The Electrophoresis of Histones in Polyacrylamide Gel and their Quantitative Determination

By E. W. JOHNS

*Chester Beatty Research Institute, Institute of Cancer Research:
Royal Cancer Hospital, London, S.W. 3*

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1. A new method has been devised for the separation of the histone fractions of calf thymus by electrophoresis in polyacrylamide gel at pH 2.4. 2. The fractions have been characterized by their relative mobilities with respect to a marker protein, bovine plasma albumin. 3. A method has been developed for the quantitative determination of the separated histone fractions by measuring the colour yields of dye-histone complexes formed in the gel.

The histones of calf thymus have been separated into five major fractions, designated f1, f2(a)1, f2(a)2, f2(b) and f3, by the selective extraction of deoxyribonucleoprotein with various solvents, followed by acetone precipitation (Johns, 1964; Phillips & Johns, 1965). These five fractions, which comprise about 20% each of the total histone, are quite distinct, and have all been characterized by total and *N*-terminal amino acid analyses, and by starch-gel electrophoresis at pH 2.3 (Johns, 1966).

It would appear from previous work in our Laboratory (Hnilica, Johns & Butler, 1962; Laurence & Butler, 1965) and from the work of others (Hnilica & Busch, 1963) that these fractions occur in all mammalian somatic cells so far examined, regardless of their differing functions. Palau & Butler (1966) have shown that these five fractions are present in trout liver, representing life prior to the emergence of mammals. The question therefore arises: do the quantities of the various histones change in different cells, depending perhaps on the state of differentiation or activity?

One approach to this problem is to attempt to measure quantitatively the separations obtained with whole histone on electrophoresis in the various stabilizing gels.

A new method was therefore developed for the electrophoresis of histones in polyacrylamide gel at about pH 2 to minimize aggregation effects, which are known to occur above pH 3 and below pH 1 (Davison & Shooter, 1956).

The present paper deals with the electrophoretic method and the quantitative determination of the bands obtained with calf-thymus histone.

Starch-gel electrophoresis was not used because of the considerable trailing effect due to the adsorp-

tion of the histones; however, Hnilica, Edwards & Hey (1966) have indicated that this may be overcome by the use of aluminium chloride.

MATERIALS AND METHODS

Reagents. Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were obtained from Kodak Ltd. (Kirkby, Liverpool) and used without further purification.

Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Silicone Repelcote water-repellent was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex). All other reagents were of A.R. grade where possible.

Solutions. The monomer solution contained acrylamide (40%, w/v) and *NN'*-methylenebisacrylamide (0.6%, w/v). Catalyst 1 solution was 0.5% (v/v) *NNN'N'*-tetramethylethylenediamine in 4.6*N*-acetic acid. Catalyst 2 solution was 0.6% (w/v) ammonium persulphate. The monomer solution and catalyst 1 solution could be stored at room temperature, but catalyst 2 solution had to be made up freshly as required. The staining solution was 0.5% (w/v) Naphthalene Black in *N*-acetic acid. The sample solvent was *m*-sucrose in 2 *MN*-acetic acid.

Preparation of whole histone. The calf-thymus histone used in these experiments was prepared as described by Johns, Phillips, Simson & Butler (1961) with the following modification. After clarification of the acid extract the histone was precipitated with 6 vol. of acetone, washed twice in acetone and dried under vacuum.

Preparation of histone fractions. The histone fractions were prepared by the methods described by Johns (1964), method 2, and Phillips & Johns (1965). The amino acid analyses and starch-gel electrophoretic patterns at pH 2.3 were similar to those described previously (Johns, 1966).

Preparation of rat-liver ribosomal protein. This was prepared by extraction with dilute HCl (Cohn & Simson, 1963) and was kindly given by Dr P. Cohn.

EXPERIMENTAL AND RESULTS

The 18-position electrophoresis apparatus used was constructed in this Laboratory, but resembles many already described (Davis, 1964; Reisfeld, Lewis & Williams, 1962). The upper buffer reservoir (18 cm. $\times$ 10 cm. $\times$ 4 cm. deep) was made of rubber and contained 18 holes in three rows of six. The holes were drilled to take the gel containers, which were 14 cm. lengths of glass tubing (10 mm. external diam. and 9 mm. internal diam.). The tubes were rendered water-repellent by immersion in silicone Repelcote and air-drying. They were held vertical in the apparatus by appropriately drilled holes in a Perspex plate positioned in the lower buffer reservoir. The electrodes were platinum and placed so that they were equidistant from all tubes. The gels were formed in the tubes in the following manner. Monomer solution (80 ml.) was mixed with 40 ml. of catalyst 1 solution and 40 ml. of catalyst 2 solution. After thorough mixing, and as little aeration as possible, the solution was poured into the tubes (previously stoppered at the lower end) to within 2 cm. of the top. After tapping to remove any small air bubbles the solutions were gently overlaid with 0.5 ml. of water, by using a motor-driven syringe running at approx. 0.5 ml./min. The solutions polymerized in about 2 hr. The water was then removed from the top of the gels, and the stoppers from the bottom of the tubes and both buffer reservoirs were filled with 0.01 N-acetic acid. The gels were then equilibrated in this solution by applying 175 v d.c. for 3-4 hr., during which period the buffer reservoirs were refilled once with fresh 0.01 N-acetic acid. The measured pH of the gel after equilibration was 2.4 and the concentration of acetic acid in the gel about 0.9 N.

After equilibration the buffer reservoirs were again refilled with fresh 0.01 N-acetic acid and the samples (whole histone, 20 μ g.; histone fractions, 8 μ g. in 0.02 ml. of sample solvent) applied by carefully underlayering with an Agla micrometer syringe. In some experiments bovine plasma albumin (5 μ g.) was added to each sample as a marker protein. Then 200 v d.c. was applied (cathode in lower reservoir) for 4-5 hr., during which time the current increased from 50 to 55 ma.

The gels were recovered by cracking the glass tubes in a vice, a procedure that has been found much more convenient than the conventional methods of rimming with hypodermic needles (Reisfeld *et al.* 1962) or using compressed air (Shepherd & Gurley, 1966). The gels are strong and are not harmed by this procedure. They were then stained overnight (18-20 hr.) in the staining solution, rinsed in water and destained electrically as follows. A layer of cotton wool about 1 cm. thick soaked in N-acetic acid was placed in the bottom

of a Perspex tray (20 cm. $\times$ 30 cm. $\times$ 5 cm. deep). Four slits (14 cm. $\times$ 0.5 cm.) were cut in the cotton wool about 6 cm. apart and gels were placed in them. Platinum electrodes (15 cm. long) were positioned at either end of the tray on the cotton wool and 175 v was applied perpendicular to the gels, giving a current of about 150 ma. Destaining was complete in about 30-40 min. The precise conditions will, of course, vary with the dimensions of the cotton-wool pad and with its content of acetic acid, but care must be taken that the gels do not overheat or dry out.

The results obtained from such an experiment are shown in Plate 1(a), gels 1-6. The fastest-moving band is fraction f2(a)1 followed by fraction f2(a)2. Fractions f3 and f2(b) then run together as the third band and the slowest-migrating histone band is f1. The bovine plasma albumin, which migrates at less than half the speed of the histones, was added to characterize the bands more fully by measuring their relative mobilities with respect to a marker protein. Relative mobility in this case is defined as distance fraction migrated/distance bovine plasma albumin migrated. However, even in polyacrylamide gel the relative mobilities of the fractions were dependent on the amount of whole histone or fraction applied. Consequently a range of values were obtained for the relative mobilities of the various histone fractions, which, however, do not overlap in the range 20-40 μ g. of applied whole histone and so may still be used to characterize the histone fractions further. The results are summarized in Table 1.

Attempts were also made to demonstrate the considerable heterogeneity of calf-thymus histones claimed by some authors (Rasmussen, Murray & Luck, 1962), by increasing the amount of whole histone applied to 150 μ g., and by increasing the time of migration to 9 hr. The results are shown in Plate 1(b), which shows that under these conditions

Table 1. *Relative mobilities of histone fractions in polyacrylamide gel*

The relative mobility is defined as distance fraction migrated/distance bovine plasma albumin migrated under the conditions given in the text. The ranges of values given are for 20-40 μ g. of applied whole histone, or 4-8 μ g. of any fraction. A 5 μ g. sample of bovine plasma albumin was always added. The ranges are the maximum and minimum values obtained under these conditions for five separate runs of whole histone and six separate runs of each of the fractions.

Fraction ...	Relative mobility				
	f1	f2(b)	f3	f2(a)2	f2(a)1
Maximum	2.20	2.60	2.60	2.85	3.10
Minimum	2.05	2.25	2.25	2.65	2.90

very few minor bands can be seen. It is estimated that 1 μ g. of protein would be visible under these conditions, and that the minor bands which are just visible would not account for more than 1–2% of the original protein applied.

To demonstrate that the method is capable of separating a more complex mixture of basic proteins, rat-liver ribosomal proteins (100 μ g. in 0.05 ml. of sample solvent) were run under the same conditions as the whole histone and histone fractions, and the result is shown in Plate 1(a), gel 7.

Quantitative determination of protein bands. The stained protein bands, and a blank of approximately the same size, were cut from the gel with a scalpel blade. This was more easily carried out if the gel was partially frozen by immersing it in a mixture of ethanol–solid carbon dioxide for about 15 sec. and then cut by using a sawing motion with the blade. The gel sections were then placed in tubes and broken up with a glass rod, and 3 ml. of dimethyl sulphoxide was added to each. The tubes were heated in a water bath at 50° for 2 hr., cooled and centrifuged, and the clear supernatants were measured in a spectrophotometer at 600 m μ against the appropriate blanks. It has since been found that the colour is extracted equally efficiently by leaving the tubes overnight (18–20 hr.) at room temperature. It is important, however, that the gel is adequately broken up in order to extract the colour completely. The colour yields of the various fractions were expressed as percentages of total colour yield. The results of one such experiment carried out in triplicate on one sample of whole histone is given in Table 2.

To convert these colour yields into relative amounts of protein it was then necessary to construct standard curves of protein applied, against colour yield, for each of the fractions. This was done by applying various amounts of each fraction (4, 8 and 12 μ g.) to the gel, running in the usual manner, staining, destaining, cutting, eluting and

measuring as described above. These experiments were all carried out in duplicate and blanks were cut from all gels. The results, which are the averages of the duplicate determinations, are shown in Fig. 1. The results given in Table 2 were then converted into protein concentration expressed as a percentage of total protein measured. These results are given in Table 3, together with the average percentage yields of the various fractions obtained by the two preparative methods (Johns, 1964), and it can be seen that they are in reasonable agreement. One assumption that had to be made was that the composite band containing fractions f2(b) and f3 contained equal amounts of these proteins, and thus an average of the two curves in Fig. 1 was taken to convert the colour density of this band into protein concentration.

Fig. 1. Standard curves of protein concentration against colour yield (600 m μ , 4 cm. cell) of the protein-dye complex, for the five histone fractions: ○, f2(a)2; △, f3; □, f2(a)1; ●, f1; ▲, f2(b).

Table 2. Measurement of stain taken up as complexes by the electrophoretically separated fractions of calf-thymus histone

The colour yields were measured at 600 m μ and are expressed as percentages of total colour yield. Three separate determinations on the same whole histone are given.

Fraction	...	Colour yield (% of total)			
		f1	f2(b)+f3	f2(a)2	f2(a)1
1		13.0	37.0	30.0	20.0
2		13.0	38.0	30.5	18.5
3		12.5	37.0	30.5	20.0

EXPLANATION OF PLATE 1

Migration of protein is from positive to negative. (a) Comparison of whole histone, the five histone fractions and rat-liver ribosomal protein. In gels 1–6 the slowest-migrating band is due to added bovine plasma albumin (5 μ g.). (1) Whole histone (20 μ g.); (2) f1 (8 μ g.); (3) f2(a)1 (8 μ g.); (4) f2(a)2 (8 μ g.); (5) f2(b) (8 μ g.); (6) f3 (8 μ g.); (7) rat-liver ribosomal protein (100 μ g.). (b) Effect of increasing the time of migration and varying the amount of whole histone added. The migration time was 9 hr., twice that of the standard procedure. (1) 20 μ g.; (2) 40 μ g.; (3) 80 μ g.; (4) 100 μ g.; (5) and (6) 150 μ g.

Table 3. *Relative yields of histone fractions*

Average values for the colour yields of the fractions given in Table 2 were converted into protein concentration by using the standard curves given in Fig. 1. All values are expressed as percentages of total yield. The corresponding values obtained by preparative methods (Johns, 1964) are given for comparison.

Histone fraction	Relative yield	
	Preparative methods (average of methods 1 and 2)	Polyacrylamide gel
f1	18	17
f2(b)	25	45
f3	21	
f2(a)2	18	19
f2(a)1	18	19

DISCUSSION

The electrophoretic method described here for the separation of histones in polyacrylamide gel is carried out in an equilibrated system with only 0.01 *N*-acetic acid for buffer solutions, in contrast with many so-called 'discontinuous systems' that have various pore sizes in the gel (Neidle & Walsch, 1964) and different buffer solutions in the gel and electrode vessels (Shepherd & Gurley, 1966). These discontinuous systems are employed to give band-sharpening and consequently more definition, but it has been pointed out by Hjertén, Jerstedt & Tiselius (1965) that equally effective results can be obtained with an equilibrated system by using the band-sharpening technique described by Haglund & Tiselius (1950). In this method the sample is applied in a solution about one-fifth of the ionic strength of the buffer solutions, giving less conductivity and therefore a higher voltage gradient across the applied sample. This technique has been applied in the method described here and found to be extremely effective.

The electrophoretic method described above also constitutes a new qualitative test for histones, and with the aid of a marker protein a particular histone fraction may be identified by its relative mobility.

It is extremely difficult to compare the mobilities of the histone fractions obtained by using these methods with those obtained by other authors. The same histone fractions, prepared in this Laboratory, when run in polyacrylamide gel at pH 2.9 according to the method of Shepherd & Gurley (1966), have entirely different relative mobilities with respect to one another. For example, under the conditions described in this paper fraction f2(a)1 is the fastest-moving band, whereas under the conditions of Shepherd & Gurley (1966) at pH 2.9 it is one of the slowest-moving bands.

These differences probably reflect the strong tendency for fraction f2(a)1 and other histones to aggregate under a variety of conditions. It is also noteworthy that under the conditions described here, i.e. with a 20% gel, at pH 2.4, all fractions migrate freely into the gel, leaving no protein at the origin, whereas at pH 4.2 Cruft (1964), using a 10% gel, found large aggregates of β -histones [arginine-rich, equivalent to fractions f3 and f2(a)1] unable to enter the gel.

The quantitative determination of the separated bands agrees reasonably well with the results obtained by preparative methods. It should now be possible to obtain an estimation of the amounts of these fractions in other tissues on quantities of whole histone as low as 50 μ g. The band containing fractions f2(b) and f3 presents some difficulty with preparations from tissues other than calf thymus where the ratio of the two will not be known and thus the colour yield cannot be converted accurately into protein concentration. However, fractions f2(b) and f3 have proline *N*-terminal groups and alanine *N*-terminal groups respectively, the two main end groups found in the whole histone, the other fractions having acetyl *N*-terminal groups (Phillips, 1963; Phillips & Johns, 1965). Thus it would be possible to obtain the molar ratio of the two in this way. For purely comparative work, however, percentage of total colour density of the various bands may be sufficient, and changes in the amounts of the fractions in whole histones from various sources could be detected in this way.

No evidence has been found with these methods for the considerable heterogeneity of calf-thymus histones suggested by some authors (Rasmussen *et al.* 1962). The relative simplicity of these electrophoretic patterns, and that of the *N*-terminal amino acids (Phillips, 1963), starch-gel electrophoretic patterns (Johns, 1966) and peptide composition (Phillips, 1966), suggests that there are a limited number of major histone components. The minor bands seen in the gel pictures of the individual fractions (Plate 1a) are probably due to a slight cross-contamination with the other known fractions, as suggested by work on the countercurrent distribution of these fractions by Butler, Power & Palau (1967). That the present method can separate a more complex mixture of basic proteins is well demonstrated by the separation of the rat-liver ribosomal proteins. It is possible that there is a microheterogeneity due to small changes in the amino acid sequences that could not be detected by these methods, but more work on the primary structure of pure fractions would be required to prove this.

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