THE FRACTIONATION OF α-HISTONES FROM CHICKEN ERYTHROCYTE NUCLEI

I. THE EFFECT OF ETHANOL CONCENTRATION AND pH ON THE FRACTIONAL PRECIPITATION OF α-HISTONES

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Summary

The isolation of a group of low molecular weight, lysine-rich α-histones from chicken erythrocyte nuclei is described. Starch-gel electrophoresis indicates that this crude α-histone complex contains 13 components. Precipitation of the complex with ethanol at pH 8·0 and 9·5 has resulted in a partial fractionation of these proteins although no clean-cut separation was obtained. All these components are of low molecular weight (sedimentation coefficients = 0·8 S) but at pH values above 8 some of these components, notably components 2, 4, and 5, aggregate. The fractionation of these histones by precipitation at pH 11·0 is described.

I. INTRODUCTION

Histones, the basic proteins of cell nuclei, have come into prominence in recent years largely as a result of the suggestion by Stedman and Stedman (1950) that histones are cell specific and that their function might be to control cell differentiation by acting as gene inhibitors.

Although in the last 4 years considerable support for this concept of histone activity has come from the work of Davis and Busch (1959, 1960), Huang and Bonner (1962), and Bonner, Huang, and Gilden (1963), the problem of the unequivocal demonstration of cell specificity has been complicated by the fact that histones, as a group of proteins, are much more heterogeneous than was hitherto supposed. The histones from the nuclei of most tissues can be demonstrated by sedimentation and electrophoresis (Cruft, Mauritzen, and Stedman 1957) or by chromatography (Johns *et al.* 1960) to contain at least six components, while more recently electrophoresis on starch or acrylamide gels has indicated the presence of from 12 to 20 histone components (Johns *et al.* 1960; Neelin and Neelin 1960; Neelin and Butler 1961; Cruft 1962; Irvin *et al.* 1963). It is by no means certain, however, whether all the bands observed in gel electrophoresis represent individual proteins or whether some are mixed aggregates of histones.

The present study, which is part of a wider investigation of cell specificity of histones, deals with attempts to fractionate one group of histones, i.e. the low molecular weight α- and γ-histones, by selective precipitation with ethanol and pH changes. All the histones used in the present work were derived from chicken erythrocyte nuclei.

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II. MATERIALS AND METHODS

(a) Isolation of Nuclei

Domestic fowls were killed by exsanguination and the blood collected in polyethylene trays containing sodium oxalate (1 g/100 ml). The blood was immediately filtered through gauze to remove small fibrin clots and feathers, and chilled to 0°C prior to removal to the laboratory.

Erythrocyte nuclei were isolated by a modification of the technique of Stedman and Stedman (1951) as follows. The blood was centrifuged for 15 min at 1200 g in a PR2 International centrifuge at 0°C. The plasma was discarded and the packed erythrocytes were washed twice by suspension and centrifugation in ice-cold physiological saline (2 volumes for each original volume of packed red cells). The erythrocytes were then lysed by adding a concentrated solution of white saponin to a final concentration of 0·3% (w/v) and stirring for 15 min. The suspension was then diluted by the addition of a further 4 volumes of physiological saline containing $CaCl_2$ (0·005M). The addition of Ca^{2+} prevented the nuclei from gelling on sedimentation.

The nuclei were then sedimented by centrifuging for 25 min at 1200 g and 0°C, and were then washed by suspension in physiological saline (equal to the original blood volume) and centrifuged for 7 min at 1200 g. Usually six to eight washings sufficed to give nuclei which were microscopically free from contamination with cell debris or whole cells. All manipulations were performed in the cold room at 2°C. The nuclei were then dried and defatted by washing successively with 50 and 70% ethanol and finally with acetone and ether, filtered at the pump, and dried in air. Drying in this way has the advantage that the non-basic proteins of the nucleus are rendered insoluble and therefore do not contaminate the histones on extraction.

(b) Extraction of Histone

Crude histone was obtained by blending the dried nuclei with 10 times its weight of ice-cold 0·1N H_2SO_4. The suspension was centrifuged and the supernatant extract filtered through Whatman No. 1 paper. The extraction procedure was repeated until no further histone was obtained from the nuclei, as judged by the appearance of a precipitate or opalescence on the addition of 6 volumes of acetone. Histone sulphate was precipitated from the combined filtered extracts, either by the addition of 6 volumes of acetone or by dialysis against ethanol. The precipitated histone sulphate was then collected by centrifugation, washed successively with acetone and ether, and dried in air.

(c) Fractionation of Histone

Most unfractionated histones have been shown to contain one or two species of histones capable of undergoing reversible aggregation (Cruft, Mauritzen, and Stedman 1957, 1958a, 1958b). These are the β- and 1·6 S γ-histones. Aggregation of the former is promoted by increasing ionic strengths and pH, while the latter only aggregates with increasing pH. Both of these histones, which together represent c. 50% of the unfractionated histone (Cruft et al. 1957), can be precipitated by low

ethanol (10–15%) concentrations at pH values around neutrality. The histones remaining soluble under these conditions are all of low molecular weight (sedimentation coefficients = 0·8 S) and have been demonstrated by moving boundary electrophoresis (Cruft et al. 1957) to contain at least four components designated α_1, α_2, α_3, and 0·8 S γ-histones. The present communication deals with the further fractionation of this crude α- and γ-histone.

(d) Preparation of Crude α-Histone

The crude histone sulphate was dissolved in water and titrated with NaOH to pH 6·5 (glass electrode). Concentrated sodium chloride–barbitone buffer (pH 6·5) was then added, and the solution was diluted to a final histone concentration of 2% (w/v) and ionic strength 0·2. One volume of ethanol was then added to each 5 volumes of histone solution. The solution was allowed to stand overnight at room temperature to facilitate aggregation, and then sedimented in a Servall SS1 centrifuge at 19,000 g for 30 min at 2°C. The water-clear supernatant was decanted and dialysed against 0·1N H_2SO_4 at 0°C to remove salt. The histone solution was then dialysed against ethanol, and histone sulphate precipitate collected and dried as described above. The sedimented pellet of β- and 1·6 S γ-histones was dissolved in water and was fractionated, initially, as described by Cruft, Mauritzen, and Stedman (1958a, 1958b) and then by exclusion chromatography (Bellair and Mauritzen 1964).

The crude α-histone sulphate was further purified by dissolving it in water, adjusting to pH 6·5 and a histone concentration of 4% (w/v), and adding ethanol to a final concentration of 20%. The small amount of precipitate which formed was removed by centrifugation and discarded, the α-histone in the supernatant being then precipitated and dried as described above.

(e) Fractionation of α-Histone with Ethanol

The fractional precipitation of α-histone with ethanol was studied at pH 8·0 and 9·5 as follows. Crude α-histone sulphate was dissolved in water, and adjusted to either pH 8·0 or 9·5 (glass electrode) and to 10% protein concentration.

Portions (0·50 ml) of chilled histone solution were added to a series of heavy-walled glass centrifuge tubes containing 4·50 ml of an ice-cold mixture of Tris buffer (pH 8·0 or 9·5) in ethanol. The content of Tris buffer was kept constant (0·2M) but the ethanol concentration was varied to give from 10 to 80% in the final mixture. The mixture was stirred vigorously during addition of the histone and allowed to stand for 1 hr. The precipitated histone was then sedimented by spinning for 30 min at 9000 g in a Servall SS1 centrifuge. The clear supernatant histone solution was poured off and its concentration determined by measuring its optical density at 230 mμ against an appropriate Tris–ethanol blank.

Measurements of histone concentration were made at 230 mμ rather than at the more usual wavelength of 280 mμ, for it is known (Cruft et al. 1957) that α-histones have very low contents of aromatic amino acids and that some are devoid of tyrosine and phenylalanine.

(f) Amino Acid Analyses

The amino acid composition of the various histone fractions was determined by the method of Spackman, Stein, and Moore (1958) with a Spinco model 120B amino acid analyser. Histone samples, usually 10 mg, were dissolved in 2 ml 5·5N HCl, frozen, sealed *in vacuo* in glass tubes, and heated for 22 hr at $110 \pm 0·5°C$.

The hydrolysates were dried over solid NaOH and dissolved in an appropriate volume of sample-diluting buffer. Most α-histones contain so large a percentage of lysine and alanine that it is difficult to obtain accurate results for all the amino acids in a single run. For this reason, duplicate analyses were regularly performed in both long and short columns on 1-ml and 4-ml samples of the diluted hydrolysates.

(g) Sedimentation

Sedimentation studies were made with a Spinco model E ultracentrifuge fitted with R.T.I.C.; all runs were performed at 20°C. As α-histones have molecular weights of the order of 10,000, a synthetic-boundary cell was used in all cases. In the sedimentation runs barbitone–acetate buffers of ionic strength 0·18 and protein concentrations of 1·2–1·6% were used.

(h) Starch-gel Electrophoresis

The apparatus used was of the vertical type (Smithies 1959) with cooling on one side. The apparatus consisted of a hollow brass cooling plate, fitted with baffles to ensure circulation of the water. This was insulated with polyester sheet 0·003 in. thick (Mellinex, I.C.I. Ltd.), to which strips of Perspex sheet were cemented to form the sides and ends of the tray. The dimensions of the tray were 0·32 cm deep by 15 cm wide and 23 cm long. Gels were prepared essentially by the method of Smithies (1955), Connaught brand starch being used at a slightly higher concentration than is customary for serum proteins, namely, 14·8 g starch/100 ml buffer. It was found desirable to increase the starch concentration to obtain maximum resolution of many of the low molecular weight histones. The gels were poured with the tray in the horizontal position. Eight sample slots, each with a capacity of approximately 5 μl, situated 4·75 cm from the uppermost end of the gel, were moulded as the gel set. After 10 min, the gel tray was transferred to a cold room at 2°C and left for 1–2 hr before use.

The histone samples (5 μl), which varied in concentration from 0·5 to 3·0% of histone in water depending on the degree of heterogeneity, were placed in the sample slots, which were then sealed with molten Vaseline and the gel covered with a sheet of $\frac{1}{16}$ in. Perspex.

A wide variety of conditions were tried, and those finally chosen were similar to those of Neelin and Neelin (1960), namely, sodium acetate–acetic acid buffer of pH 4·9 and ionic strength 0·08 in the presence of 4M urea. It was found that if low ionic strengths (0·01–0·02) were used, the mobility of the histone components was not reproducible and was, as expected, dependent on the histone concentration. 4M urea was included in the buffer to minimize the formation of aggregates.

A bridge solution of 0·15M sodium chloride was used. Electrophoresis was carried out for 4 hr, with a voltage-stabilized power supply giving 220 V across the gel. Cooling water at 0°C was pumped through the brass plate at 2 litres/min. On completion of electrophoresis, the gel was sliced and the lower half stained by immersion for 30 min in an 0·1% solution of amido black 10B in water. The excess dye was then removed by washing overnight in running tap water. It was not found necessary to use an ethanol–acetic acid mixture for staining or washing the gel, as the histone was firmly bound to the starch. The gel patterns were photographed by transmitted light on Adox 14 35-mm film.
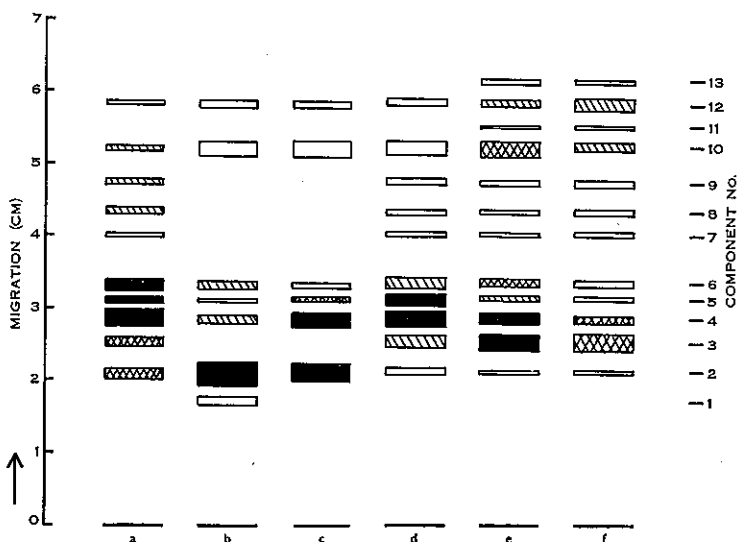

Fig. 1.—Diagrammatic representation of separation of various α-histone fractions by starch-gel electrophoresis: *a*, 17% soluble; *b*, 20% precipitate; *c*, 32% precipitate; *d*, 70% precipitate; *e*, 70% (−20°C) precipitate; *f*, 70% soluble. For description of fractions and experimental conditions, see text.

III. RESULTS

The pattern obtained on starch-gel electrophoresis of the crude α-fraction is depicted in Figure 1*a*. From Figure 1 it can be seen that 13 bands are present. As the mobilities of these components were readily reproducible under the conditions used, they have been arbitrarily numbered 1–13. Thus it should be noted that, although components 1, 11, and 13 are not shown as being present in the crude α-histone, they could be seen as faint bands if a high concentration of histone were used. Not all the components were visible in a single run, for, if the histone concentration was sufficiently high (3%) to detect the presence of components 7–13, then the bands of components 2, 3, 4, 5, and 6 overlapped to such an extent that individual bands could not be detected. For this reason, starch-gel electrophoresis was normally performed at both 0·5 and 3% histone concentrations.

The bands corresponding to components 1–6 stained blue, but on standing in water for a few days, one of these bands (component 3) gradually changed colour from blue to purple. Components 7–13, on the other hand, are observed as blue-green bands. A typical gel pattern is illustrated in Plate 1.
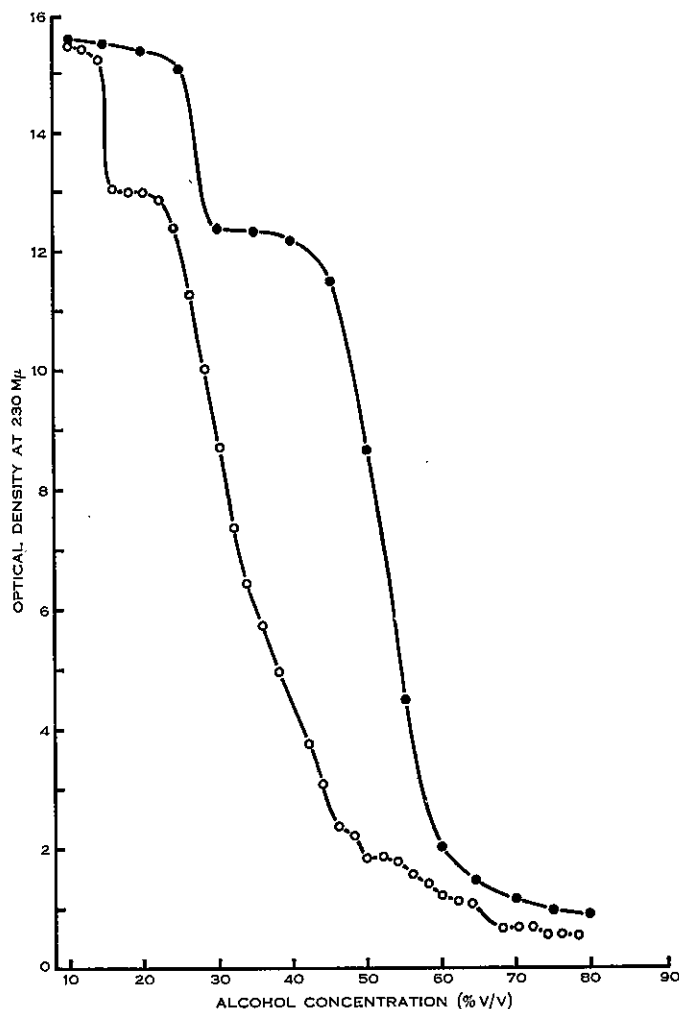

Fig. 2.—Precipitation of α-histone with ethanol at pH 8·0 (●) and pH 9·5 (○).

The precipitation of crude α-histone at pH 8·0 and 9·5 with varying ethanol concentrations is shown in Figure 2, where the histone concentration in the supernatant solution, as measured by absorption at 230 mμ, is plotted against ethanol concentration. At pH 9·5 a sharp precipitation of histone occurred between concentrations of 14 and 16% ethanol; whilst between 24 and 54% there was a gradual and continuous precipitation of histone with no obvious plateaux. Above 55%, there was little further precipitation of histone with increasing ethanol concentration.

The precipitation curve at pH 8·0 was similar to that obtained at pH 9·5, except that a higher ethanol concentration was required to produce the same degree of protein precipitation.

In order to follow the ethanol fractionation in terms of electrophoretic components, it was decided to isolate the fractions which precipitated with 20, 32, and 70% ethanol at pH 9·5.

Crude α-histone sulphate (14 g), prepared as described above, was dissolved in water (concn. c. 2%) and adjusted with NaOH to pH 9·5. The solution was then diluted with water and mixed with concentrated Tris buffer (pH 9·5) and finally with ethanol, so that the final concentrations were: histone 1%, ethanol 20%, Tris buffer, 0·1M. After standing overnight at 2°C the precipitate which formed was
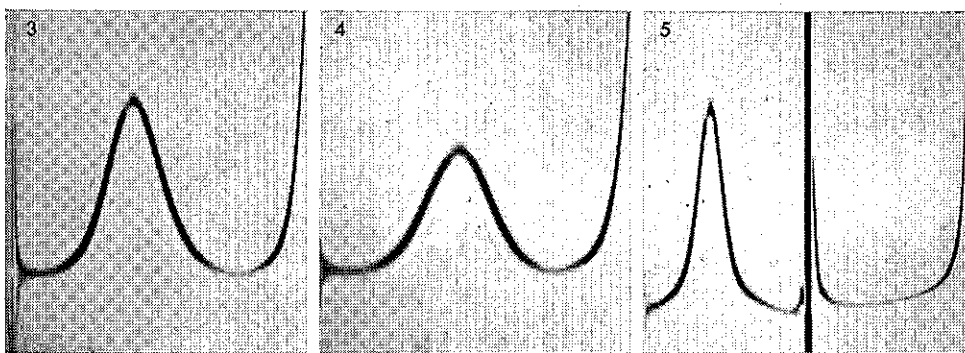
Figs. 3–5.—Sedimentation patterns of α-histone fractions 163, 128, and 12 min, respectively, after reaching 59,780 r.p.m. Sedimentation from left to right in each case, diaphragm angles being 50, 50, and 60°, respectively. 3, 32% precipitate at pH 8·0, 0·2M Tris buffer, histone concentration 1·3%; 4, 70% (−20°C) soluble fraction at pH 8·0, 0·2M Tris buffer, histone concentration 1·2%; 5, 70% precipitate at pH 11·0, 0·2M NaOH–glycine buffer, histone concentration 1·2%.

removed by centrifuging and designated "20% precipitate". The supernatant was then dialysed for 2 days at 2°C against three 6-litre changes of 32% ethanol in 0·1M Tris buffer to yield a "32% precipitate". A "70% precipitate" was obtained in the same way by dialysis of the "32% supernatant" against a 70% ethanol–Tris buffer mixture. It was noticed that the supernatant from the "70% precipitate", while initially clear, gave rise to a further small precipitate on standing at −5°C. The solution was therefore chilled to −20°C and the precipitate, which formed after 4 hr, was removed by centrifugation at this temperature. The 20%, 32%, 70%, and 70% (−20°C) precipitates (yields 2·8, 3·1, 3·7, and 0·5 g, respectively) and protein from the final supernatant (yield 1·4 g) were dissolved in water, dialysed against 0·05N H₂SO₄ to remove Tris buffer, and recovered as histone sulphates as described above.

These fractions were submitted to starch-gel electrophoresis and the results are shown in Figure 1, b–f. It can be seen that the 20% precipitate represents a considerable enrichment of component 2, while the 32% precipitate is largely composed of components 2 and 4. Component 1 was only observed in the 20% precipitate, while the components of higher electrophoretic mobility were present, in trace

amounts only, in both 20% and 32% precipitates. The 70% precipitate contained components 4 and 5 in major amounts with smaller amounts of components 3 and 6, while histones of high mobility were present, again, in trace amounts.

It should be noted that the existence of component 1 is somewhat doubtful, for while this component was only clearly observed in the 20% precipitate fraction it did not resolve well from component 2.

The two fractions obtained at the highest ethanol concentration, the 70% (—20°C) precipitate and 70% soluble, were characterized by increased amounts of the components of higher mobility, particularly components 10 and 12, the former of these two fractions also being particularly rich in component 3. It will also be seen from these patterns that component 4 was present in them all and that ethanol fractionation does not appear to be a useful means of separating it from other α-histones.

When subjected to ultracentrifugation at pH 8·0 in barbitone–acetate buffer of ionic strength 0·2, no component with a sedimentation coefficient greater than 0·8 S was observed. Typical patterns are shown in Figures 3 and 4.

When an ethanol precipitation curve similar to those made at pH 8·0 and 9·5 was attempted at pH 10·5, it was found that on titrating a 10% histone solution to this pH, the solution set to a gel before the titration could be completed.

As there had been previously no indication of any components of the α-fraction undergoing aggregation reactions, the sedimentation of the crude α-fraction and the various ethanol fractions was studied at pH values between 8·5 and 11·0. In every case, a component of high sedimentation value was observed at pH values of 10·5 or greater.

This aggregated component was present in highest concentrations in the 20% and 32% precipitates, where it gave rise to an intense Tyndall effect and was found to sediment between 10,000 and 20,000 g. There was much less aggregated material in the other fractions (70% and 70% (—20°C) precipitates and 70% soluble), the amount being very low in the latter case. In these fractions the sedimentation coefficient of the aggregated material was much lower, and it sedimented as a single, sharp boundary with sedimentation coefficients which decreased from 45 S, in the case of the 70% precipitate, to 23 S in the 70% soluble fraction. The pattern obtained with the 70% precipitate fraction is shown in Figure 5.

All the fractions obtained above by ethanol precipitation were therefore subjected to a further fractionation step by aggregation at pH 11·0.

Solutions (1%) of the histone fraction in 0·1M glycine–sodium hydroxide buffer at pH 11·0 were allowed to aggregate overnight at room temperature. The aggregated components were then removed by sedimentation in a Spinco model L ultracentrifuge for 8 hr at 78,000 g (30,000 r.p.m. in rotor 30). The histone in the gel pellets and supernatants was then dialysed to remove buffer, and recovered as described above as histone sulphate.

As indicated earlier, the 20% and 32% precipitate fractions consisted largely of material which precipitated at pH 11·0, while at the other extreme, the 70% soluble fraction contained less than 5% of material which would aggregate at pH 11·0.

The starch-gel electrophoresis patterns of the pH 11 aggregate and supernatant from the various fractions are shown in Figures 6A and 6B. From these it can be seen that the pH 11 aggregates consisted largely of components 2 and 4 with traces of 1, 5, and 6. As components 3 and 7–12 were completely missing from the
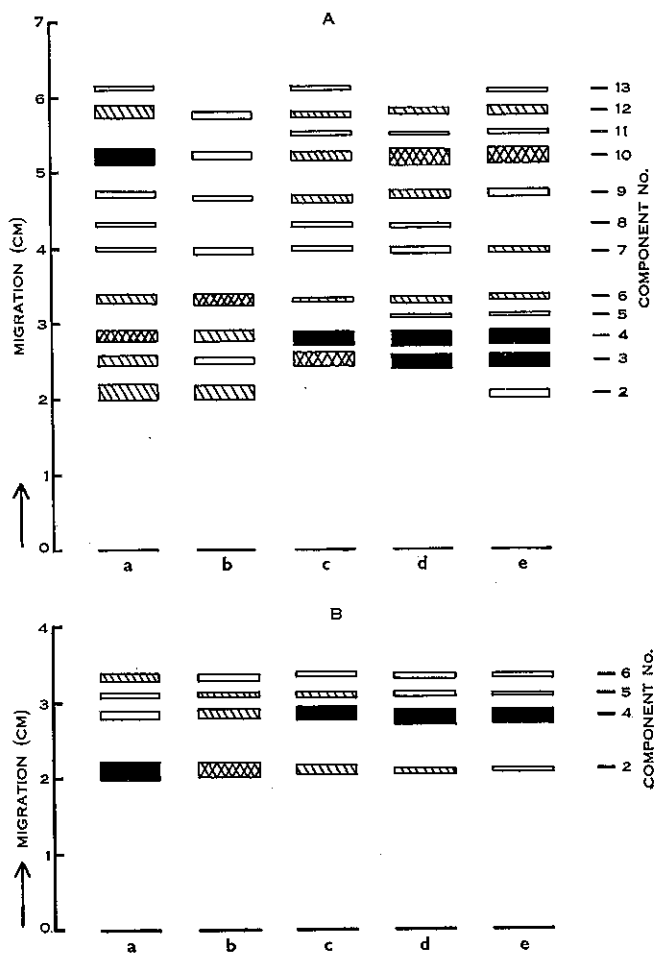

Fig. 6.—Diagrammatic representation of the separation by starch-gel electrophoresis of the various α-histone fraction supernatants (A) and precipitates (B) obtained at pH 11·0 from the following fractions: a, 20% precipitate; b, 32% precipitate; c, 70% precipitate; d, 70% (−20°C) precipitate; e, 70% soluble.

aggregated material, the presence of components 1, 5, and 6 could not be attributed to mere inclusion of supernatant solution in the gel pellet. All of these components, i.e. 1, 2, 4, 5, and 6, were found in the supernatants, which suggested that aggregation did not go to completion. Component 4 particularly was found in quite high concentration in the pH 11 supernatant from the 70% soluble fraction, and this suggested

that, under the conditions used, either it did not possess as high a propensity to aggregate as component 2 or it aggregated more readily in the presence of component 2, which was in low concentration in this fraction.

The amino acid composition of some of these fractions is given in Table 1. Columns 1 and 2 of this table give analyses of fractions which were largely composed of components 2 and 4 respectively, and it will be seen from the composition of both these histones that they are typical α-histones, rich in lysine and alanine, of the α_3 and $0 \cdot 8$ S γ-type described by Cruft *et al.* (1957).

TABLE 1

AMINO ACID COMPOSITION OF STARCH-GEL ELECTROPHORETIC COMPONENTS OF α-HISTONE FRACTIONS

Values as molar percentage of total amino acids

Amino Acid	Fraction			Amino Acid	Fraction		
	20% Precipitate	70% Precipitate (pH 11·0)	70% Supernatant (pH 11·0)		20% Precipitate	70% Precipitate (pH 11·0)	70% Supernatant (pH 11·0)
Component:*	**2**(4, 6)	**4**(2, 5)	**4, 2**(6–13)		**2**(4, 6)	**4**(2, 5)	**4, 2**(6–13)
Lysine	16·5	20·3	30·8	Valine	5·3	4·4	4·3
Histidine	1·8	1·9	0·4	Methionine	0·5	0·4	0·0
Ammonia	4·5	4·3	3·1	Isoleucine	4·3	3·8	1·1
Arginine	8·2	9·5	8·1	Leucine	7·0	5·6	3·3
Aspartic acid	2·9	2·6	1·4	Tyrosine	2·4	1·9	0·6
Threonine	4·4	3·1	3·6	Phenylalanine	1·0	0·8	0·5
Serine	9·1	13·8	9·2	Lysine+histidine			
Glutamic acid	7·2	5·3	3·0	+arginine (B)	22·9	26·6	39·3
Proline	4·8	5·4	8·3	Aspartic acid+			
Glycine	7·1	6·4	5·8	glutamic acid (A)	10·0	7·9	4·4
Alanine	13·2	14·6	19·9	B+amide−A	17·4	21·0	31·8

* Numerals in bold-face type indicate principal component(s), numerals in parenthesis indicate minor components.

IV. DISCUSSION

Previous work of Cruft, Mauritzen, and Stedman (1957, 1958*a*, 1958*b*) has demonstrated the existence of two groups of histones capable of undergoing aggregation. The first, β-histone, aggregates with increasing pH and ionic strength; the aggregation of the second type, $1 \cdot 6$ S γ-histone, is promoted by increasing pH but not by high ionic strengths, which can in fact reverse to some extent the effects of high pH.

The present results indicate that there exists a third group of histones which can undergo aggregation at high pH. The aggregation in this case, however, is more probably a form of isoelectric precipitation for, unlike β- and $1 \cdot 6$ S γ-histones, which commence aggregation at pH values remote from their isoelectric points, the α-histones in the present case aggregate only at or near their isoelectric point.

Nevertheless, this aggregation or precipitation represents a useful step in the separation of histone components of low molecular weight. The analytical results suggest that the histones which aggregate at pH $11 \cdot 0$ are probably of the α_3 and $0 \cdot 8$ S γ-types.
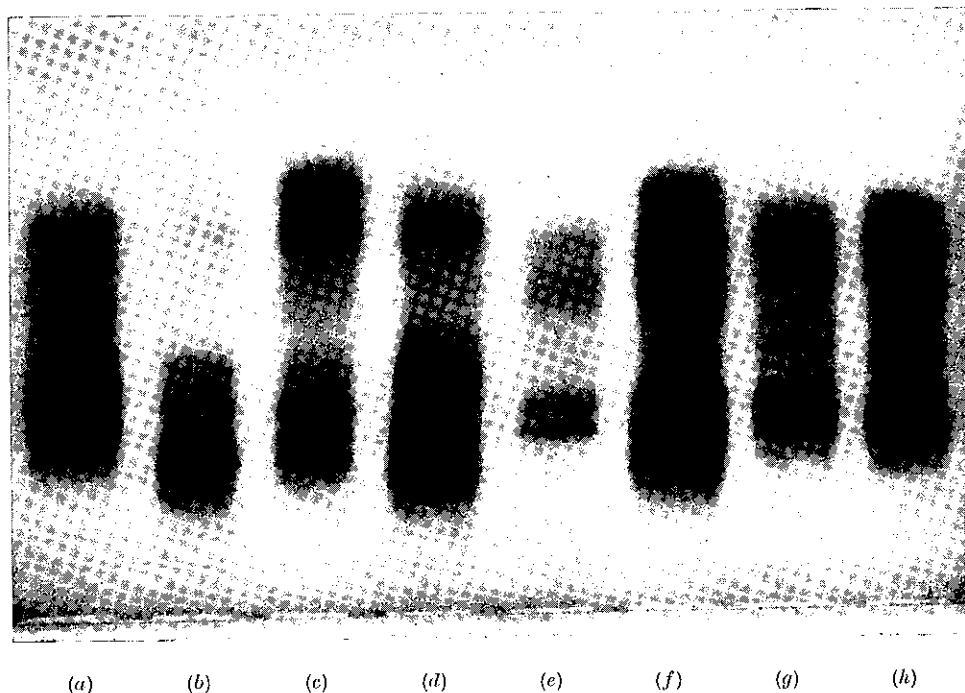
Precipitation of α-histones with ethanol at pH $9 \cdot 5$ gives useful enrichment of components 2 and 4 but is probably not as effective as precipitation at pH $11 \cdot 0$, although it is more convenient to perform.

V. Acknowledgments

VI. References

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α-HISTONES FROM CHICKEN ERYTHROCYTE NUCLEI. I

(a)   (b)   (c)   (d)   (e)   (f)   (g)   (h)

Pattern obtained by starch-gel electrophoresis of various fractions of α-histone: (a) crude α-histone, i.e. histone soluble in 17% ethanol at pH 6·5; (b) histone precipitated with 20% ethanol at pH 9·5; (c) supernatant from (b) at pH 11·0; (d) precipitate with 70% ethanol from 32% ethanol supernatant at pH 9·5; (e) supernatant from (d) at pH 11·0; (f) supernatant with 70% ethanol at pH 9·5; (g) precipitate formed at −20°C from (f); (h) supernatant from (g) at pH 11·0.