HISTONES WITH HIGH LYSINE CONTENT

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This paper deals with some basic proteins of cell nuclei which differ in a number of ways from the classical histones of Kossel. Two characteristics of a histone noted by Kossel are its high arginine content and its way of being precipitated on addition of an excess of ammonium hydroxide (1–2). A high arginine content would tend to give a protein a basic isoelectric point and precipitability in ammonium hydroxide is also evidence of a basic isoelectric point. That a histone actually has a basic isoelectric point is shown by its being precipitated at about pH 10.6 by addition of sodium hydroxide and by its redissolving when an excess of alkali is added (3). In Kossel's time an excess of hydrochloric or sulfuric acid was always used to free a histone from its combination with desoxyribonucleic acid and it might be thought that the insolubility of a histone at its isoelectric point should be attributed to the denaturing effect of treatment with strong acid. More recently, however, histone has been dissociated from nucleic acid by a high concentration of sodium chloride at neutrality and then separated from the nucleic acid by high speed centrifugation, which sediments the nucleic acid, leaving histone in the supernate (3). Histone prepared in this way was also found to be insoluble at its isoelectric point so that there is no reason to attribute this property to denaturation.

The basic proteins to be described in this paper are, like the classical histones, derived from the cell nucleus but differ from them in having a low arginine content, an exceedingly high lysine content, and in being soluble in the isoelectric region.

We first prepared these lysine-rich proteins nearly 3 years ago and most of their properties which we shall describe were already noted then. On looking over the older literature at that time we noted that lysine-rich material having much in common with the substances which we were preparing had already been described by Felix in 1921 (4). The next paper on this subject was by Kossel and Schenk in 1928 (5). In this paper a "cyprinodipeptone" prepared from the testes of the carp was described. Analysis showed that 46.80 per cent of the nitrogen of this substance was lysine nitrogen and 0.36 per cent was arginine nitrogen. We have returned to this subject during the past year because experiments on N^{15} uptake and retention have shown that the synthesis of histone (and here we mean the protein isolated by precipitation at pH 10.6) is in some

way connected with over-all protein synthesis in pancreas, liver, and kidney tissues. These experiments should be extended to include a consideration of the lysine-rich basic proteins of nuclei. For this purpose a better method of preparation is required. While devising another method of preparation a further study of the lysine-rich protein was made.

In the method of preparation first used basic proteins were separated from nucleic acid by adding an excess of sulfuric acid to isolated nuclei. Arginine-rich and lysine-rich histones were then separated from each other. Arginine-rich histone was precipitated at pH 10.6 and what failed to precipitate was then salted-out with saturated NaCl, leaving lysine-rich histone in solution.

In the more recently devised procedure no strong acid is used. Histones are dissociated from DNA at neutrality by concentrated NaCl. A solution of 1 M NaCl and 0.05 M citric acid extracts histone from isolated nuclei, leaving the DNA within the nuclei. The quantity of histone extracted depends upon the concentration of NaCl, increasing rapidly as the concentration of NaCl is raised from 0.75 M to 1.25 M. In 0.05 M citric acid containing 0.5 M NaCl no more than traces of the arginine-rich histones are extracted, but under these conditions lysine-rich histone is extracted. The quantity of lysine-rich histone extracted does not depend on the salt concentration, for the same amount is extracted between 0.14 M and 0.50 M NaCl. Concentration of citric acid is a factor influencing the quantity of lysine-rich histone extracted, increasing amounts being extracted as the concentration is raised from 0.025 to 0.10 M. Indeed some lysine-rich histone may have been extracted from the nuclei while they were being isolated, although in the course of this procedure the citric acid concentration was held down to 0.005 M with the pH of the medium at 3.5. The relative ease with which lysine-rich histone dissociates from desoxyribonucleic acid may well be one of its most significant properties.

The quantity of lysine-rich histone prepared from isolated thymus nuclei by extraction with H_2SO_4 is about 20 per cent of the arginine-rich histone isolated. The quantity extracted with citric acid represents about 8 per cent of the nuclear mass. These yields are about the same.

The composition of lysine-rich histone extracted from thymus nuclei with H_2SO_4 is given in Table I. Composition of arginine-rich histone precipitated at pH 10.6 is given for comparison. It will be seen that the two proteins differ with respect to their contents of other amino acids as well as in their arginine and lysine contents. Unlike the arginine-rich histone, the lysine-rich histone contains no histidine or cystine. The two types of histone contain similar amounts of valine, while the lysine-rich histone contains more proline, alanine, and serine, and less of the other amino acids than the arginine-rich histone.

The lysine-rich material, analysis of which is given in Table I, was subjected to zonal electrophoresis on glass in a phosphate buffer at pH 6.8. The pattern obtained showed that a number of components were present but one especially

sharp peak was found. The basic amino acid composition of this material was determined (No. 1 of Table II) and it was found to contain about one-half the arginine and somewhat less lysine than was in the unfractionated material.

Lysine-rich proteins extracted with citric acid from isolated nuclei were analyzed for their basic amino acid contents. The results are given in Table II

TABLE I

Amino Acid Composition of the Arginine-Rich and Lysine-Rich Histones

The amino acid composition was determined chromatographically (6–8). Starch columns were used, and the basic amino acid composition of the lysine-rich histone was confirmed using a column of dowex-50. Proteins were hydrolyzed by refluxing for 20 hours with 200 times the sample weight of 6 N HCl twice distilled in glass. The nitrogen contents of these preparations were 19.2 per cent for the arginine-rich histone and 17.4 per cent for the lysine-rich histone.

Amino acid	Moles amino acid per 100 moles N		Gm. amino acid residue per 100 gm. protein	
	Arginine-rich histone	Lysine-rich histone	Arginine-rich histone	Lysine-rich histone
Leucine	5.11	3.28	7.93	4.64
Isoleucine	2.16	0.99	3.35	1.41
Phenylalanine	1.42	0.55	2.86	1.01
Valine	2.72	3.00	3.70	3.71
Methionine	0.67	0.15	1.21	0.24
Tyrosine	1.60	0.55	3.58	1.12
Proline	2.51	5.09	3.34	6.18
Glutamic acid	6.18	2.66	10.9	4.29
Alanine	7.60	12.7	7.41	11.3
Threonine	3.85	2.92	5.33	3.69
Aspartic acid	3.82	1.60	6.02	2.30
Serine	3.17	4.02	3.79	4.38
Glycine	6.13	4.62	4.80	3.30
Ammonia	4.97	2.99		
Arginine	7.07	1.98	15.1	3.86
Lysine	7.22	22.4	12.7	35.9
Histidine	1.18	0.0	2.22	0.0
Cystine	0.28	0.0	0.74	0.0
Nitrogen recovery	98.7	97.8		

(Nos. 2 to 5). The lysine contents, 25 to 26 per cent, though high, are definitely lower than that found for the protein extracted with sulfuric acid. Similarity in the yields obtained by extraction with sulfuric and citric acids would indicate that the materials have much in common. If this is indeed the case, then the lower lysine content of the material extracted with citric acid could be explained either by presence of a contaminant with low lysine content or by the loss of lysine from the protein during the preparation.

Histones have been found to be lacking in tryptophane (3). Absence of tryptophane is also a characteristic of the lysine-rich basic proteins.

Considering the absence of tryptophane from both the arginine-rich and the lysine-rich histones and the presence of 3 times as much tyrosine in the former as in the latter, it would be expected that at the protein maximum of 276 mμ the arginine-rich histone would have a much higher extinction. Its extinction

TABLE II

Amino acid composition of the histones was determined by chromatography on dowex-50 (8).

Preparation No.	Nitrogen content	Moles amino acid per 100 moles N			Gm. amino acid residue per 100 gm. protein		
		Histidine	Lysine	Arginine	Histidine	Lysine	Arginine
	per cent						
1	16.1		17.5	0.99		26.0	1.78
2	15.6	0.23	18.7	1.72	0.35	26.6	2.98
3	15.7	0.33	17.5	0.99	0.50	25.2	1.54
4	15.1	0.56	18.5	1.40	0.82	25.5	2.35
5	16.0	0.61	17.1	1.65	0.95	24.9	2.95
6	16.6	1.10	7.47	4.84	1.77	11.4	9.00
7	16.0	1.28	7.90	5.93	2.03	11.6	10.6
8	17.0	1.03	6.81	5.91	1.71	10.6	11.2

coefficient at this wave length is in fact about 3 times that of the lysine-rich histone.

We have referred to the lysine-rich protein as being a nuclear constituent. One reason for doing so is that the protein was prepared from nuclei isolated by the citric acid procedure. This reason could, however, be misleading, for if the lysine-rich protein were a cytoplasmic constituent, being basic, an amount equal to 8 per cent of the nuclear mass could well be taken up by the free acidic groups of DNA in much the same way that a basic dye or extra histone combines with these isolated nuclei. Proof that the lysine-rich protein is present in the intact cell nucleus is that this protein is found in thymus nuclei isolated by the Behrens procedure; *i.e.*, in non-aqueous media (9). Once it is known that the lysine-rich protein is a nuclear constituent, it seems probable that such a basic protein would be located in the chromosomes, combined with DNA.

Proteins similar to the lysine-rich histone of thymus nuclei have been prepared from nuclei isolated from beef liver and turtle erythrocytes. It may well be, therefore, that lysine-rich histones are as widely distributed as are arginine-rich histones.

We have referred to arginine-rich and lysine-rich histones as if there were a definite cleavage between two groups of histones. The possibility, however, should be considered that there is a continuous series of histones of which the

types we have considered are the extremes at either end. We have looked for such intermediate types but have not found them. Since lysine-rich histone is more readily dissociated from combination with DNA than is arginine-rich histone, we looked for intermediate types under conditions when the arginine-rich histone is just beginning to be released from combination with DNA.

These conditions are of two kinds: in one the sodium chloride concentration has reached a point at which arginine-rich histone begins to be extracted from isolated nuclei; in the other the citric acid concentration is increased to a point at which it begins to release some arginine-rich as well as lysine-rich histone. In 0.05 M citric acid, 0.50 M NaCl removes lysine-rich histone and only traces of histone precipitable at pH 10.6. Nuclei which have been extracted with 0.05 M citric acid–0.50 M NaCl, release a moderate quantity of histone on extraction with 0.05 M citric acid–0.73 M NaCl. When the same nuclei are extracted still another time, now with 0.05 M citric acid–0.89 M NaCl another fraction of histone is extracted. The fractions extracted in 0.73 M and 0.89 M NaCl are rapidly dialyzed in the cold and lyophilized. Their basic amino acid contents are given in Table II (Nos. 6 and 7). It can be seen that the lysine contents of these two fractions are the same, but that the 0.89 M NaCl fraction has a higher arginine content. There is here no evidence for an intermediate between lysine-rich and arginine-rich histones but there is evidence showing that the latter are heterogeneous with respect to their arginine contents. When sodium chloride concentration is kept at 0.14 M and citric acid concentration is increased, in 0.2 M citric acid enough histone precipitable at pH 10.6 is extracted to collect and analyze. The basic amino acid contents of this histone are given in Table II (No. 8). Here again there is no evidence for an intermediate; the arginine content is high and the lysine content is about the same as in other arginine-rich histones.

A number of properties of lysine-rich histone will now be described, a comparison being made in each instance with arginine-rich histone.

Lysine-rich histone, like previously prepared histones, combines with polymerized DNA to give a fibrous precipitate. This combination occurs at neutrality and also in a medium as basic as pH 8.5, indicating clearly that these histones have distinctly basic properties. Satisfactory conditions for observing formation of a fibrous precipitate are the following: 0.5 ml. containing 2 mg. DNA is mixed with 1.0 ml. of a 0.1 M buffer (phosphate if the final pH is to be 7.0 and veronal if it is to be 8.5) and to the mixture is added 1 ml. of histone (containing 2.4 mg.).

An arginine-rich histone is not precipitated when a solution containing it is added to an equal volume of 0.34 M $HgSO_4$–1.88 M H_2SO_4 and the mixture is then heated to 60°C. Since the generality of proteins does precipitate under these conditions the non-precipitability of histones by Hg^{++} in acid is a distinguishing characteristic (3). Perhaps this property of histones provides for

a need which Kossel had in mind when he wrote, "One would base the classification (of histones) upon one or more reactions by which the histones would be distinguished from the other proteins, but such a reaction is not known." The value of the reaction with Hg^{++} in acid when classifying histones is seen when globin is considered. This protein which occupies a place in cells quite different from that of histone has, nevertheless, frequently been classified with the histones. Among the reasons for not accepting this classification, one of the clearest is the fact that globins are precipitated by Hg^{++} in acid. The lysine-rich basic proteins of the cell nucleus, like other histones, are not precipitated by Hg^{++} in acid.

Another protein precipitant, trichloroacetic acid (TCA), precipitates both arginine-rich and lysine-rich histones but a much higher concentration of TCA

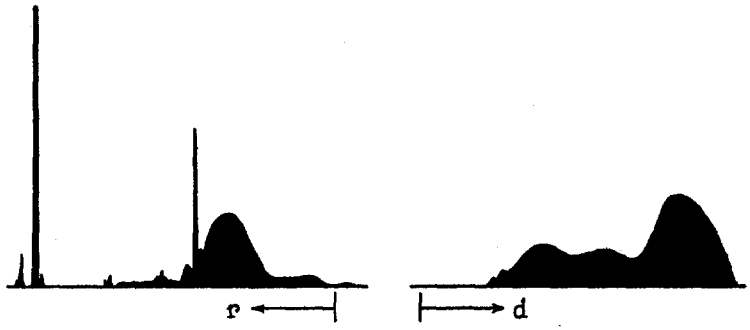

FIG. 1. Patterns of arginine-rich histone (preparation 7 of Table II) after electrophoresis in potassium phosphate buffer pH 6.8, $\mu = 0.2$ for 200 minutes at 9.0 volts/cm. in the apparatus described by Longsworth (10). r = rising pattern, d = descending pattern.

is required to precipitate the latter. It takes almost twice the concentration of TCA (13 per cent final concentration) to precipitate lysine-rich histone as suffices to precipitate arginine-rich histone (7.5 per cent).

A further difference between the two types of histone appears when their color reactions with ninhydrin are compared. For the same quantity of protein the lysine-rich histone gives about twice the optical density obtained with arginine-rich histone.

Lysine-rich histone prepared by extraction of nuclei with sulfuric acid is strikingly deliquescent. Neither arginine-rich histone nor the lysine-rich histone prepared by extraction with citric acid shows this property.

The electrophoretic behavior of the two types of histone was investigated, and the patterns produced by the two types under the same conditions are quite different. The rising and descending patterns of both histones are far from being mirror images of each other and therefore the results must be

interpreted with caution. Both types of histone appear to consist of a mixture of components. The arginine-rich histone produces two major boundaries, one very sharp, on the rising side, and a series of broad peaks on the descending side (Fig. 1). The lysine-rich histone shows one principal exceedingly sharp boundary on both rising and descending sides with a number of minor boundaries. The major peak may consist of a number of components as it appears to break up in time on the descending side (Fig. 2). The large stationary boundary shown by the lysine-rich histone is an anomaly, as it was not observed in zone electrophoresis.

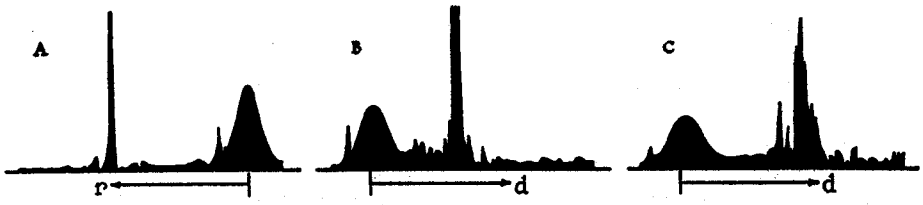

FIG. 2. Patterns of lysine-rich histone (preparation 5 of Table II) after electrophoresis in potassium phosphate buffer pH 6.8, $\mu = 0.2$ at 9.1 volts/cm. A, rising pattern after 144 minutes; B, descending pattern after 144 minutes; C, descending pattern after 211 minutes. The rising pattern after 211 minutes is not shown because there is no change in the pattern except for the distance of migration.

EXPERIMENTAL

The histones for which analyses are given in this paper were prepared from thymus nuclei isolated in citric acid (3). Fresh calf thymus was obtained as quickly as possible after the death of the animal, transported to the laboratory in ice, and all further operations were carried out in a cold room at 2°C. except when otherwise indicated.

Preparation of the Lysine-Rich Histone Extracted with Sulfuric Acid.—Thymus tissue was minced, placed in a Waring blendor with an equal weight of ice and 2½ volumes of 0.05 M citric acid, and blended for 6 minutes. The homogenate was diluted with about 2 volumes of 0.01 M citric acid, strained through gauze and flannelette, and then centrifuged. The sediment, which contained the nuclei, was washed about four times by resuspension in 0.01 M citric acid and centrifugation at lower speed. By this procedure a microscopically clean preparation of nuclei is obtained.

The histones were extracted by adding to the suspension of nuclei an equal volume of 0.5 N H_2SO_4 and stirring for 10 minutes. The suspension was centrifuged and the supernatant dialyzed overnight against distilled water. The solution was centrifuged and the clear supernatant was brought to pH 8.5 with alkali. At this point a scanty precipitate usually forms which is removed by centrifugation. The supernatant was brought to 30°C. and alkali added to pH 10.6 to precipitate the arginine-rich histone. After standing for 1 hour at 30°C., the suspension was centrifuged. (The precipitate

of arginine-rich histone may be purified by resolution in acid, dialysis, and reprecipitation at pH 10.6.)

The supernatant from the first precipitation at pH 10.6, which contains the lysine-rich histone, was brought to pH 6 with acid and 3 volumes of alcohol were added to precipitate the histone. The suspension was allowed to stand overnight at room temperature. The precipitate was centrifuged, redissolved in water with the addition of a few drops of HCl, and dialyzed against distilled water overnight in the cold. The solution was brought to pH 6 with acetic acid, and then saturated with NaCl to remove traces of arginine-rich histone. After standing overnight the suspension was centrifuged, the supernatant dialyzed against distilled water, and then lyophilized.

A sample of this preparation was subjected to zone electrophoresis on glass powder in a phosphate buffer, pH 6.8, ionic strength 0.2 (11). The dimensions of the block of glass powder were 70 × 8 × 1 cm^3. 320 volts were applied for 98 hours; the current was 14 milliamperes. After electrophoresis the block was cut into 2 cm. segments which were extracted with water; protein was located by means of the extinction of the solutions at 230 mμ. An especially sharp peak was found to have migrated 10 cm. toward the cathode. The two fractions containing most of this material were combined, dialyzed against water to remove buffer, and precipitated with 3 volumes of alcohol. The precipitate was washed with alcohol and ether and dried. The protein obtained in this way represented about 10 per cent of the starting material. It was hydrolyzed and its basic amino acid composition is given in Table II (preparation 1).

The Arginine-Rich Histone of Table I.—This preparation was made from isolated thymus chromosomes (12). The chromosomes were extracted with 1 M NaCl containing 1 per cent citric acid. The suspension was centrifuged, the clear supernatant dialyzed against distilled water overnight, and the histone precipitated by the addition of alkali until maximum precipitation was obtained (pH 9.2). The precipitate of histone was washed with alcohol and ether and dried at room temperature *in vacuo*.

Other Histone Preparations of Table II

Preparations 2, 6, and 7.—Citric acid nuclei were prepared by the procedure described at the beginning of the experimental section. The nuclei were washed once with 0.05 M citric acid and resuspended in 0.05 M citric acid. A solution of NaCl in citric acid was added slowly with stirring to the suspension to bring the final concentration to 0.5 M NaCl–0.05 M citric acid. The suspension was stirred for 1 hour and then centrifuged. The supernatant solution contained the lysine-rich histone (No. 2, Table II). The nuclear residue was washed once with 0.5 M NaCl–0.05 M citric acid and then extracted with 0.75 M NaCl–0.05 M citric acid (final concentration). This extract contained preparation 6 of Table II. After one washing with the solution used for the second extraction the residue was extracted with 0.89 M NaCl–0.05 M citric acid. This extract contained preparation 7 of Table II. All extracts were dialyzed against 0.025 N HCl in the cold for 3 days. Dilute acid was used for the dialysis in order to prevent autolysis.

The extract containing the lysine-rich histone was brought to pH 10.6 to precipitate traces of arginine-rich histone; the supernatant was brought to pH 8.2, dialyzed against distilled water, and lyophilized. The extracts in 0.75 and 0.89 M NaCl, which

contained arginine-rich histones, were dialyzed against 0.025 N HCl and then against distilled water and finally lyophilized.

Preparations 3 and 4.—These histones were prepared from nuclei isolated in 0.005 M citric acid. Except for the change in citric acid concentration the details of the preparation of nuclei were the same as those given at the beginning of the experimental section. For preparation 3, the nuclei were extracted with 0.05 M citric acid–0.125 M NaCl (final concentration); for preparation 4, nuclei were extracted with 0.1 M citric acid–0.125 M NaCl. The suspensions were stirred for 15 minutes and centrifuged. The supernatants were dialyzed against distilled water overnight, brought to pH 10.6 to remove traces of the arginine-rich histone, brought to pH 7.3, dialyzed against distilled water for 2 days, centrifuged, and lyophilized.

Preparation 5.—This preparation was made in the same way as No. 4.

Preparation 8.—Nuclei were isolated in 0.005 M citric acid and extracted with 0.2 M citric acid–0.125 M NaCl. The extract was dialyzed against water overnight. The small amount of arginine-rich histone which is extracted along with the lysine-rich histone under these conditions was precipitated at pH 10.6. The precipitate was collected, dissolved in water with the addition of acid, and dialyzed against water for 2 days. The pH of the solution was brought to 8, the slight precipitate which formed was centrifuged and discarded. The supernatant was brought to pH 10.6, the precipitate was washed with alcohol and ether and dried (preparation 8).

SUMMARY

1. The preparation and properties of lysine-rich histones, which differ in a number of respects from the classical arginine-rich histones, have been described.

2. Lysine-rich histones, like those previously known, are located in cell nuclei.

3. Lysine-rich histones dissociate more readily from combination with nucleic acid than do other histones.

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