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Countercurrent-Distribution Studies on Histones

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1. The possibilities of fractionating histones and histone fractions by means of countercurrent distribution between two phases formed by water and butan-2-ol, in the presence of various concentrations of trichloroacetic acid, have been examined. 2. Although the principal histone fractions differ considerably in their partition ratios, a satisfactory resolution of the principal histone fractions from the whole histone has not been achieved. 3. The histone fractions obtained by other methods can be resolved with suitable concentrations of trichloroacetic acid. Besides the main peak several subsidiary peaks are obtained in most cases, the composition of which corresponds with others of the main fractions. 4. The method is therefore capable of removing from the principal fractions as previously prepared contamination by other fractions. 5. Except in one case, no fraction with composition unlike other fractions has been obtained. In several cases the material isolated from the principal peak behaves as a single component on running again. In two cases fractions with similar compositions were distinguished by countercurrent distribution.

Histones are a group of basic proteins which have a considerable range of composition, and exhibit distinctive physicochemical characteristics which can be made use of in fractionation procedures. The methods of fractionation hitherto investigated are (1) chromatography on suitable resins, (2) gel filtrations, (3) differential extraction procedures from the intact nucleoprotein, (4) differential precipitation by organic solvents. These have been reviewed by Phillips (1962), Butler (1964) and Murray (1963, 1965). The two last-named methods depend partly on the ratios of polar to non-polar groups in the molecule. In this Laboratory a number of fractions have been obtained by methods (3) and (4) or combinations of them which have characteristic and, within limits, repeatable compositions (Johns, Phillips, Simson & Butler, 1960; Johns & Butler, 1962; Phillips & Johns, 1965; Johns, 1965). The questions arise whether these fractions are reasonably homogeneous or if methods could be found for further fractionation. Since the total number and relative quantities of histone species are of considerable interest in assessing the role of histones, it appeared to be necessary to see whether a further degree of fractionation could be brought about by other procedures and for this purpose their behaviour in a countercurrent-distribution process has been examined. Since histones are insoluble in the solvents usually used in these processes, which are incompletely miscible in water, it is necessary to confer sufficient solubility in the non-aqueous phase by adding an acid which is soluble in both phases. We have used low concentrations of trichloroacetic acid for this purpose, with butan-2-ol as the organic phase (*cf.* Hausmann & Craig, 1958).

Trichloroacetic acid produces a four-component system that complicates the system considerably, so that the behaviour of the histones depends to a great extent on the concentration of trichloroacetic acid present. If the concentration of the acid were too low the histone remained largely in the aqueous phases of the first tubes; if too high it was carried rapidly to the final tubes. We did not find a solvent which was very suitable for fractionating the whole histone, but useful separations have been obtained in some cases with fractions obtained by the methods already recorded.

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EXPERIMENTAL

Distribution coefficients of histone fractions between water and butan-2-ol containing trichloroacetic acid. As a guide to further work, approximate distribution coefficients of the different histone fractions were determined between phases prepared by shaking aqueous trichloroacetic acid solutions at the concentrations stated with an equal volume of butan-2-ol at about 20°. The initial concentration of the histone in the aqueous solution was 1 mg./ml. in each case. After equilibration the amounts of nitrogen in 1 ml. of each phase were determined by the Kjeldahl method. The ratios

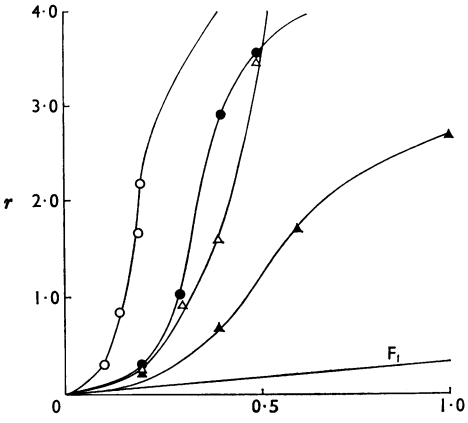

Fig. 1. Partition ratios of histone fractions in phases made from aqueous trichloroacetic acid solutions of the concentrations given and butan-2-ol. ○, F2a1; △, F2a2; ▲, F2b; ●, F3.

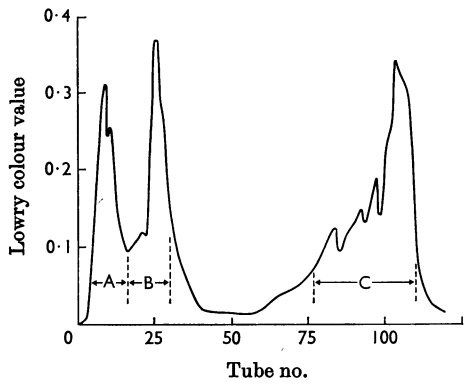

Fig. 2. Countercurrent-distribution pattern of the whole histone (102mg.) from calf thymus with 0·7% trichloroacetic acid and 115 transfers.

of concentration in the two phases are shown in Fig. 1. These ratios cannot be expected to remain constant with different amounts of histone owing to the interaction between basic groups of the histones with the acid. Nevertheless they indicate that considerable differences in the partition ratios occur in most cases in concentrations between 0·3 and 1·0% of trichloroacetic acid without the disparity being too great to be compatible with a reasonable speed of transfer.

Distribution of histones between water and butan-2-ol with other acids present. Some observations on the distribution were made in systems containing hydrochloric acid (0·1N), perchloric acid (1%) and butyric acid (5%). The differences between the different histone fractions are smaller than with trichloroacetic acid and these systems were not investigated further. With monochloroacetic acid and dichloroacetic acid the histones remained to a great extent in the aqueous phases ($r < 0·1$).

Analyses. Determinations of total amino acids and of *N*-terminal amino acids, for which we are indebted to Miss P. Simson, were carried out as described by Phillips & Johns (1965). The analyses are reported as moles of each amino acid/100moles of all amino acids. The overall reproducibility for most amino acids is ±3%, but with amino acids present in very small proportions the variation may be greater. No corrections have been applied for hydrolytic losses. Cysteine is given as cysteic acid and cystine formed during the hydrolysis. Protein concentrations in the countercurrent-distribution tubes were determined by adding 1ml. of ethanol to each tube after the completion of a series of transfers, and the protein concentration was then determined in a portion by the method of Lowry, Rosebrough, Farr & Randall (1951). The colour developed in 30min. was determined at 750mμ in a Unicam spectrophotometer.

Starch-gel electrophoresis patterns. These were obtained with nearly all fractions by the method described by Johns, Phillips, Simson & Butler (1961). The results are described when they provide additional information.

Countercurrent distribution. The apparatus used was the automatic model manufactured by J. W. Towers and Co., with 120 tubes, each containing 3ml. of both phases. The two solvent phases were equilibrated with each other at the temperature of the apparatus (approx. 20°) and 3ml. of each was put in the tubes. After running for six transfers, the histone dissolved in 3ml. of lower phase, which had been previously equilibrated with 3ml. of upper phase, was introduced into tube no. 4 and the desired number of transfers effected.

To isolate the protein present in a number of tubes, their contents were united and dialysed against 0·01N-hydrochloric acid and the protein was precipitated by adding 5vol. of acetone. The precipitate was then washed three times with acetone.

RESULTS

Experiments with whole histone. Initially some experiments were made with the whole histone, extracted from calf thymus with 0·2N-hydrochloric acid. It was hoped that peaks corresponding to the fractions would be sufficiently separated to enable their quantities to be determined. In preliminary experiments it was found that countercurrent-distribution curves with 0·7% trichloroacetic acid showed the best separation. Fig. 2 shows the curve obtained in this solvent. Peak A (Fig. 2) is undoubtedly F1, as this has always been found to be the most slowly moving peak in all systems. Peak B was analysed and found to correspond closely with F2b. The final peak C must contain the other fractions which are not clearly differentiated. The study of whole histone was not pursued as the separations (except F1 and F2b) did not seem to be sufficient to be useful.

Countercurrent-distribution experiments with F3.

Experiments were next done with the arginine-rich fraction F3, which has been in previous work the most refractory to further fractionation. It has also been somewhat variable in composition, owing to its tendency to carry with it other fractions, especially F2a2, on precipitation. The preparation used in all experiments was a large one (yield of F3, 5·9g. from 1200g. of calf thymus) made by the method of Johns *et al.* (1960). Its arginine content (12·3%, see Table 3) was found to be lower than that of other preparations by this and other methods (approx. 14%; see Phillips, 1962). A fractionation of a similar sample of F3 on Sephadex columns has been attempted by Hnilica & Bess (1965), but the main fractions obtained did not differ in composition and were attributed to different states of aggregation.

To ascertain suitable conditions, countercurrent-distribution runs were carried out with a range of trichloroacetic acid concentrations originally present in the aqueous phase from 0·1 to 1·0% (w/v). The amount of histone introduced and the number of transfers effected were varied in different experiments. Up to 0·4% trichloroacetic acid the greater part of the histone remained in the earlier tubes and no useful fractionation resulted. However, in a longer series of transfers some predominantly arginine-rich material was carried into the later tubes.

With 0·5% trichloroacetic acid a more clearly defined fractionation was obtained (Fig. 3, curve I). The amino acid compositions of the regions A–D as marked are given in Table 1. It can be seen that these regions differ considerably in composition. A is a lysine-rich material which cannot be identified with any known fraction. The composition of region B approximates to that of F2a2, but is complex on starch-gel electrophoresis. C and D are both arginine-rich fractions with similar composition, C having a lysine/arginine ratio as low as any obtained by other methods, but the starch-gel-electrophoresis pattern still showed the presence of F2b.

A somewhat similar pattern was obtained with 0·6% trichloroacetic acid (Fig. 3, curve II). A was not analysed but B again approximates to F2a2, and C is an arginine-rich protein similar to F3 fractions with the lowest lysine/arginine ratio previously obtained (Table 1).

In 0·7% trichloroacetic acid a similar pattern of fractionation was obtained (Fig. 4, curve I). Amino acid analyses of the regions A, B and C (Table 1) show that A is a lysine-rich fraction intermediate between A and B of Fig. 3, curve I. Fraction B of Fig. 4 (curve I) now approximates in composition to F2b2, and C is an arginine-rich fraction which, according to the starch-gel picture, still contains some F2b.

Finally the countercurrent-distribution pattern was obtained in 1·0% trichloroacetic acid with 100 transfers (Fig. 4, curve II). Only two peaks were observed, which were widely separated. A is intermediate in composition between F2a2 and F2b and B contains all the more arginine-rich fractions, i.e.

Table 1. *Amino acid compositions of fractions obtained by countercurrent-distribution experiments with a sample of F3*

For details of the method of expressing results in the Tables see the Experimental section.

	Original fraction	Fig. 3 (curve I)				Fig. 3 (curve II)		Fig. 4 (curve I)			Fig. 4 (curve II)	
	F3	A	B	C	D	B	C	A	B	C	A	B
Aspartic acid	4·9	3·2	5·6	3·9	6·7	5·8	4·2	3·5	5·8	4·2	4·0	4·8
Glutamic acid	10·3	7·3	9·7	11·3	11·2	9·5	10·8	9·1	9·4	11·1	9·6	10·6
Glycine	7·4	6·3	9·1	5·5	6·6	9·1	6·0	5·8	9·1	6·1	6·2	7·2
Alanine	13·9	19·7	13·2	13·4	11·4	12·6	12·8	16·7	12·6	13·6	15·5	13·1
Valine	7·1	3·9	6·1	4·6	5·4	5·8	4·8	4·4	6·3	4·9	4·8	5·3
Leucine+isoleucine	13·6	7·7	16·3	14·6	15·5	15·5	14·6	11·2	15·6	14·9	12·2	15·3
Serine	3·6	4·3	3·9	3·7	4·4	4·1	3·7	4·2	4·4	3·6	4·1	3·7
Threonine	5·9	7·3	4·8	7·2	6·4	4·9	7·0	6·8	4·7	6·8	6·7	6·2
Phenylalanine	1·6	1·4	1·4	3·0	3·2	1·9	3·5	2·2	1·4	2·8	2·5	2·4
Tyrosine	2·3	0·9	2·3	2·1	2·3	2·5	2·4	1·4	2·3	2·0	1·8	2·1
Proline	4·7	7·3	3·4	4·1	3·9	3·9	4·3	6·5	4·3	4·1	6·2	4·2
Methionine	—	—	0·3	1·1	—	0·6	1·3	0·6	0·2	1·0	0·8	0·8
Histidine	2·3	0·8	2·6	1·5	1·9	2·8	1·7	1·2	2·7	1·7	1·3	2·0
Lysine	9·8	20·3	10·5	8·7	8·1	10·2	8·6	15·5	10·6	8·8	12·6	9·1
Arginine	12·3	9·0	10·7	14·1	12·0	10·6	13·9	10·4	10·1	13·4	11·0	12·6
Cystine (half)	0·4	0·6	0·3	0·1	1·1	—	—	0·5	0·4	0·3	0·6	0·6
Lysine/arginine	0·79	2·25	0·98	0·62	0·67	0·95	0·62	1·49	1·05	0·66	1·15	0·72

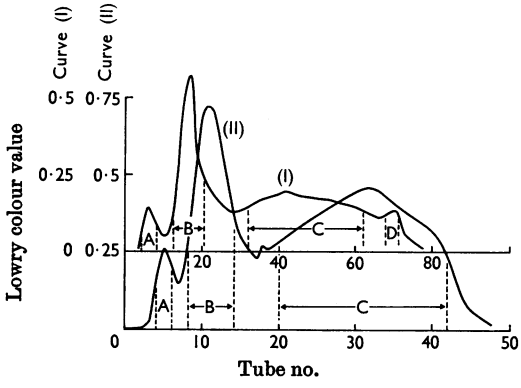

Fig. 3. Countercurrent-distribution patterns obtained with: (I) 142mg. of fraction F3 with 0·5% trichloroacetic acid with butan-2-ol after 74 transfers; (II) 118mg. of fraction F3 with 0·6% trichloroacetic acid with butan-2-ol after 50 transfers.

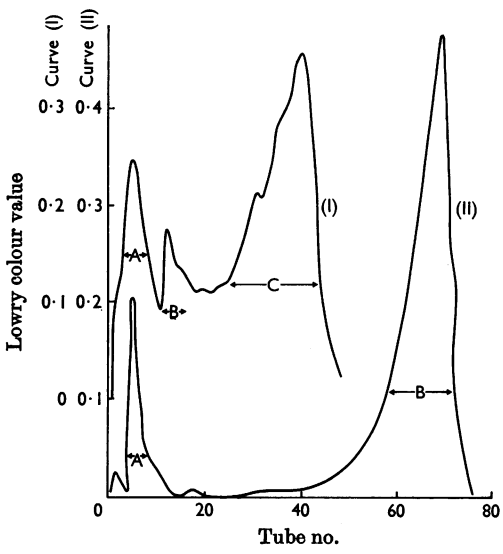

Fig. 4. Countercurrent-distribution patterns: (I) 113mg. of fraction F3 with 0·7% trichloroacetic acid with butan-2-ol after 45 transfers; (II) 108mg. of fraction F3 with 1·0% trichloroacetic acid with butan-2-ol after 100 transfers.

the predominantly lysine-rich fractions remain near the start and the predominantly arginine-rich fractions are moved to near the end of the series.

It is evident that the most effective fractionation is in the intermediate range, with about 0·5–0·7% trichloroacetic acid. In this range a clear separation into three fractions: (a) arginine-rich, lysine/arginine 0·65; (b) intermediate, lysine/arginine 1·0;

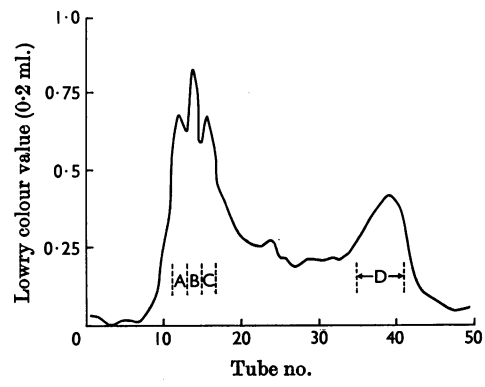

Fig. 5. Countercurrent-distribution pattern of 103mg. of F2a with 0·6% trichloroacetic acid.

(c) lysine-rich, lysine/arginine 1·5–2·0. None of these products is homogeneous as judged by starch-gel electrophoresis. Apart from the lysine-rich fraction the products appear to be similar to other fractions previously isolated.

The experiments show that the method is capable of purifying a crude fraction, which is contaminated by other fractions, but the purified F3 obtained is not greatly different from specimens of F3 which have been previously obtained. It is possible that in a longer series of transfers C (Fig. 3, curves I and II) could be further resolved.

Countercurrent-distribution experiments with fraction F2a. This fraction was originally obtained as the portion of histone extracted with acidic 95% ethanol together with F3, from which it was separated by fractional precipitation with acetone. It was later separated by acid–acetone fractionation into two fractions designated F2a1 and F2a2 (Phillips & Johns, 1965). A similar fractionation was reported independently by Hnilica (1965) and by Hnilica & Bess (1965). Fractions having a somewhat similar composition to F2a2 have been described as fraction 1·6Sγ by Cruft, Hindley, Mauritzen & Stedman (1957) and as fraction IIb$_1$ b Rasmussen, Murray & Luck (1962).

The principal characteristics of these fractions (Table 2) are F2a1, arginine/lysine 1·27, glycine (exceptionally high), 15·1%, alanine, 7·4%; F2a2, arginine/lysine 0·91, glycine, 10·3%, alanine, 12·5%. These fractions are also characterized by a low content of free *N*-terminal amino acids, and a high acetyl content (Phillips, 1963; Phillips & Johns, 1965).

In the first place a countercurrent distribution was attempted with the whole of the fraction F2a, with butan-2-ol equilibrated with 0·6% trichloroacetic acid (Fig. 5). The small regions A, B, C and

Table 2. *Compositions of fractions of F2a obtained by countercurrent distribution in water–butan-2-ol with 0·6% trichloroacetic acid*

See also Fig. 5.

F2a	F2a1*	F2a2†	Fractions of F2a (Fig. 5) A	B	C	D
Aspartic acid	5·9	6·7	5·3	5·6	6·2	5·6
Glutamic acid	7·4	9·3	6·7	7·9	8·7	8·5
Glycine	15·1	10·3	13·9	12·6	11·6	11·8
Alanine	7·4	12·5	8·4	10·3	12·2	8·1
Valine	8·4	6·4	7·6	7·1	7·3	7·3
Leucine	8·3	11·2	8·5	10·5	11·9	9·7
Isoleucine	2·4	4·7	5·4	4·9	5·0	5·5
Serine	2·4	3·1	2·7	3·2	2·4	2·7
Threonine	6·4	4·0	6·2	5·2	4·0	5·8
Phenylalanine	2·2	1·3	2·9	1·5	1·2	2·5
Tyrosine	3·4	2·5	4·0	3·1	2·5	3·4
Proline	1·6	4·1	2·0	2·4	3·0	2·3
Methionine	0·7	0·2	0·9	0·7	0·2	1·1
Histidine	2·6	2·9	2·0	2·5	2·8	2·0
Lysine	10·1	10·8	10·7	10·7	10·6	9·2
Arginine	12·8	9·9	12·8	11·9	10·4	13·0

* From Phillips & Johns (1965).
† Analysis as given in Table 3.

D (of Fig. 5) were analysed and their principal characteristics are given in Table 2. It can be seen that the composition in A corresponds fairly closely to that of F2a1, B is intermediate, whereas C is similar to F2a2 and D is an arginine-rich fraction approaching F3 in composition. It is evident that the peaks are not sufficiently separated for effective fractionation. It seemed better to go as far as possible with the previously established fractionations before resorting to the countercurrent-distribution process. The fraction F2a2 prepared as described by Phillips & Johns (1965) was therefore examined. It was found that no further fractionation of this occurred in countercurrent-distribution experiment with 0·2% trichloroacetic acid (Fig. 6, curve I). However, in 0·5% trichloroacetic acid separation occurs (Fig. 6, curve II) and the analyses of the regions A, B and C are given in Table 3.

It can be seen that fractions A and B do not differ appreciably except in one or two respects which may be due to experimental errors. The reason for the difference in countercurrent-distribution behaviour of fractions A and B is not evident. It may possibly be a difference of the state of aggregation or conformation. The amount of *N*-terminal amino acid in both these fractions was small, and in fraction B has been practically eliminated. Fraction C has a markedly greater arginine/lysine ratio and its composition approaches that of F3, and the main result of the process has thus been to separate from

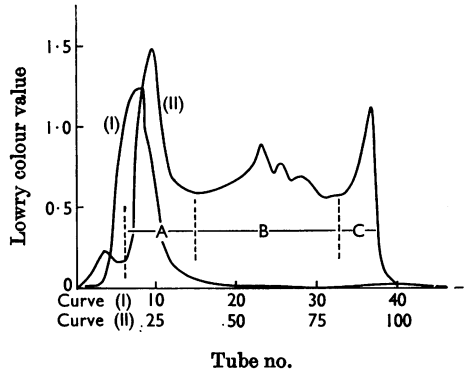

Fig. 6. Countercurrent-distribution patterns of F2a2: (I) with 0·2% trichloroacetic acid; (II) with 0·5% trichloroacetic acid.

F2a2 a quantity of material which approaches the arginine-rich fraction F3 in composition.

Experiments with F2b. The fraction F2b is characterized by a high lysine/arginine ratio (approx. 1·8), a mainly proline *N*-terminal group, and a high serine content. It has been fractionated to some extent by successive fractional precipitation with acetone in 0·02N-hydrochloric acid (Johns, 1965). Of the five fractions obtained, B and C amounting to 25% and 56% of the total recovery

Table 3. *Composition of histone fractions of calf-thymus fraction F2a2 obtained by countercurrent distribution in water–butan-2-ol with 0·5% trichloroacetic acid*

N.D., not determined.

	Original material F2a2	Fractions (Fig. 6, curve II)		
		A	B	C
Aspartic acid	6·7	6·5	6·4	5·6
Glutamic acid	9·3	9·4	9·6	10·2
Glycine	10·3	10·2	10·7	9·6
Alanine	12·5	12·7	12·7	10·9
Valine	6·4	6·2	6·2	6·6
Leucine	11·2	10·6	11·7	9·6
Isoleucine	4·7	4·5	4·3	5·6
Serine	3·1	5·0	3·6	3·2
Threonine	4·0	4·5	4·1	5·7
Phenylalanine	1·3	1·1	1·0	2·4
Tyrosine	2·5	2·5	2·3	2·7
Proline	4·1	4·1	4·2	2·9
Methionine	0·2	0·5	0·1	1·2
Histidine	2·8	2·8	3·0	2·0
Lysine	10·8	9·9	9·8	7·8
Arginine	9·9	9·6	10·2	13·3
Cystine (half)	—	—	—	0·6
N-terminal amino acids (μmoles/g. of protein)				
Proline	N.D.	10·3	—	N.D.
Alanine	N.D.	1·4	2·5	N.D.
Glycine	N.D.	0·9	0·4	N.D.
Others	N.D.	2·8	1·7	N.D.

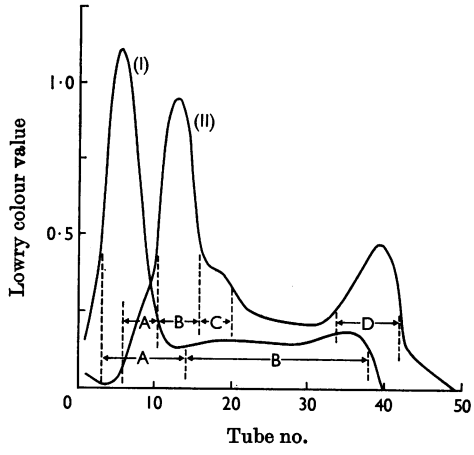

Fig. 7. Countercurrent-distribution patterns of F2b: (I) in 0·6% trichloroacetic acid (series 17) with 40 transfers; (II) in 1·0% trichloroacetic acid with 50 transfers.

resembled the initial fraction in general characteristics.

We have examined by countercurrent distribution both the original material F2b and the fraction C prepared from it by Dr E. W. Johns.

With 0·4 or 0·6% trichloroacetic acid F2b was not well resolved. The distribution obtained with 124mg. of F2b with 0·6% trichloroacetic acid in 40 transfers is shown in Fig. 7 (curve I). There was, however, a marked difference of composition between the sections marked A and B, A having a lysine/arginine ratio 2·08 with 8·0% serine, and B had lysine/arginine 0·74 with 3·9% serine (Table 4). Both A and B gave complex starch electrophoretic patterns.

A second experiment with 121mg. of F2b with 0·4% trichloroacetic acid and with 100 transfers gave a very similar picture with lysine/arginine 2·7 and serine 10·7%, in the peak corresponding to A of Fig. 7 (curve I) above (see A′ in Table 4). The countercurrent-distribution treatment had thus split F2b into two fractions with lysine/arginine ratios respectively higher and lower than the original material.

Better resolution was obtained when the trichloroacetic acid concentration was increased to 1·09%. Fig. 7 (curve II) shows the distribution of 105mg. of F2b with 50 transfers. The analyses of regions A, B and D are given in Table 4. A (Fig. 7, curve II) has a greater lysine/arginine ratio than B and is probably contaminated with lysine-rich histones belonging to the group F1. D, with lysine/arginine 0·8, is clearly an arginine-rich

Table 4. *Composition of fractions obtained by countercurrent distribution of fraction F2b in aqueous trichloroacetic acid–butan-2-ol*

Details of the method are described in the text.

	Original F2b	Fig. 7 (curve I) A	A′	B		Fig. 7 (curve II) A	B	D
Aspartic acid	5·5	5·7	4·7	5·4		5·7	5·9	5·1
Glutamic acid	9·5	9·4	8·8	10·1		9·5	8·9	9·8
Glycine	7·8	7·2	5·0	8·7		8·1	6·0	8·5
Alanine	12·4	11·1	10·8	11·3		14·8	10·2	11·2
Valine	6·9	6·7	8·5	5·8		5·0	6·4	5·9
Leucine + isoleucine	12·2	10·9	9·2	14·9		9·0	9·5	15·3
Serine	6·1	8·0	10·7	3·9		6·6	9·2	4·0
Threonine	5·7	5·5	5·7	6·8		5·3	5·8	6·1
Phenylalanine	1·5	1·7	1·4	2·5		1·8	2·2	2·6
Tyrosine	2·8	3·3	3·8	2·5		1·6	3·9	2·9
Proline	4·9	4·8	4·8	3·6		6·2	5·1	3·6
Histidine	2·4	2·5	2·7	1·9		1·2	2·3	2·2
Lysine	13·4	14·8	17·4	9·2		17·2	16·1	9·3
Arginine	8·3	7·1	6·5	12·5		7·1	6·7	11·7
Cystine (half)		0·2		0·7	Lys/Arg	2·4	2·4	0·8
N-terminal groups (%)								
Proline	82	—	—	—		—	82	—
Alanine	12	—	—	—	(Ser)	—	7	—
Glycine	1	—	—	—		—	7	—
Others	5	—	—	—		—	4	—

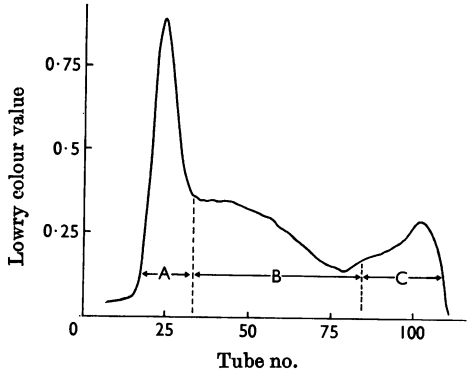

Fig. 8. Countercurrent-distribution pattern of fraction F2b-C (Johns, 1965) with 0·7% trichloroacetic acid.

fraction. Some fractionation has been achieved. On starch-gel electrophoresis B shows a single front with some slowly migrating material which may be an 'aggregate'. The percentage of *N*-proline terminal in this fraction has not been increased, but the proportions of the minor constituents are different.

The countercurrent-distribution pattern with 0·7% trichloroacetic acid of the fraction C of F2b of Johns (1965) described above is shown in Fig. 8. The material taken had a composition very similar to that of B of Fig. 7 (curve I) as given in Table 4, with about 84% proline as *N*-terminal. Analyses of the fractions obtained are given in Table 5. It can be seen in Fig. 8 that the section corresponding to A of Fig. 7 (curve II) has disappeared but otherwise the pattern is similar. The fraction B (Fig. 8) has a somewhat smaller content of arginine, but in other respects resembles A very closely. Proline is over 98% of the total *N*-terminal. The final fraction C resembles F2a.

A further countercurrent-distribution experiment with the same material in 1·5% trichloroacetic acid gave an initial peak with composition (A†) (Table 5), remarkably similar to peak A (Table 5) and with *N*-terminal proline 94·5% of all *N*-terminal amino acids. Fraction A† (14mg.) was collected and on being run again in 0·7% trichloroacetic acid was not further fractionated.

These results suggest that the fractions A and B of F2b (Fig. 8 and Table 5) are practically homogeneous proteins. They do not differ much in composition or in *N*-terminal amino acid. They also have similar starch-gel patterns consisting of a strong band in the position of the main band of F2b, and a fine band near the origin. However, the greater lysine content in A appears to be real. In this case it would appear that besides removing some contaminating F2a, the countercurrent-

Table 5. *Composition of histone fractions prepared by countercurrent distribution in 0·7% trichloroacetic acid–butan-2-ol of calf-thymus histone fraction F2b-C*

Fractions A, B and C are those shown in Fig. 8. N.D., not determined.

	Original F2b-C	Fractions A	A†	B	C
Aspartic acid	5·7	5·9	5·3	5·0	6·7
Glutamic acid	9·0	9·0	8·6	8·7	10·7
Glycine	6·2	5·4	5·8	5·9	8·0
Alanine	11·2	10·7	10·7	10·8	12·3
Valine	7·5	6·8	7·1	7·5	5·9
Leucine+isoleucine	10·3	4·8	5·0	4·9	9·9
		4·6	4·8	5·1	5·2
Serine	8·8	9·8	9·6	10·4	5·2
Threonine	5·5	6·0	5·9	6·4	5·3
Phenylalanine	2·1	1·9	1·8	1·6	1·8
Tyrosine	3·4	3·8	3·9	4·0	2·2
Proline	5·2	4·5	4·4	4·9	4·6
Methionine	N.D.	1·6	1·6	1·5	0·7
Histidine	2·2	2·3	2·4	2·3	2·3
Lysine	15·6	16·3	16·2	14·1	9·6
Arginine	7·3	6·5	6·8	6·9	9·3
Cystine (half)	N.D.	+	—	—	0·3
Lysine/arginine	2·1	2·5	2·4	2·04	1·03
N-terminal amino acids (μmoles/g. of protein)					
Proline	60·0 (84%)	N.D.	86·5	90·0	N.D.
Alanine	4·3	N.D.	—	—	N.D.
Glycine	2·9	N.D.	1·8	—	N.D.
Others*	4·3	N.D.	2·7	1·0	N.D.

* Includes aspartic acid, glutamic acid, serine and lysine.

† Fraction A from a countercurrent-distribution experiment in which 1·5% trichloroacetic acid–butan-2-ol was used.

distribution process has split F2b into two proteins having very similar composition, apart from the lysine content. The difference of behaviour may be due to the different lysine contents, but it is possible that it may be due to different states of aggregation or conformation occurring in the two subfractions. Moreover, as the amount of *N*-terminal amino acids in both corresponds to a molecular weight about 11,000, it may be assumed that proteins with an acetyl termination have been effectively removed from these fractions.

DISCUSSION

It is evident that the countercurrent-distribution method with a suitable two-phase system provides a fairly powerful means of fractionating histone mixtures. The system described is not really useful in fractionating the whole complex of histones extractable from natural sources, as some of the fractions are not sufficiently distinguished. However, when applied to material which has already been fractionated by other methods into the principal fractions already described, it is evident that in some cases useful further separations can be obtained. The components separated from a main fraction in such cases do not usually appear to be new fractions but components similar, at least, to those already known, which were evidently present in the fraction as originally prepared. Under the conditions stated both a lysine-rich component and a component of intermediate composition in addition to the main arginine-rich material can be obtained from F3. The lysine-rich component does not agree in composition with any previously known fraction. It follows that in the fractionation processes previously used some co-precipitation or co-extraction has occurred, the amount in different preparations being variable. We do not wish to suggest that such contaminants could not be removed by other methods, such as fractional precipitation with acetone in some cases, but the methods developed here are undoubtedly a useful means of removing such contaminants and also of obtaining new fractions in certain cases.

Fractions have now been obtained from F2a and F2b which not only give a single peak on repeated subjection to countercurrent distribution, but have

nearly 100% of a single *N*-termination. It is probably too soon to claim that such fractions are entirely homogeneous, but any further variation could only be of a minor character, and would in any case be difficult to demonstrate.

To sum up, in most cases the countercurrent-distribution method is useful in reducing the cross-contamination of fractions by each other. In three cases further fractionation has been achieved: (1) with F3 a new lysine-rich fraction was obtained; (2) F2a2 gave two fractions differing in counter-current-distribution behaviour but of almost identical compositions; (3) F2b gave two fractions differing significantly in lysine content. The nature of the differences between these fractions will require further study.

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