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A Method for the Selective Extraction of Histone Fractions f2(a)1 and f2(a)2 from Calf Thymus Deoxyribonucleoprotein at pH7

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1. A new method has been developed for the specific extraction of histone fraction f2(a) from calf thymus deoxyribonucleoprotein at pH 7 by using a mixture of ethanol and guanidinium chloride. 2. Fraction f2(a) has been separated into the subfractions f2(a)1 and f2(a)2 by acetone precipitation from acid solution, and at pH 7. 3. Modifications of existing electrophoretic methods are described that enable these fractions to be more easily characterized.

The histones of calf thymus have been separated into four fractions, designated f1, f2(a), f2(b) and f3, by the selective extraction of deoxyribonucleoprotein with various solvents at low pH values (Johns, 1964). More recently fraction f2(a), the largest of the four fractions, has been separated into two subfractions, designated f2(a)1 and f2(a)2, by acetone precipitation (Phillips & Johns, 1965). These five fractions, which constitute about 20% each of the total calf thymus histone, have all been characterized by total and *N*-terminal amino acid analyses, and by starch-gel and polyacrylamide-gel electrophoresis (Johns, 1966, 1967). Fractions f1 and f2(b) are lysine-rich histones, fractions f2(a)1 and f3 are arginine-rich histones and fraction f2(a)2 is an intermediate type having a lysine/arginine molar ratio about 1:1.

Specific extraction methods for fraction f1, the lysine-rich histone, have been reported by many authors (Davison & Butler, 1954; Johns & Butler, 1962; De Nooij & Westenbrink, 1962; Hindley, 1964; Murray, 1966), as this fraction appears to have the least affinity for DNA (Johns & Butler, 1964). The method described by Hindley (1964) has the advantage that the extraction is at pH 7 and thus the remaining deoxyribonucleoprotein may be used for studies on its ability to act as a template for RNA synthesis (Georgiev, Ananieva & Kozlov, 1966) or for conformational studies.

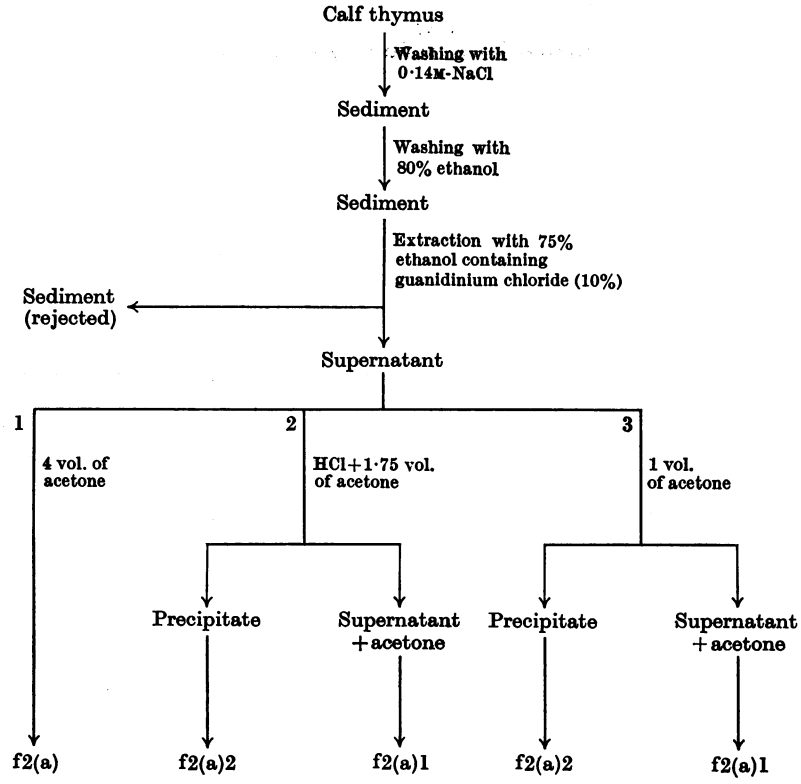
It is therefore of interest to be able to remove other histone fractions specifically at pH 7 and such a method for fraction f2(a) is detailed below. This selective extraction, however, when followed by acetone fractionation also constitutes a new method for the preparation of the subfractions f2(a)1 and f2(a)2 in large amounts, and it is with the preparation of these subfractions that this paper is mainly concerned.

EXPERIMENTAL AND RESULTS

All operations up to the preparation of the clarified extract were carried out at 4°. The precipitation of all proteins and the washing and drying of precipitates was carried out at room temperature. A summary of the method is given in Scheme 1.

Method. Minced calf thymus (100g.) was homogenized with 700ml. of 0·14M-NaCl at top speed for 2min. in an MSE Ato-Mix. The homogenate was centrifuged at 1100*g* for 30min. and the supernatant discarded. The sediment was washed five times in a similar manner but with homogenizing for 30sec. and centrifuging for 15min. The sediment from the last washing was stirred with 500ml. of ethanol–water (4:1, v/v) and centrifuged at 1100*g* for 5min. This ethanol washing was repeated twice more to ensure that the ethanol–guanidinium chloride solvent used for the subsequent extraction was not diluted with water from the wet residue. The sediment was then transferred to a 500ml. polythene bottle with 500ml. of ethanol–water (3:1, v/v) containing guanidinium chloride (10%, w/v). This solution was made up by first preparing 40% (w/v) guanidinium chloride in water, adjusting to pH 7 and then adding 3vol. of ethanol. About 15 porcelain balls (2cm. diam.) were added to the polythene bottle, which was then rotated on rollers at about 20 rev./min. for 18hr. The contents, excluding the balls, were then centrifuged at 1100*g* for 30min. and the sediment extracted twice more in a similar manner but with 200ml. of solution and extracting for 2hr. The combined supernatants (890ml.), which contained fraction f2(a) [i.e. subfractions f2(a)1 and f2(a)2], were then clarified by filtering through a no. 4 sintered-glass funnel. At this stage in the preparation the clarified extract can be treated in three ways.

(1) Precipitation of fractions f2(a)1 and f2(a)2 together at pH 7. Acetone (4vol.) was added to the clarified extract, and the precipitate that formed was washed twice in



Scheme 1.

ethanol–water (17:3, v/v). The precipitate was then washed three times in acetone and dried under vacuum. The yield of fraction f2(a) was approx. 900mg.

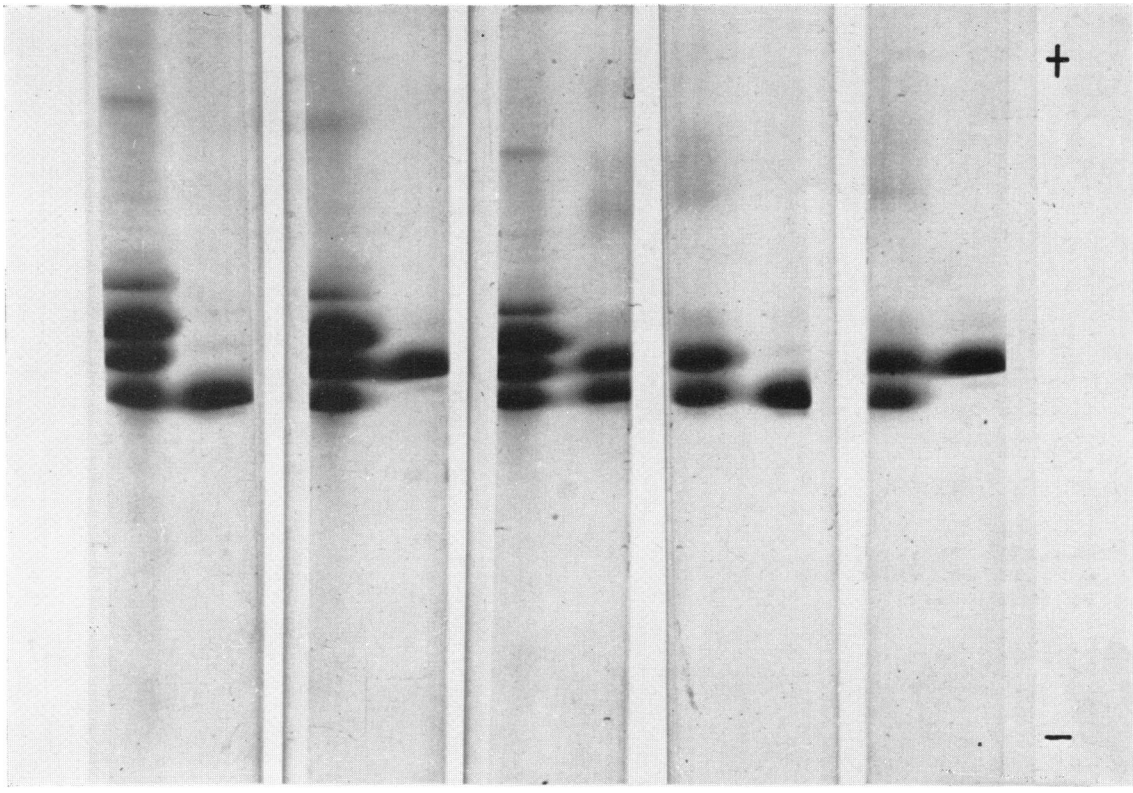
(2) Separation and isolation of fractions f2(a)1 and f2(a)2 under acid conditions. Conc. HCl (16ml.) was added to the clarified extract with rapid stirring, followed by 1·75vol. (1560ml.) of acetone. The precipitate that formed was recovered by centrifugation and washed once in 300ml. of a solution similar to that from which it was precipitated [i.e. ethanol–water (3:1, v/v) containing guanidinium chloride (10%), with 1·75vol. of acetone added]. The precipitate was then washed once in 200ml. of acidified acetone (200ml. of acetone plus 0·1ml. of conc. HCl), three times in acetone and dried under vacuum. The yield of fraction f2(a)2 was approx. 460mg. The supernatant remaining after the precipitation of fraction f2(a)2 contained fraction f2(a)1, which was recovered by adding 1l. of acetone. The precipitate was recovered by centrifugation, washed three times in acetone and dried under vacuum. The yield of fraction f2(a)1 was 400mg.

(3) Separation and isolation of fractions f2(a)1 and f2(a)2 at pH7. Acetone (890ml.) was added to the clarified supernatant, and the precipitate that formed was recovered by centrifugation, washed three times in acetone and dried under vacuum. The yield of fraction f2(a)2 was 560mg. The supernatant remaining contained fraction f2(a)1, which was isolated by adding 1·8l. of acetone. The precipitate was washed and dried as described above. The yield of fraction f2(a)1 was 660mg. The higher yields obtained with this method at pH7 are due to a non-protein contaminant that is apparently not precipitated from acid solutions. This may be removed by redissolving the proteins in water (1mg./ml.) and precipitating with 8vol. of acetone. The analyses given in this paper, however, are from fractions that have not been reprecipitated in this way.

The amino acid analyses of all fractions are given in Table 1. These may be compared with the analyses of corresponding fractions prepared under acid–ethanol extraction conditions given previously (Johns, 1964; Phillips & Johns, 1965).

Starch-gel electrophoresis. Starch-gel electrophoresis at pH2·3 (Johns, Phillips, Simson & Butler, 1961) does not differentiate between fractions f2(a)1 and f2(a)2. However, if the 0·01N-HCl used in this method for the gel, electrode vessels and sample application also contains 0·01N-trichloroacetic acid, fraction f2(a)1 aggregates and cannot enter the gel. The two fractions can be well differentiated by this method and all other histone fractions enter the gel under these conditions. Fraction f2(a)1 may also be specifically detected in a mixture of histones, or in whole histone, in this way.

Polyacrylamide-gel electrophoresis. The method used has been described recently (Johns, 1967) and clearly separates the two subfractions f2(a)1 and f2(a)2. However, to make



EXPLANATION OF PLATE 1

Migration of protein is from positive to negative. Polyacrylamide-gel electrophoresis at pH 2·4. A comparison of whole histone, fractions f2(a), f2(a)1 and f2(a)2. For details see the text.

Left-hand side	Right-hand side
(1) Whole histone (20 μg.)	Fraction f2(a)1, method 2 (5 μg.)
(2) Whole histone (20 μg.)	Fraction f2(a)2, method 2 (5 μg.)
(3) Whole histone (20 μg.)	Fraction f2(a), method 1 (10 μg.)
(4) Fraction f2(a), method 1 (10 μg.)	Fraction f2(a)1, method 3 (5 μg.)
(5) Fraction f2(a), method 1 (10 μg.)	Fraction f2(a)2, method 3 (5 μg.)

Table 1. *Amino acid analyses of the various histone fractions*

Amino acids are expressed as moles/100 moles of all amino acids found.

Amino acid	Method 1 (pH 7)	Method 3 (pH 7)		Method 2 (low pH)	
	f2(a)	f2(a)1	f2(a)2	f2(a)1	f2(a)2
Asp	5·5	5·2	6·0	4·4	6·1
Glu	8·9	7·5	9·9	8·1	9·7
Gly	12·3	13·7	9·7	13·7	10·0
Ala	11·0	9·0	12·5	9·0	13·1
Val	6·8	7·6	6·0	7·1	5·9
Leu	10·4	8·7	11·3	9·0	12·0
Ile	5·2	5·5	4·5	5·9	4·6
Ser	2·7	2·5	3·6	2·5	3·5
Thr	4·8	6·0	4·9	6·5	4·1
Phe	1·7	2·3	1·4	2·4	1·1
Tyr	2·6	3·6	2·3	3·5	2·3
Pro	3·1	1·7	4·1	2·1	4·1
Met	0·3	0·7	0·3	0·8	0·1
His	2·7	2·4	2·9	2·1	3·1
Lys	9·7	10·5	9·8	9·7	10·0
Arg	11·9	13·1	10·3	13·0	10·3

comparisons between the various fractions, without using marker proteins, the following modification was developed in which two samples were applied to the same gel. The samples [whole histone, 20 μg./μl.; fraction f2(a), 10 μg./μl.; fractions f2(a)1 and f2(a)2, 5 μg./μl.] were applied to opposite edges of a disk (8 mm. diam.) of Whatman no. 1 filter paper, which was then placed on the surface of the gel. Sample solvent (M-sucrose, 2 mN-acetic acid) was then layered carefully on to the filter paper to a depth of 1 cm. followed by 0·01 N-acetic acid, which is also used for the electrode vessels. The electrophoresis was then carried out as described above. The results for the fractions prepared as described above are shown in Plate 1. The whole histone used was prepared as described previously (Johns, 1967).

Amino acid analyses. Total amino acid analyses were performed by the method of Spackman, Stein & Moore (1958) with a Technicon Auto-Analyser. The samples were hydrolysed for 24 hr. in 6 N-HCl at 110°. No corrections were made for hydrolytic losses. The *N*-terminal amino acid analyses were carried out by the modification of Sanger's (1945) method described by Phillips (1958) and Phillips & Johns (1959). The yield of *N*-terminal groups was, however, very small (approx. 1 mole/150000 g. of protein). It has been reported (Phillips, 1963) that fraction f2(a) histones have a low content of *N*-terminal amino acids and it is now concluded (Phillips, 1966) that this is due to the presence of α-*N*-acetyl groups in these proteins.

DISCUSSION

The histone fractions f2(a)1 and f2(a)2 together constitute the fraction originally described as fraction f2(a) (Johns & Butler, 1962). This was extracted together with fraction f3 by treating deoxyribonucleoprotein with an ethanol–hydrochloric acid solvent and subsequently separated from fraction f3 by dialysis against ethanol. The method described above is a selective extraction of fraction f2(a) only at pH 7, leaving the remaining histone fractions f1, f2(b) and f3 presumably attached to the DNA.

During the preliminary work on the selective extraction of fraction f2(a) it was found that it could be removed quite effectively by using ethanol–water (3:1, v/v) containing sodium chloride (2%). To isolate the proteins more easily, and to avoid the problems associated with the removal of inorganic salts, the guanidinium chloride system was chosen because of its solubility in acetone. However, the ethanol–sodium chloride system may be more convenient for workers who are interested in the remaining histone-depleted deoxyribonucleoprotein, rather than in the isolation of histone fractions.

Fraction f1, the lysine-rich histone, can also be removed from the remaining deoxyribonucleoprotein by using dilute sodium chloride solution (Hindley, 1964) if the guanidinium chloride is first washed out with ethanol and the ethanol subsequently removed by washing with 0·14 M-sodium chloride. This, however, has not been investigated in any detail.

Histone fraction f2(a) is the most hydrophobic of the histone fractions as judged by the criteria of Hatch (1965) and this is probably the reason for its selective extraction with ethanol. It is also the fraction that gave the highest histone/DNA ratio during recombination experiments (Johns & Butler, 1964), and it has been suggested (Johns, 1966) that this fraction may play an important part in maintaining the condensation or supercoiling of the

deoxyribonucleoprotein molecules. It might be predicted therefore that the selective removal of fraction f2(a) will result in the loss of such a structure. Laurence (1966) has also shown that fraction f2(a) has the greatest ability of all the histone fractions to bind the anionic fluorescent dye 8-anilinonaphthalene-1-sulphonic acid, which appears to combine only with the hydrophobic portions of the protein molecule.

Although fraction f2(a) and the subfractions prepared as described above have never been below pH 7, no differences in analyses or electrophoretic patterns have been observed between them and the corresponding fractions prepared by acid–ethanol extraction (Phillips & Johns, 1965). Detailed conformational studies have yet to be carried out, but preliminary results show that fraction f2(a) prepared at pH 7 is completely free of the extended antiparallel β-conformation (E. M. Bradbury & C. Crane-Robinson, personal communication).

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