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Structural Studies of Calf Thymus F3 Histone. II. Occurrence of Phosphoserine and ε-*N*-Acetyllysine in Thermolysin Peptides†

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ABSTRACT: Thermolysin digestion of a large cyanogen bromide peptide (CNBr-1) from calf thymus F3 histone yields three peptides which contain ε-*N*-acetyllysine and two peptides which contain phosphoserine, one of which is also acetylated. Only a small proportion of the purified peptides were modified with either acetate (<5–33%) or phosphate (20–22%). One peptide was obtained with 22% in the phosphorylated form, 33% in the acetylated form, and 45% in the unmodified form. There was no evidence of doubly modified peptides. These experiments establish that extensive microheterogeneity exists in F3 histones in that region of the molecule which contained few aliphatic amino acids.

The present study is designed to partially characterize some of the modified amino acids in the F3 histone from calf thymus. Only the cyanogen bromide peptides containing the modified amino acids have been examined.

Methods

Labeling of F3 histone with [^{14}C]acetate and [^{32}P]phosphate, its purification, analysis by acrylamide gel and paper electrophoresis, chromatography, amino acid analysis, and preparation of cyanogen bromide peptides, CNBr-1 and CNBr-1A, have been described previously, Marzluff and McCarty (1972a).

Thermolysin Digestion. Samples of either carboxymethylated F3 histone or the fractionated cyanogen bromide peptides were dissolved in deionized water (2–4 mg/ml). The pH was adjusted to 8.0 with 0.1 N NaOH, and 0.1 volume of 25 mM $CaCl_2$ was added. Thermolysin was added as a freshly prepared solution (2 mg/ml in 25 mM $CaCl_2$) at a final concentration of 2:100 by weight. The pH was maintained between 7.5 and 8.0 for 3.5 hr, after which the pH was adjusted to 4 with 30% acetic acid, and the solution was lyophilized.

Paper Chromatography and Electrophoresis. Paper chromatography was performed as described by DeLange *et al.* (1969a). Solvent systems A–D were butanol–acetic acid–water: A, 200:30:75; B, 100:60:75; C, 90:60:75; D, 70:60:75. Solvent system E was pyridine–butanol–acetic acid–water in proportions 15:10:3:12. Electrophoresis at pH 1.9 was performed in 8.7% acetic acid–2.5% formic acid. Electrophoresis was at 1000 V for 100 min.

Full size (43 × 57 cm) Whatman No. 3M paper was used. The papers were scalloped at the bottom edge to insure an even solvent flow. In preparative runs the sample was applied across the width of the paper. An edge of the sample was cut and sprayed with ninhydrin for identification and cut into 1-cm pieces for scintillation counting. Efficiency for ^{14}C under these conditions was about 40%. The strips containing the peptides were cut out, hung in the opposite direction in a chromatography tank, and eluted with 30% acetic acid for 12–18 hr. A volume of 0.5–1.0 ml was collected in a test tube, diluted, and lyophilized. The yield of this procedure varied from 50 to 70% as judged by recovery of the radioactivity.

Results

Digestion of Cyanogen Bromide Peptides from Calf Thymus F3 Histones. Carboxymethylated F3 histone labeled with either [^{14}C]acetate or inorganic $^{32}PO_4$ was digested with thermolysin, and the peptides were resolved by gel filtration on Sephadex G-25. Ten per cent of alternate fractions were assayed for radioactivity, and the radioactive fractions pooled as shown (Figure 1A,B). The same radioactive peptides were obtained whether one digested the whole molecule or isolated peptides CNBr-1 or CNBr-1A with no evidence of label in CNBr-2 or CNBr-3 (Marzluff and McCarty, 1972b). The radioactive profiles of the acetylated peptides differ in size distribution from those peptides that are phosphorylated. For example, the thermolysin peptide fractions from 128–139 ml, designated as fraction I (for analysis see Figure 2), were phosphorylated (Figure 1B) but not acetylated (Figure 1A), whereas the thermolysin peptide fractions from 158–169 ml, designated as fraction IV, were acetylated but not phosphorylated.

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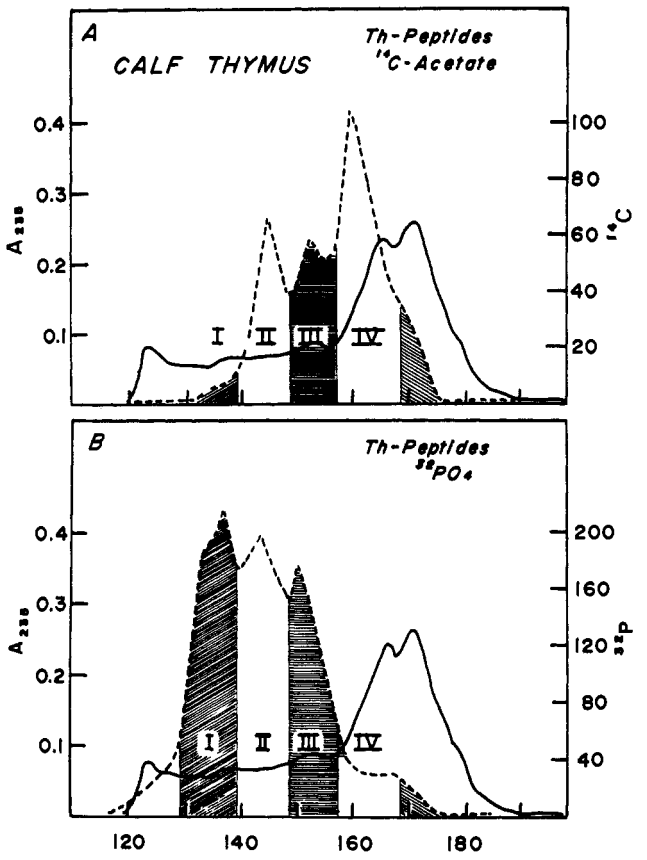

FIGURE 1: Resolution of calf thymus ^{14}C-acetylated F3 histone thermolysin peptides. A. ^{14}C-Acetylated calf thymus F3 histone (1 μmole) was digested with thermolysin, and the peptides were resolved by chromatography on Sephadex G-25 (2.4 × 100 cm), equilibrated with 0.1 N acetic acid eluted at a rate of 12 ml/hr. The specific activity of the F3 histone was 800 cpm/mg. Ten per cent of every other fraction was counted, and the fractions were pooled as indicated: fractions I, II, III, and IV. B. $^{32}PO_4$-Labeled calf thymus F3 histone (1 μmole) was purified, digested with thermolysin, resolved by chromatography on Sephadex G-25, and fractionated as in A. Ten per cent of every other fraction was counted and the fractions were pooled as shown: I, II, III, and IV. The optical density is demonstrated by the solid line, the radioactivity by the dotted lines in Figure 1A and IB.

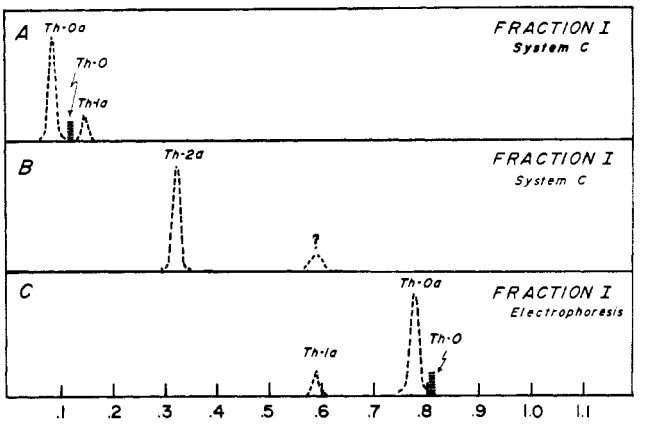

FIGURE 2: Analysis of fraction I (see Figure 1) thermolysin peptides by paper chromatography and electrophoresis. The dotted line represents the percentage of radioactivity in ^{32}P, and the bar graphs represent the location of the ninhydrin peptides. The abscissa represents the relative migration rates in reference to leucine as 1.0 for the chromatography systems. Paper electrophoresis was performed in an acetate–formate buffer (87 mg of glacial acetic acid–27 ml of formic acid per liter) at pH 1.9. The abscissa represents the relative migration rates of the peptides in reference to lysine as 1.0. Peptide Th-0A represents the largest phosphorylated peptide. When this peptide is further digested with additional thermolysin and rechromatographed as shown in Figure 1A, a single major phosphorylated peptide appears that migrates at the same rate as Th-2A and has the same amino acid composition as thermolysin peptide 2. Thus thermolysin 0A represents an incompletely digested peptide of which the major radioactive component is Th-2A.

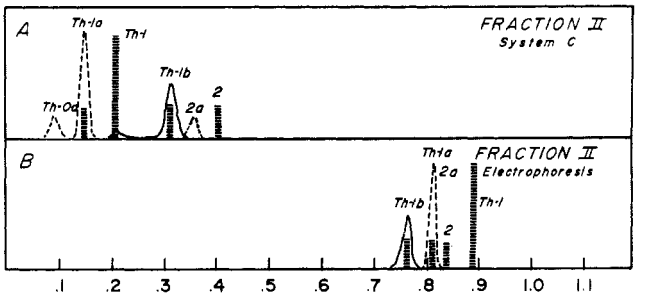

FIGURE 3: Chromatographic analysis of fraction II (from Figure I). The phosphorylated peptides are represented as dotted lines. The solid line represents the acetylated peptides and the bar graph represents the ninhydrin components. The electrophoresis and chromatography are as described in the legend to Figure 2.

Each of the three acetylated thermolysin peptides fractionated on Sephadex G-25 (fractions II, III, IV) contained single major radioactive peptides. Fraction II contained the acetylated thermolysin peptide Th-1b (Figure 3A,B) which represented 15% of the total radioactivity, whereas fraction III contained the acetylated thermolysin peptide Th-3b (Figure 4A,B), which contained 25% of the total radioactivity, and fraction IV contained thermolysin peptide Th-5b (Figure 5B), which contained 60% of the total radioactivity. Some overlap of radioactivity in fraction IV (Figure 5A) was the result of incomplete resolution of the peptides Th-3b and Th-5b on Sephadex G-25.

The presence of acetyllysine in a peptide reduces its polarity. During paper chromatography the reduction in polarity results in a more rapid migration, dependent on the solvent system employed. Thus the mobilities of ϵ-*N*-acetyllysine and lysine relative to leucine (R_{Leu}) in the different solvent systems were: solvent system B, acetyllysine 0.71, lysine 0.38; solvent system C, acetyllysine 0.84, lysine 0.52; solvent system E, acetyllysine 0.58, lysine 0.28. In contrast, electrophoresis of the acetylated peptide would result in a retardation relative to the unacetylated peptide, the amount dependent on the original charge of the peptide at that pH, and therefore the knowledge of relative chromatographic and electrophoretic behavior of the ϵ-*N*-acetyllysine *vs.* lysine was helpful in identifying the partially acetylated peptides.

Characterization of ^{14}C-Acetylated Thermolysin Peptides from Fraction III. The peptides in fraction III were purified from contaminants of fraction IV by an initial preparative paper chromatography in either system B or system E. The acetylated peptide in the purified fraction migrated as a single zone, Th-3b in two different solvent systems (Figure 4A,B) with one major and four minor peptides, all of which migrated slower than the acetylated peptide, Th-3b (Figure 4B). The major ninhydrin component in system B was the unmodified peptide Th-3 in systems B and E (Figure 4A,B), and also on electrophoresis at pH 1.9 (Figure 4C). Peptide Th-2a was a phosphorylated peptide.

TABLE I: Composition of Thermolysin Peptides.[a]

Amino Acid	Th-0	Th-1	Th-2+	Th-3	Th-4	Th-5	Th-6
Lysine	3.3 (3)	2.6 (3)	2	2.1 (2)	0.9 (1)	1.2 (1)	0.8 (1)
Histidine	0.2 (0)	0.3 (0)	1	0.2 (0)	0	0	0
Arginine	3.1 (3)	1.8 (2)	2	1.2 (1)	0.4 (0)	1.0 (1)	1.1 (1)
Aspartic acid	0.3 (0)	0	0	0.4 (0)	1.0 (1)	0.2 (0)	0.7 (1)
Threonine	2.1 (2)	2.3 (2)	1	1.0 (1)	0.1 (0)	0.3 (0)	1.0 (1)
Serine	1.3 (1)	1.1 (1)	1	0.3 (0)	0.2 (0)	0	0
Glutamic acid	1.9 (2)	0.8 (1)	0	1.3 (1)	1.4 (1)	0.2 (0)	1.1 (1)
Proline	0.4 (1)	1.1 (1)	1	0	0.2 (0)	1.0 (1)	1.1 (1)
Glycine	2.4 (2)	1.9 (2)	2	0.3 (0)	1.0 (1)	1.2 (1)	1.0 (1)
Alanine	2.7 (3)	2.3 (2)	2	2.7 (3)	2.0 (2)	0.9 (1)	1.3 (1)
Valine	0	0	0	0	0.2 (0)	0	1.0 (1)
Isoleucine	0	0	0	0	1.0 (1)	0.4 (0)	0
Leucine	0.8 (1)	0	0	1.0 (1)	0.3 (0)	0.3 (0)	0.4 (0)
Phe, Tyr, Cys Met	0	0	0	0	0	0	0
Total residues	18	14	11	9	7	5	9

[a] The amino acid composition of the thermolysin peptides was determined after hydrolysis in 6 N HCl for 20 hr. The details of purification are given in Methods and Results. Several peptides were present in multiple forms. Peptide Th-1 exists in three forms, unmodified, phosphorylated, and acetylated. All of these forms had the same amino acid composition and were obtained in 20, 10, and 13% yields, respectively. The peptide Th-2 exists in two forms, unmodified and phosphorylated, obtained in 20 and 7% yields estimated from the content of serine residues. The peptide Th-0 was a partial digestion product which contained peptide Th-2. Peptide Th-0 also existed in the phosphorylated form, although the two forms were not sufficiently resolved to permit accurate calculations of the yields of each form. The peptide Th-3 existed as the unmodified and acetylated derivative. The yield was estimated assuming one residue of leucine per molecule. The unmodified form was obtained in 25% yield and the acetylated form in 7% yield. Peptide Th-5 existed in both the unmodified and acetylated derivative. The unmodified form was obtained in 25% yield. The small amounts of the acetylated derivative prevented precise determination of its yield and composition. Peptide Th-6 was neither acetylated or phosphorylated and was purified in system E giving a 20% yield.

The amino acid composition and the mobility of the acetylated peptide Th-3b on chromatography and electrophoresis were consistent with its being derived from peptide Th-3. Although the best preparation of the acetylated peptide Th-3b was obtained by chromatography in system B, this preparation was probably still impure as judged by small amounts of serine, histidine, and aspartic relative to proline, Table I. The yield of this peptide could be estimated, however, assuming one residue of leucine. The fact that digestion of this preparation with pronase resulted in only a small reduction in the total amount of lysine in the mixture, approximately equal to the amount of leucine present, suggested an incomplete acetylation of peptide Th-3b. The small amount of any contaminating peptide which cochromatographed with Th-3b was not characterized in these studies.

Peptide Th-3 was the unmodified peptide and peptide Th-3b, the acetylated peptide. Although several unlabeled peptides with greater mobility than the acetylated Th-3b were detected, they were not investigated.

Characterization of ^{14}C-Acetylated Thermolysin Peptides from Fraction IV. Not all of the unlabeled peptides in this fraction were characterized because of the large number of components in this fraction. The peptides were first purified by chromatography in system E from any contaminants, such

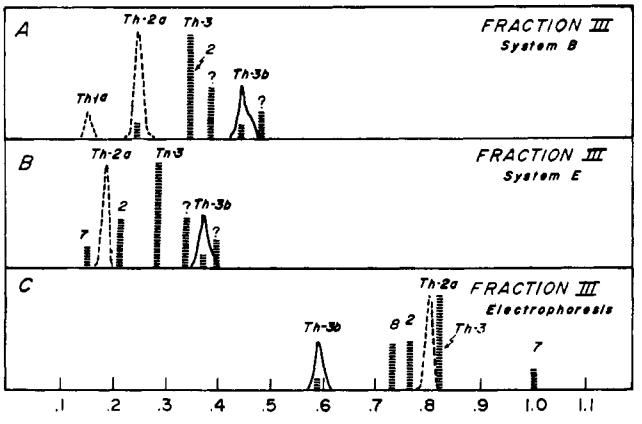

FIGURE 4: Analysis of fraction III as described in Figures 1, 2, and 3.

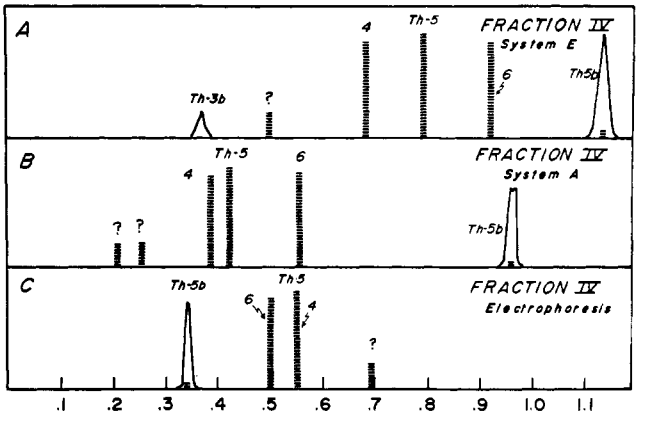

FIGURE 5: Analysis of fraction IV as described in Figures 1–4.

TABLE II: Composition of Modified Calf Thymus F3 Peptides.[a]

		Form		
		PO_4 (%)	Acetate (%)	Unmodified (%)
Th-1	Ala, Arg, Thr, Lys, Glu, Thr, Ala, Arg, Lys, Ser, Pro, Gly, Gly, Lys	22	33	45
Th-2	Ala, Thr, Ser, Pro, Gly_2, Ala, Lys_2, Arg_2	20	0	80
Th-3	Arg, Lys, Glu, Leu, Ala, Thr, Lys, Ala, Ala	0	25	75
Th-5	Pro, Ala, Gly, Lys, Arg	0	<5	>95

[a] Table II shows the composition of modified calf thymus F3 peptides from Table I. The percentage of each form is given.

as the acetylated Th-3b peptide from fraction III, as shown in Figure 4 and 5A. All the radioactivity of Th-5b migrated as a single zone in systems E and A (Figure 5B). For simplicity, only peptides with R_{Leu} greater than 0.25 in system A and 0.45 in system E were characterized. Peptide Th-4 was present in a zone centered at R_{Leu} 0.68 in system E and was further purified by chromatography in system A from other peptide contaminants. When fractionated the peptide Th-4 was observed to be greater than 80% pure as judged by amino acid analysis.

Peptide Th-5, the unmodified peptide, was further purified by rechromatography in system A. Peptide Th-6 was purified by rechromatography in system E. No other peptides with R_{Leu} greater than 0.25 in system A were present in these fractions.

The acetylated peptide Th-5b had a very high specific activity. Analysis of this pentapeptide after chromatography in system E followed by rechromatography in system A (Figure 5B) reproducibly showed the presence of lysine, arginine, proline, alanine, and glycine. The composition of this peptide and its behavior on electrophoresis and chromatography are consistent with its being derived from the unmodified peptide Th-5.

On electrophoresis at pH 1.9, the peptides in fraction IV suggest that peptide Th-5 would have a positive charge of +3, and peptide Th-4 and Th-5b would each have a positive charge of +2, Figure 5C.

The composition of these peptides are given in Table I.

Characterization of $^{32}PO_4$-Labeled Thermolysin Peptides. The $^{32}PO_4$-labeled carboxymethylated histone F3 was digested with thermolysin, and the peptides were fractionated on Sephadex G-25 (Figure 1B). The fractions were pooled as shown and analyzed by paper chromatography and electrophoresis. The presence of phosphoserine in a peptide would alter its chromatographic behavior, since the phosphorylated peptide would be more polar and would be retarded on chromatography, an effect opposite to that seen for the acetylated peptides. On electrophoresis at pH 1.9, however, the phosphorylated peptide will also be retarded, as it has an extra (partial or complete depending on the pK of the phosphate group in the peptide) negative charge. The relative mobilities of phosphoserine and serine in systems C are R_{Leu} phosphoserine 0.50:serine 0.65, in systems E R_{Leu} phosphoserine: 0.36: serine 0.50.

Purification of $^{32}PO_4$-Labeled Peptides in Fraction I. The $^{32}PO_4$-labeled peptides in fraction I were purified by chromatography in system C (Figure 2A). Of the radioactivity 80% migrated as a single component Th-0a, and the remaining 20% migrated in the position of Th-1a. The majority of the radioactivity also was a single component in system D (7.8 cm in 18 hr) not shown here and electrophoresis at pH 1.9 (R_{Lys} 0.78), (Figure 2C).

A single large ninhydrin-positive peptide was detected, Figure 2, and was obtained in lower yields than any of the other smaller peptides and was probably a product of incomplete digestion. For this reason, the largest phosphorylated peptide Th-0a was isolated and digested further with thermolysin and analyzed by rechromatography in system C. Of the radioactive phosphate 80% now migrated as a single zone (Figure 2B) in the same position as peptide Th-2a, the major phosphorylated peptide found in fraction III (Figure 4).

Purification of $^{32}PO_4$-Labeled Peptides in Fraction II. The ninhydrin-positive peptides present in fraction II were the same as those present in fraction II of the acetylated digest. The majority of the radioactivity (60–70%) migrated in a single zone Th-1a as shown in Figure 3A. The rest of the radioactivity (30–40%) was due to some contamination with fraction I, Th-0a, and fraction Th-2a.

Purification of $^{32}PO_4$-Labeled Peptides Present in Fraction III. The ninhydrin-positive peptides present in fraction III were similar to those found in fraction III of the acetylated peptides. The $^{32}PO_4$ radioactivity was detected in peptide Th-2a as a single major zone in systems B, C, and E, Figures 2B, 4. The radioactive peptide and the parent unmodified peptide in this fraction were never completely purified because only small amounts of peptide material were present in the zone containing the radioactivity. Peptide Th-2 probably represents the unmodified form of the phosphorylated peptide. Analysis of the small amount of material associated with the $^{32}PO_4$ showed a high glycine and alanine content in agreement with this assignment.

Discussion

These experiments establish that extensive microheterogeneity in histone F3 may exist, due to modification of preformed histones, as a result of postsynthetic modifications; see Table II. Two lysyl residues are demonstrated to be partially acetylated in peptides Th-1, Th-3, Th-5, two seryl residues partially phosphorylated in peptides Th-1, Th-2, and in addition, at least one lysyl residue may be partially methylated (DeLange *et al.*, 1970). In addition to this microheterogeneity, we have demonstrated that histone F3 may also exist in two different forms differing in the number of half-cystine residues (Marzluff and McCarty, 1972b; Marzluff *et al.*, 1972b). Thus, although this histone is apparently a single polypeptide chain whose structure has been rigidly conserved during evolution

(Fambrough and Bonner, 1968), this histone may exist in many different forms with a variety of different, as yet unknown, functions.

Sequence studies of other histones also suggest that there is a specific region of these proteins which is more basic than the whole histone and which contains few bulky aliphatic and aromatic amino acids. This region has been suggested to represent a major DNA binding site. Similar areas deficient in aliphatic and aromatic amino acids have been shown to be present at the amino terminal of histone F2a1 (DeLange *et al.*, 1969b) and histone F2b (Hayashi and Iwai, 1970). Measurement of histone–DNA binding (Shih and Bonner, 1970) suggests in addition that not all of the basic residues of histone F2a1 are involved in binding DNA, and a similar conclusion has been reached by Johns and Hoare (1970) for histone F3. Li and Bonner (1971) have recently shown that the amino-terminal half of histone F2b (the more basic half) binds more strongly to DNA than the carboxyl-terminal half does.

All the modified residues are present in peptide CNBr-1, and it is tempting to speculate that they may be found in a DNA binding site. Since two of the acetyllysine residues and all the phosphoserine residues were present in large basic thermolysin peptides, it is not unreasonable to postulate that they were derived from a region of the molecule which contained few aliphatic amino acids. In this regard it is interesting to note that in histone F2a1 all the modified amino acids are also present in the amino-terminal region (DeLange *et al.*, 1969a; Sung and Dixon, 1970; Dixon *et al.*, 1969). Acetyllysine is also found in the basic amino-terminal region of histone F2b (Marzluff *et al.*, 1972a). Preformed, chromatin-bound histones are modified (Kleinsmith *et al.*, 1966; Vidali *et al.*, 1968; Marzluff and McCarty, 1970). The effect of modification of specific residues on the reconstruction of chromatin structure and its effect on transcription and replication remain for further experimentation.

Many different forms of calf thymus histone F3 could theoretically exist, arising from different combinations of modifications in the same molecule. Figure 4 suggests that a great many of the ^{14}C-acetylated molecules contained more than 1 modified amino acid as about 50% of the radioactivity migrated slower than expected for a single difference in positive charge. No obvious correlation of occurrence of phosphoserine and acetyllysine in the same molecule was found for the one thermolysin peptide Th-1, which contained both acetyllysine and phosphoserine.

The total content of acetyllysine, judging from the yields of the peptides, was 0.60–0.75 residue per molecule and that of phosphoserine about 0.4 residue. This is the first report of phosphoserine occurring in histone F3, although Hayashi and Iwai (1970) have found alkali-labile phosphate associated with this histone. Acetyllysine in calf thymus histone F3 has been reported previously (Vidali *et al.*, 1968). While this work was in progress DeLange *et al.* (1970) reported the sequence of two maleylated tryptic peptides containing acetyllysine and one containing methyllysine. In addition, they reported the sequence of peptide CNBr-3, the carboxyl terminus of the protein. Our results are consistent with their results. The acetyllysine-containing peptides they found correspond to peptide Th-1 and Th-3. It is possible that Th-5 represents five of the same amino acid residues also present in the eleven amino acid peptide Th-2. While it has not been rigorously proven here that only a single lysyl residue in each peptide was acetylated, this is likely, both from the sequence studies of DeLange *et al.* (1970) and the fact that the same acetylated tryptic peptides are found in digests of ^{14}C-acetylated Th-1 and Th-3 from different species (Marzluff and McCarty, 1972b). Further information on the structural relationships of the modified residues to one another will be forthcoming from further sequence studies.

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