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The Heterogeneity of Histones. I. A Quantitative Analysis of Calf Histones in Very Long Polyacrylamide Gels*

Sakol Panyim and Roger Chalkley

ABSTRACT: The heterogeneity of calf histones is limited. The histones are divided into five major electrophoretic groups, several of which are further subdivided to make a total of twelve species of histone molecule. The heterogeneity described is probably not due to impurity (though this is hard to assess), to failure of extraction, to degradation during extraction, or to multiple polymerization through disulfide bonds.

The relative amounts of each of the twelve components is reported. No significant differences in relative quantitation are observed for bovines of varying age and sex. A tissue-specific lysine-rich histone is reported; other major groups of histone show tissue specificity only in terms of variation in the amount of each histone fraction present.

Calf thymus histones are thought to be a mixture of relatively few homogeneous proteins. Johns has described five major components separable by chemical means (Johns, 1964; Phillips and Johns, 1965). Kinkade and Cole (1966) have shown that one of these groups (the lysine-rich histones) is further divided into three or four components.

Histones, it has been argued, may play a role in the regulation of genetic activity of higher organisms. As a method of studying this matter several workers analyzed the similarity or otherwise of histones from different organs and various species (Bustin and Cole, 1968; Fambrough *et al.*, 1968; Hnilica *et al.*, 1966; McGillivray, 1968), and from active and inactive chromatin (Littau *et al.*, 1964). There were, however, a number of severe problems encountered in this work: nucleoprotein preparations are often contaminated by highly active proteolytic enzymes (Reid and Cole, 1964; Furlan and Jericijo, 1967; Panyim *et al.*, 1968); there was a lack of resolution in the various techniques for separating histones (Shepherd and Gurley, 1966; Rasmussen *et al.*, 1962; Cruft, 1961) and there was a need for a more precise quantitation of histone fractions which were only insufficiently resolved. Cole and his coworkers have largely solved these problems for the lysine-rich (F1) histone fractions; however, their procedure is lengthy, and precise quantitation difficult because of overlap of column effluent fractions and moreover at this time is restricted to only one of the five groups of histones described by Johns.

We wanted to determine whether any other of the five major components of calf thymus histones showed further subdivision. Also, we wanted to quantitate all the various subfractions of an unseparated histone preparation in a single experimental system to facilitate comparison studies between the entire histone complement of different organs of a given creature and between histones of different species.

We have used a gel electrophoretic technique (Panyim and Chalkley, 1969) which, coupled with microdensitometric scanning and electronic curve analysis, permits us to reliably quantitate histone fractions which differ in electrophoretic

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mobility by less than 1%. In addition, this approach has the merit of simplicity of operation, speed, and also permits a comparative study of all the components contained in an unfractionated histone preparation.

We find under maximal resolution that there are twelve histone bands upon electrophoresis of calf thymus histones. However, more than 96% of the total mass is found in ten of these bands. We have extended these techniques to a study of other calf tissue histones. Although the amount of histone in a given band may vary somewhat with tissue, we will show that eleven of the bands are found with identical mobilities in all the tissues examined. One band, from a lysine-rich histone, is present only in rapidly replicating tissues (thymus, intestinal mucosal cells) and is totally absent from nonreplicating tissues. Conversely, a new band (possibly a lysine-rich histone) moving with a mobility faster than the bulk of the lysine-rich material is found in nonreplicating tissues and is absent from rapidly replicating cells.

Materials and Methods

Nucleoproteins were isolated in a number of ways, and these are specified in the text. However, they all had in common the presence of 0.05 M sodium bisulfite as an inhibitor of proteolysis unless it is specifically mentioned that it was omitted. Calf histones were isolated from nucleoprotein preparations by blending vigorously with H_2SO_4 (0.4 N), sedimenting at 18,000 rpm/20 min, and by precipitating from the resulting supernatant with four volumes of ethanol at −20°.

For electrophoretic analysis the histone sample was dissolved in 15% sucrose, 0.9 N acetic acid, and applied to preelectrophoresced 15% polyacrylamide gels (in 2.5 M urea–0.9 N acetic acid, pH 2.8). The gel dimensions were either 0.6 × 25 or 0.6 × 8.5 cm. Electrophoresis was at 200 V for 16 hr for 25-cm gels or at 130 V for 3.5 hr for 8.5-cm gels. The 25-cm gels were removed by cracking the glass tubes and stained with Amido Black (0.1% in Amido Black–20% ethanol–7% acetic acid). After destaining the gels were scanned in a microdensitometer (Gilford, Model 2000, gel scanner) and the resulting curves were analyzed on a DuPont electronic curve analyzer.

Results

Optimum Conditions for Histone Extraction. Histones are extracted at low pH from nucleoprotein complexes obtained by disrupting isolated nuclei. The electrophoretic patterns obtained strongly depend upon the techniques used for isolating nuclei as shown in Figure 1 for histones isolated from chromosomal material obtained from calf thymus, kidney, and liver nuclei. Analysis of the gels of Figure 1 enables us to develop an operational definition of histones as those nuclear, acid-soluble chromosomal proteins which maintain a constant mass ratio to one another no matter what nuclear isolation technique is employed. Thus essentially all of the slower moving bands are not classified as histone. It is immediately apparent that calf thymus histones are relatively free from contamination no matter what method of isolation is used, though calf liver and kidney extracted by standard techniques are grossly contaminated. However, if liver is extracted with citric acid (Allfrey, 1959) or by sedimentation through 2.4 M sucrose, it is seen that the histone pattern now much resembles

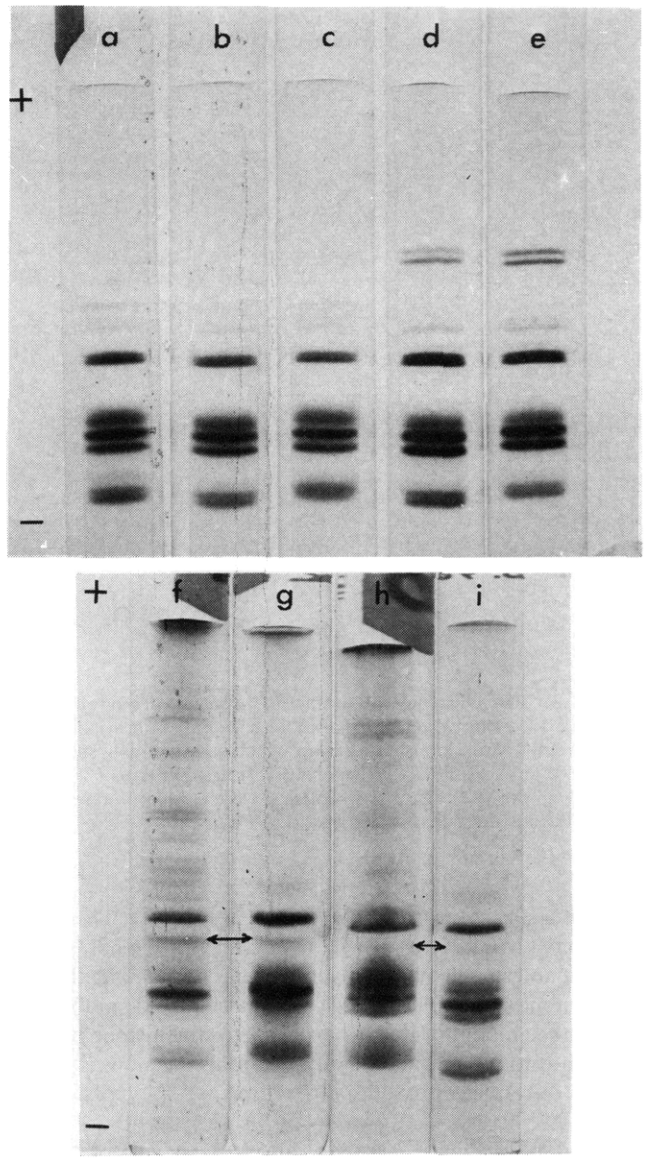

FIGURE 1: The effect of the mode of isolation on the purity of histones. Histones from calf thymus extracted using several modifications of standard techniques: (a) standard procedure (Bonner *et al.*, 1968); (b) isolation included sedimenting nuclei through 2.4 M sucrose; (c) isolation in the presence of citric acid (Allfrey, 1959); (d sedimentation through 2.4 M sucrose containing 0.5% Triton X-10 (e) by washing nuclei twice in the grinding medium containing 0.5 Trition X-100 (no sucrose density degradients). Histones from calf liver extracted by various modifications of the standard technique: (f) standard technique; (g) standard technique modified to include sedimenting the nuclei through 2.4 M sucrose. Histones from calf kidney extracted by standard technique (h); by sedimenting the nuclei through 2.4 M sucrose in the presence of 0.5% Triton X-100; histones were electrophoresed in 2.5 M urea (pH)–2.7–0.9 N acetic acid for 3.5 hr at 130 V. The slower moving bands in d and e are due to oxidation–polymerization of a thiol-containing component of one of the histone bands as discussed below.

that of calf thymus and most of the slower moving material has been removed. We have observed that the yield of kidney nuclei through high-density sucrose solutions is very low because of adhering cytoplasmic material, and it is preferable if kidney nuclei are extracted in the presence of Triton X-100

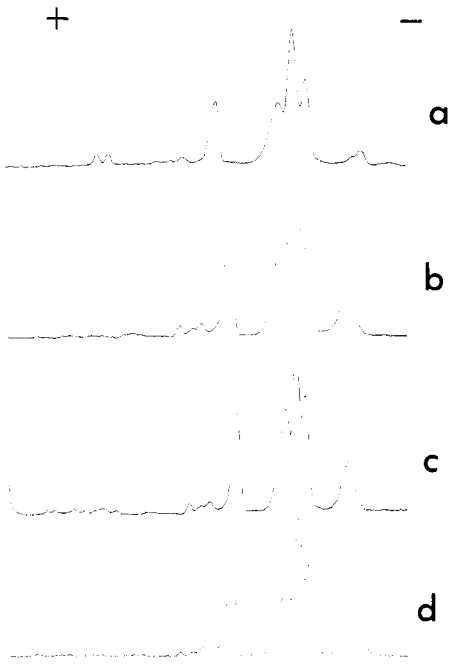

FIGURE 2: The efficiency of extracting histones from chromatin material. Densitomer patterns of calf thymus histones isolated by sulfuric acid extraction of chromatin which as previously suspended in (a) 0.01 M Tris (pH 8), (b) water, (c) 4 M urea, and (d) 1% sodium dodecyl sulfate. The histones so isolated were electrophoresed under the conditions described in the legend to Figure 1.

prior to a sucrose treatment as shown in Figure 1. There is an additional band (arrowed) in liver and kidney not seen in thymus and it is likely from the above criteria that this is a real histone band and not a contaminating acid-soluble protein as the amount of this band relative to the histone bands is independent of the method of isolation. We now routinely isolate our nucleoprotein preparations in the presence of Triton X-100.

The data of Figure 2 are typical of a wide survey we have made concerning the most reproducible method of extracting histones from nucleoprotein preparations. Nucleoproteins are normally extracted from an ionic strength of approximately 0.01, in which they have a compact conformation (Zubay and Doty, 1959). At this ionic strength a direct acid extraction sometimes gives variable yields, particularly of the fast-moving histones. Uniformly reproducible preparations were obtained if the nucleoprotein was solubilized and extended by lowering the ionic strength below 5×10^{-4}, or by treating with 4 M urea or sodium dodecyl sulfate (1%). Because of our recent finding that low ionic strengths (2×10^{-4}) inhibit the protease associated with thymus nucleohistone (J. A. Bartley and R. Chalkley, unpublished observations) we have employed this method most extensively in obtaining the bulk of the data presented below.

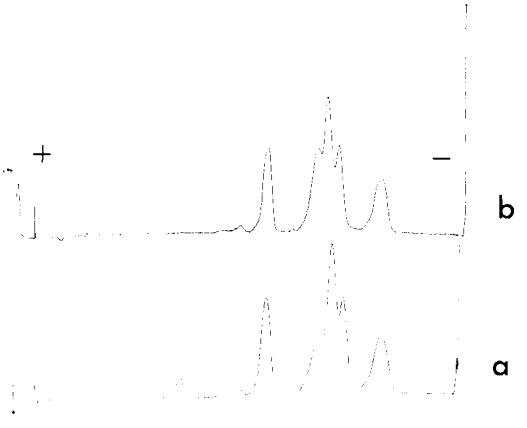

FIGURE 3: Formation of disulfide bonds from cysteine-containing histone. Densitometer patterns of calf thymus histones which had been (a) dialyzed for 18 hr at 25° in 0.9 N acetic acid; (b) subsequently treated with 0.5 M mercaptoethanol in 8 M urea–0.9 N acetic acid, for 18 hr before electrophoresis. The electrophoretic conditions were those described in Figure 1.

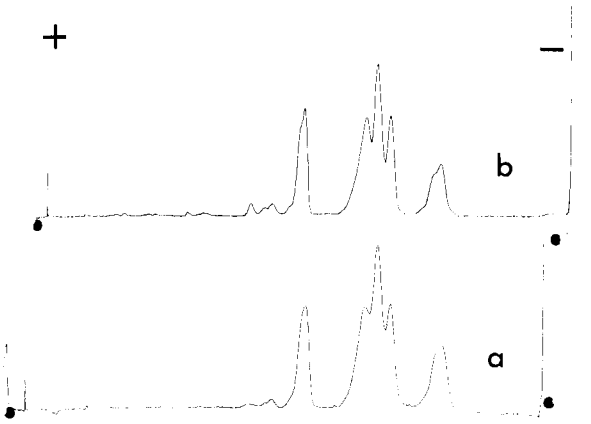

FIGURE 4: The amount of histones in the disulfide form *in vivo*. Densitometer patterns of the electophoretic banding of calf thymus histone obtained from nucleoprotein isolated (a) in the presence of sodium bisulfite (0.05 M) and (b) isolated at pH 4 in the absence of sodium bisulfite; all solutions were evacuated prior to use at all stages in the isolation procedures to avoid aerial oxidation.

Disulfide Bonds in Native Histones. The histones in electrophoretic group 2 (bands 2, 2′, and 2″; the nomenclature has been defined previously (Panyim and Chalkley, 1969) and is reiterated in Figure 5, *vide infra*) contain thiol groups (Fambrough and Bonner, 1968) which can be oxidized to form disulfide bonds simply by dialyzing a histone solution. The formation of disulfide-linked histone 2 gives rise to a new double band (at the expense of histone 2) moving more slowly than the bulk of the histones (Figure 3). Treatment of the oxidized histone with β-mercaptoethanol prior to electrophoresis removes the slower moving bands, returning the thiol-containing histones to band 2 (Figure 3).

We were concerned that if disulfide bonds existed among histone fraction 2, *in vivo*, an additional contribution to the heterogeneity of histones might come from the use of sodium bisulfite as an inhibitor of proteolysis during the isolation. Sodium bisulfite, a reducing agent, breaks disulfide bonds linking two proteins to yield two differently charged protein species (Gutte and Merrifield, 1969; Bailey and Cole, 1959). One has an extra negative charge due to thiosulfite formation and the other would have no extra charge as a sulfhydryl group is formed. Such a charge difference would be easily detected in this system (*vide infra*).

We have compared our normal preparations of histone, isolated in the presence of sodium bisulfite with preparations

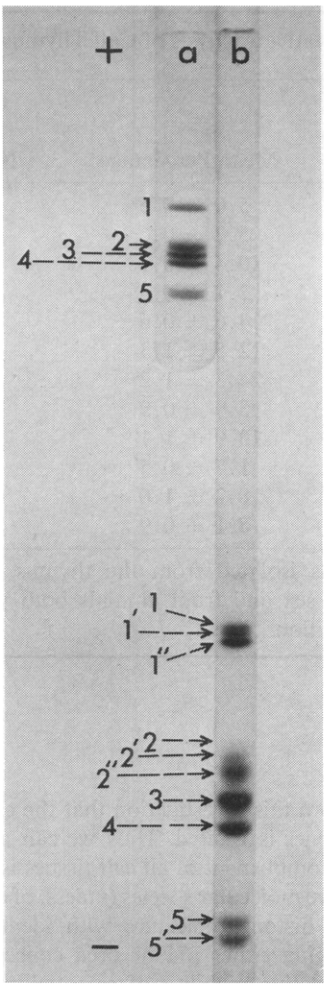

FIGURE 5: Polyacrylamide electrophoresis of calf thymus histone on a long gel. The electrophoresis in both short and long gels was performed as described in Materials and Methods. (a) The shorter gel is 9 cm long and is the same as used for the densitometer traces in previous figures; (b) the long gel was 25 cm long. The nomenclature is that described in a previous paper (Panyim and Chalkley, 1969).

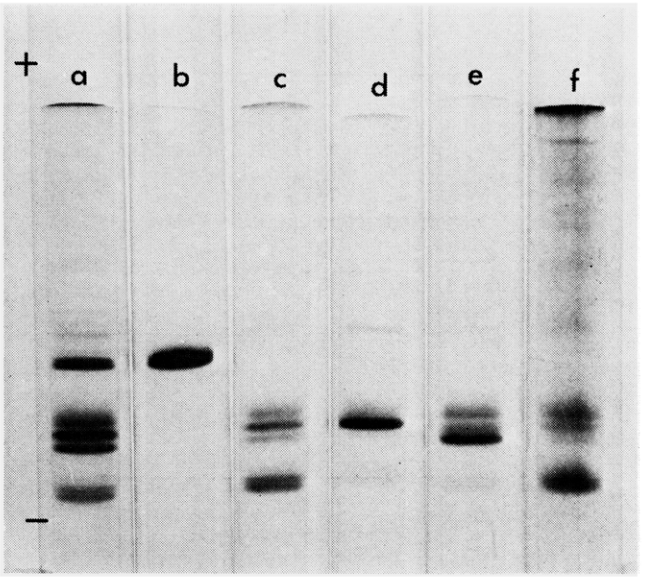

FIGURE 6: Polyacrylamide gel electrophoresis of whole calf thymus histone and fractions separated by method 1 of Johns (1964). (a) Whole calf thymus histone, (b) F1, (c) F2a1, (d) F2b, (e) F2a2, and (f) F3. The gels were electrophoresed at 130 V for 3.5 hr in 2.5 M urea–0.9 N acetic acid. Recently we have obtained much improved fractionation using method 2 of Johns (1964).

isolated at pH 4.0 where proteolysis is also inhibited under which conditions we would expect any disulfide bonds existing *in vivo* to survive intact. Histones were therefore isolated from calf thymus in the presence of either sodium bisulfite or at low pH (in which case all solutions were thoroughly deaerated prior to use to avoid oxidation). After acid extraction all solutions employed (*e.g.*, ethanol for precipitation) were deaerated, as was the buffer in which the histones were dissolved prior to electrophoresis. The absolute requirement for preelectrophoresis to remove ammonium persulfate (the oxidant involved in acrylamide gel formation) was observed as part of our normal technique. The result of this experiment is shown in Figure 4, demonstrating that in calf thymus, *in vivo*, there is no detectable presence of the disulfide form of histone 2; that isolation of histones in the presence of sodium bisulfite in no way changes the pattern of bands produced, and that the heterogeneity we are describing is not due to oxidation–polymerization of thiol-containing proteins.

Identification of Histone Subfractions. If histones are isolated in the appropriate manner as described above, the data of Figure 5a show that the electrophoretic system is capable of separating whole calf thymus histone into five major groups, at least three of which show signs of further resolution. The bands in this experiment were obtained after electrophorescing histones through about 8.5 cm. In order to investigate the further resolution of these bands, we have used longer polyacrylamide gels as described in the Materials and Methods section. The electrophoretic separation obtained by this technique is shown in Figure 5b, in which we visibly see that calf thymus histones can be resolved into ten bands. Two additional weak bands can be detected with a densitometer to give a total of twelve bands in all.

In order to relate this work to that previously reported, we have separated histones according to the method of Johns (Johns, 1964; Phillips and Johns, 1965) and the electrophoretic patterns of these fractions are shown in Figure 6. Fractions F1, F3, F2a1, and F2a2 are resolved into multiplet bands and are equivalent to fractions 1, 2, 4, and 5 in our nomenclature; however, fraction F2b appears to be homogeneous and is equivalent to fraction 3. It is clear from Figure 6 that fractions F2a1, F2a2, and F3 (nomenclature of Johns) are not fully separated one from the other. Johns has previously reported a similar conclusion (Johns, 1967). The resolution of this system is such that on the long gels band F2b has a band width of 2 mm after traveling 20 cm and therefore contains within the single main band only those species which do not differ in electrophoretic mobility by greater than 1%.

Relative Yield of Separate Bands Is Independent of Total Input of Histones. Fambrough *et al.* (1968) have recently shown that several separate pea histone fractions have a linear color response to Amido Black. In Figure 7 we show that when total calf histone is electrophoresced, the relative yields of the various fractions are independent of input concentration of total histone up to an initial amount of 50 μg of histone/gel.

Quantitation of Calf Thymus Histones. A microdensitometer trace of a typical calf thymus histone fractionation on a

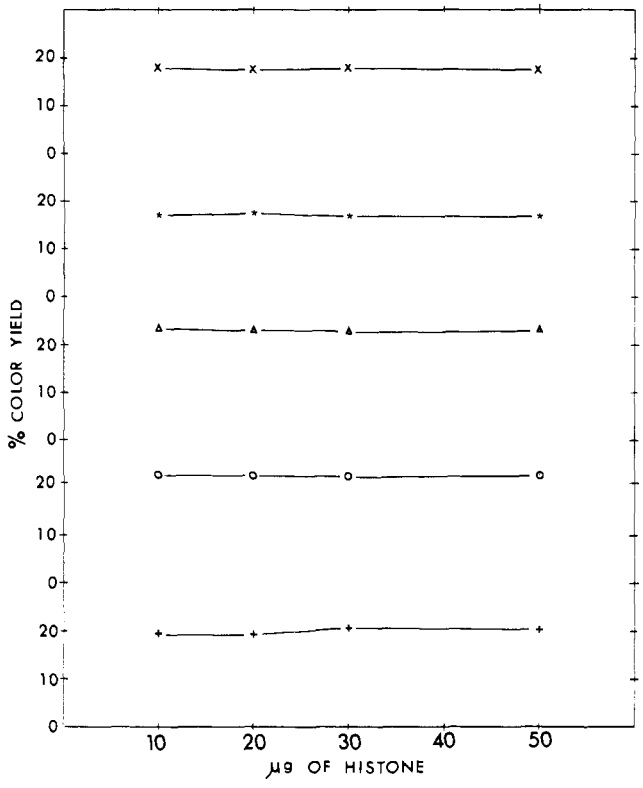

FIGURE 7: The dependence of the yield of histone fractions upon amount of protein electrophoresed. Identical gels were prepared and increasing amounts of total histone were applied. After electrophoresis and staining, densitometer traces of the bands were obtained and the per cent yield under each peak was determined using a curve analyzer (see below). (+—+) Histone 1, (the lysine-rich histone); (○—○) histone 2; (×—×) histone bands 5; (△—△) histone 3; (☆—☆) histone 4.

long gel is shown in Figure 8. This curve was analyzed on a DuPont curve analyzer and resolved into twelve gaussian bands, and the fraction of the total area was computed (electronically) for each band (Figure 8). The resulting tabulation of the relative amounts of each component is presented in Table I. This was compiled with data from several different thymus glands, extracted in a variety of ways as described in the legend to the table.

Electrophoresis of Calf Tissue Histones on Long Gels, and Their Quantitation. Histones were also extracted from the brain, lung, spleen, liver, kidney, endometrium, and intestinal mucosal cells of the calf. They were electrophoresced through long gels and quantitated as described above. The complete electrophoretic patterns are shown in Figure 9 demonstrating the over-all similarities in mobility. The lysine-rich histone region is the sole place where bands of tissue-dependent mobility are found. This region is shown more clearly in Figure 10. The tentative identification of the characteristic band (1°) of nonreplicating tissues (Figure 11) as a lysine-rich histone is based upon its total extractibility into either 5% perchloric acid or 5% trichloroacetic acid, a property shown by only the lysine-rich histones. Confirmation of this idea must await the isolation and purification of this fraction, a task upon which we are currently engaged. Quantitation of the relative yields of histone fractions from the various calf tissues studied is shown in Table II.

TABLE I: Quantitative Analysis of Calf Thymus Histones.[a]

Histone Band	Mean Percentage	Phillips' and Johns' Nomenclature
1	5.0 ± 0.5[b]	
1′	5.2 ± 0.6	F_1
1″	10.4 ± 0.7	
2	2.1 ± 0.3	
2′	4.6 ± 0.6	F_3
2″	12.5 ± 1.3	
3	24.3 ± 1.2	F_{2b}
4	3.9 ± 0.9	
4′	13.9 ± 1.1	$F_{2(a)_2}$
5[0]	1.7 ± 0.5	
5	8.2 ± 1.0	$F_{2(a)_1}$
5′	8.1 ± 0.9	

[a] Histone was isolated from the thymus glands of beef stock of either sex and from animals both young and old. [b] Standard deviation.

Discussion

It is clear from this investigation that the extent of heterogeneity of histones is limited. Thus we can assert that 99% of the histone complement of all calf tissues is made up of no more than twelve molecular species (unless, of course, there are some different histone molecules with identical mobilities; however, changing either pH or urea concentration, which modifies the relative mobility of essentially all the electrophoretic groups of histone (Panyim and Chalkley, 1969), has never revealed the presence of another component).

We do not consider it likely that the multiplicity of histone bands is a reflection of a contamination of the system. The bands described as histone were obtained in constant mass ratios, one to another, no matter what isolation technique was employed. It is of interest that purification of nuclei in the neutral detergent Triton X-100 did, however, completely remove several additional slow-moving bands from the electrophoretic system. Though, except for calf liver and kidney histone preparations, these slower moving bands have never constituted more than 2 or 3% of the whole.

Nor do we think that the heterogeneity of calf histones is due to polymerization *via* disulfide bands or to the effect of sodium bisulfite upon cysteine residues. In fact, we were unable to obtain evidence that any of the sulfhydryl-containing histone groups (band 2) is in the oxidized form *in vivo*. However, it is noteworthy that oxidation is easily achieved upon dialysis of a solution of whole histone at a final concentration of *ca.* 2 mg/ml.

Calf histones are divided into five electrophoretic classes. The calculation of the relative amounts from the color intensities is based upon the observation that Beer's law is obeyed for histone–dye color intensities and that all the histone fractions complexed with Amido Black in acrylamide gels have identical molar absorbance (Fambrough *et al.*, 1968; Hnilica, 1966). Each class of histone is, with one exception, further subdivided. Quantitation of each subfraction has

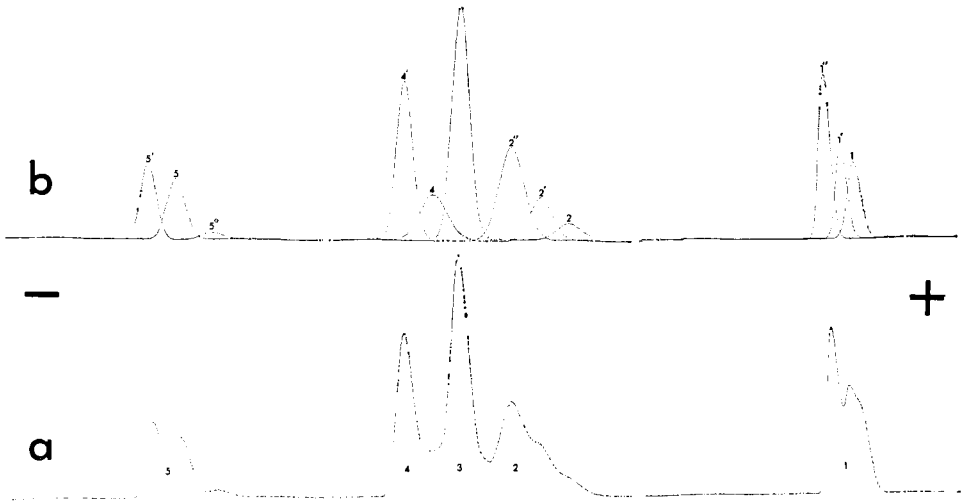

FIGURE 8: Densitometer tracing of calf thymus histone electrophoresed on a 25-cm gel (a), and the resolution of such a pattern using the DuPont curve analyzer (b).

TABLE II: Quantitation of Calf Tissue Histones.

Band	Thymus	Intestine	Spleen	Lung	Brain	Liver	Endometrium	Kidney
1	5.4	1.9	1.7	0	0	0	0	0
1′	4.4	6.3	5.4	6.1	2.8	2.7	2.3	3.4
1″	9.7	10.0	15.9	12.2	16.0	13.8	11.0	15.6
2	1.7	1.9	0.8	1.7	3.6	1.2	2.2	2.1
2′	4.1	1.8	1.7	3.2	7.6	3.9	6.3	6.5
2″	13.8	17.6	9.0	11.1	14.0	10.2	12.2	13.3
3	24.6	22.9	26.9	27.8	22.7	32.4	28.4	26.7
4	5.6	4.9	4.7	6.2	5.0	2.6	5.8	3.9
4′	12.5	14.3	9.7	13.1	10.7	11.4	11.1	7.9
5^0	2.1	1.5	1.7	0.5	1.0	1.0	1.9	1.4
5	8.4	8.1	10.2	5.0	5.9	7.0	4.9	6.5
5′	7.8	8.0	11.2	10.3	9.2	12.7	10.5	11.1
1^0	0	0.7	1.1	2.8	1.6	2.2	3.0	1.5

been achieved and is recorded in Tables I and II. We find that there are ten different protein molecules making up the bulk (96%) of calf thymus histones, and these, together with two minor components constitute the entire complement. It is unlikely that any of these bands is a product of even slight proteolytic degradation of another histone for two reasons. (1) Great care was taken to isolate histones in the presence of inhibitors of proteolysis in this system; (2) recent studies upon this system exploiting a controlled endogenous proteolysis have shown that the earliest signs of degradation are a minor loss of lysine-rich histone (histone 1) with the concomitant production of a new band running slightly ahead of the lysine-rich histone. The band is not observed in our histone preparations. Further the constancy in the yield of the lysine-rich histone from many thymus glands (Table I) argues against their proteolytic destruction.

We have chosen to consider calf thymus histones as consisting of five major electrophoretic groups divided into subfractions, rather than twelve independent distinct species for several reasons. (1) If the electrophoretic conditions are modified (*e.g.*, by changing pH or urea concentration), then all the subfractions within a given group behave in a parallel fashion which is distinct from the subfractions within another group (Panyim and Chalkley, 1969). (2) Chemical isolation of the major electrophoretic groups by the methods of Johns and his coworkers shows that the subfractions within the group are always coisolated suggesting a group-dependent chemistry. (3) Cole and his coworkers have independently shown the lysine-rich histones are subdivided into several lysine-rich proteins.

Because of the chemical similarities within a given electrophoretic group, it is reasonable to suppose that substantial portions of these molecules are identical or at least very similar. Acetylation of a basic amino acid as has been postulated to occur in histones 2, 4, and 5 *in vitro* by Vidali *et al.* (1968) would certainly give closely related species differing by small

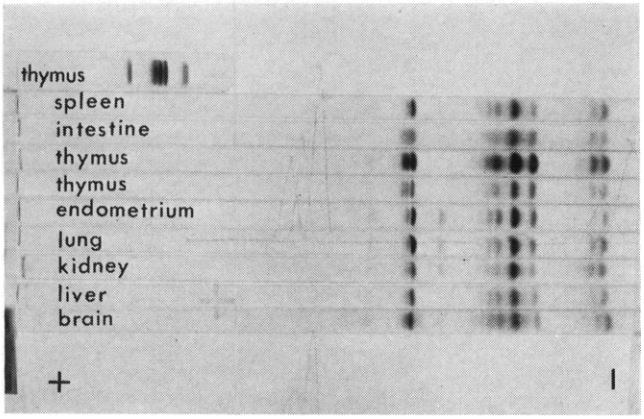

FIGURE 9: Electrophoretic patterns of histones isolated from calf tissues. Electrophoresis on long gels was performed as described in Materials and Methods.

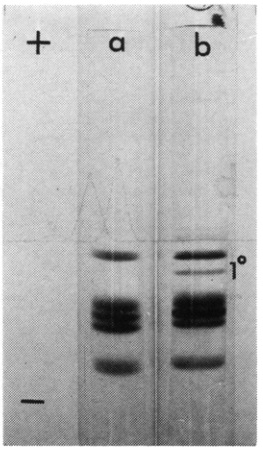

FIGURE 11: Electrophoretic band patterns of histone showing band (1°) found only in nonreplicating cells. (a) Calf thymus histones and (b) calf lung histone.

amounts in their electrophoretic mobility. Ord and Stocken (1968) have reported a phosphorylation of the lysine-rich histones. We have recently found in our laboratory (D. W. Sherod and R. Chalkley, unpublished observations) that, following *in vivo* labeling, increasing amounts of ^{32}P are associated with each more slowly moving member of the lysine-rich group, again arguing in favor of a microheterogeneity imposed upon a parent lysine-rich histone molecule by increasing levels of phosphorylation.

Further, Delange *et al.* (1969) have isolated the entire fraction 5 material for amino acid sequence studies. Their observations indicate that there is only one polypeptide chain in this group, though one lysine residue was found to be only 50% acetylated. This observation is most easily rationalized in terms of our data and that of Delange *et al.* if the subfractions of 5 and 5′ represent the parent molecule (5′) and the parent molecule with one lysine residue acetylated (5). It is unlikely that there is a difference of greater than one acetyl group because of the separation of bands 5 and 5′ (5 mm between band centers after 5′ had migrated 20 cm).[1] Confirmation of this idea will require a preparative isolation of bands 5′ and 5, an operation currently underway.

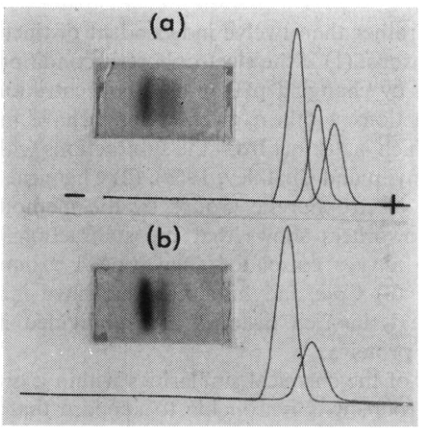

FIGURE 10: Variation in lysine-rich histone bands. Electrophoretic bands and curve resolution of densitometer scan of lysine-rich region of calf thymus (a) and calf lung (b). Photographed in close-up from gels included in Figure 9.

Observations of histones extracted from thymus glands from different bovines of either sex and over a range of ages show that the composition of calf thymus histones is independent of the animal from which the initial tissue was taken. The composition is simply a property of the calf thymus gland itself.

This last observation, coupled with the technical aspects of histone isolation and criteria of purity, puts us in a position to assess the quantitative aspects of histone heterogeneity in the various organs of the calf and of other mammals.

Previous workers have argued that, on the one hand, there was a tissue specificity of histones (Purkayastha and Neelin, 1966; Mauritzen and Stedman, 1959; Bustin and Cole, 1968) or that there is no histone tissue specificity (Crampton *et al.*, 1957; Bellair and Mauritzen, 1967; Hnilica *et al.*, 1966; Hnilica, 1966), though the latter authors report quantitative variation from tissue to tissue.

The data described in Table II show that there is a tissue specificity of histones, though this is only found for the lysine-rich histone group (this was the histone group studied by Bustin and Cole, 1968). The other histone groups (and their subfractions) show no tissue specificity in as much as no histone with a tissue-specific electrophoretic mobility was found; however, there is a significant tissue specificity with respect to the amount of each histone present.

Several major trends emerge from the quantitation in the various tissues. These are (1), fraction 3, (F2b), is invariably present to the largest extent, and it appears to be homogeneous. (2) In the other histone groups in which subdivision does oc-

[1] The molecular weight of this histone is 11,200 and it contains 27 positively charged amino acids/molecule. If one positive charge were lost through acetylation, we would expect the separation between the band centers of the two proteins to be $^1/_{27} \times l$, where l is the distance moved by the more highly charged species. Thus, after electrophoresis through 18 cm, we would predict a separation of 0.6 cm. Similarly, if two positive charges were lost through acetylation, a separation of 1.2 cm would be expected.

cur the major contributor to each group is the same in all tissues. (3) The slowest moving lysine-rich band is found only in those tissues with a high rate of cell division. (4) The fastest moving lysine-rich band is found only in those tissues with a very low rate of cell division. (5) Rapidly dividing cells have roughly equal amounts of 5 and 5′ (the parent and acetylated form of F2a1), whereas very slowly dividing tissues have more of the unacetylated form (5′) relative to the acetylated molecule (5). (6) When assayed as described above (including isolation in the absence of bisulfite) for disulfide-bridged histone, we found no significant quantity in this form, indicating that the dimer may not be of any biological importance, at least for differentiated tissues.

The electrophoretic approach is of course limited in that it measures only mobility which is dependent upon molecular weight and the presence of charged amino acids; and confirmation of the identity of the subfractions must await preparative isolation and tryptic fingerprinting. However, Hnilica has described the identity of F2b histone molecules from calf thymus and from a Walker carcinoma, and it seems not unlikely that the calf histone molecules of identical mobility will have identical primary structures. The recent work of Delange *et al.* (1969) would support this concept. Such an identity of histones from several calf tissues supports the idea of a passive role of histones in gene regulation and perhaps strengthens the concept of an active role as a chromosomal structural protein. These results also suggest that the subfractions of histone I (lysine rich) are connected in some way with cell replication, though whether this is a cause or an effect is at this time unknown.

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