Evidence has recently accumulated that histone preparations, particularly those derived from the nuclei of calf thymus, may contain a mixture of different basic proteins. Variations in the amino acid composition of histone fractions extracted and precipitated by different means have been demonstrated by Stedman and Stedman (2), Daly, Mirsky, and Ris (3), Eadie and Leaf (4), Hamer (5), Davison and Butler (6), and Daly and Mirsky (7). Electrophoretic heterogeneity has been shown by Grégoire and Limozin (8) and Butler, Davison, James, and Shooter (9, 10). Studies with the ultracentrifuge have indicated that various fractions possess different sedimentation rates ((9, 10), Ahlström (11), Luck et al. (12)). Methods which permit the isolation of individual, well characterized histones from the mixture have not been available, however. The fact that ion exchange chromatography has been employed successfully for the purification of basic proteins such as cytochrome c (13), ribonuclease (14), lysozyme (15), and chymotrypsinogen (16) prompted an attempt to chromatograph histones in a similar manner. Initial trials with the carboxylic acid resin IRC-50 in the sodium form were not successful. The histones were bound much more strongly than the proteins previously studied and could not be eluted unless very alkaline eluents were used.¹ In order to achieve increased eluting power, calcium and barium acetate buffers of gradually increasing ionic strength were employed with the corresponding salt of the resin. Some, but not all, of the components of calf thymus histone could be chromatographed under these conditions. The present report describes the purification and properties of the proteins comprising the less basic half of crude calf thymus histone.

¹ We are indebted to Dr. M. M. Daly and Dr. A. E. Mirsky for information indicating that the published IRC-50 procedures were not applicable to the fractionation of histones. The negative results with the sodium salt of the resin led us to investigate modifications which might extend the scope of the method to basic proteins of this type.
CHROMATOGRAPHY OF HISTONES

EXPERIMENTAL

Calf Thymus Histones—Preparations of the total histone mixture used in the present study were obtained by a previously described modification (17) of earlier methods in which Hammarsten's procedure is employed for dissociating histone from nucleic acid with NaCl and ethanol. The protein fraction thus prepared contained 17.4 per cent N (Kjeldahl) and 1.93 per cent S (Parr bomb) on an ash- and moisture-free basis (ash, 1.28 per cent). A sample of calf thymus histone isolated by dissociating the proteins from nucleic acid by extraction with 0.2 N HCl by the method of Mirsky and Pollister (18) was also used in some studies.2

Preparation of Tissue Extracts—In order to minimize the possibility that the chromatographic results obtained with the isolated histones might arise from artifacts formed during their preparation, crude tissue extracts were also chromatographed. These were obtained in one step by homogenizing thymus tissue with barium acetate and adding ethanol in sufficient quantity to precipitate barium nucleates and most neutral proteins. Fresh tissue was brought from the slaughter-house in cracked ice and processing was begun within 90 minutes of the death of the animal. The extractions were carried out at 4°. The thymus was trimmed and 10 gm. portions were homogenized in a Waring blender with 90 ml. of water for 60 seconds. Enough solid Ba(OAc)2 (15.3 gm.) was added to make the homogenate 0.6 M in Ba.3 The mixture was allowed to stand for 30 minutes, and 2 volumes of 95 per cent ethanol were added in two equal portions with vigorous shaking. After an additional 30 minutes, the resulting fibrous precipitate was lifted from the mixture and squeezed until compact with the aid of a porcelain spatula in order to express the occluded fluid. After filtration to remove a granular precipitate, the filtrate (260 ml.) containing the histones was taken to dryness on a rotary evaporator (19), the bath temperature of which was kept below 40°. The residue was dissolved by the addition of 520 ml. of water, and an aliquot of 6 ml. was removed at this point for chromatographic analysis. The sample was adjusted to pH 6.7 with 2 N HOAc and centrifuged before being chromatographed. The remaining extract was dialyzed (27/32 inch NoJax casing) against cold running tap water for 24 hours and against distilled water at 4° for 18 hours. The solution was centrifuged and lyophilized. The yield was 450 mg. of material per 10 gm. of thymus tissue (wet weight).

2 Calf thymus histone, lot No. 532, from the Worthington Biochemical Corporation, Freehold, New Jersey.

3 Separate experiments showed that 0.6 M Ba(OAc)2-ethanol caused the dissociation of a maximal amount of ninhydrin-positive material from isolated nucleohistone, 85 per cent of the ninhydrin color of the nucleohistone being recovered in the ethanolic extract.
A number of dialyzed and lyophilized 2.6 M NaCl-ethanol extracts of calf thymus homogenates were prepared exactly as described above except that enough NaCl (15.2 gm.) to render the extract 2.6 M was added to the aqueous homogenate instead of the Ba(OAc)$_2$.

Chromatography—The sodium salt of IRC-50 (XE-64) was prepared in the manner already described (14) and screened through a 200 mesh sieve (16, 20). The acid form of the resin was washed on a Büchner funnel with 2 M Ba(OAc)$_2$ (or Ca(OAc)$_2$) until the effluent was about pH 6.5 (pH 6.0 when Ca was used). The bed was washed with water, followed by several bed volumes of 0.1 M Ba(OAc)$_2$ buffer of pH 6.7 or 0.13 M Ca(OAc)$_2$ buffer of pH 6.2. The resin was suspended in the 0.1 M buffer and transferred to a 4 X 30 cm. chromatograph tube. For convenience in the final washing about 6 liters of buffer per 4 X 15 cm. resin bed were passed through over a 24 to 48 hour period to bring the pH of the effluent to the same value as that of the influent. The final equilibrations and the chromatography were performed at about 25°. Before reuse, the resin was cycled through the acid and sodium forms.

The analytical chromatograms were performed with columns 0.9 cm. in diameter and of the desired height, prepared from suspensions of the resin in the given buffer by the procedures already described (14, 21). Chromatograph tubes with sealed in filter plates (20) were employed. To promote regular flow of solvent, a roll of silver gauze was inserted into the delivery tip of the chromatograph tube (14).

The sample to be analyzed contained 5 to 10 mg. of histone in 1 to 6 ml. of the initial buffer. Before the sample solution was placed on the column, an aliquot was removed and analyzed by the ninhydrin method (19) to permit subsequent calculation of the per cent recovery of ninhydrin color in the chromatographic analysis. Chromatography was carried out with eluents of gradually increasing ionic strength through the use of a small magnetically stirred mixing device (cf. (20)). In most instances the mixing chamber containing the buffer of lower ionic strength was made from a 30 ml. flat bottomed flask attached to the upper joint of a wash bottle, the assembly having a total capacity of about 50 ml. Changes in the rate at which the ionic strength of the eluent was increased were effected by altering the strength of the solution in the separatory funnel.

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4 The buffer was prepared by adding 0.67 ml. of glacial acetic acid to 10 liters of 0.1 M Ba(OAc)$_2$. The stronger buffers were prepared with similar proportions. All pH values were checked with the glass electrode. Since the chromatographic behavior of the histones proved to be much more dependent on the Ba$^{++}$ concentration than on pH, the relatively low buffering capacity of the eluents has not caused the results to vary unduly.

5 It has been noted that silicone grease and glass cloth both adsorb appreciable quantities of histone. Adsorption by the sintered disk seems not to occur, however.
feeding into the mixing chamber. In order to minimize upward flow of the less dense buffer, a capillary was inserted between the two chambers (20) and the stop-cock of the separatory funnel was only partly opened. The columns could be operated at flow rates of from 1.5 to 9 ml. per hour without affecting their performance. The effluent was usually collected in 1 ml. fractions which were analyzed by the photometric ninhydrin method with 1 ml. of the modified ninhydrin reagent (22). The blank readings gradually increase as the Ba(OAc)₂ concentration in the effluent rises. The base-line for each peak on the effluent curve is estimated from the readings immediately preceding or following the peak.

The preparative scale experiments were carried out with a load of about 400 mg. of protein on columns 4 cm. in diameter, a 1 liter mixing chamber being employed. Since the cross-sectional area of the column is about 20-fold that of the columns 0.9 cm. in diameter, the effluent volumes at which the changes in eluent were made (and at which the peaks emerged) in the analytical scale chromatograms were multiplied by 20. The effluent was collected in 15 ml. fractions at a rate of 75 ml. per hour, and 0.5 ml. aliquots were pipetted for ninhydrin analysis. Fractions corresponding to a given protein peak were pooled and transferred to Visking casings for dialysis against cold running tap water and distilled water at 4°. After dialysis, the histones were obtained by lyophilization.

Determination of Amino Acid Composition of Isolated Histones—Samples of the lyophilized preparations were simultaneously weighed for the determination of moisture (dried at 100° over P₂O₅ for 3 hours at 0.1 mm. pressure), ash (residue on ignition), nitrogen (Kjeldahl), and amino acid composition. For hydrolysis, the samples (about 2 mg.) were heated at 110° with 6 N HCl in evacuated, sealed tubes for 22 and 70 hours in the manner already described for the analysis of ribonuclease by Hirs et al. (23). The amino acid analyses were performed by the use of 150 cm. columns of Dowex 50-X4 (20). An approximate value for tryptophan was obtained, after alkaline hydrolysis, by chromatography on starch columns with 0.1 N HCl as eluent (cf. (23)).

Results

Chromatography of Preparations of Thymus Histone—Attempts were first made to chromatograph the histone fraction on columns of sodium IRC-50. As eluents, sodium acetate buffers (pH 6.4) varying in ionic strength from 0.2 to 4 M were tried. The results indicated that more than 90 per cent of the histone preparation was being held tenaciously by the resin. When greater eluting power was achieved by the use of calcium ions, a series of peaks was obtained as illustrated by the effluent curve shown in Fig. 1. The resin was equilibrated initially with 0.13 M calcium
acetate at pH 6.2. Previous test-tube experiments (for the procedure see Hirs et al. (14)) had shown that at this calcium concentration the resin binds nearly all of the histone in the dialyzed preparations from calf thymus. In contrast to the results obtained with sodium, however, higher concentrations of calcium proved able to elute portions of the bound histone. Thus, as the concentration of calcium acetate in the influent buffer was gradually increased from 0.13 toward 2 M, three principal peaks were eluted, accounting for 4, 24, and 20 per cent of the total ninhydrin color applied to the column. Even if the experiment was continued to 150 effluent ml., or if the concentration of the influent buffer was changed directly to 2 M, no further peaks appeared. No more than about 50 per cent of the starting material (on a ninhydrin basis) could be recovered from the chromatogram. Similar results were obtained with a commercial sample of histone chloride.

The low yield does not result from the irreversible binding by the resin of major quantities of Fraction A or B, because, when a portion of the material isolated from Peak A was rechromatographed under the same conditions, it gave a single peak in the original position in 88 per cent yield. Similarly, material from Peak B, upon rechromatography, gave at

\[ pH \text{6.2} \quad [0.13\text{M}(50\text{ml})] \rightarrow 2\text{M} \]

If the Ca concentration of the initial buffer is above 1 M, a much greater percentage of the ninhydrin color is recovered in a single unretarded peak at the column volume. Thus, there is a part of the histone mixture which, once it has been bound by the resin, can no longer be eluted by a buffer possessing an ionic strength sufficiently high to have prevented binding of this histone fraction initially.
its original position a single peak in a yield of 83 per cent. Apparently the low yield results from the presence in the histone preparation of additional components that are not eluted from the resin under these conditions. It is probable that the fraction which is retained is composed of proteins more basic than Fraction B, which, in turn, is more basic than Fraction A. This supposition is in accord with the results of the amino acid analyses subsequently obtained in the present study. The fact that the positions of the peaks did not change upon rechromatography supported the conclusion that the substances obtained were not artifacts induced by the column and that competition and displacement effects were not playing a rôle in the fractionation process. Thus, for the proteins comprising the least basic half of the histone mixture, the IRC-50 columns appeared to provide a suitable means of fractionation.

When further study was made of the variables which determine the resolving power of the system, it was found that the results were not dependent upon the flow rate (up to 15 ml. per hour per sq. cm. of cross-sectional area of the column), upon the temperature in the range from 4–25°, or upon the pH in the range from pH 6.0 to 7.5. The chromatographic behavior of histones on calcium IRC-50 is thus markedly less sensitive to changes in the pH of the eluent than was found to be the case for the basic proteins previously studied with the sodium form of the resin (14–16). If the height of the column was doubled, but the rate of change of calcium concentration in the influent kept the same, the separation of Fractions A and B was improved. A less rapid rate of change in the composition of the influent buffer also yielded better separation, but was accompanied by a broadening of the peaks which approached impractical limits (zones 50 to 100 ml. wide) when the rate of change was reduced to one-fifth of that used for the experiment shown in Fig. 1.

Barium acetate, with the barium form of the resin, was found to possess stronger eluting power than the corresponding calcium buffer and was, therefore, adopted for the subsequent chromatograms.

The data obtained thus far do not provide decisive information as to the mechanisms operating to produce the separations. When ribonuclease is chromatographed on sodium IRC-50, the pH and ionic strength are maintained constant, and there can be no doubt that a true reversible distribution of the protein between the buffer and the resin phase occurs (14, 24). To chromatograph histones, however, a changing concentration of Ca or Ba in the influent buffer is necessary. The effluent does not reflect exactly the change in the influent because the IRC-50 column is not quite saturated and continues to take up small amounts of Ca++ or Ba++ from the buffer as the cation concentration is raised. Therefore, in order to determine whether the protein has a finite distribution coefficient between
buffer and Ca or Ba IRC-50, it will be necessary to perform single stage, test-tube experiments at constant pH and ionic strength with purified histone fractions (cf. (14)).

Chromatography of Extracts of Tissues and Isolated Nuclei—In Fig. 2, the chromatographic behavior (with the barium system) of a total histone preparation isolated by the modified Hammarsten procedure (17) is compared with that of a tissue extract prepared by the simple one-step Ba(OAc)$_2$-ethanol precipitation described in the experimental section.

![Chromatogram of Histone Preparations](image)

**Fig. 2.** Comparison of the chromatographic behavior of a total histone preparation (a) with that of a crude extract of the whole tissue prepared by a single step barium acetate-ethanol precipitation (b). Dialysis of the extract used in (b) removed the constituents responsible for the large peak before histone Fraction A and for the smaller peak after histone Fraction B.

Two peaks, A and B, appear at nearly the same positions on each chromatogram. The insensitivity of the IRC-50 columns to salts and other constituents in tissue extracts (14) makes feasible the chromatographic analysis of the crude histone solution. The peaks preceding and following Peaks A and B in Fig. 2, b largely disappear if the crude tissue extract is dialyzed prior to chromatography.7

Chromatography of a number of dialyzed and lyophilized 2.6 M NaCl-ethanol extracts consistently showed the presence of major components, the behavior of which was similar to that of Fractions A and B. If the

7 Under the conditions of the chromatograms, all of the amino acids present in a mixture simulating the composition of a hydrolysate of bovine serum albumin have emerged by 25 effluent ml.
aqueous homogenate was allowed to autolyze for 4 hours at 29° prior to extraction with NaCl-ethanol, the chromatographic picture given by the final preparation was qualitatively the same as that shown in Fig. 2, b, except that the amounts of Fractions A and B were less. Autolysis of the extract did not lead to increased heterogeneity.

Calf thymus nuclei, which had been isolated by a modified Behren's technique (Allfrey, Stern, Mirsky, and Saetren (25)\textsuperscript{8}), were extracted with 0.6 M Ba(OAc)\textsubscript{2}-ethanol and the extract was chromatographed under the same conditions that were used for the experiment illustrated in Fig. 3.

Components emerged which corresponded both in position and relative amounts to histone Fractions A, B, and C present in total histone preparations.

The results obtained indicate that the histone Fractions A, B, and C probably preexist in the nuclei of the gland and are not formed as a result of enzymatic action or other changes occurring during the preparation of the histones.

Isolation of Histone Fractions A, B, and C—In the preparative scale experiments and in the analytical scale chromatograms that served as models, a longer column (28 cm.) was employed and the change in the cat-

\* We are indebted to Dr. A. E. Mirsky and Dr. V. Allfrey, who, through a generous gift of isolated nuclei, prompted us to perform this experiment.
ion concentration in the influent was made more gradually. The results of such an analysis are shown in Fig. 3. The increased resolution observed in this experiment was fully reproducible when the same histone preparation was analyzed and was also obtained with numerous total histone preparations isolated in a similar manner. In addition to the major Peaks A and B, the two smaller peaks before and after Peak A were found repeatedly. The least basic half of crude calf thymus histone contains, therefore, five or more chromatographically differentiable proteins.

In an experiment paralleling the one shown in Fig. 3, the proteins responsible for three of the peaks (A, B, and C) were isolated from the effluent after chromatography of 400 mg. of total histone on a column 4 cm. in diameter. The pooled fractions corresponding to only the central part of Peak A were taken, representing 13 per cent of the total ninhydrin color added to the column. The yield of dialyzed, lyophilized protein was 21 mg. In order to obtain information as to the homogeneity of Peak B, independent amino acid analyses were performed on samples taken from the left-hand half and the right-hand half of the peak. The second portion taken corresponded to 11 per cent of the total ninhydrin color and yielded 39 mg. of Fraction B. A sample of Fraction C corresponding to 4 per cent of the ninhydrin color gave 8 mg. of histone. During the removal of the Ba(OAc)$_2$ by dialysis there were losses of 6, 19, and 1 per cent for histone Fractions A, B, and C, based on the recovery of ninhydrin color. From the weight of the histone obtained, and the per cent of the total ninhydrin color in each of the peaks on the curve, it is calculated that, on a weight basis, the proteins in Fractions A, B, and C represent a minimum of approximately 10, 22, and 3 per cent of the histone preparation. The sizes of the peaks on the effluent curve in Fig. 3 convey the relative amounts of proteins on a ninhydrin color basis but not on a weight basis, since the individual proteins have different ninhydrin color values per mg., as a result of differences in amino acid composition.

Fraction A contained 17.95 per cent N (Kjeldahl) corrected for moisture and for 2.08 per cent ash. Fraction B similarly contained 16.78 per cent N and 7.86 per cent ash on ignition. The ash content probably could have been reduced by redialysis. Fraction C, which was not isolated in sufficient quantity for moisture and ash determinations, gave 13.99 per cent N on the lyophilized but not dried preparation.

**Amino Acid Composition of Isolated Histones**—In order to characterize further the proteins responsible for Peaks A, B, and C in Fig. 3, amino acid analyses of the isolated fractions were performed. It was also hoped to obtain in this manner additional evidence as to the homogeneity of the fractions.

The analytical data obtained by chromatographing 22 and 70 hour hy-
### Table I

**Amino Acid Composition of Hydrolysates of Calf Thymus Histone and Derived Fractions**

The compositions were determined by chromatography of the 6 N HCl hydrolysates on columns of Dowex 50-X4 (17, 20). Analysis of an alkaline hydrolysate showed that the total histone mixture contained less than 0.08 per cent of tryptophan.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Total histone mixture</th>
<th>Histone Fraction A</th>
<th>Histone Fraction B</th>
<th>Histone Fraction C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22 hrs.</td>
<td>70 hrs.</td>
<td>22 hrs.</td>
<td>70 hrs.</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.10</td>
<td>1.52</td>
<td>1.52</td>
<td>3.60</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.31</td>
<td>2.65</td>
<td>2.62</td>
<td>5.49</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.44</td>
<td>4.99</td>
<td>5.19</td>
<td>5.37</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.75</td>
<td>17.80</td>
<td>17.66</td>
<td>7.49</td>
</tr>
<tr>
<td>Valine</td>
<td>3.98</td>
<td>3.77</td>
<td>3.81</td>
<td>4.16</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.02</td>
<td>3.41</td>
<td>3.31</td>
<td>5.49</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.82</td>
<td>0.77</td>
<td>0.77</td>
<td>2.83</td>
</tr>
<tr>
<td>Serine</td>
<td>3.13</td>
<td>4.25</td>
<td>2.94</td>
<td>4.04</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.41</td>
<td>3.81</td>
<td>3.33</td>
<td>3.20</td>
</tr>
<tr>
<td>Cystine</td>
<td>&lt;0.06</td>
<td>0.06</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.63</td>
<td>0.06</td>
<td>0.51</td>
<td>0.56</td>
</tr>
<tr>
<td>Proline</td>
<td>3.23</td>
<td>6.79</td>
<td>6.67</td>
<td>2.39</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.36</td>
<td>0.42</td>
<td>0.47</td>
<td>0.98</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.53</td>
<td>0.45</td>
<td>0.38</td>
<td>1.77</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.87</td>
<td>&lt;0.49</td>
<td>&lt;0.15</td>
<td>5.03</td>
</tr>
<tr>
<td>Lysine</td>
<td>19.11</td>
<td>39.27</td>
<td>36.09</td>
<td>15.81</td>
</tr>
<tr>
<td>Arginine</td>
<td>21.47</td>
<td>6.74</td>
<td>18.60</td>
<td>19.15</td>
</tr>
<tr>
<td>Ammonia</td>
<td>4.99</td>
<td>2.74</td>
<td>4.51</td>
<td>6.56</td>
</tr>
</tbody>
</table>

* The analyses were performed on samples pooled from the left side (Sample I) and right side (Sample II) of the histone B peak on the effluent curve. The agreement among the analytical values is supporting evidence that histone Fraction B is chromatographically homogeneous. The values in Table II are based on the analyses of Sample II.

† The values for leucine and lysine in Table II are based on the results obtained for the 70 hour hydrolysate of histone Fraction B. These results were confirmed by a duplicate analysis of a 70 hour hydrolysate, which yielded values of 5.64 for leucine and 16.77 for lysine.

‡ The methionine figures include the 10 per cent correction for losses during chromatography (20).

§ The 70 hour histidine value was used for the calculations in Table II. Further analyses would be required to verify whether the rate of liberation of histidine is actually slow in this case.

∥ The analysis was lost.
drolysates of Fractions A, B, and C on columns of Dowex 50 (20, 23) are given in Table I, together with an analysis of the initial total histone preparation. Hydrolysis for two different lengths of time has been shown by several earlier investigations (23, 26–28) to permit more accurate estimates both of those amino acids that decompose detectably on acid hydrolysis and of those that may not have been fully liberated after the shorter time. The results in Table I are expressed in terms of amino acid nitrogen as per cent of total nitrogen and are thus independent of the nature and the quantity of any anion that might be present in the various preparations. If, as seems likely, these very basic proteins do bind anions, such as acetate, the presence of 1 acetate per ε-amino group of lysine and per guanido group of arginine would contribute as much as 10 to 15 per cent to the weight of the preparation. Results expressed in terms of amino acid nitrogen as per cent of total nitrogen are also independent of the ash content of the various preparations. The ash content of Fraction B was 7.86 per cent after ignition (presumably to BaO), but this figure does not take into account the mode of combination of the metal ion. The contribution of the ash to the weight of the original preparation would be different, depending upon whether the metallic cation existed as the salt of the free carboxyl groups of aspartic and glutamic acids or as barium acetate.

In Table II are given the amino acid compositions of Fractions A, B, and C (in terms of amino acid N as per cent of total N) derived from the analytical data in Table I. The values for serine, threonine, and tyrosine, and for proline in Fractions B and C have been calculated by extrapolating the results for the 22 hour and 70 hour hydrolysates to zero time, on the assumption that the decomposition follows first order kinetics in each case (cf. (23)). Leucine and lysine in Fraction B appear to be the only amino acids incompletely liberated after 22 hours of hydrolysis. The values for 70 hours have, therefore, been used in Table II. The remaining figures in Table II are averages of the values obtained for the 22 and 70 hour hydrolysates. Tryptophan has been assumed to be absent from Fractions A, B, and C, inasmuch as a negligible amount (less than 0.08 per cent) of this amino acid was found in the total histone preparation by chromatography of an alkaline hydrolysate on starch (23). The amide-NH₃ values have been estimated from the NH₃ content of the 22 hour hydrolysate corrected for the NH₃ calculated to have arisen by the decomposition of serine and threonine in this period (23). As can be seen from the bottom of Table II, the amino acid analyses account for more than 90 per cent of the nitrogen of the preparations in each case. More accurate results and more complete recoveries might be possible if a more extensive series of analyses was performed.
The differences in the amino acid compositions of the three isolated frac-
tions are readily apparent from the data in Table II. In Fraction A, lysine N represents 37.7 per cent of the total N of the protein, corresponding to over one-fourth of all of the amino acid residues. Alanine and

**Table II**

*Amino Acid Composition of Calf Thymus Histone and Derived Fractions*

Based on the analytical data in Table I.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Total histone mixture</th>
<th>Histone Fraction A</th>
<th>Histone Fraction B</th>
<th>Histone Fraction C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>3.10</td>
<td>1.52</td>
<td>3.53</td>
<td>2.23</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.31</td>
<td>2.64</td>
<td>5.49</td>
<td>3.60</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.44</td>
<td>5.09</td>
<td>5.37</td>
<td>4.94</td>
</tr>
<tr>
<td>Alanine</td>
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<td>17.73</td>
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</tr>
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</tr>
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<td>3.36</td>
<td>5.42</td>
<td>4.04</td>
</tr>
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<td>Isoleucine</td>
<td>2.82</td>
<td>0.77</td>
<td>2.95</td>
<td>1.58</td>
</tr>
<tr>
<td>Serine</td>
<td>3.77*</td>
<td>5.05†</td>
<td>4.65†</td>
<td>4.47†</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.70*</td>
<td>4.07†</td>
<td>3.29†</td>
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<td>Cystine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.63</td>
<td>0</td>
<td>0.60</td>
<td>0.29</td>
</tr>
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<td>Proline</td>
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<td>6.73</td>
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</tr>
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<td>0.80</td>
<td>0.91</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.64*</td>
<td>0.48†</td>
<td>2.04†</td>
<td>1.33†</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.87</td>
<td>0</td>
<td>4.85</td>
<td>2.07</td>
</tr>
<tr>
<td>Lysine</td>
<td>19.11</td>
<td>37.68</td>
<td>16.07</td>
<td>21.80</td>
</tr>
<tr>
<td>Arginine</td>
<td>21.47</td>
<td>6.74</td>
<td>19.46</td>
<td>12.31</td>
</tr>
<tr>
<td>Amide-NNH₂</td>
<td>4.06</td>
<td>1.68</td>
<td>4.40</td>
<td>2.36</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>97.26</strong></td>
<td><strong>97.78</strong></td>
<td><strong>94.82</strong></td>
<td><strong>90.01</strong></td>
</tr>
</tbody>
</table>

* The values for serine, threonine, and tyrosine in the 22 hour hydrolysate of the total histone mixture were corrected for the average percentage losses of these amino acids found for histone Fractions A and B.

† Values obtained from the data in Table I by extrapolation, based on the assumption that the decomposition of the amino acids follows first order kinetics (cf. (23)).

proline (accounting for 17.7 and 6.7 per cent of the total N, respectively) are unusually abundant, and four amino acids are absent, namely cystine, methionine, histidine, and tyrosine. The absence of these amino acids indicates that Fraction A has been separated from other histones that contain methionine and histidine.

The amino acid composition of Fraction B is much closer to that of the total histone than is that of Fraction A. With the exception of glycine,
phenylalanine, arginine, and probably serine and methionine, the values for the total material are intermediate between those for Fraction A and those for Fraction B. Evidence favoring the homogeneity of Fraction B is provided by analyses of a sample from the front half (Sample I, Table I) and the back half (Sample II, Table I) of the peak obtained from the IRC-50 column. The analyses of the 22 hour hydrolysates agreed well, except for serine and threonine, which are subject to varying degrees of decomposition. If a mixture of proteins were emerging from the column, some difference should be detected in the amino acid composition of fractions collected from different portions of the peak. There is, for example, a great difference in the amino acid compositions of the two neighboring Fractions A and C (Table II). Fraction C contains a significant quantity of histidine, and only a little more than half the lysine of Fraction A, but twice the phenylalanine and tyrosine.

DISCUSSION

The data presented in this communication emphasize that major structural differences may exist among the proteins conventionally classified as histones. The amino acid analyses of Fractions A and B isolated from a thymus histone mixture by chromatography on IRC-50 indicate that two of these proteins may have been obtained in homogeneous form. Comparison of the arginine content of Fractions A, B, and C with that of the total histone mixture shows that the proteins which it has not so far been possible to chromatograph on IRC-50, and which are retained by the resin, must be higher in arginine content than the isolated fractions.

Proteins or polypeptides rich in lysine have been isolated previously from calf thymus. Bloom, Watson, Cromartie, and Freed (29) obtained a "polylysine" fraction which inhibited the growth of the anthrax bacillus. Of the nitrogen of this preparation, 36.7 per cent was lysine N, 7.36 per cent was arginine N, and 1.84 per cent was histidine N. The complete amino acid composition was not determined. The lysine and arginine values are very similar to those of Fraction A. Because it contains no histidine, Fraction A clearly differs from the "polylysine" fraction of Bloom et al. Davison and Butler (6) have published preliminary amino acid analyses of a histone fraction of high lysine content prepared by fractionation of crude histone with ammonia and acetone (9) or by extraction of nucleoprotein with dilute citric acid (10). Daly and Mirsky (7) have described the isolation of lysine-rich histone fractions prepared by partial extraction of calf thymus nuclei with NaCl and dilute citric acid.

Hirsch and Dubos (30) have recently reported the isolation from calf

9 It has not been possible thus far to chromatograph substances known to have a high arginine content, such as salmine, on columns of Ba IRC-50.
thymus of a basic peptide fraction with antimycobacterial activity. On analysis 23.8 per cent of the N of the preparation was lysine N and 30.3 per cent was arginine N. The components of this mixture were shown to be dialyzable, however, and hence would not have been included in the total histone preparations utilized in the present study.

The authors are greatly indebted to Miss Joyce F. Scheer for technical assistance and to Mr. S. Theodore Bella who performed the microanalyses reported in this paper.

SUMMARY

A total histone preparation from calf thymus has been fractionated by chromatography on columns of the carboxylic acid resin IRC-50. The material was bound firmly by the sodium form of the resin, but chromatography could be achieved when calcium or barium acetate buffers (pH 6.2 or 6.7) of continuously increasing ionic strength (0.1 to 2.0 M) were employed with the Ca or Ba form of the resin. Under these conditions, only the less basic half of the total histone mixture could be eluted. Even the increased eluting power of the Ca or the Ba system did not serve to elute the most basic components, which were still retained by the resin. The chromatograms showed that the portion of the total histone eluted was composed of five or more proteins. Three of these components have been separated chromatographically and isolated by dialysis and lyophilization. The three protein fractions, A, B, and C, represent a minimum of about 10, 22, and 3 per cent of the total mixture. Each preparation has been characterized by complete amino acid analyses by chromatography on columns of Dowex 50-X4. Component A is unusually rich in lysine and alanine (37.7 and 17.7 N as per cent of total N, respectively) and contains no cystine, methionine, histidine, or tryptophan. The results of the amino acid analyses, taken in conjunction with the behavior of the proteins on the columns of IRC-50, indicate that histone Fractions A and B may have been isolated in homogeneous form. The chromatographic procedure is applicable to crude extracts of calf thymus prepared by treatment of an aqueous homogenate with Ba(OAc)$_2$ and ethanol. In this manner evidence has been obtained that histones A and B are present in tissue extracts freshly prepared by a one-step fractionation procedure. The chromatographic technique may also provide a means for comparing the less basic histones obtained from various tissues and from different species.

BIBLIOGRAPHY

CHROMATOGRAPHIC FRACTIONATION OF CALF THYMUS HISTONE
Charles F. Crampton, Stanford Moore and William H. Stein


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