# THE FRACTIONATION OF α-HISTONES FROM CHICKEN ERYTHROCYTE NUCLEI

## II. FRACTIONS OBTAINED BY EXCLUSION CHROMATOGRAPHY

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*Summary*

Crude α-histone, obtained from the original histone complex by precipitation of the β- and γ-histones with ethanol, has been shown by starch-gel electrophoresis to contain 13 components. The fractionation of α-histone by exclusion chromatography on Sephadex G-200 is reported. While none of these components have been obtained pure in the present study, considerable purification of components 2, 3, 4, 5, 8, 9, 10, and 11 has been achieved, and their amino acid composition leaves no doubt that α-histones represent a much larger family of "lysine-rich" proteins than was hitherto supposed.

## I. Introduction

In a previous paper (Bellair and Mauritzen 1964) the fractionation of α-histones by means of ethanol and isoelectric precipitation was reported. The present paper deals with further attempts to separate these histones by exclusion chromatography.

The separation of histones by exclusion chromatography is complicated by the observation that, even at low pH and low ionic strengths, histones tend to aggregate, and the possible formation of mixed aggregates cannot be eliminated (Cruft, Mauritzen, and Stedman 1958*a*). Cruft (1961), who used Sephadex G-75 to separate the histones of calf thymus, suggested that when whole histone is fractionated on Sephadex, with 0·02N HCl as eluant, mixed aggregates of β- and α-histones are in fact formed. For this reason, the present studies were performed on the crude α-histone fraction from chicken erythrocyte nuclei, which had been obtained by the removal of the two principal aggregating histones, namely, the β- and 1·6 S γ-components.

## II. Materials and Methods

### (*a*) *Histones*

The histone fractions used in this study were prepared as follows:

(1) A crude α-histone was obtained as the supernatant when a 2% solution of whole histone was adjusted to pH 6·5 and a final ethanol concentration of 17%. This fraction was further purified by adjusting a 4% solution at pH 6·5 to a final ethanol concentration of 17% and removing the small amount of precipitate which formed.

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(2) The "70% soluble" $\alpha$-histone fraction, which contained components of high mobility on starch-gel electrophoresis, was obtained by precipitation at pH 9·5 and a final ethanol concentration of 70% and removal of the bulk of the $\alpha$-histones which formed.

The isolation of erythrocyte nuclei and of these histone fractions has already been described in Part I of this series (Bellair and Mauritzen 1964). Starch-gel electrophoresis, amino acid analysis, and ultracentrifugation were carried out as described in Part I.

### (*b*) *Exclusion Chromatography*

Sephadex G-200 (particle size 40–120 $\mu$) in bead form was used in most of this work, although several separations were made with G-75 (medium size). The columns used were of glass or Perspex, having an internal diameter of 6 cm, a length of 150–180 cm, and a total packed volume of 3·5–4·4 litres. Smaller columns of 0·5 and 1 litre bed volume were used for re-running fractions obtained with the larger columns. The Sephadex was allowed to swell for 24 hr in 0·1M NaCl, then washed by settling and decantation (six times) to remove fines, and finally equilibrated with the solvent to be used (0·025N HCl in aqueous solution or in 0·1M NaCl). The columns were mounted vertically, filled with solvent, and then packed with Sephadex by allowing the gel to settle from a slurry poured into an 8-in. filter funnel mounted on top of the column. After the column had been packed, a thin polyethylene ring, which had four internal lugs and which fitted tightly inside the column, was pushed into the column so that the top of the polyethylene insert was just level with the top of the Sephadex bed. A circle of filter paper 1 mm thick was placed on top of the insert, which served to prevent the filter paper from sinking into the Sephadex. The column space above the paper disk (*c.* 200 ml) was then filled with solvent. The columns were operated at room temperature under a pressure head of approximately 200 cm of water and, after packing, were equilibrated with solvent until one hold-up volume had been passed.

Histone samples were dissolved at a concentration of between 5 and 10% in solvent containing 5% sucrose, and were layered on the filter paper above the Sephadex bed and below the supernatant solvent by slowly injecting the histone solution through a narrow-bore polyethylene tube which terminated 1–2 mm above the filter paper disk.

The filter paper prevented the histone solution from pitting the surface of the Sephadex during sample application, while the presence of 5% sucrose in the sample increased the density of the solution so that a sharp boundary was formed between the histone and the supernatant solvent. In this way, the histone sample was loaded onto the Sephadex as a sharp, narrow band only a few millimetres thick. The initial flow rates of the columns were between 90 and 120 ml/hr. The void volume (23% of the total bed volume of G-200) was discarded before starting fraction collection. A time-operated Locarte machine was used and adjusted to collect 10–15-ml fractions.

### (*c*) *Histone Estimations*

Optical density measurements were made directly at 280 and 230 m$\mu$, the latter, where necessary, on diluted aliquots.

Ninhydrin estimations were performed by heating a 0·5-ml sample, diluted if required with 2·0 ml of ninhydrin reagent (Spackman, Stein, and Moore 1958), for exactly 20 min at 100°C on a water bath and diluting the solutions to 20·0 ml with a 1 : 1 ethanol–water mixture. The resultant colours were read against a water blank on an EEL colorimeter with a green (OGRI) filter.

### (*d*) *Histone Recovery*

The contents of appropriate tubes from each Sephadex separation were bulked, adjusted to pH 4–5 (test paper) with alkali, and the histone absorbed on small columns (30 ml packed volume) of water-washed carboxymethylcellulose (CM-cellulose) in the acid form. The histone was then recovered in a concentrated solution by elution with 20 ml of 0·2N $H_2SO_4$ followed by 50 ml of 0·1N $H_2SO_4$. 80–95% of the histone was found in the first 20–25 ml of eluant after the acid breakthrough. The histone was precipitated from this concentrate either directly by the addition of 6 volumes of acetone, or by dialysis against ethanol; the precipitated histone sulphate was then spun down, washed by centrifugation with acetone and ether, and dried in air. In those cases where the histone concentration was high, i.e. where the optical density at 230 mμ exceeded 4·0, prior concentration on CM-cellulose was not necessary and the histone could be precipitated directly by dialysis against ethanol containing a few drops of 2N $H_2SO_4$ per litre. In some cases, where the histone fractions were rather soluble in ethanol the neutralized solutions were concentrated directly by freeze-drying.

Samples of the histones from the various fractions were analysed for their components by starch-gel electrophoresis and also for their amino acid composition as described previously (Bellair and Mauritzen 1964).

## III. Results

### (*a*) *Fractions Obtained by Exclusion Chromatography*

The results obtained from exclusion chromatography of histones by using various Sephadex gels were highly reproducible for any given histone fraction, but were dependent on the type of Sephadex used and on the solution used for elution. In general, while the elution patterns suggest the presence of several components, no clear-cut separation of any component was obtained.

The elution pattern of crude α-histone from Sephadex G-75 is shown in Figure 1, and while some fractionation has occurred, it was inferior to that obtained with a similar fraction from Sephadex G-100 (Fig. 2). The α-histones used in these two experiments were not identical: that used in the experiment depicted in Figure 1 differed from that in Figure 2 in that much of the ethanol-soluble α-histone had been removed from the former preparation. In the early chromatography runs 0·025N HCl was used as eluant, and it was found that as the histone entered the column, the Sephadex bed contracted to approximately 95% of its initial volume while the flow rate dropped to half or less than half of its starting value at the point at which protein emerged from the columns. If subsequent separations were attempted without repacking the columns it was found that the flow rate fell even further, and in some cases the column flow stopped altogether.

A reduced flow rate on protein breakthrough and a reduction in bed volume were observed in almost every case where Sephadex G-75, G-100, or G-200 was used, whether glass or Perspex columns were used and irrespective of the solvents used

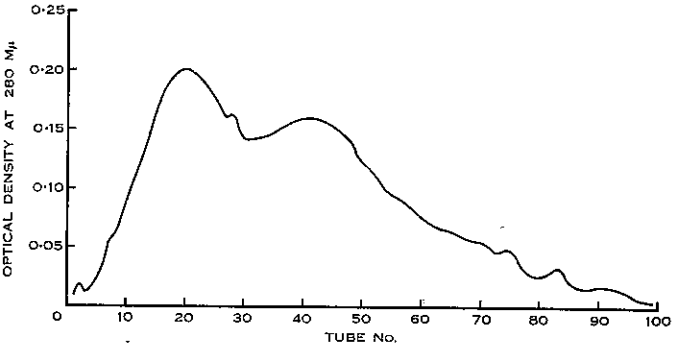


Fig. 1.—Elution pattern obtained on Sephadex G-75 with crude α-histone from which the component soluble in 40% ethanol at pH 10 had been removed. Solvent: 0·025N HCl.

(dilute HCl, 0·1M NaCl, or 4M urea) or the column diameter. When columns were operated under reduced hydrostatic pressure (20 cm water) it was noticed that, while reduction in bed volume still occurred to much the same extent when histone penetrated the Sephadex, the reduction in flow rate on protein breakthrough was not as pronounced as when the columns were operated at higher hydrostatic pressures.

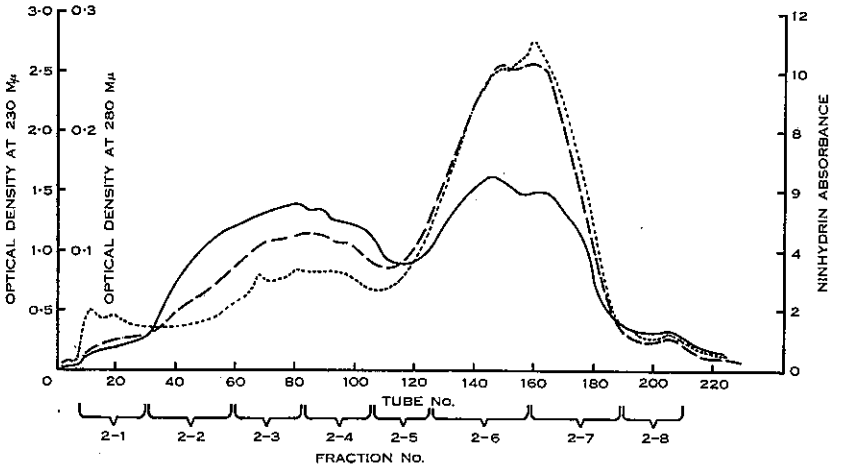


Fig. 2.—Elution pattern obtained with 850 mg crude α-histone on Sephadex G-100. Solvent: 0·025N HCl. —— Ninhydrin absorbance. – – – Optical density at 230 mμ. ···· Optical density at 280 mμ.

The elution patterns obtained for a given histone fraction, Sephadex type, and solvent were identical whether the columns were operated at low or high hydrostatic pressures.

It will be seen from Figure 2, where the elution pattern of histone as measured by absorption at 230 and 280 mμ and by ninhydrin estimation is recorded, that the ratios of the three measurements do not coincide throughout the separation. Thus,

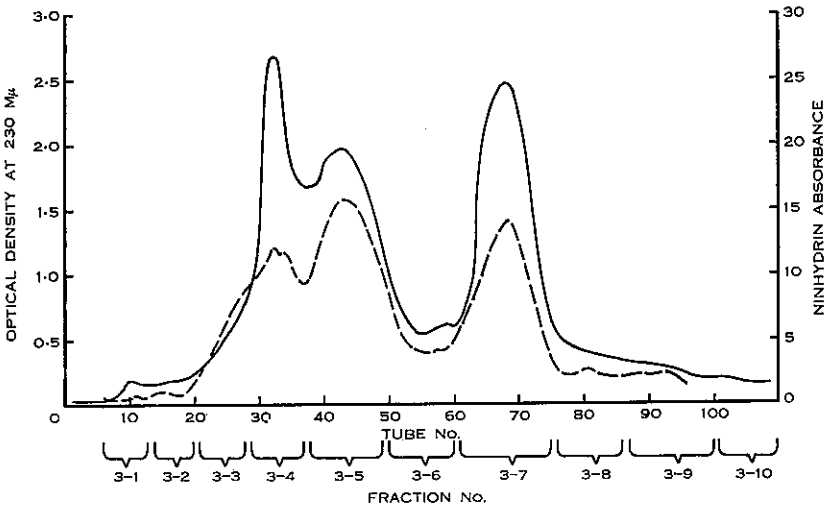


Fig. 3.—Elution pattern obtained with 630 mg crude α-histone on Sephadex G-100. Solvent: 0·025N HCl in 0·1M NaCl. —— Optical density at 230 mμ. – – – Ninhydrin absorbance.

the histone which is almost totally excluded from the Sephadex and is eluted early had an optical density ratio (230 mμ/280 mμ) of almost 5 : 1 while in later fractions this ratio increases to about 10 : 1. In a similar way, the ninhydrin value relative to

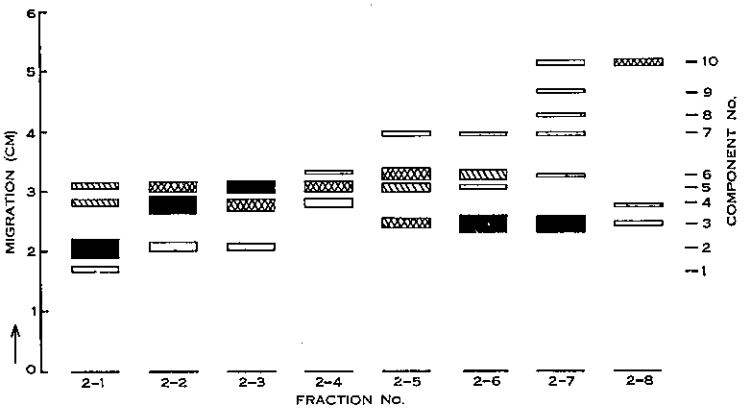


Fig. 4.—Patterns obtained on starch-gel electrophoresis of α-histone fractions 2-1 to 2-8 from run 2 on Sephadex G-100 (see Fig. 2).

the absorption at 230 mμ rises rapidly (fraction 2), falls (fraction 5), and then rises again (fraction 8). It was therefore possible by using all three measurements to interpolate rather more peaks in the elution patterns than if a single type of estimation

were performed. That this interpolation was justified is shown later when the starch-gel electrophoretic patterns of the fractions are considered.

A typical elution pattern obtained by using 0·025N HCl in 0·1M NaCl is shown in Figure 3. A comparison of the pattern in Figure 3 with that in Figure 2 (the same histone fraction run in 0·025N HCl) might suggest that a superior fractionation was obtained with acid in the presence of dilute sodium chloride. However, as mentioned below, starch-gel electrophoretic patterns of the various fractions from the two Sephadex runs did not support this (see Figs. 4 and 5).

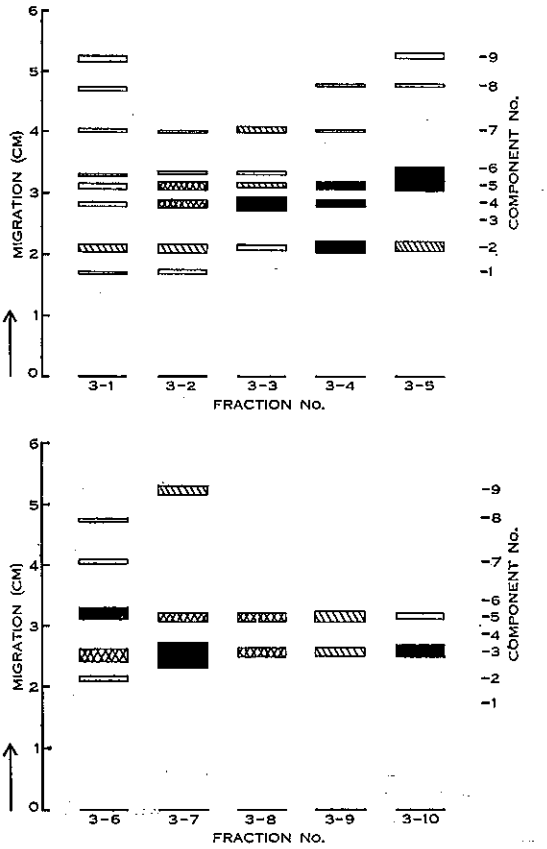


Fig. 5.—Patterns obtained on starch-gel electrophoresis of α-histone fractions 3-1 to 3-10 from run 3 on Sephadex G-100 (see Fig. 3).

As explained earlier (Bellair and Mauritzen 1964) it was not usually possible to determine all the electrophoretic components in a single run, for if the concentration of histone applied to the gel were sufficiently high to detect the presence of trace components (5% total histone), then the bands representing the major components frequently overlapped to such an extent that they could not be distinguished. For this reason, electrophoresis was usually carried out on both 0·5 and 3·0% histone

solutions. The electrophoretic components have been arbitrarily numbered 1–13 in increasing order of mobility.

It will be seen from Figure 4 (Sephadex run 2, Fig. 2) that (with one exception, component 3) the α-histones with the lowest mobilities are partially excluded and therefore eluted from Sephadex before the components of higher electrophoretic mobility. Conversely, it should also be noted from Figure 5 that in the separation with acid in presence of dilute sodium chloride (Sephadex run 3, Fig. 3) the fractions 3-1, 3-2, 3-3, and 3-4 are, unlike the corresponding fractions 2-1 and 2-2 from run 2 (in acid only), contaminated with appreciable amounts of fast electrophoretic components. It will be seen from Figures 4 and 5 that in both separations components 4 and 5 are spread over rather large effluent volumes. This is particularly true of component 5 in the acid–sodium chloride solvent, where it is present, albeit in varying concentrations, in every fraction.

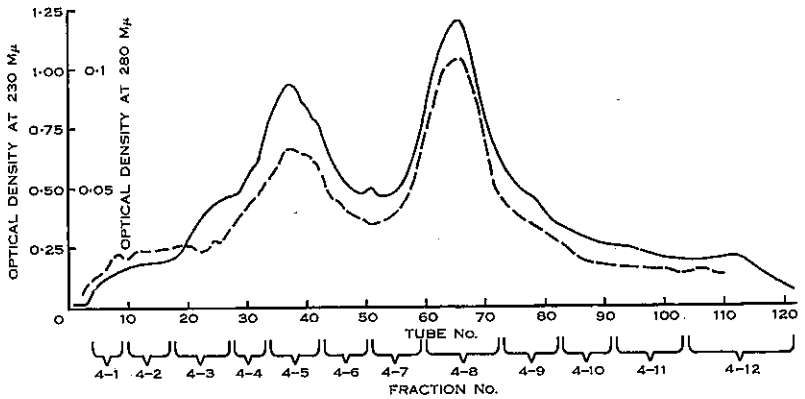


Fig. 6.—Elution pattern of 500 mg crude α-histone on Sephadex G-100. Solvent: 0·025N HCl in 4M urea. ——Optical density at 230 mμ. – – –Optical density at 280 mμ.

Component 3, which is present in fractions 3-6 to 3-10, is the principal component of both 3-7 and 3-10, despite the fact that these two fractions emerged at considerably different effluent volumes. The results of separation in presence of dilute sodium chloride might therefore suggest some form of association of histone components in this solvent, although sedimentation studies on the fractions with the same solvent as was used for chromatography did not reveal the presence of components with sedimentation coefficients greater than 0·8 S, and hence did not support the idea of simple mixed aggregation. Amino acid analyses of fractions 3-7 and 3-10 suggests that the component 3 observed in both consists of two different histones. However, as neither fraction was uncontaminated by other histones this point cannot be resolved at present.

It should be noted that not all 13 components known to be present in the crude α-histone were observed in the fractions obtained by chromatography on Sephadex. Components 11, 12, and 13 are present in the crude α-histone in extremely small amounts (Bellair and Mauritzen 1964) and it is probable that they were present in some fractions but undetected under the conditions used.

The separation of crude α-histone obtained on Sephadex G-100 with 0·025N HCl in 4M urea (included to minimize possible aggregation) is shown in Figure 6, and was similar to that obtained with dilute acid in the absence of urea (Fig. 2). The presence of 4M urea in the solvent precluded ninhydrin estimation in this case. The elution pattern with this solvent was similar to, but not identical with, that obtained with the same histone fraction in 0·025N HCl in the absence of 4M urea.

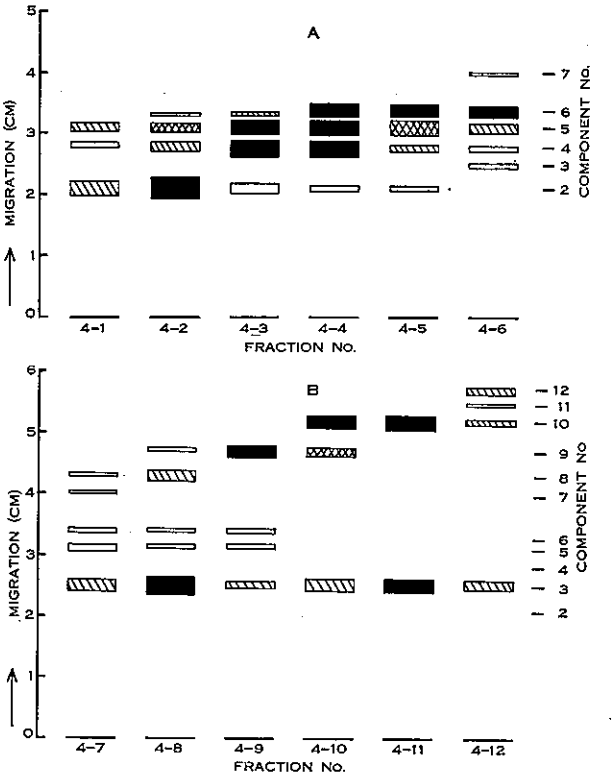


Fig. 7.—Patterns obtained on starch-gel electrophoresis of α-histone fractions 4-1 to 4-12 from run 4 on Sephadex G-100 (see Fig. 6).

The patterns of the 12 fractions from separation 4 on Sephadex are depicted in Figures 7*A* and 7*B*. These patterns are very similar to those obtained from run 2 on Sephadex. In this case, however, component 3 emerges in high concentration in two separated fractions, 4-8 and 4-11, which suggests again that this component might be two distinct histones.

### (*b*) *Amino Acid Composition of Fractions*

The amino acid compositions of the fractions obtained from the separation on Sephadex shown in Figure 2 are recorded in Table 1. The values are calculated for each amino acid as moles per 100 moles of total amino acid exclusive of ammonia.

The table also records total basic amino acids (B), total dicarboxylic acids (A), and the net positive charge. The ratios of certain amino acids are included in the table, for it is suggested that some of these, notably lysine : arginine, might be used to distinguish the various α-components. Finally, the table records for each fraction the major and minor components observed on starch-gel electrophoresis.

TABLE 1

AMINO ACID COMPOSITION OF α-HISTONE FRACTIONS OBTAINED BY EXCLUSION CHROMATOGRAPHY ON SEPHADEX G-100

Values as molar percentage of total amino acids exclusive of ammonia

| Fraction: | 2-1 | 2-2 | 2-3 | 2-4 | 2-5 | 2-6 | 2-7 | 2-8 |
|---|---|---|---|---|---|---|---|---|
| Starch-gel component:* | **2**(4, 5) | **4**(2, 5) | 5(2, 4) | 5(4, 6) | **3**, 6(5, 7) | **3**(5, 6, 7) | **3**(6–10) | **10**(3, 4) |
| Lysine (B) | 22·0 | 27·0 | 24·6 | 24·8 | 21·9 | 17·3 | 18·6 | 26·1 |
| Histidine (B) | 1·1 | 0·5 | 1·0 | 1·4 | 1·7 | 2·1 | 1·9 | 1·4 |
| Arginine (B) | 7·0 | 3·8 | 8·1 | 10·1 | 9·6 | 8·3 | 7·6 | 9·1 |
| Amide $NH_3$ | 6·3 | 2·9 | 3·7 | 3·7 | 4·7 | 6·5 | 6·2 | 5·7 |
| Aspartic acid (A) | 2·8 | 1·9 | 2·0 | 1·8 | 2·9 | 5·1 | 4·9 | 3·0 |
| Glutamic acid (A) | 5·5 | 3·9 | 4·4 | 4·2 | 5·6 | 7·9 | 7·9 | 5·1 |
| Threonine | 4·1 | 3·9 | 3·7 | 3·3 | 3·9 | 4·3 | 5·1 | 3·7 |
| Serine | 9·0 | 6·9 | 9·6 | 11·2 | 10·5 | 6·8 | 8·3 | 9·7 |
| Proline | 7·5 | 8·4 | 7·7 | 6·9 | 6·2 | 5·3 | 5·2 | 6·7 |
| Glycine | 6·4 | 5·7 | 5·6 | 5·3 | 5·9 | 7·6 | 7·1 | 5·5 |
| Alanine | 19·7 | 25·7 | 20·1 | 17·1 | 15·4 | 13·6 | 13·0 | 15·5 |
| Valine | 5·0 | 4·7 | 4·1 | 4·2 | 4·6 | 5·5 | 5·5 | 5·0 |
| Methionine | 0·0 | Trace | 0·2 | 0·4 | 0·5 | 0·6 | 0·6 | Trace |
| Isoleucine | 2·6 | 1·4 | 2·3 | 2·7 | 3·3 | 4·0 | 4·2 | 2·9 |
| Leucine | 5·3 | 4·7 | 4·7 | 4·4 | 5·2 | 7·8 | 6·5 | 4·2 |
| Tyrosine | 1·1 | 0·8 | 1·2 | 1·6 | 2·0 | 2·8 | 2·7 | 1·5 |
| Phenylalanine | 1·0 | 0·6 | 0·6 | 0·6 | 0·8 | 1·0 | 1·1 | 0·6 |
| B (Lys+Arg+His) | 30·1 | 31·3 | 33·7 | 36·3 | 33·3 | 27·6 | 28·1 | 36·6 |
| A (Asp+Glu) | 8·3 | 5·8 | 6·4 | 6·1 | 8·5 | 13·0 | 12·8 | 8·1 |
| B+amide−A | 27·1 | 28·4 | 31·0 | 34·0 | 29·5 | 21·1 | 21·5 | 34·2 |
| Lys : Arg | 3·1 | 7·1 | 3·1 | 2·4 | 2·3 | 2·1 | 2·5 | 2·9 |
| Lys : His | 19·3 | 58·4 | 23·8 | 18·0 | 12·8 | 8·5 | 9·3 | 18·7 |
| Lys : Asp | 8·0 | 14·4 | 12·2 | 13·5 | 7·6 | 3·4 | 3·9 | 8·7 |
| Lys : Ala | 1·1 | 1·0 | 1·2 | 1·5 | 1·4 | 1·3 | 1·5 | 1·7 |
| Leu : Ileu | 2·1 | 3·4 | 2·0 | 1·7 | 1·6 | 1·9 | 1·6 | 1·5 |
| Ser : Pro | 1·2 | 0·8 | 1·3 | 1·6 | 1·7 | 1·3 | 1·6 | 1·5 |

* Principal component in bold-face type, minor components in parenthesis.

It will be seen from these results that all the histone fractions possess compositions typical of α-histones and are characterized by a high net positive charge and by high contents of lysine, alanine, serine, and proline, which together account for 51–66% of the total residues in these histones.

It should be emphasized that while none of these fractions are pure when judged by starch-gel electrophoresis, they do in several cases represent one principal

component. Thus fractions 2-1, 2-2, 2-3, 2-6, and 2-8 are principally components 2, 4, 5, 3, and 10 respectively. The net positive charge fo these fractions is, with the exception of component 3, in fair agreement with their observed mobilities.

The amino acid composition of component 3 would suggest that it corresponds to the 0·8 S $\gamma$-histone described by Cruft *et al.* (1957), which also appears to be synonymous with component F2b of Hnilica, Taylor, and Busch (1963) and RPL2 of Davis and Busch (1960).

Similarly, the composition of components 2 and 4 is reminiscent of $\alpha_2$- and $\alpha_3$-histones (Cruft *et al.* 1957). That component 2 has a lower mobility than component 3, despite its apparently higher net positive charge, may be explained by its higher

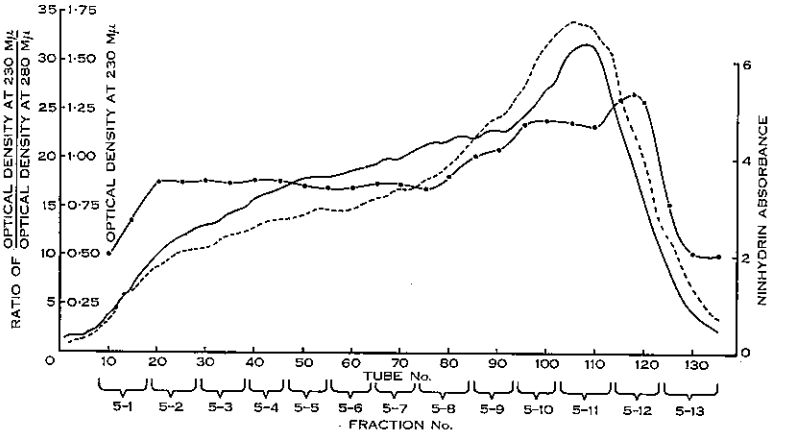


Fig. 8.—Elution pattern obtained with 1200 mg of α-histone fractions soluble in 70% ethanol on Sephadex G-200. Solvent: 0·025N HCl. —— Optical density at 230 mμ. – – – Ninhydrin absorbance. ·—· Ratio of absorbance at 230 mμ to absorbance at 280 mμ.

absorption at 260 mμ than at 280 mμ. This could imply the presence of an attached nucleotide. Component 5 is probably similar to the low molecular weight histone from chicken erythrocytes described by Stedman and Stedman (1951), which contained an appreciable content of arginine.

Fraction 2-8 is complex, containing components 3, 5, and 7–13, and little can be deduced from the analysis of this fraction. It does comprise the α-histones extremely soluble in ethanol (Bellair and Mauritzen 1964) and it is to be expected that the extremely lysine-rich $\alpha_1$-histone will be found in this fraction.

When the various fractions obtained by exclusion chromatography were re-run on Sephadex, considerable purification was achieved. As yet, however, it is not possible to say whether the complete separation of the individual components will be effected by repeated exclusion chromatography.

The elution patterns obtained when the α-histone soluble in 70% ethanol at pH 9·5 (Bellair and Mauritzen 1964) was separated on Sephadex G-200 are shown in Figure 8; the corresponding starch-gel electrophoretic patterns of the fractions are shown in Figure 9.

The α-histone soluble in 70% ethanol at pH 9·5 is particularly rich in components 7–13, and therefore the elution pattern (Fig. 8) obtained with this fraction is what might have been expected from the previous results. Starch-gel patterns of all the fractions have not been included in Figure 9, but the fractions omitted are, in general, intermediate in composition between those shown. Fraction 5-7 is the only fraction to contain component 8, while the principal component of fraction 5-9 is 10. The position regarding component 3 is somewhat clarified by this separation, for it is the principal component of fractions 5-1 to 5-3, and dwindles in concentration in fractions 5-4 to 5-7. It is not present in fraction 5-8, but reappears in fractions 5-10 to 5-12. It is therefore tentatively proposed to name these components 3*A* and 3*B* respectively.

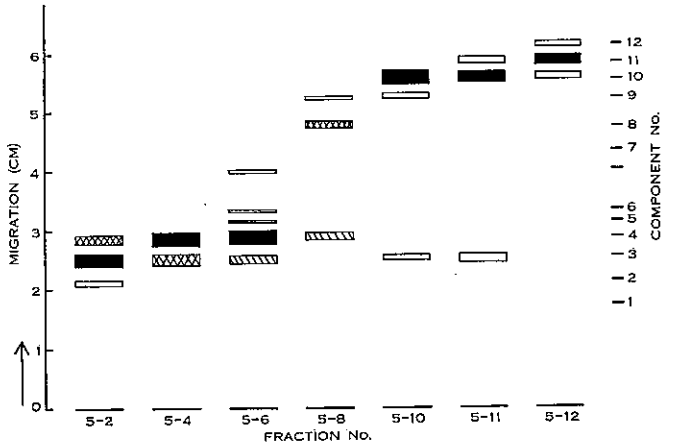


Fig. 9.—Starch-gel patterns obtained on starch-gel electrophoresis of some of the α-histone fractions from run 5 on Sephadex G-200 (see Fig. 8).

The amino acid composition of fractions 5-8 to 5-12 are recorded in Table 2. It will be noted that all these histones have an extremely high net positive charge (30–40% of total residues). Bearing in mind the low molecular weight of these histones (of the order of 10,000) the values for histidine, aspartate, isoleucine, tyrosine, and phenylalanine clearly indicate contamination and indicate that component 11 is probably devoid of these amino acids. The composition of component 11 is therefore very similar to $\alpha_1$-histone isolated from calf thymus (Cruft *et al.* 1957), with one difference in that the $\alpha_1$-histone of calf thymus contains 40% lysine and is devoid of both histidine and arginine, while the present component 11 contains both arginine and lysine. Components 8, 9, 10, and 11 are also characterized by high contents of lysine, alanine, serine, and proline, which comprise from 65 to 73% of the total residues of these fractions.

The fractional elution volumes, i.e. the fraction of the total bed volume at which the various components emerge in highest concentration from the column, have been calculated from a series of separations of α-histone on Sephadex G-100 and G-200 with 0·025N HCl as eluent, and are recorded in Table 3.

## IV. Discussion

The separation of individual histone components has proved to be difficult, for while a large number of different separation procedures have been tried (for references see Phillips 1961), no single method or combination of methods has as yet been found successful. Aggregation or precipitation methods are certainly capable of resolving crude histone into three or more principal fractions, but these in turn are heterogeneous. Ion-exchange chromatography on CM-cellulose or IR C-50 has proved

Table 2

Amino acid composition of α-histone fractions 5-8 to 5-12 obtained by exclusion chromatography on Sephadex G-200

Values as molar percentage of total amino acids

| Fraction: | 5-8 | 5-9 | 5-10 | 5-11 | 5-12 |
|---|---|---|---|---|---|
| Starch-gel components:* | **8** (4, 10) | **9** (3B, 4, 9) | **10** (3, 9) | **10** (3, 12) | **11** (10, 12) |
| Lysine } B | 26·5 | 28·1 | 30·1 | 31·2 | 29·9 |
| Histidine } B | 0·3 | 0·4 | 0·4 | 0·6 | 0·5 |
| Arginine } B | 5·1 | 6·0 | 6·3 | 7·2 | 9·2 |
| Amide $NH_3$ | 3·8 | 3·3 | 2·4 | 4·6 | 2·7 |
| Aspartic } A | 2·2 | 1·8 | 1·5 | 1·2 | 0·8 |
| Glutamic } A | 4·1 | 3·1 | 2·6 | 2·8 | 3·1 |
| Threonine | 4·0 | 3·6 | 3·3 | 3·8 | 4·3 |
| Serine | 8·6 | 8·3 | 9·1 | 9·1 | 9·2 |
| Proline | 8·0 | 8·7 | 9·0 | 8·9 | 8·2 |
| Glycine | 6·4 | 4·7 | 3·6 | 4·0 | 4·8 |
| Alanine | 22·7 | 25·1 | 25·5 | 22·3 | 22·5 |
| Valine | 4·7 | 4·5 | 4·5 | 4·6 | 4·0 |
| Cystine (½) | — | — | — | — | — |
| Methionine | Trace | 0·7 | Trace | — | — |
| Isoleucine | 1·4 | 1·1 | 1·1 | 1·2 | 0·6 |
| Leucine | 5·0 | 3·3 | 2·4 | 2·4 | 2·2 |
| Tyrosine | 0·7 | 0·4 | 0·4 | 0·5 | 0·3 |
| Phenylalanine | 0·5 | 0·4 | 0·2 | 0·3 | 0·3 |
| B (Lys+His+Arg) | 31·9 | 34·5 | 36·8 | 39·0 | 39·6 |
| A (Asp+Glu) | 6·3 | 4·8 | 4·1 | 4·0 | 3·9 |
| B+amide−A | 29·4 | 33·0 | 35·1 | 39·6 | 38·4 |

* Principal component in bold-face type, minor components in parenthesis.

disappointing, though this may be due to the fact that, because of aggregation effects, such separations have been conducted at or below the p$K$ of the resin or cellulose rather than near the isoelectric points of the histones. Many separations have been attempted by the present authors on CM-cellulose under a variety of conditions, including some in the region pH 10–12 in the presence of urea, and the results, when judged by starch-gel electrophoresis of the fractions obtained, have been disappointing.

From the results presented it appears that exclusion chromatography will be a very useful method for separating histone components. It was not to be expected,

in view of the large numbers of histone components present and their close similarity in properties, that a single passage through a Sephadex column would completely resolve any one histone component, and this in fact did not occur. On the contrary, considering that exclusion chromatography on Sephadex is usually held to depend essentially on differences in particle size, and that all the histone components in the fractions used had molecular weights of the order of 10,000, it is perhaps surprising that such a range of elution volumes was observed. While it is true that some histones, particularly components 2 and 4, can undergo aggregation at high pH, no evidence was found of any histone with a sedimentation coefficient greater than 0·8 S at pH values below 8·0 (Bellair and Mauritzen 1964). The explanation of why histones of low molecular weight are prevented from entering the gel matrix of G-100 and G-200 therefore presumably depends on some charge effect or, possibly, charge distribution of the histone micelle. In this connection, it should be pointed out that if distilled water is used as solvent, extremely poor histone recovery results, presumably owing to binding of histone by the slight negative charge which Sephadex possesses.

TABLE 3

FRACTIONAL ELUTION VOLUME* OF VARIOUS STARCH-GEL COMPONENTS ON EXCLUSION CHROMATOGRAPHY

| Starch-gel component: | 2 | 3A | 3B | 4 | 5 | 6 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| Fractional elution volume: | 0·26† | 0·49† | 0·77‡ | 0·32† | 0·36† | 0·42† | 0·69‡ | 0·73‡ | 0·77‡ | 0·82‡ |

* Elution volume as fraction of total bed volume. † On Sephadex G-100. ‡ On Sephadex G-200.

While the above investigation has not resulted in the clear-cut separation of any one α-histone, several broad conclusions can be drawn.

The results of gel electrophoresis by many groups of workers, notably Neelin and Neelin (1960), Cruft (1961), Johns *et al.* (1961), Neelin and Butler (1961), Busch *et al.* (1962), Rasmussen, Murray, and Luck (1962), and Irvin *et al.* (1963), have suggested that a range of 12–20 histone components is found in the nuclei of any given tissue. This is many more than has ever been suggested by amino acid analysis of isolated components or by other separation procedures, and the possibility that starch-gel electrophoresis was merely separating a series of mixed aggregates could not be excluded. The present study was confined to the low molecular weight histones, the crude α- +0·8 S γ-histones of Cruft, Mauritzen, and Stedman (1958*b*), and this fraction was found to resolve into 13 components on starch-gel electrophoresis. While the studies mentioned above have repeatedly demonstrated the polydispersity of histones on starch-gel electrophoresis, it was not possible hitherto to give any indication of composition of the various components. (The amino acid analyses, recorded in Tables 1 and 2, indicate that lysine is the predominant basic amino acid in all these components, and that they are further characterized by relatively high contents of alanine, serine, and proline.)

While the amino acid composition of the fractions are broadly similar, differences with respect to many amino acids are shown by these fractions; these differences

are more striking if the ratios of certain amino acids to each other (viz. lysine : arginine, lysine : histidine, or leucine : isoleucine) are considered, and leaves little doubt that the crude α-histone consists of a family of closely related proteins.

Cruft (1961) has claimed that the mobilities on moving boundary electrophoresis are reversed on starch-gel electrophoresis. When gel electrophoresis is performed at pH 4·9 and an ionic strength of 0·08 in the presence of 4M urea, this reversal of mobilities was not observed except in one case, where component 3 was found to have a greater mobility than component 2 although the latter has the higher apparent net positive charge.

Of the solvent systems studied, it would appear that 0·025N HCl alone is the most satisfactory, as the inclusion of sodium chloride in the elution medium resulted in a poorer separation of components. The presence of 4M urea in the solvent did not improve the separation of α-histones, although its presence has proved to be of benefit in exclusion chromatography of β- and 1·6 S γ-histones (Bellair and Mauritzen, unpublished data).

The results also suggest that while separations of crude α-histone can be achieved on Sephadex gels, it is probably of advantage to use fractions which have been enriched by selective ethanol or pH precipitations.

Finally, the large number of members of the histone family has a bearing on the contentious question of cell specificity of histones. It is obvious that the existence of 10 or more closely similar α-histones in the nuclei from a given tissue will add to the problem of distinguishing, by physicochemical means, the individual histones derived from different cell types.

Since this work was prepared for publication a short note by Hnilica (1964) has appeared. This author has isolated some "lysine-rich" histones from chicken erythrocyte nuclei by a combination of selective histone extraction, chromatography on CM-cellulose, and exclusion chromatography on Sephadex G-75. Like ours, his fractions are still impure when judged by starch-gel electrophoresis, but his amino acid analyses leave no doubt that the fractions which he designates as F1, F2B, and F2C are identical with components 4, 3, and 5 respectively of the present study.

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