates of fractions IIb (except from preparation B, where fraction IIa is a contaminant) or II (obtained by chromatography with barium acetate solution). The presence of an unknown compound with similar chromatographic properties was observed by Crampton *et al.* (1957) in the hydrolysates of the later fraction B preparations.

Histone fractions Iaa and Ix are similar in amino acid composition, but, rather surprisingly, in this respect they resemble fraction IIb much more than they do fractions Ia and Ib, which they resemble in their behavior on chromatography with guanidinium chloride solution. Development of a chromatogram with barium acetate solution did not yield fractions Iaa and Ix, but the reason for this may be that they were eluted together with fraction IIb, in the presence of which their detection would be difficult. Starch-gel electrophoresis experiments showed that each is a complex mixture, and their differences prove that they are separate fractions and not the extremities of a single broad chromatographic peak which overlaps fractions Ia and Ib. The presence of at least nine components was indicated in both fractions Iaa and Ix. Fractions IIaa and IIa also closely resemble each other in amino acid composition, although fraction IIa must be heavily contaminated with fraction IIb. Both fractions resemble fractions III and IV, rather than than fraction IIb, in their amino acid content, particularly in that they contain the unknown compound which was eluted from the chromatography column immediately after lysine. Again, both fractions were shown by starch-gel electrophoresis experiments to be complex mixtures; fraction IIaa contained at least eleven components and fraction IIa contained ten components.

The last fractions eluted from Amberlite IRC-50 by guanidinium chloride solution are fractions III and IV, which are the histone fractions richest in arginine. These fractions apparently were not eluted from Amberlite IRC-50 with barium acetate solution, but when guanidinium chloride solution was used after barium acetate solution two further fractions were eluted (Fig. 2). Fraction III + IV from preparation A and fractions III and IV from preparation C were all found to be very complex mixtures when examined by starch-gel electrophoresis. They each contained from eight to eleven components and were particularly rich in the very slowly moving components.

Because of their heterogeneity, no attempt has been made to calculate the minimum molecular weight of the various histone fractions. Starch-gel electrophoresis experiments suggest that histone fractions Ia_s, Ib_s, and IIb may contain fewer components than the other fractions, and for this reason they are to be preferred, initially, for further studies. The two major components of fraction IIb have been obtained by a direct elution method of preparative starch-gel electrophoresis. The separation of these components is shown in Figure 7. Although the first component (fraction IIb_1) should have been free from the second (IIb_2), the latter may have been slightly contaminated with the former even though a generous allowance was made for the overlap region. Studies of fractions IIb_1 and IIb_2 have commenced with the determination

[8] The arginine values previously reported for these fractions from this laboratory (Satake *et al.*, 1960) differ from those reported here. The earlier values were obtained by means of a modified Sakaguchi reaction, and the values reported here in Table I are the more reliable because they were obtained by the use of the ninhydrin reaction with an automatic recording apparatus.

[9] Eluate fractions in the volume range 3700 to 4100 ml were taken for the recovery of fraction IIb in the experiment represented by Figure 1.

of their amino acid composition, and the data are given in Table III. A difference in relative lysine and arginine contents was anticipated because the two components differ in mobility on starch-gel electrophoresis. Large differences were also found in the serine, glycine, methionine, leucine, and tyrosine contents of fractions IIb_1 and IIb_2, but the significance of these cannot be discussed at this stage.

The extreme complexity of thymus histones, which was demonstrated by the starch-gel electrophoresis experiments of Neelin and Neelin (1960), has been emphasized by the studies on various chromatographic fractions of histone reported here. Only in the case of histone fraction IIb has the authenticity of the components indicated by starch-gel electrophoresis been established by a second electrophoresis as described by Neelin and Neelin (1960). The various chromatographic histone fractions contain a multiplicity of components, and the formidable task of establishing the difference or similarity of corresponding electrophoretic components in different chromatographic fractions is of importance in illustrating the true complexity of histone. Studies of partial enzymatic hydrolysates of various electrophoretic bands by the fingerprinting method of Ingram (1956) or the chromatographic procedure of Crampton *et al.* (1957) may provide the most convenient approach to this problem.

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NOTE ADDED IN PROOF: Since the preparation of this manuscript, we have found that the unidentified compound in the hydrolysates of histone fractions IIaa, IIa, III, and IV resembles ϵ-*N*-methyllysine in its behavior on ion-exchange chromatography. We are grateful to Dr. F. Sanger for a gift of flagellin.

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Dialysis Studies. IV. Preliminary Experiments with Sugars*

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Thin-film dialysis experiments have been made with a variety of sugars. From the data it has been concluded that where adsorption does not play a role the factors which determine the rate of free diffusion also determine the rate of dialysis. However, in comparative studies with different solutes thin-film dialysis appears to amplify greatly the differences noted in free diffusion. The data also indicate that differences in effective molecular diameters of the order of 2% or less can be detected by dialysis.

The dialysis studies under way in this laboratory were originally undertaken with a rather limited objective in mind, that of making simple dialysis a more useful separation technique (Craig and King, 1955) for small-scale laboratory work. The inherent selectivity and nature of the process soon suggested other possibilities, however, and led us to broaden our objective considerably. The method now offers one of the most convenient and informative procedures for determination of the homogeneity (Craig *et al.*, 1957a) with respect to size of a given preparation. In addition, when such dialysis data (Goldstein and Craig, 1960) are supported by data from ultracentrifugation and other techniques, much can be learned in regard to the state of association in a given solution and of the changes in association and molecular shape that occur when the solvent is altered as

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