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The Stepwise Removal of Histones from Chicken Erythrocyte Nucleoprotein

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1. A fractionation of chicken erythrocyte histones was achieved simultaneously with their extraction from saline-washed nuclei by stepwise titrations to progressively lower pH values. 2. Different acids and dilute buffer solutions of comparable pH behaved similarly in stepwise extractions of histones. 3. The histone preparations so obtained were characterized by their amino acid composition and behaviour on zone electrophoresis in starch gels. 4. The fractionation by titration was quite sharp at appropriate pH ranges, and the histone fraction that is apparently unique to avian erythrocytes was obtained without contamination by other histone fractions. 5. Histones prepared by stepwise titration were fractionated further by cation-exchange and exclusion chromatography. The chromatographic behaviour and amino acid composition of the components permitted comparison with histones prepared by other methods. 6. Histone fraction IIb was resolved into its subfractions IIb_1 and IIb_2 by exclusion chromatography on Bio-Gel P-60. 7. Histone fractions III and IV, previously reported to be absent from chicken erythrocyte nuclei, were found in extracts made at pH 1.

Histone fractions may be removed from deoxyribonucleoprotein by a variety of procedures. In aqueous systems there are essentially two approaches. Different fractions may be dissociated from the native complex in salt solutions of appropriate molarity (Daly & Mirsky, 1955; Hindley, 1964; Bonner & Huang, 1966). Alternatively, the relative ease of removal of different fractions from the native deoxyribonucleoprotein as a function of acid concentration may be used (Bigwood, 1959; Bijvoet, 1957; de Nooij & Westenbrink, 1962; Johns & Butler, 1962; Johnson, Driedger & Marko, 1964; Satake, Rasmussen & Luck, 1960). It has been shown recently that with calf thymus nucleohistone this process is largely a function of the pH of the extraction mixture (Murray, 1966).

Chicken erythrocyte nuclei and calf thymus nuclei have most histone fractions in common, but avian erythrocyte nuclei contain a histone fraction, of distinctive amino acid composition, which appears to be characteristic of this source (Neelin, 1964; Hnilica, 1964). It was therefore decided to apply the stepwise titration procedure (Murray, 1966) to chicken erythrocyte nuclei, both as a potential preparative method and because the system could be useful for studies of some aspects of the structure of native deoxyribonucleoprotein. The present paper describes the selective removal of histone fractions from chicken erythrocyte deoxyribonucleoprotein as a function of pH.

A summary of the results has appeared in an abstract (Murray, Vidali & Neelin, 1966).

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MATERIALS

Preparation of chicken erythrocyte deoxyribonucleoprotein. Pooled blood was collected from the production-line slaughter of chickens within 1 min. of the birds' throats being cut. During collection of the blood (which was generously given by J. Sainsbury Ltd., Bury St Edmunds, Norfolk), 10% (w/v) trisodium citrate solution was added (100 ml./l. of blood) to minimize clotting. Bottles of blood were transported to the laboratory in melting ice. All subsequent operations were carried out in the cold-room (4°) and centrifuges were refrigerated at about 2°. The blood was centrifuged at 1800 rev./min. for 30 min. (MSE refrigerated centrifuge, 4 × 1 l. swing-out rotor). Serum was discarded and the erythrocytes were stirred with sufficient cold 0·14 M-NaCl–0·01 M-sodium citrate to restore the volume to the original value. Erythrocytes were recovered by centrifugation as before, resuspended in cold 0·14 M-NaCl–0·01 M-sodium citrate, strained through eight thicknesses of

butter muslin and centrifuged again. Washing by suspension and centrifugation was repeated once more. The erythrocytes were then lysed by vigorous stirring with an equal volume of white saponin soln. [0·6% (w/v) in 0·14M-NaCl]. The lysate was allowed to stand for about 2hr., with occasional stirring, then diluted with an equal volume of 0·14M-NaCl–0·01M-sodium citrate and centrifuged at 1800rev./min. for about 1hr. In later experiments centrifugation was prolonged to 4–5hr. to improve recovery of nuclei. The nuclear pellet was then washed 10–12 times with 0·14M-NaCl–0·01M-sodium citrate; initially the pellet was dispersed and stirred with a glass rod, but after the first four washes the suspension was blended for about 2min. at half speed in an MSE Ato-Mix homogenizer. The pellet recovered from the final washing by centrifugation corresponded to crude deoxyribonucleoprotein and was used as 'washed nuclei'.

Extraction of the nucleoprotein with acid. All operations other than titration were carried out at 2–4°.

(*a*) Small-scale parallel extractions. About 20ml. of washed nuclei was blended in an MSE Ato-Mix homogenizer with distilled water (250ml.) for about 2min. at full speed. Portions (30ml.) of the viscous creamy-coloured mass were transferred to 50ml. beakers, stirred at room temperature (17°) with magnetic stirrers at very high speed and titrated to the desired pH with 0·2N-H_2SO_4 (or 3·5N-H_2SO_4 when very low pH was required), which was slowly added dropwise at the centre of the vortex. The pH was measured with a direct-reading pH-meter (Electronic Instruments Ltd., Richmond, Surrey) standardized at pH4·0. When the desired pH was attained, the suspension was stirred in the cold-room (4°) for a further 30–60min. and then centrifuged at 11000rev./min. (MSE 17 centrifuge) for 30–45min. Ethanol (2·5vol.) was added to the clear supernatants. Precipitates were allowed to settle overnight, then collected by centrifugation and washed two or three times with ethanol. In some cases they were then dissolved in water (10ml.), acidified with 1 drop of 0·2N-H_2SO_4, reprecipitated with ethanol (25ml.) and collected as before. Ethanol-washed precipitates were washed twice with acetone and dried *in vacuo*.

(*b*) Large-scale serial extractions. Washed nuclei from 4l. of blood were homogenized in the cold-room (4°) with distilled water (about 700ml.) in an Ato-Mix homogenizer at half speed for about 2min. and at full speed for a further 3min. (In later experiments 1mM-NaCl was used instead of water to decrease gel formation.) The viscous solution was transferred to a 2l. beaker, stirred vigorously with a magnetic stirrer and titrated at room temperature with 0·2N-H_2SO_4. When the desired pH was attained, the suspension was stirred for a further 20min. before centrifugation at 2000rev./min. for 20min. The centrifuged pellet was blended in an MSE Ato-Mix homogenizer with distilled water (about 250ml.) for about 2min., titrated as before to the same or lower pH and stirred for a further 30min. The sequence of titration and centrifugation was repeated several times to gradually lower pH values. A sample (5ml.) of each supernatant was retained for measurement of ultraviolet absorption, and ethanol (2·5vol.) was added to the remainder. After overnight precipitation, the histone sulphate was collected by centrifugation, washed three times with ethanol and twice with acetone and then dried *in vacuo* at room temperature.

In some experiments HCl was used instead of H_2SO_4 for titration. In these cases histone was precipitated from the supernatants by addition of acetone (10vol.) and the precipitates were washed only with acetone.

METHODS

Measurements of ultraviolet absorption. These were made with a Unicam SP.500 spectrophotometer.

Amino acid analyses. Histone samples were dissolved in 6N-HCl (10mg./ml.) and heated in evacuated sealed tubes at 105° for 22hr.; in some cases various longer periods of time were used also. The hydrolysates were evaporated to dryness *in vacuo* over NaOH and dissolved in 0·2M-sodium citrate buffer, pH2·2, for analysis on an automatic amino acid analyser (Beckman–Spinco model 120) based on that of Spackman, Stein & Moore (1958); an accelerated flow system was used with a 6cm. column for basic amino acids and a 50cm. column for neutral and acidic amino acids. When N^{ϵ}-methyl-lysine was determined (Murray, 1964) the analysis of basic amino acids was done on 22cm. columns. Amino acid compositions of histones in chromatographic fractions were determined as described by Neelin, Callahan, Lamb & Murray (1964).

Qualitative analyses for esters of serine and threonine were carried out by two-dimensional electrophoresis by the procedure of Murray & Milstein (1967).

Zone electrophoresis in starch gels. The procedures and apparatus used were as described previously (Neelin & Connell, 1959; Murray, 1962). Gels were made in 0·01M-sodium acetate buffer, pH4·1, made 4M with respect to urea.

Chromatography. Histone preparations were applied to a column (55cm.×4·5cm. diam.) of Amberlite CG-50 (type 1) cation-exchange resin (100–200 mesh) (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in 9% (w/v) guanidinium chloride soln. (in 0·1M-sodium phosphate buffer, pH6·8), and chromatograms were developed with buffered guanidinium chloride solutions (Satake *et al.* 1960). Protein was assayed in the column eluate by measurement of the turbidity at 400mμ, after addition of 5vol. of 1·1M-trichloroacetic acid. Within the range 0·03–0·3mg. of histone a reproducible linear relationship between turbidity and histone concentration exists for each fraction in all the media used in these chromatograms (G. Vidali & J. M. Neelin, unpublished work). To some extent the relation between turbidity and histone concentration depends on the type of histone.

Histone fractions were recovered from the guanidinium chloride soln. by desalting on columns of Bio-Gel P-2 (66cm.×6·0cm. diam.), eluted with 10% (w/v) acetic acid, followed by concentration under reduced pressure and freeze-drying.

Exclusion chromatography was carried out on a column of Bio-Gel P-60 (165cm.×2·5cm. diam.) in 0·01N-HCl (Hnilica & Bess, 1965).

RESULTS

Titration of the heavy gelatinous aqueous suspensions of washed chicken erythrocyte nuclei with acid caused a gradual fall in viscosity until, at about pH5–4·5, the gelatinous mass had degenerated to a fluid creamy suspension. Since the ultraviolet absorption (at 230mμ) of the super-

Table 1. *Ultraviolet absorption of supernatant solutions from samples of washed chicken erythrocyte nuclei titrated to various* pH *values with* 0·2N-*sulphuric acid*

Titrated suspensions were clarified by centrifugation (see the Methods section).

pH	E_{230}	E_{277}/E_{230}
7·4	0·618	0·47
5·3	0·500	0·49
4·7	0·420	0·42
4·1	0·380	0·32
3·5	0·341	0·21
3·05	0·412	0·26
2·8	0·414	0·20

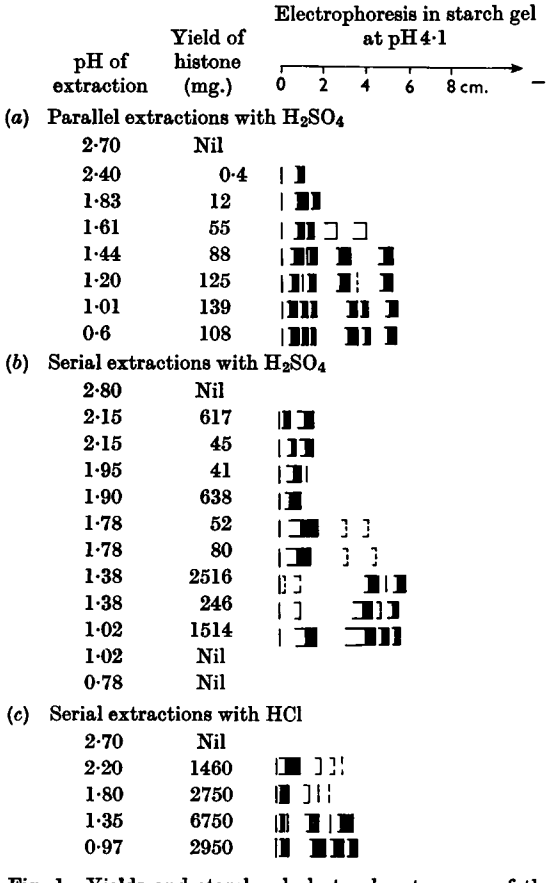

Fig. 1. Yields and starch-gel electrophoretograms of the histones obtained from washed nuclei of chicken erythrocytes by titration with acid to various pH values. (Amino acid analyses of the main H_2SO_4 extracts are given in Table 2.) Experimental details are given in the text. The quantities of material extracted were: (*a*) about 2·5ml. of a pellet of washed nuclei for each extraction; (*b*) the washed nuclei from 4l. of blood; (*c*) the washed nuclei from 8l. of blood.

natant solution decreased steadily as the pH of the suspension was lowered from pH 7·4 to about pH 4·5 (Table 1), the decrease in viscosity was due to precipitation of deoxyribonucleoprotein and not to dissolution of some component of the complex (such as the so-called 'residual protein'; see, e.g., Dounce & Hilgartner, 1964).

Histone was not recovered from supernatants of suspensions that had been titrated to pH 2·5 or above (Fig. 1); this observation is similar to those on calf thymus nuclei, where histone was found in extracts made at pH 2·6 but not above (Murray, 1966).

Parallel extractions of nuclear suspensions on a small scale and examination of the products by zone electrophoresis in starch gels (Fig. 1*a*) and amino acid analysis (Table 2) showed that increasingly complex histone mixtures were obtained as washed nuclei were titrated to lower pH values. Each of these experiments (Fig. 1) was corroborated by a similar set of titrations on nuclei from a different batch of blood. In general only the first extract of each preparation approached electrophoretic homogeneity. According to its amino acid composition (Table 2), histone of high lysine content was extracted about pH 2·4 and above. This fraction was resolved into three components (other than the small quantity of unretarded material) by cation-exchange chromatography (Fig. 2). These did not differ significantly in amino acid composition (Table 3) from each other, or from fractions 1, 2 and 3 of chicken erythrocyte histone reported by Neelin *et al.* (1964). With progressive titration to lower pH, the extracted histones decreased in content of lysine, proline and alanine (Table 2) and increased in content of arginine, glycine, leucine, isoleucine and the aromatic amino acids.

On the basis of the small-scale experiments, preparative experiments were carried out in which the nuclear suspension was extracted serially to gradually lower pH values. Typical pH steps used in these experiments are shown in Fig. 1(*b*), along with the yield of histone and the starch-gel-electrophoretic pattern obtained at each step. These results, like those in the small-scale experiments, were confirmed with similar preparations, and suggested that the extraction alone had effected a sharp fractionation of the histones. This selectivity of extraction was substantiated by the amino acid composition of the four major extracts at pH 2·15, 1·90, 1·38 and 1·02 (Table 2).

Differences in the content of aromatic amino acids between the four extracts were reflected in their ultraviolet spectra (Fig. 3). It is obvious from the great differences in specific extinction coefficient of the various preparations that, though measurement of extinction may provide an adequate means for

Table 2. *Amino acid compositions of histone extracts obtained by titration of chicken erythrocyte nucleoprotein suspensions to various* pH *values* (*Fig.* 1)

Amounts of amino acids are expressed as moles/100 moles of total recovered amino acids; no corrections were applied for hydrolytic losses of any of the amino acids, or for the formation of *O*-sulphates of serine and threonine (Murray & Milstein, 1967).

Extraction	Parallel (with H_2SO_4)				Serial (with H_2SO_4)			
pH of titration ...	2·40	1·90	1·70	0·80	2·15	1·90	1·38	1·02
Amino acid								
Lys	27·7	27·6	17·8	10·4	28·1	23·5	13·7	10·5
N^{ϵ}-Methyl-Lys	—	—	0	0	0	0·7	0	—
His	0	0	2·0	1·6	Trace	2·2	2·4	1·6
Arg	2·7	6·6	9·6	11·5	3·6	10·3	8·4	11·1
Asp	2·4	1·9	3·8	4·7	2·2	1·9	5·8	4·9
Thr	4·0	3·4	4·1	6·8	3·7	2·9	5·2	6·4
Ser	6·7	8·2	9·8	4·6	6·5	13·0	5·5	3·9
Glu	4·5	3·9	6·5	9·5	3·5	4·0	9·1	9·2
Pro	10·0	8·2	5·7	3·6	9·0	7·2	4·3	3·5
Gly	6·0	5·1	6·8	9·4	6·0	5·1	8·6	9·9
Ala	25·7	22·0	14·6	11·4	25·1	16·1	13·1	11·4
CyS	0	0	0	0	0	0	0	0·3
Val	4·6	4·5	5·0	5·8	4·9	4·6	6·2	6·0
Met	0	0·3	0·6	1·2	0·1	0·5	0·9	1·3
Ile	0·8	2·1	3·9	5·3	1·3	3·4	4·9	5·2
Leu	4·3	4·6	7·0	8·6	4·7	4·6	6·8	8·7
Tyr	0·3	1·1	2·3	3·1	0·7	1·7	3·2	3·3
Phe	0·3	0·6	0·9	2·5	0·5	0·6	1·7	2·8
Arg/Lys ratio	0·10	0·24	0·54	1·11	0·13	0·44	0·61	1·06

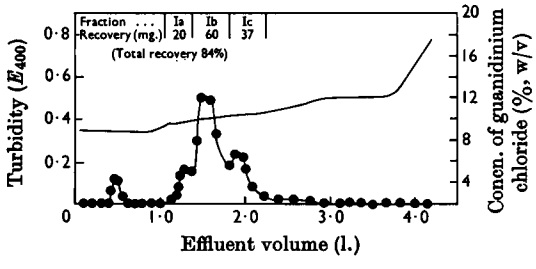

Fig. 2. Chromatographic fractionation of chicken erythrocyte histone extracted with H_2SO_4 at pH 2·4 on Amberlite CG-50 with a gradient of guanidinium chloride buffered at pH 6·8. ——, Concn. of guanidinium chloride in effluent; ●—●, turbidity (at 400 mμ) of effluent sample after addition of 5 vol. of 1·1 M-trichloroacetic acid. Fractions are labelled as for calf thymus histone (Rasmussen *et al.* 1962) with the addition of fraction Ic and, in Fig. 4, fraction V; fraction V is peculiar to avian erythrocytes. Yields (by weight) of fractions taken from the chromatogram are indicated at the top. (Amino acid analyses of these fractions are given in Table 3.)

qualitative monitoring of some fractions eluted from a column, it cannot be used (at any wavelength) for the determination of histone concentration unless one knows the fraction under examination. Turbidity in trichloroacetic acid, as used here, is somewhat less vulnerable to variation with the particular type of histone (G. Vidali & J. M. Neelin, unpublished work), but conversion of turbidity readings into concentration values still requires calibration against the particular histone involved.

The four major histone preparations from the preparative extractions were examined in some detail. The zone-electrophoretic behaviour of the product extracted at pH 2·15, its lysine and alanine contents, the low amounts of arginine, acidic and aromatic amino acids and the near absence of histidine and methionine were characteristic of calf thymus histone fraction Ia or Ib (arginine/lysine ratio 0·1; Rasmussen, Murray & Luck, 1962). Chromatographic fractionation of the material extracted at pH 2·15 on Amberlite CG-50 (Fig. 4*a*) furnished four components, three of which were eluted at low guanidinium chloride concentrations. That these three components are analogous to fractions Ia, Ib and Ic (Fig. 2 and Table 6) is confirmed by the composition (Table 4) of the main component (fraction 1b). These three peaks undoubtedly represented fractions 1, 2 and 3 with identical electrophoretic properties, described in chicken erythrocytes by Neelin *et al.* (1964). The chromatographic subfractionation of this type of histone, sometimes referred to as 'lysine-rich

Table 3. *Amino acid compositions of histones extracted from chicken erythrocyte nucleoprotein at* pH 2·4 *and chromatographed on Amberlite CG-50 (Fig. 2)*

Amounts of amino acids are expressed as moles/100 moles of total recovered amino acids; no corrections were applied for hydrolytic losses of any amino acids, or for the formation of *O*-sulphates of serine and threonine.

Fraction	Ia	Ib	Ic
Amino acid			
Lys	26·6	26·9	26·0
His	Trace	Trace	Trace
Arg	2·2	2·0	2·4
Asp	1·7	1·6	1·8
Thr	3·4	3·5	3·5
Ser	5·7	6·7	5·7
Glu	3·4	3·5	3·8
Pro	9·4	9·1	9·3
Gly	6·5	6·2	6·5
Ala	29·2	29·5	29·2
CyS	0	0	0
Val	5·1	4·9	5·0
Met	0	0	0
Ile	0·8	0·9	0·9
Leu	5·2	5·1	5·2
Tyr	0·2	0·1	0·1
Phe	0·6	0·5	0·5

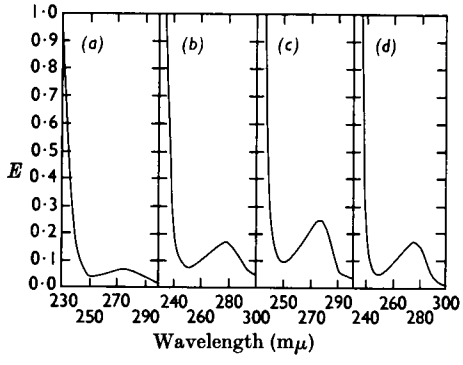

Fig. 3. Ultraviolet spectra of histones obtained from chicken erythrocyte nuclei by serial extraction (Fig. 1) with H_2SO_4 at pH 2·15 (*a*), pH 1·90 (*b*), pH 1·38 (*c*) and pH 1·02 (*d*). Concentrations (mg./ml.) of the respective solutions were 0·55, 0·64, 0·63 and 0·53.

histone', is well known (Satake *et al.* 1960; Kinkade & Cole, 1966). The fourth component resolved from the pH 2·15 extract was in fact the histone reported to be characteristic of avian erythrocytes (Neelin *et al.* 1964). The presence of this fraction, suspected from the zone-electrophoretic pattern of the pH 2·15 extract (Fig. 1*b*), was confirmed by the chromatographic behaviour (Fig. 4*a*) and amino acid composition (Table 4) of the component described here as fraction V (previously termed fraction 5; Table 6).

The zone-electrophoretic behaviour (Fig. 1) and amino acid composition (Table 2) of the histone extracted at pH 1·9 were clearly typical of the component (fraction V) that is peculiar to avian erythrocytes (Neelin, 1964). The identity and homogeneity (with respect to other histone fractions) of the pH 1·9 extract was confirmed by cation-exchange chromatography (Fig. 4*b*), which gave a single component, fraction V, and by amino acid analysis (Table 4), which demonstrated relatively high contents of serine, lysine, arginine and alanine, as previously reported (Neelin, 1964; Hnilica, 1964; Neelin *et al.* 1964).

The electrophoretic properties (Fig. 1) and amino acid composition (Table 2) of the histone extracted at pH 1·38 were very reminiscent of those of histone fraction IIb of calf thymus (Rasmussen *et al.* 1962) or of chromatographic fraction 4 of chicken erythrocyte histone (Table 6). Analysis of the extract by cation-exchange chromatography (Fig. 4*c*) showed that, besides fraction IIb, only a small amount of a more retarded fraction (IV) was evident. Amino acid analysis (Table 4) confirmed the identity of the main fraction as fraction IIb.

The histone preparation obtained by extraction at pH 1·02 represented the remainder of the protein extractable by acid. It exhibited complex electrophoretic behaviour, and in this respect and in its amino acid composition it was similar to, but not identical with, fractions III and IV of calf thymus histone (Rasmussen *et al.* 1962). Chromatography of the pH 1·02 extract on Amberlite CG-50 (Fig. 4*d*) separated a minor component (fraction IIb_1) from the major fraction, which was itself partly resolved into two fractions (III and IV) having similar amino acid compositions (Table 4). These fractions, however, did not precisely correspond in amino acid composition to fractions III and IV of calf thymus histone (Rasmussen *et al.* 1962); in particular, they contained more glycine. The pattern of peptides produced from a tryptic digest of the pH 1·02 extract was also different from that obtained from fractions III or IV of calf thymus histone.

The amino acid compositions and starch-gel electrophoretograms of fractions IIb, III and IV of chicken erythrocyte histone indicated that they were mixtures. This heterogeneity was confirmed by exclusion chromatography on Bio-Gel P-60. Fraction IIb of the pH 1·38 extract was resolved into two major components, IIb_1 and IIb_2 (Fig. 5*a*); these two components were not analysed, but analogous fractions from goose erythrocyte histones (G. Vidali & J. M. Neelin, unpublished work) had the amino acid compositions of fractions IIb_1 and IIb_2 of calf thymus histone (Rasmussen *et al.*

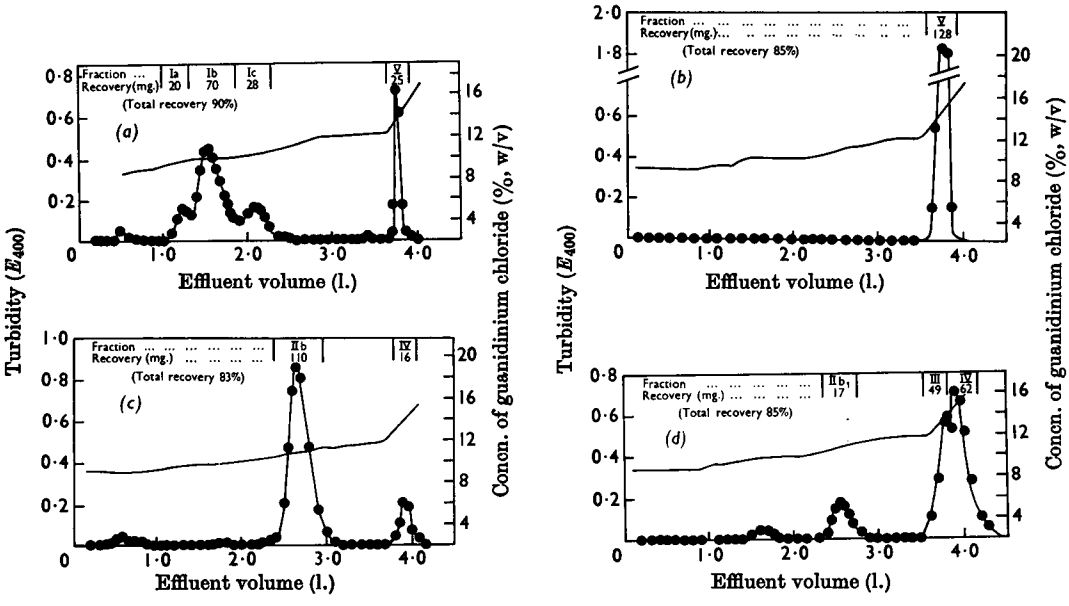

Fig. 4. Cation-exchange chromatography of chicken erythrocyte histones obtained by serial extraction (Fig. 1) at pH 2·15 (*a*), 1·90 (*b*), 1·38 (*c*) and 1·02 (*d*). Chromatography, symbols and the labelling of the fractions are the same as in Fig. 2. (Amino acid analyses are given in Table 4.)

Table 4. *Amino acid compositions of fractions of chicken erythrocyte histone obtained by chromatography on Amberlite CG-50 of extracts from serial extractions at* pH 2·15, 1·90, 1·38 *and* 1·02 (*Fig.* 4)

Amounts of amino acids are expressed as moles/100 moles of total recovered amino acids; no corrections were applied for hydrolytic losses of any amino acids, or for the formation of *O*-sulphates of serine and threonine.

pH of extract chromatographed ...	2·15		1·90	1·38	1·02		
Fraction	Ib	V	V	IIb	IIb_1	III	IV
Amino acid							
Lys	27·1	20·0	22·7	15·8	11·0	10·2	9·4
His	Trace	1·6	1·8	2·4	2·2	1·5	1·6
Arg	1·8	11·8	11·5	7·5	7·2	13·3	13·3
Asp	1·7	2·0	1·8	4·0	6·6	4·4	4·7
Thr	3·4	3·4	3·2	5·4	5·5	6·4	7·2
Ser	6·7	11·8	12·5	6·8	6·4	2·5	3·7
Glu	3·6	4·3	4·1	8·9	10·2	9·6	9·6
Pro	9·2	7·1	6·7	5·8	4·6	3·6	2·6
Gly	6·2	5·4	4·4	9·3	8·7	11·3	10·3
Ala	28·7	17·4	16·3	12·6	11·5	11·5	11·0
CyS	0	0	0	0	0	0	0
Val	5·3	4·7	4·4	5·8	6·6	6·0	6·0
Met	0	0·3	0·5	0·6	1·1	0·3	1·4
Ile	0·9	3·2	5·3	3·9	4·8	5·8	5·4
Leu	4·5	4·5	4·3	6·9	8·5	8·7	7·6
Tyr	0·3	1·6	2·0	2·0	2·7	2·8	3·0
Phe	0·5	1·0	0·8	2·2	2·3	2·2	3·2

1962). These correspond to fractions F2a2 and F2b of other nomenclatures (Table 6).

After treatment with 0·2% (w/v) β-mercaptoethanol, the pH 1·02 extract (which was mainly fractions III and IV) was resolved into two components by exclusion chromatography on Bio-Gel P-60. The first of these components was eluted as a relatively diffuse zone and therefore the eluted

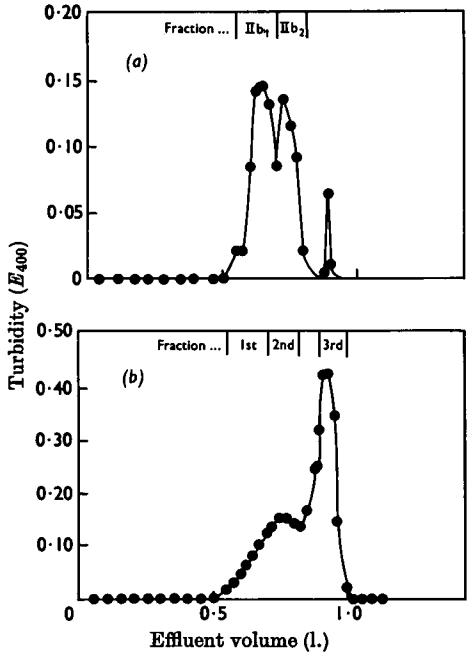

Fig. 5. Exclusion chromatography of the chicken erythrocyte histone, serially extracted from washed nuclei (after prior extraction at pH 1·78; Fig. 1), at pH 1·38 (*a*) and 1·02 (*b*). The pH 1·02 extract was adjusted to contain 0·2% β-mercaptoethanol. Chromatography was performed on Bio-Gel P-60 in 0·01 N-HCl. (Amino acid analyses of the three subfractions from the pH 1·02 extract are given in Table 5.)

components were subdivided, to give three fractions in all (Fig. 5*b*). Amino acid analysis (Table 5) showed that the first fraction was essentially a contaminating portion of fraction IIb (mostly fraction IIb_1), whereas the second part was more akin to fraction III or IV. The third (and major) fraction from the Bio-Gel P-60 column had a much higher glycine content than the first or second fractions, and in general its amino acid composition resembled that of the subfraction f2a1 of calf thymus histone (Phillips & Johns, 1965; see also Table 6). Fractions III and IV were not found in previous work on chicken erythrocyte histones (Neelin *et al.* 1964), presumably because the extracts had not been made at sufficiently low pH.

The starch-gel-electrophoretic pattern of the chromatographically purified fractions III and IV from the pH 1·02 extract included the same series of fast, intermediate and slowly migrating zones as did the corresponding fraction of calf thymus histone (Neelin *et al.* 1964). The other chromatographic fractions isolated from the pH 2·15, 1·90 and 1·38 extracts, herein termed Ia, Ib, Ic, IIb and V

Table 5. *Amino acid compositions of chromatographic fractions from Bio-Gel P-60 (Fig. 5) of the chicken erythrocyte histone extracted at* pH 1·02

Amounts of amino acids are expressed as moles/100 moles of total recovered amino acids; no corrections were applied for hydrolytic losses of any amino acids, or for the formation of *O*-sulphates of serine and threonine.

Fraction	First	Second	Third
Amino acid			
Lys	13·4	12·5	11
His	2·6	2·4	1·8
Arg	8·1	10·7	12·8
Asp	6·3	4·6	4·7
Thr	4·8	6·6	6·4
Ser	5·5	5·3	2·1
Glu	8·7	10·6	7·9
Pro	4·3	4·4	3·0
Gly	8·6	6·2	14·8
Ala	12·7	12·6	8·8
CyS	0	0	0
Val	6·4	5·5	7·0
Met	0·3	0·5	0·9
Ile	5·2	5·6	5·8
Leu	9·2	7·6	8·1
Tyr	2·5	2·6	3·2
Phe	1·2	2·4	1·7

(Figs. 4*a*, 4*b* and 4*c*) furnished the same electrophoretograms as those reported for chicken erythrocyte histones 1, 2, 3, 4 and 5 respectively (Table 6).

Qualitative analyses for esters of serine and threonine were carried out on the four major extracts. *O*-Phosphoserine was present in the first two extracts (i.e. pH 2·15 and pH 1·9 extracts) and in trace amounts in the pH 1·38 extract, but it was not found in the pH 1·02 extract. *O*-Phosphothreonine was not detected in any of the preparations (Murray & Milstein, 1967). All the extracts, however, were shown to contain the sulphates of serine and threonine after acid hydrolysis and subsequent evaporation (Murray & Milstein, 1967). To prevent the formation of sulphate esters, a series of extractions of washed nuclei was made with hydrochloric acid instead of sulphuric acid. The fractionation with respect to pH (Fig. 1*c*) was essentially the same as discussed for the sulphuric acid extracts; yields were similar and the extracts had similar chromatographic and gel-electrophoretic properties.

Although the fractionation of histones achieved by extraction at certain pH values was quite sharp, there was a certain amount of overlap of components and variability in their proportions in replicate extractions. Duration of extraction, frequency of manipulation and local variations in pH may be factors responsible for this variability.

Table 6. *Identity of the principal histone fractions obtained from chicken erythrocyte nucleoprotein by serial extraction with acid, and a correlation with other nomenclatures for histone components*

	Equivalent fractions			
Chromatographic fractions (Figs. 2 and 4) ...	Ia, Ib, Ic	IIb	III, IV	V
pH of extraction	2·1–2·5	1·3–1·8	1·0–1·4	1·8–2·1
Nomenclatures for avian erythrocyte histones				
Neelin (1964); Neelin *et al.* (1964)	1, 2, 3	4	—	5
Bellair & Mauritzen (1964, 1967)	α(4)	α(3)	β5+β7	α(5)
Hnilica (1964)	F1	F2b	—	F2c
Nomenclatures for calf thymus histones				
Rasmussen *et al.* (1962)	Ia, Ib	IIb (IIb_1+IIb_2)	III, IV	—
Johns (1964); Phillips & Johns (1965)	F1	f2a2+f2b	f2a1+f3	—

For example, repeated extractions at pH 2·15 tended to release slightly more histone fraction V along with the remaining histone fractions I. Similarly, a single extraction of avian erythrocytes with 0·10 M-citric acid in 0·125 M-sodium chloride (pH 2·2–2·5) yielded virtually uncontaminated histone fraction I, but a washing in the same extractant (pH 2·1) contained appreciable histone fraction V. Goose erythrocyte nuclear extracts in 5% (v/v) perchloric acid contained both histone fractions I and V. The pH, rather than the nature of the acid, is clearly the prime factor in extractability of each histone component.

This conclusion was confirmed in an attempt to eliminate local 'overshooting' of pH during titration; in a small-scale experiment a small quantity of washed nuclei was extracted serially with acetate buffers (Britton, 1955) at pH 3·1, 2·3, 1·85, 1·35 and 0·90. The products from these extracts were examined only by zone electrophoresis in starch gels, but this showed that a stepwise removal had been effected essentially the same as that by titration with acid to these pH values.

DISCUSSION

The stepwise titration method provides a useful means for the simultaneous preparation and fractionation of histones on a small or a large scale. In particular, this approach affords a convenient method for the preparation of fraction V with a minimal exposure to potentially deleterious agents, such as solvents, multivalent buffer salts and hydrogen-bonding agents. The suspension of deoxyribonucleoprotein is titrated to pH 2·0 and the residue from this extraction (preferably after two washings at this pH) is subsequently titrated to pH 1·9 to extract the purified fraction V. Some losses in prior washings are inevitable, but this fraction provides a valuable control on more quantitative, or purified, preparations.

A correlation of the histone fractions discussed in this paper with those obtained by other methods is summarized in Table 6. Fraction V is the histone component peculiar to avian erythrocytes and is characterized by its unusually high serine content. In earlier publications this fraction was designated chromatographic peak 5 (Neelin, 1964; Neelin *et al.* 1964) and it has been identified with starch-gel-electrophoresis zone 15 (Neelin & Butler, 1961; Purkayastha & Neelin, 1966).

Stepwise removal methods of the type described here (particularly at low temperatures) should be of value in studies of nucleoprotein structure, and application of the procedure in cytochemical experiments may well be useful as a means of studying different histone fractions in chromosomes.

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REFERENCES

Bellair, T. & Mauritzen, C. M. (1964). *Aust. J. biol. Sci.* **17**, 1001.

Bellair, T. & Mauritzen, C. M. (1967). *Biochim. biophys. Acta*, **133**, 263.

Bigwood, M. (1959). *Nucleoproteins*, p. 103. New York: Interscience Publishers Inc.

Bijvoet, P. (1957). *Biochim. biophys. Acta*, **25**, 502.

Bonner, J. & Huang, R. C. (1966). In *Ciba Found. Study Group no. 24: Histones*, p. 18. Ed. by de Reuck, A. V. S. & Knight, J. London: J. and A. Churchill Ltd.

Britton, H. T. S. (1955). *The Hydrogen Ions*, vol. 1, chapter 16. London: Chapman and Hall Ltd.

Daly, M. M. & Mirsky, A. E. (1955). *J. gen. Physiol.* **38**, 405.

de Nooij, E. H. & Westenbrink, G. H. K. (1962). *Biochim. biophys. Acta*, **62**, 608.

Dounce, A. L. & Hilgartner, C. A. (1964). *Exp. Cell Res.* **36**, 778.

Hindley, J. (1964). *Abstr. 6th int. Congr. Biochem., New York*, 1–82. London: Pergamon Press Ltd.

Hnilica, L. S. (1964). *Experientia*, **20**, 13.

Hnilica, L. S. & Bess, L. G. (1965). *Analyt. Biochem.* **12**, 421.

Johns, E. W. (1964). *Biochem. J.* **92**, 55.

Johns, E. W. & Butler, J. A. V. (1962). *Biochem. J.* **82**, 15.

Johnson, L. D., Driedger, A. & Marko, A. (1964). *Canad. J. Biochem.* **42**, 795.
Kinkade, J. M. & Cole, R. D. (1966). *J. biol. Chem.* **241**, 5790.
Murray, K. (1962). *Analyt. Biochem.* **3**, 415.
Murray, K. (1964). *Biochemistry*, **3**, 10.
Murray, K. (1966). *J. molec. Biol.* **15**, 409.
Murray, K. & Milstein, C. (1967). *Biochem. J.* **105**, 491.
Murray, K., Vidali, G. & Neelin, J. M. (1966). *Biochem. J.* **101**, 35P.
Neelin, J. M. (1964). In *The Nucleohistones*, p. 66. Ed. by Bonner, J. & Ts'o, P. O. P. San Francisco: Holden–Day Inc.
Neelin, J. M. & Butler, G. C. (1961). *Canad. J. Biochem. Physiol.* **39**, 485.
Neelin, J. M., Callahan, P. X., Lamb, D. C. & Murray, K. (1964). *Canad. J. Biochem.* **42**, 1743.
Neelin, J. M. & Connell, G. E. (1959). *Biochim. biophys. Acta*, **31**, 539.
Phillips, D. M. P. & Johns, E. W. (1965). *Biochem. J.* **94**, 127.
Purkayastha, R. & Neelin, J. M. (1966). *Biochim. biophys. Acta*, **127**, 468.
Rasmussen, P. S., Murray, K. & Luck, J. M. (1962). *Biochemistry*, **1**, 79.
Satake, K., Rasmussen, P. S. & Luck, J. M. (1960). *J. biol. Chem.* **235**, 2801.
Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.