An Electrophoretic Comparison of Vertebrate Histones*

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SUMMARY

We have studied histones from a wide range of vertebrates with high resolution electrophoresis. There are five major electrophoretic fractions of histone in all somatic tissues in all species examined. Two fractions of histone have a constant electrophoretic mobility no matter what their source. Two fractions vary somewhat from class to class of vertebrate; and one fraction, the lysine-rich fraction, varies considerably in mobility, indicating substantial changes in its primary structure during evolution. This interpretation is confirmed by separation of the individual histone fractions and amino acid analysis.

Electrophoretic heterogeneity is found within several of these fractions in good agreement with previous reports on calf histones (Panyim and Chalkley, Biochemistry 8, 3972 (1969)). The electrophoretic heterogeneity within Fractions F2a and F3 is manifested by bands which, while varying in relative intensity from species to species, nonetheless always have a constant mobility. On the other hand the extent of the electrophoretic heterogeneity of the lysine-rich histone group varies among species indicative of a much less stringent demand for a constancy of primary sequence of these molecules relative to the more arginine-rich histone molecules.

There has been no totally unequivocal demonstration of a biological function for histones. It has been variously argued that they are structural proteins of chromosomes (1-3) or that they have a role in the genetic activity of higher organisms (4-6). In the meantime it is clear that additional information on the nature of histones in a diverse group of organisms is needed.

Several workers have examined the similarities or differences of histones from different organs and various species (7-10), and from active and inactive chromatin (11). There were, however, a number of major problems encountered in this work: nucleoprotein preparations are often contaminated with highly active proteolytic enzymes (12-14), the various techniques for separating and analyzing histones were not sufficiently sensitive (15-17), and there was a need for a more precise quantitation of histone fractions. These problems in previous studies have often led to contradictory results. Thus it has been reported on the one hand that histones are essentially constant from species to species (8, 9, 18), and on the other that they can be species and tissue specific (19-22).

We have previously compared histones from several calf tissues (23). If appropriate precautions are taken to ensure the purity of the sample and that it is not degraded, it is possible to describe the complement of histones in terms of five major electrophoretic fractions most of which upon close examination show heterogeneity. The lysine-rich histone fraction however was the only fraction to show a tissue-specific electrophoretic microheterogeneity. This result is also supported by the data of Kinkade and Cole (19) and of Kinkade (20).

This paper describes an extension of the previous work, and data are presented for the total complement of histones of a wide range of vertebrates showing that two histone fractions (F3 and F2a) have electrophoretic mobilities which are independent of the origin of the histones, that two histone fractions (F2b and F2a) show small changes in mobility among different classes of vertebrate, and that histone F1 displays an electrophoretic behavior which is almost species specific. Some of the implications of these results are discussed.

MATERIALS AND METHODS

In general, methods for chromosome isolation followed those described previously (23). However, in previous publications we have simply indicated the modifications of earlier procedures. As the method in its entirety has proved applicable to chromatin isolations from any vertebrate tissue it is presented in detail. Specific modifications for different species are also given.

Tissue

Mammalian tissues were collected immediately after the animals were killed, frozen, and stored at -17°. All other animals were killed by pithing through the foramen magnum. The animal was rapidly dissected, the major vein returning to the heart was severed, and cold 0.9% NaCl solution-0.01 M oxalate injected into the heart. Blood was collected through the severed vein. The NaCl oxalate solution was injected until the blood vessels in most organs turned pale. Normally such a perfusion is complete in about 5 min.

Solutions for Chromatin Isolation

Grinding Medium—This medium contained 0.25 M sucrose, 0.01 M MgCl₂, 0.01 M Tris-HCl, pH 8, 0.05 M NaHSO₃ (added as crystals immediately prior to use).
Washing Medium—This medium contained 0.25 M sucrose, 0.01 M MgCl₂, 0.01 M Tris-HCl, pH 8, 0.05 M NaH₂SO₄, and 0.2 to 0.5% Triton X-100 (depending on particular tissue, the breakage of more fragile nuclei can be reduced by reducing the concentration of Triton X-100).

EDTA-Tris-Bisulfite Solution—This solution contained 0.02 M EDTA, pH 8, 0.01 M Tris-HCl, pH 8, and 0.05 M NaH₂SO₄; mixed without further adjustment of the pH.

Isolation of Nuclei and Chromatin

The frozen tissues were cut into small pieces, soaked in the grinding medium (approximately 1 g of tissue per 20 ml) for a short while before being blended in a Waring blender at (a) 30 volts for 3 min and (b) 60 volts for 1 min. The solution was filtered through four layers of cheesecloth and then two layers of Miracloth.* The filtrate was centrifuged at 480 × g for 10 min. The supernatant was discarded and the pellet was washed with the washing medium (approximately 10 ml of washing medium per g of tissue; although this is not very crucial and larger volumes can be used) by mixing at 20 volts for 2 to 3 min in a Waring blender. The solution was centrifuged at 480 × g for 10 min. The washing process is repeated until the supernatant is clear; normally two washings are adequate. The pellet was mixed in approximately 10 volumes of 2.2 M sucrose solution which contains the same ionic components as the washing medium. After homogenizing (two to three strokes) with a Potter-Elvejem homogenizer (this will not break the nuclei), the nuclei in the sucrose solution were layered on top of a 2.4 M sucrose solution containing the same ionic composition as the washing medium. The suspension of nuclei in 2.2 M sucrose occupies about 70% of the volume of the centrifuge tube. The solution was centrifuged at 40,000 × g for 2½ hours at 4°C in a Beckman model L2-65B preparative ultracentrifuge. After removing the sucrose solution, the nuclear pellet was thoroughly homogenized with a hand homogenizer in 40 to 50 volumes of EDTA-Tris-bisulfite solution and centrifuged at 4,300 × g for 10 min. The supernatant was discarded and the washing with the EDTA-Tris-bisulfite solution repeated. After centrifugation, the sticky pellet was homogenized in 40 to 50 volumes of redistilled, deionized water, and sedimented at 12,100 × g for 10 min. The pellet was treated again with the same amount of water. At this stage all nuclei are broken and the solution should be very viscous. It is advisable to monitor all steps in the preparation with a light microscope and staining with aceto-carmine. All operations described above were performed at 4°C.

Isolation of Histone

The viscous chromatin solution was sheared at maximum speed for 5 min in a VirTis homogenizer to give a clear, nonviscous solution. Histones were extracted from the solution (adjusted to A₂₈₀ = 5.0) by adding 2 N H₂SO₄ to a final concentration of 0.4 N, and stirring in the cold for 1 hour. After centrifugation (12,100 × g for 20 min) histone was precipitated from the supernatant either by dialysis against 4 volumes of ethanol (if a small amount of histone is involved) or by adding 4 volumes of ethanol and standing at −10°C for 24 hours. The histone was recovered by centrifugation at 12,100 × g for 15 min, and washed two times with cold 95% ethanol and dried under vacuum. The yield per g of tissue varied depending on the particular tissue.

Separation of Histone Fractions

The procedure for the separation of whole histones into the five major fractions was based essentially on the methods originally devised by Johns (24) and Phillips and Johns (25) as modified by Panyim (26).

Polyacrylamide Gel Electrophoresis of Histones

Electrophoresis was performed as described in detail by Panyim and Chalkley (27). After completion of electrophoretic separating, the gels were scanned at 600 ml with a Gilford model 2000 spectrophotometer fitted with a gel scanner. The peaks obtained were quantitated with a DuPont electronic curve analyzer. The average values of two independent analyses were used.

RESULTS

Many of the animals used were caught locally. If they were not standard laboratory animals they were kept for several days to ensure their good health. This precaution is of particular importance when isolating histones from cold blooded vertebrates. We have observed a correlation between the appearance of degraded histones upon isolation and the poor health of the animal before killing. Thus, if fish are killed while there is still gill action, but at a time when they are clearly disabled (swimming invertedly or floating, and showing glazing of eyes), then

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1 In the case of very fragile nuclei, the Triton X-100 in "washing medium" was cut down to 0.2% and the first washing accomplished by gently stirring in the centrifuge tubes instead of using a Waring Blender.

2 Chicopee Manufacturing Company, New Jersey.
the histones will give the variable electrophoretic patterns characteristic of degradation.

Fig. 1 shows that five electrophoretic groups of histones are found in the nucleated erythrocytes of healthy buffalo fish, whereas a dramatic and characteristic change in the electrophoretic band pattern is seen when histones are from sick fish. Furthermore, the extent of histone degradation within a given dying fish is clearly tissue dependent as seen in Fig. 2, again showing histones isolated from a sick buffalo fish. Liver histones are relatively intact, erythrocyte histones moderately damaged, and spleen and kidney histones are severely degraded. We would emphasize that this is not a reflection of a tissue-specific histone pattern in vivo, for if histones are isolated from the tissues of healthy fish they consist of the five major histone groups with only minor quantitative differences between the tissues as discussed below.

The changes in band patterns in Figs. 1 and 2 are primarily in decreases in the amounts of some fractions, and in the appearance.
ance of faster moving bands generating as many as seven bands in degraded erythrocyte histones. We have recently described a somewhat similar phenomenon in the calf thymus histone system as a result of the proteolytic degradation of some of the histone fractions (28).

We observed a similar phenomenon with frogs and turtles which were damaged by heat in the mail (so that several control animals died within 36 hours); on the other hand, turtles and frogs from the wild showed characteristically intact histone patterns, notably particularly for their clarity and sharpness. That the histone preparations we describe as degraded do indeed reflect changes away from the patterns of intact histones is further supported by experiments in which we deliberately per-

![Figure 4: Dependence of fish histone mobility on gel urea concentration. The procedure was identical with that described in Fig. 3 except that the urea concentration was varied as indicated. The 0 M urea gel was electrophoresed for 24 hours, the 2.5 M urea gel for 24 hours, and the 6.0 M urea gel for 48 hours; all at 100 volts.

![Figure 5: Fractionation of fish histone. Demonstration of purity by electrophoresis. Fish histones were fractionated by a modification of the method of Johns (36). Unequivocal assignment is made by adding together differing amounts of various fractions.](http://www.jbc.org/)

| Amino acids | Calf thymus histone | Fish (buffalo) liver histone |
|-------------|---------------------|-----------------------------|
|             | F1 | F2b | F3 | F2a | F3 | F1 | F2b | F3 | F2a | F3 |
| Lysine      | 27.3 | 14.3 | 11.4 | 10.7 | 8.7 | 30.4 | 14.8 | 11.2 | 8.9 | 9.5 |
| Histidine   | 0 | 2.3 | 3.0 | 2.4 | 1.8 | 0.9 | 2.3 | 1.7 | 2.7 | 1.8 |
| Arginine    | 2.1 | 8.5 | 10.3 | 13.4 | 13.7 | 2.9 | 7.7 | 9.4 | 13.3 | 13.0 |
| Aspartic acid | 2.6 | 5.4 | 5.6 | 4.7 | 4.4 | 2.9 | 5.4 | 6.4 | 8.4 | 4.2 |
| Threonine   | 6.0 | 6.1 | 4.3 | 6.6 | 6.6 | 3.6 | 6.6 | 4.9 | 6.5 | 6.6 |
| Serine      | 6.0 | 7.5 | 3.5 | 2.9 | 4.3 | 6.4 | 8.1 | 3.0 | 2.7 | 4.6 |
| Glutamic acid | 4.7 | 10.3 | 9.6 | 8.0 | 12.6 | 4.5 | 9.7 | 10.0 | 8.1 | 12.1 |
| Proline     | 8.4 | 4.9 | 3.9 | 1.5 | 4.6 | 8.9 | 4.4 | 4.5 | 1.9 | 4.4 |
| Glycine     | 6.7 | 7.1 | 10.8 | 15.1 | 5.5 | 5.0 | 7.8 | 10.4 | 13.9 | 5.9 |
| Alanine     | 25.4 | 10.2 | 12.0 | 7.8 | 12.6 | 20.5 | 10.1 | 13.5 | 8.0 | 12.6 |
| Cysteine    | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Valine      | 6.0 | 6.4 | 6.6 | 7.9 | 4.7 | 6.3 | 6.0 | 6.6 | 7.0 | 4.8 |
| Methionine  | Trace | 1.2 | 0.4 | 0.7 | 1.3 | 0.3 | 1.2 | Trace | 0.9 | 1.2 |
| Isoleucine  | 0.8 | 4.2 | 4.1 | 4.9 | 5.0 | 1.7 | 4.9 | 3.3 | 5.1 | 5.7 |
| Leucine     | 3.9 | 6.9 | 11.0 | 7.9 | 9.3 | 3.6 | 6.3 | 12.3 | 8.6 | 9.2 |
| Tyrosine    | 0.6 | 2.7 | 2.6 | 3.2 | 2.1 | 0.9 | 2.6 | 1.7 | 3.5 | 2.1 |
| Phenylalanine | 0.6 | 1.9 | 0.9 | 2.2 | 3.0 | 0.7 | 2.0 | 1.1 | 2.4 | 2.6 |

The data in Table I show that the amino acid compositions of the calf thymus histones are quite different from those of the fish liver histones. The differences are particularly striking in the case of alanine, valine, methionine, and isoleucine, which are all present in much higher concentrations in the fish liver histones. The data also show that the calf thymus histones are more rich in lysine and histidine, and less rich in aspartic acid and threonine, than the fish liver histones. This suggests that the calf thymus histones are more rich in basic amino acids and less rich in acidic amino acids, and that the fish liver histones are more rich in acidic amino acids and less rich in basic amino acids.

Fig. 3 shows the electrophoretic mobility of at least one sample from each of the above animals compared to that of calf thymus histones. A
FIG. 6. High resolution electrophoresis of vertebrate histones. Histones were electrophoresed some 20 to 25 cm as described previously (27). Only the bottom 25% of the gels containing the bands are shown.

more detailed analysis of several tissues from each animal is presented in subsequent tables.

It is clear from the data of Fig. 3 that the electrophoretic mobility of some fractions differs and depends upon the species.
from which they were isolated. Thus we are confronted with the problem of identifying fractions of different mobility and relating them to similar histone fractions of calf thymus. We are encouraged in this aim by the observation that there are five major electrophoretic groups in essentially all species examined (birds are a key exception to this rule); although this is not immediately apparent from the data of Fig. 3, in which, on occasion, we see four major electrophoretic species in the standard gel system. However, by exploiting our earlier observations (27) on the effect of urea concentration upon mobility, it is possible to show the existence of five bands in these systems. This is shown in Fig. 4 for fish histones in which only four bands are seen in 2.5 M urea, but in either 0 or 6 M urea the full complement of five histone bands are apparent.

We have described and numbered the five major groups in calf thymus histones previously. That similar fractions make up essentially all species examined (as cited under "Materials and Methods") and comparing them to calf histones. Very similar amino acid analyses are observed (Table I) although it is clear that the lysine-rich histones of fish are significantly different from that of the calf. By running control gels the histones of fish are easily defined electrophoretically; these are shown and numbered in Fig. 5.

The isolation of individual histone fractions is a laborious and time-consuming task, which, moreover, is not easily applied to small amounts of tissue. Since fish contain five histone fractions of comparable chemistry to those of calf thymus we have felt justified in interpolating the data of creatures between fish and cows on an evolutionary scale to ascertain which histone is which. The criteria applied are simple and direct: (a) The doublet band, Fraction F2a1, has an identical mobility in fish and cows (and moreover has exceedingly little primary sequence modification even in a comparison of this histone from a plant and a vertebrate (29)). Thus if an intermediate animal has a doublet band of mobility identical with Fraction F2a1 of calf histone, it is designated as F2a1. (b) Band F3 also has mobility in fish and cow histones, and also invariably contains cysteine (the only histone fraction which does (30)). Thus any band of mobility identical with calf histone F3 and which contains cysteine is denoted as histone F3. (c) Histone F2b of calf and fish histones invariably stains a distinctive grayer tone than any other fraction. It is always present in the largest amount. (d) Histone F1 (the lysine-rich histone) is easily identified, not only is it the slowest moving histone, but also it is quantitatively and selectively extracted by 5% perchloric acid from either chromosomal preparations or from isolated whole histones. (e) The remaining fraction is relatively featureless and is usually identified by the elimination of the other four possibilities. Support for its assignment can be obtained from the fact that it

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### TABLE II

**Percentage of individual histone fractions in vertebrate tissues**

When a given histone fraction is heterogeneous the quantitation of the bands is from the slowest moving to the fastest. The sub-bands of F3 and F2a1 are all identical in mobility. We stress that the sub-bands of F1 are NOT identical in mobility from species to species and cannot be compared directly.

| Source of histones | Quantity of fractions |
|-------------------|-----------------------|
|                   | F1 | F3 | F2b | F2a1 | F2a2 |
|                   | %  | %  | %   | %    | %    |
| Mammal            |    |    |     |      |      |
| Dog spleen        | 4.2, 21.7, 0.91 | 1.6, 7.6, 11.1 | 26.3 | 13.1 | 3.9, 8.5 |
| Dog thymus        | 0.9, 22.6, 9.0  | 2.8, 5.6, 9.0  | 32.3 | 15.9 | 6.7, 6.2 |
| Dog lung          | 0, 12.4, 1.0     | 2.9, 7.0, 10.8 | 33.6 | 12.2 | 8.4, 10.8 |
| Opossum lung      | 12.5, 4.2, 3.1   | 3.8, 7.5, 11.2 | 28.3 | 15.7 | 5.2, 8.1 |
| Opossum spleen    | 14.7, 3.9, 1.1   | 4.0, 8.8, 10.4 | 27.9 | 12.3 | 6.1, 10.6 |
| Opossum thymus    | 7.8, 4.4, 0      | 3.1, 6.1, 11.8 | 27.0 | 19.6 | 4.1, 5.7 |
| Opossum kidney    | 5.6, 2.9, 3.4    | 2.2, 6.8, 10.2 | 34.4 | 18.7 | 4.5, 11.7 |
| Bird              |    |    |     |      |      |
| Hen spleen        | 4.5, 6.6, 4.9, 2.2, 1.5 | 2.1, 5.9, 12.1 | 26.3 | 14.5 | 4.5, 10.2 |
| Hen blood         | 2.5, 1.6, 1.4, 0.9, 23.2 | 0.8, 6.0, 11.9 | 23.4 | 14.1 | 3.7, 9.6 |
| Hen liver         | 2.1, 3.6, 8.8, 1.7, 6.7 | 1.4, 7.0, 11.9 | 25.7 | 15.0 | 5.8, 8.7 |
| Hen kidney        | 2.4, 3.4, 4.0, 0.8, 5.0 | 2.7, 7.7, 12.5 | 30.9 | 18.2 | 4.3, 10.1 |
| Reptile           |    |    |     |      |      |
| Snapping turtle kidney | 2.9, 1.0, 6.4, 9.8, 1.8 | 3.1, 5.4, 12.5 | 28.4 | 21.0 | 1.9, 5.7 |
| Snapping turtle lung | 1.1, 0.8, 4.2, 5.4, 0.5 | 2.8, 5.5, 9.1 | 30.2 | 15.8 | 8.9, 15.8 |
| Snapping turtle liver | 1.0, 0.8, 6.0, 8.8, 4.2 | 5.2, 6.4, 10.9 | 28.0 | 15.2 | 2.7, 5.4 |
| Snapping turtle blood | 2.5, 1.5, 5.7, 6.0, 1.9 | 2.6, 4.9, 11.0 | 28.8 | 19.9 | 2.3, 8.9 |
| Amphibian         |    |    |     |      |      |
| Bull frog lung    | 14.1, 7.6, 1.6    | 1.6, 4.5, 15.3 | 24.9 | 16.3 | 3.0, 10.8 |
| Bull frog intestine | 12.0, 5.8, 2.1 | 2.0, 7.3, 12.6 | 26.4 | 16.0 | 3.3, 12.4 |
| Bull frog liver   | 10.5, 4.4, 1.9   | 1.9, 7.7, 14.9 | 26.8 | 18.3 | 5.8, 13.8 |
| Bull frog blood   | 15.1, 6.0, 2.1    | 1.3, 6.9, 12.9 | 22.1 | 14.9 | 4.2, 12.7 |
| Fish              |    |    |     |      |      |
| Carp intestine    | 4.9, 5.2, 2.6     | 1.4, 6.0, 11.5 | 29.8 | 17.2 | 4.7, 16.4 |
| Carp liver        | 6.2, 11.2, 2.7    | 2.7, 7.0, 13.0 | 22.3 | 16.5 | 5.8, 12.6 |
| Carp blood        | 5.2, 5.8, 1.7     | 8.2, 6.9, 12.1 | 26.0 | 13.9 | 5.0, 15.0 |
is usually found in proximity to F3 and F2b and also from its behavior in gels with different urea concentrations (27). Bands (usually minor) which do not fall into this classification pattern have been investigated in their own right.

Thus an examination of the histone band patterns of a range of vertebrates in a variety of urea-containing gels reveals that the histone complement is made up of five major groups, that two of the five fractions have identical mobility in all systems and that the other three fractions may vary in mobility in different species.

Quantitative Analysis of Vertebrate Histones—To examine the electrophoretic properties of the histone fractions further and to study if the heterogeneity of individual fractions resembled that previously reported for calf thymus, the above samples were subjected to electrophoresis on very long polyacrylamide gels. A selection of typical band patterns is presented in Fig. 6. Microdensitometer traces of several typical gels are shown in Fig. 7. The quantitation of the various histone fractions used the approach defined previously (23) and the quantitative data for many of the species and tissues examined are presented in Table II.

Variation in Mobility of Histone Fractions Other Than Lysine-rich Histone Fraction—The data of Figs. 6 and 7 indicate that F3 and F2a1 have not changed in electrophoretic mobility during the course of vertebrate evolution. On the other hand the mobilities of F2b and F2a2 have changed slightly during the same time period. Recent observations in this laboratory indicate that there are no changes in molecular weights of these fractions (26) and the change in mobility is therefore ascribed to minor changes in charge density.

Although the mobility of F2b and F2a2 may change from class to class, within tissues of a given species they are invariably constant. With the exception of the reptilia they are class specific.

Variation in Mobility of Lysine-rich Histone Fractions (F1)—Lysine-rich histones differ from the other histone fractions in several ways. Not only are there significant differences in the mobility of such histones isolated from all classes of vertebrates but the extent of the electrophoretic heterogeneity may vary from order to order. Further, if different tissues within a given animal are examined it is seen that although the nature of the electrophoretic heterogeneity is essentially constant in that all bands are present, the relative intensity of different bands can be tissue dependent. This is shown specifically for turtle and chicken tissues in Fig. 8, although it is a good generalization to say that it holds true for all creatures examined, and this is documented in Table II. The extent of lysine-rich histone electrophoretic heterogeneity appears to be significantly greater in reptiles (four or five bands) than in fish and amphibia (two or three bands).

The mobilities of lysine-rich histones from different species appear primarily to reflect their differences in over-all positive charge as seen from their amino acid composition shown in Table III. This table shows again that if the other four histones are

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**Fig. 8.** The microheterogeneity of lysine-rich histones from different tissues of the turtle and the hen. High resolution gel electrophoresis of several tissues from turtles and hens was followed by microdensitometer analysis as described above. The positions of the various peaks indicate their relative mobility under the conditions of the electrophoresis.
Table III
Amino acid analysis of lysine-rich histone (F2)
from vertebrate animals

| Amino acids | Calf thymus | Hen blood | Hen liver | Snap- ping turtle liver | Bull frog blood | Fish (carp) blood | Fish (carp) liver |
|-------------|-------------|-----------|-----------|------------------------|----------------|------------------|------------------|
|             | mole %      | mole %    | mole %    | mole %                 | mole %         | mole %           | mole %           |
| Lysine      | 27.3        | 26.6      | 27.2      | 26.0                   | 28.6           | 30.3             | 30.4             |
| Histidine   | 0           | Trace     | Trace     | 0.8                    | 0.7            | Trace            | 0.9              |
| Arginine    | 2.1         | 5.4       | 3.5       | 2.3                    | 2.1            | 2.7              | 2.9              |
| Aspartic acid | 2.6        | 2.0       | 2.3       | 2.5                    | 3.6            | 2.0              | 2.9              |
| Threonine   | 0.0         | 3.8       | 4.3       | 4.2                    | 4.4            | 3.9              | 3.6              |
| Serine      | 0.9         | 8.0       | 6.2       | 8.4                    | 8.3            | 7.7              | 6.4              |
| Glutamic acid | 4.7        | 4.3       | 4.5       | 4.5                    | 4.7            | 3.7              | 4.5              |
| Proline     | 8.4         | 8.0       | 8.6       | 10.0                   | 9.2            | 8.5              | 8.9              |
| Glycine     | 0.7         | 0.8       | 6.7       | 0.2                    | 6.1            | 5.4              | 5.0              |
| Alanine     | 23.4        | 22.8      | 25.3      | 20.9                   | 19.9           | 22.8             | 20.5             |
| Cysteine    | 0           | 0         | 0         | 0                      | 0              | 0                | 0                |
| Valine      | 6.0         | 4.6       | 4.1       | 6.1                    | 5.5            | 5.2              | 4.3              |
| Methionine  | 0           | 0         | 0         | 0.1                    | 0.1            | 0                | 0.3              |
| Isoleucine  | 0.8         | 1.5       | 1.2       | 2.0                    | 2.4            | 1.5              | 1.7              |
| Leucine     | 3.9         | 4.5       | 4.7       | 4.5                    | 3.2            | 4.3              | 3.6              |
| Tyrosine    | 0.6         | 0.9       | 0.7       | 0.8                    | 0.6            | 0.8              | 0.9              |
| Phenylalanine | 0.6       | 0.6       | 0.7       | 0.8                    | 0.6            | 0.8              | 0.7              |

characterized by conservation of primary structure, the lysine-rich histone is not.

The heterogeneity of the lysine-rich histones can be distributed among one or two distinct electrophoretic groups. As seen in Fig. 6, frog, snake, lizard, and most mammalian organs (excluding thymus) have two groups of lysine-rich histones, both of which can exhibit heterogeneity. On the other hand fish, alligators, snapping turtles, most bird tissues, and the mammalian thymus contain only a single heterogeneous group of lysine-rich histones.

Erythrocyte-specific Histones—The presence of a substantial proportion of an additional histone fraction in nucleated avian erythrocytes has been previously documented (31). We wondered if this histone fraction was present in other avian organs, and also if nucleated erythrocytes from other vertebrates contained another, similar histone fraction.

Direct extraction of all chicken organs reveals the presence of the additional histone. However, since most organs contain a considerable amount of blood, the histone could be present because of contamination with an erythrocyte-specific histone. We have therefore perfused chickens as efficiently as possible and extracted histones from blood-depleted organs (Fig. 9). It is clear that perfusion reduces the amount of this histone fraction to low levels and we have obtained avian histones from tissues in which this fraction was almost totally absent.

An examination of nucleated erythrocytes and other tissues from reptiles, amphibia, and fish reveals the absence of significant amounts of an erythrocyte-specific histone in these classes. Erythrocytes are very sensitive to changes in the general health of the animal and rapidly provide evidence of degradation of lysine-rich histones. Thus initially there was electrophoretic evidence of an erythrocyte-specific histone band in buffalo fish, a species which dies rapidly after capture, although it is in well aerated tanks. Further if the fish is maintained out of water for a short period the intensity of this band increases. However, if blood is isolated from buffalo fish within seconds after capture and frozen on Dry Ice, a subsequent isolation of histones reveals a dramatic reduction in the amount of the putative erythrocyte-specific histone, and we ascribe its presence in the earlier isolations to an unusually active protease. On the other hand erythrocytes from the pike (which is much more healthy following capture) show no significant amounts of this fraction.

Fig. 9. The erythrocyte-specific histone of birds. (a), chicken erythrocyte histone; (b), chicken kidney histone; (c), duck erythrocyte histone; (d), duck liver histone.

DISCUSSION

Vertebrate histones in general can be divided into three classes based on their electrophoretic behavior. One class consisting of F2a1 and F3 shows a constancy of electrophoretic mobility, including the sub-bands, in all the species and samples studied. The electrophoretic heterogeneity of F2a1 is caused by acetylation of lysine 16 (29), and the multiple heterogeneity of F3 is likewise very probably caused by acetylation (32). These data are entirely consistent with the recent data in which conservation of primary structure of F2a1 is a notable feature (29). We may well expect a similar conservation for F3, although we have already documented the introduction of another cysteine residue during mammalian evolution (30).

Histone Fractions F2b and F2a2 show occasional mobility changes during the evolution of the vertebrates. This is shown in Fig. 6 and is presented diagrammatically in Fig. 10. A definite trend is discernible as one progresses towards the mammals. F2b becomes progressively slower and F2a2 progressively faster moving. In general we have observed that these two fractions have a constant mobility within a given class of animals. The reptilian class is unusual in this respect. F2a2 characteristic of
turtles and alligators is different from that of the order Squamata (snakes and lizards). F2b is constant in reptiles and birds with the single exception of the snake in which it moves fractionally slower. This may be indicative of a slow, but nonetheless higher rate of mutational change than seen for F2a1 and F3. An identity of electrophoretic mobility, as seen for F2a2 of birds and the snakes, for example, does not necessarily indicate the same changes in primary structure from the F2a2 of the primitive Chelonia (turtles).

The lysine-rich group of histones shows considerable variation in electrophoretic mobility which is reflected in changes in amino acid composition (see Table III). Thus the mobility of the F1 histones increases in the order calf < alligator < carp; comparing well with the increase in over-all positive charge at pH 2.7 (the pH of electrophoresis) calculated from Table III. With the exception of the reptiles the lysine-rich histones within a given class of vertebrates vary only slightly among different orders. This is best seen for several mammalian orders shown in Fig. 6. It is not known whether the microheterogeneity is caused by such factors as phosphorylation or acetylation, or whether lysine-rich molecules of different sequences are elaborated by the closely related species. There is most likely a contribution from both possibilities (26).

The additional lysine-rich histone of mammalian tissues appears to correlate with the absence of cell division in the tissue (33). However, all frog and snake tissues had two groups of lysine-rich histones, whereas alligators and fish showed only one group in all tissues (Fig. 6). The extra band in birds, which is associated with the nucleated erythrocyte cells, should probably be classed as a lysine-rich histone rather than as an additional serine-rich histone (31), since it is isolated in the lysine-rich fraction in chemical separations and its amino acid analysis is quite similar to that of non-erythrocyte lysine-rich histones. It appears that when the erythrocyte-specific lysine-rich band is found in high yield, the lysine-rich group characteristic of other avian tissues is much decreased in amount. This observation has also been noted by Johns (34). We found no evidence for a significant amount of an erythrocyte-specific histone in any other vertebrate we have examined.

What do these observations tell us concerning the function of histones? Clearly the function of the arginine-rich histones (F2a1 and F3) demands a precise constancy in primary sequence which is not so stringently required for F2b and F2a2. The primary structure of F1 is quite variable and there have been substantial changes in positive charge density. It is thus probable that histones F2a1, F3, F2a2, and F2b may have common functions which will differ substantially from that of F1.

It is instructive to inquire what the interphase chromosomes of calf and fish have strictly in common. They contain a negatively charged DNA molecule, with a hydrophobic interior and a common topography with respect to major and minor grooves. The normally rigid DNA molecule also has to be packed into the nucleus. Recognition of common features in DNA and the subsequent packing effect as a result of interaction is not likely to have changed dramatically since the evolution of the eucaryotes. Thus it would not be unreasonable to suppose that the biological function of the four conservative histone fractions (which likewise have not changed dramatically) is to act as chromosomal structural proteins recognizing these common features of all DNA molecules in a precise and specific fashion and as a result modifying the tertiary structure of DNA within the interphase chromosome. Although this proposal does not preclude a role for the conservative histone fractions as regulators of genetic activity (specific or otherwise), it seems to us less than certain.

Since the demands for a constant primary sequence of the lysine-rich histones are evidently much less stringent, one might be tempted to argue that this indicates a possibility for an inherent difference in specificity between the lysine-rich histones of different species. However, one cannot ignore the possibility that the function of the lysine-rich histone involves structural conformations where there are not too precise demands on certain residues so that they are more free for mutational change. Presumably such a mutation could offer no selective advantage and would only survive if the creatures carrying the mutation also developed an advantageous mutation elsewhere in the organism.

It has been argued that the lysine-rich histones have a role in cross-linking interphase chromosomes (35, 36). If this is true one might expect that those regions of the molecule interacting with DNA would have a conservative sequence and that other regions spanning nucleohistone molecules might permit a more frequent modification of the primary sequence. Since almost half of the calf lysine-rich histone has been sequenced, and since pure fish F1 is available, it should soon be possible to test this idea.

Intuitively it would seem to be biologically meaningful if the sequence of histone fractions on a given chromosome were to be exactly replicated on a daughter chromosome. Since histones do not turnover significantly, it would not be too surprising if they could in some way dictate the positioning of newly synthesized histone molecules onto the new DNA molecule. This would be a function which would require a recognition of much of the length of the histone molecule and also might permit little primary sequence variation. However, the molar ratios of the four
conservative histone fractions vary widely in different times and no regular pattern can be discerned. Thus if a histone molecule can specify the position of another histone molecule it is not a case of one fraction simply recognizing another fraction (which would require constant molar ratios between various pairs of histones), although it does not exclude the likelihood that each histone fraction might recognize and specify the chromosomal position of identical molecules.

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Calculation of molar ratios was based upon the yields in Table II assuming the following molecular weights: F2a1, 11,200 (29); F2a2, 12,500 (26); F2b, 13,700 (37); F3, 14,000 (26); F1, 21,000 (38).

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