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The Synthesis of Diacetylated Histone H4-(1–37) for Studies on the Mechanism of Histone Deacetylation[1]

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The heptatriacontapeptide [Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}]histone H4-(1–37) was synthesized by the automated solid phase method. This dual-labeled peptide was designed for studies on the mechanism of histone deacetylation. The synthesis was monitored by an automated picrate method; and the product, purified by affinity chromatography and ion exchange chromatography, was homogeneous by polyacrylamide gel electrophoresis and gave excellent amino acid and radiolabel ratios. A purified calf thymus histone deacetylase released acetyl groups from this synthetic peptide at essentially the same rate as from a diacetylated 1–37 peptide derived from native calf thymus histone H4. The relative rate of release of [^{14}C]acetyl from Lys^{12} and of [^{3}H]acetyl from Lys^{16} was 1.03 ± 0.03 throughout the time course of the enzymatic assay. Based on these results, possible mechanisms of histone deacetylation are proposed.

Histones are subject to several biochemical modifications that occur after ribosomal synthesis of the protein is completed. These postsynthetic modifications alter the primary structure and, consequently, the physical and chemical properties of the histone molecules. Quantitative changes in such modifications, under cellular regulation, are thought to provide mechanisms for controlling the functional properties of histones in chromatin (*1*). The principal postsynthetic modifications are acetylation of lysine (*2*), *N*-methylation of lysine, arginine, and histidine (*3*, *4*), and phosphorylation of serine, threonine, lysine, and histidine (*5*).

Acetylation of histones is correlated with their reduced ability to inhibit RNA synthesis *in vitro* (*6*) and is an indicator of transcriptional activity *in vivo* (*7*). The acetylation of *N*-terminal serine (*8*) occurs while the peptide chain is still bound to the ribosome and is probably an irreversible change. In contrast, the acetylation of lysine residues is a dynamic, enzymatically regulated process, which occurs primarily in the nucleus. Once incorporated into the nucleosome, the histones of the core H2a H2b, H3 and H4 become subject to a controlled series of acetylation and deacetylation reactions (*9*). Enzymes capable of effecting these changes have been detected and purified (*10–14*) and their substrate specificity studied (*13*, *15*, *16*). The major sites of reversible acetylation of H4 (*17*) are known to be on the ε-amino groups of lysine residues 5, 8, 12, and 16 (*1*, *18*, *19*) (Fig. 1), but very little is known about the sequence of addition or removal of the acetyl groups or of the substrate requirements of the enzymes involved. It is of considerable interest to know whether the same enzyme

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[3] I had great admiration for George Kenner, and am pleased to have been counted among his friends (RBM).

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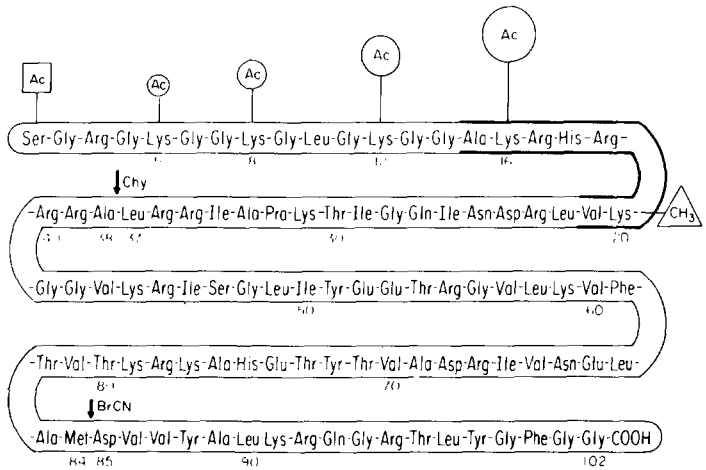

FIG. 1. Amino acid sequence of calf thymus histone H4. The sites of postsynthetic modifications in this and other species are indicated. The points of cleavage by CNBr and chymotrypsin are indicated by the arrows.

acts on all of the histones and at all of the acetylatable sites and whether the acetyl groups are removed randomly or in a particular sequence. We have undertaken to answer some of these questions by a chemical approach in which pure synthetic peptides containing differentially labeled N^{ϵ}-acetyl groups at defined positions are used as substrates for a purified nuclear deacetylase.

It has already been found that the small synthetic monoacetylated heptapeptide H-Ala-Lys([^{14}C]Ac)-Arg-His-Arg-Lys-Val-OH corresponding to histone H4-(15–21) (*16*) and the diacetylated dodecapeptide histone H4-(10–21) (*15*) derived by enzymatic digestion of natural histone are not substrates for the deacetylase. However, larger histone fragments derived from H4 by cyanogen bromide cleavage (residues 1–84) or by chymotryptic digestion (residues 1–37) can be enzymatically deacetylated (*16*).

For the present study we have synthesized [Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}]histone H4-(1–37)-heptatriacontapeptide and have compared the rates of release of the two labels by the calf thymus histone deacetylating enzyme.

EXPERIMENTAL[4]

Materials

Commercial reagents included: *tert*-butyl azidoformate (Pierce), Boc-amino acids (Beckman Instruments, Bachem, Chemical Dynamics, Fox Chemical Co.), Affigel 501 (Bio-Rad Laboratories), trifluoroacetic acid (Halocarbon Products), diisopropylethylamine, dicyclohexylcarbodiimide, and N^{ϵ}-acetyl-L-lysine (Aldrich Chemical),

[4] Abbreviations: Nomenclature for amino acids, peptides, and derivatives follows the recommendations of IUPAC–IUB *J. Biol. Chem.* **241**, 2491 (1966) and **242**, 555 (1967). In addition: Mbh, 4,4′-dimethoxybenzhydryl; Sulfmoc, 9(2-sulfo-fluorenyl)methyloxycarbonyl; Dnp, 2,4-dinitrophenyl; Cl_2Z, 2,4-dichlorobenzyloxycarbonyl; MeOBzl, 4-methoxybenzyl; PAGE, polyacrylamide gel electrophoresis; TEMED, tetraethylmethylene diamine; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; histone H4 in the system of Bradbury is equivalent to histone F2A1 in the system of Johns and Butler.

dichloromethane and picric acid (Eastman), chloromethyl-copoly(styrene-1%-divinylbenzene) resin (Lab Systems Inc), ultrapure urea (Schwartz Mann), acrylamide (Eastman), bis-acrylamide, TEMED and ammonium persulfate (Bio Rad), and [^{3}H]Ac_2O and [^{14}C]Ac_2O (New England Nuclear).

N^{α}-*tert*-Butyloxycarbonyl-N^{β}-4,4′-dimethoxybenzhydryl-L-asparagine and N^{α}-*tert*-butyloxycarbonyl-N^{β}-4,4′-dimethoxybenzhydryl-L-glutamine were prepared according to König and Geiger (*20*) and Hodges and Merrifield (*21*) and N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-2,4-dichlorobenzyloxycarbonyl-L-lysine was synthesized as previously described (*22*). Organomercurialagarose was prepared by the method of Cuatrecasas (*23*) as described by Ruiz-Carillo (*24*). When packed in a column, this affinity support was determined by Ellman's reagent to bind 0.9 to 1.2 μmol of thiol per ml.

The solid phase synthesis was carried out on an automated Beckman 990 peptide synthesizer. Automatic picrate monitoring was performed using an instrument constructed in this laboratory (*25*). Amino acid analyses were performed on a Beckman 121 analyser and radioactivity was measured on a Beckman LS-350 liquid scintillation spectrometer. HF reactions were carried out in a Diaflon HF line from Toho Co., Osaka. Column effluents were continuously monitored at 206 or 280 nm on a Uvicord III (LKB Instruments). nmr spectra were obtained on a Varian 220 MHz Fourier Transform Spectrometer. Microanalyses were by Mr. S. T. Bella, Microanalytical Laboratory, Rockefeller University.

Thin layer chromatography was performed on Analtech Silica G and GF plates in one of four solvent systems: (A) $CHCl_3$:MeOH:HOAc (85:10:5), (B) $CHCl_3$:MeOH (9:1), (C) $CHCl_3$:MeOH (99:1), (D) *n*-BuOH:pyridine:HOAc:H_2O (30:20:6:24). Compounds were visualized with 254 nm light, by spraying with ninhydrin in acetone and heating, or by spraying with bromcresol green solution. Boc groups were removed on tlc plates by exposure to HCl vapor in a closed tank before spraying with ninhydrin.

Peptides were hydrolyzed in 6 *N* HCl containing 1% thioglycolic acid in sealed evacuated tubes for 22 hr at 110°C. For accurate measurement of cysteine and methionine, peptides were oxidized with performic acid before hydrolysis (*26*).

Polyacrylamide gels were sliced into 1-mm fractions with a Mickel gel slicer. Each slice was placed in a glass vial and covered with 0.2 ml of 30% H_2O_2 and heated 18 hr at 50°C. After cooling, 10 ml of Aquasol was added for scintillation counting. Alternatively, gel slices were oven-dried at 60°C for 1 hr and combusted in a Packard sample oxidizer before counting the separated [^{3}H]H_2O and [^{14}C]CO_2.

N^{α}-tert-Butyloxycarbonyl-N^{ϵ}-acetyl-L-lysine

N^{ϵ}-Acetyl-L-lysine (941 mg, 5.0 mmol) was suspended in 8 ml of 1:1 dioxane–H_2O and adjusted and maintained at pH 9.8 with NaOH while *tert*-butyl azidoformate (1.5 ml, 10.6 mmol) was added. After 18 hr the mixture was extracted with ether, adjusted to pH 3.9 with citric acid, and extracted with ethyl acetate. The dried solution was evaporated to small volume and allowed to crystallize. Yield, 965 mg (67%); mp, 137.5–138.5°C (lit (*27*), 137–138°C). tlc (System A): single ninhydrin-positive spot, R_f 0.56. nmr (220 MHz) showed no free acetic acid.

Anal. Calcd for $C_{13}H_{24}N_2O_5$: C, 54.15; H, 8.39; N, 9.72. Found: C, 54.22; H, 8.27; N, 9.80.

N^{α}-tert-Butyloxycarbonyl-N^{ϵ}-[^3H]acetyl-L-lysine

[^{3}H]Acetic anhydride (25 mCi, 0.50 mmol) and 7.6 mmol of unlabeled acetic anhydride were dissolved in 10 ml of dry CH_2Cl_2 and cooled in an ice bath. A 1.5-ml aliquot was then added slowly to a screw-capped test tube containing N^{α}-Boc-Lys (246 mg, 1.0 mmol) and after shaking vigorously for 10 min at 0°C the vessel was shaken for 5 hr at room temperature, after which time all of the N^{α}-Boc-Lys had dissolved. The CH_2Cl_2 was evaporated, water added, and the solution lyophilized. The lyophilization from water was repeated. tlc (System A) showed a major component by ninhydrin (90% of counts) at R_f 0.56, and small spots at R_f O, and R_f 0.94. Approximately 2% of free acetic acid could be detected by nmr (220 MHz) in D_2O at 2.1 ppm (CH_3). The CH_3 protons of N^{ϵ}-acetyllysine were at 2.0 ppm. Addition of NaOD shifted the CH_3 protons of the acetate upfield to 1.9 ppm. Addition of acetic acid to the synthetic product before and after NaOD confirmed the identity of the peaks.

The crude Boc-N^{ϵ}-Ac-Lys was dissolved in EtOAc and extracted several times with an equal volume of 0.5 *M* citrate buffer (pH 3.5). The combined aqueous phases were then reextracted with EtOAc several times. Finally, the combined EtOAc extracts were washed three times with 0.1 volume of saturated aqueous NaCl. After drying over Na_2SO_4 the EtOAc was evaporated to an oil. Yield, 73%; specific activity, 3.55×10^6 dpm/μmol. On tlc (System A), this product showed a single ninhydrin-positive component at R_f 0.56, which contained 99% of the total ^{3}H counts. No citric acid was observed after bromcresol green spray of the tlc plate and no acetic acid was detected by nmr.

N^{α}-tert-Butyloxycarbonyl-N^{ϵ}-[^{14}C]acetyl-L-lysine

This compound was prepared in homogeneous form from 1 mCi of [^{14}C]Ac_2O and 0.5 mmol of N^{α}-Boc-Lys by the same procedure described for the [^{3}H]acetyl derivative. A 69% yield of product with a specific activity of 1.55×10^6 dpm/μmol was obtained. It was dissolved in CH_2Cl_2 and used directly for the peptide synthesis.

Preparation of Tritiated Acetyl Calf Thymus Histone H4

Nuclei were isolated from 100 g of fresh calf thymus according to the procedure of Vidali *et al.* (*28*) and incubated with sodium [^{3}H]acetate (10 mCi) for 15 min. The histones were then extracted from the labeled nuclei with 0.24 *N* HCl, centrifuged at 16 000*g* for 15 min and precipitated by addition of 10 volumes of acetone. The individual histones were obtained by gel filtration according to Böhm *et al.* (*29*). Thus, the sample of mixed histones (130 mg) was dissolved in 7 ml of a solution containing 8 *M* urea, 1% mercaptoethanol, 0.02 *N* HCl, 0.05 *M* NaCl, and 0.02% NaN_3, pH 1.7. It was applied to a 1.5 × 100-cm Bio-Gel P-60 column and eluted with a solution of 0.02 *N* HCl, 0.05 *M* NaCl, and 0.02% NaN_3, pH 1.7. The histones were eluted in the order H1, H3 + H2A, H2B, H4. All fractions except H1 were labeled with ^{3}H. Polyacrylamide gel electrophoresis followed by counting of the bands showed that the histone H4 contained 1.2% of triacetylated, 11.0% diacetylated, 42.1% monoacetylated, and 45.7% unacetylated molecules [see Wangh *et al.* (*30*)]. Furthermore, the specific activity of the subgroups increased in proportion to the number of [^{3}H]acetyl groups present. The acetyl group of the monoacetylated H4 is known to be on the N^{ϵ}-amino group of lysine-16. However, the homogeneity of the di- and triacetylated subgroups in

relation to the distribution of the acetyl groups on lysine residues 5, 8, 12, and 16 is not known, although it is clear that the N^{α}-acetyl group is not labeled by this procedure (*1*).

Preparation of Natural Mono [N^{ϵ}-[^3H]acetyllysine]histone H4-(1–37) and Di[N^{ϵ}-[^3H]-acetyllysine]histone H4-(1–37)

Chymotryptic digestion of 200 mg of purified [^{3}H]acetate-labeled histone H4 was performed by the method of De Lange *et al.* (*17*). The digest was fractionated on a 0.9 × 60-cm carboxymethylcellulose (Cellex-CM) column using two linear NaCl gradients (*16*). The method resolved the peptides on the basis of their varying states of acetylation. Many peaks were detected by absorbance at 230 nm, but only three major radioactive peaks were found. Fractions 168–173 (peak I), 176–182 (peak II), and 192–200 (peak III) were separately pooled and desalted on Bio-Gel P-2. Amino acid analyses of each peak agreed with that expected for residues 1–37 of histone H4 (*16*). Gel electrophoresis showed that peaks I, II, and III primarily contained, respectively, the diacetylated, monoacetylated, and nonacetylated histone H4-(1–37) fragments in the ratios 0.15:1.0:1.0.

Preparation of Histone Deacetylase

Calf thymus histone deacetylase was isolated and purified as described by Vidali *et al.* (*14*) from 100 g of fresh, cold thymus glands. The procedure involved the homogenization of minced tissue in 100 ml of buffer A [0.14 *M* NaCl–0.05 *M* phosphate buffer, pH 6.8], followed by centrifugation at 100 000*g* for 60 min. The protein was precipitated by addition of three volumes of cold acetone and collected at 30 000*g*. The protein was extracted from the pellet by buffer, clarified by centrifugation, and purified on a Sepharose 4B column. An approximately 120-fold purification was achieved.

Histone Deacetylase Assays

Enzyme assays for the deacetylation of histone H4 and its fragments were based on the kinetics of release of radioactive acetate (*13*, *14*), which were determined using one of two methods. (A) Aliquots (1 ml) of acetyl histone or acetyl peptide (1 mg/ml in buffer A) and 1 ml of enzyme solution were incubated at 37°C for 1 to 60 min with gentle mixing. The reaction was terminated by addition of 0.2 ml of 0.1 *M* HCl containing 0.05 *M* unlabeled acetic acid as a carrier. Ethyl acetate (3.0 ml) was added and shaken vigorously to extract the released [^{3}H]acetic acid. Radioactivity was measured on a 1.5-ml aliquot of the organic layer by scintillation counting. When samples were double labeled with [^{3}H] and [^{14}C], they were either counted directly by liquid scintillation with appropriate corrections for quench and efficiency or by combustion followed by individual counting of the separated [^{3}H]H_2O and [^{14}C]CO_2. (B) The acidified incubation mixture was applied directly to an 0.7 × 20-cm Bio-Gel P-2 column and eluted with 5% acetic acid at 12 ml/hr to separate the released radioactive acetic acid from unreacted substrate and enzyme. The individual tubes (0.5 ml) were then counted as described for method (A).

Polyacrylamide Gel electrophoresis (PAGE)

Three types of gel procedures were used, depending on their specific application. They

were derived from the acetic acid–urea–PAGE system of Panyim and Chalkley (*31*, *32*) or as modified by Wangh *et al.* (*30*) and Lewis *et al.* (*33*). Bulk-grade acrylamide was recrystallized and decolorized with charcoal before use.

(A) Histone H4 was characterized on 0.6 × 25-cm gels in a 15% PAGE system (*30–32*) composed of 2 parts 60% (w/v) acrylamide and 0.3% (w/v) bisacrylamide in H_2O; 5 parts 10 *M* urea and 0.2% (w/v) $(NH_4)_2S_2O_8$ in water (freshly prepared), and 1 part 1% (w/v) TEMED and 43.2% (v/v) glacial acetic acid in H_2O. All solutions were millipore filtered, degassed, mixed just before use, and poured at room temperature. The gels were preelectrophoresed in 0.9 *N* HOAc at 175 V for 12 hr or until the amperage was constant. The protein was dissolved in 10 *M* urea–2 *N* HOAc at 1 mg/ml and approximately 20 μg was applied to the gel. They were run at constant current of 1.5 mA for 20 hr. Staining was with amido black in 7% HOAc–35% MeOH.

(B) Histone H4-(1–37) peptides were not resolved on the 15% gels, but were well resolved on an 18% polyacrylamide gel containing 0.7% bisacrylamide, which was composed of 3 parts 54% (w/v) acrylamide and 2.1% (w/v) bisacrylamide in H_2O, 5 parts 10 *M* urea and 2.25% (w/v) $(NH_4)_2S_2O_8$, and 1 part 2% (w/v) TEMED and 48.6% (v/v) acetic acid in water.

(C) Small peptides >9 residues were resolved on a 30% gel, which was composed of 41.6 g acrylamide, 0.4 g bisacrylamide, 8 ml glacial acetic acid, 23 g ultra-pure urea and 64 ml of H_2O. This solution was degassed *in vacuo* for 5 min and millipore filtered. TEMED (0.25 ml) was added and then 0.25 g ammonium persulfate in 0.5 ml H_2O. If tubes smaller than 0.5 cm in diameter were poured, they were polymerized for 1 hr in the cold room, to avoid bubbles and cracks, and the polymerization was then completed at room temperature. The gels were stained for 2.5 to 5 hr only, using 0.5% amido black in 9.5% HOAc–40% EtOH–50% H_2O. These gels had to be removed by cracking the glass columns.

RESULTS AND DISCUSSION

Automated Solid Phase Synthesis of [*Lys*($[^{14}C]Ac$)^{12}, *Lys*($[^{3}H]Ac$)^{16}] *Histone H4-(1-37)*

The general methods of solid phase peptide synthesis (*34–37*) were applied, but with several modifications. The carboxyl-terminal residue was attached to the solid support, followed by the automated stepwise addition of the remaining 36 residues of protected amino acids. Unreacted peptide chains were terminated by acetylation with acetylimidazole after each synthetic cycle to convert any potential deletion peptides into terminated peptides. The coupling and deprotection reactions were monitored for completion by the automated picrate titration method (*25*). The 1–37 sequence was then extended by two more residues, Met and Cys(MeOBzl), to permit purification by a new affinity method that was developed for this synthesis (*38*). Finally, the peptide was cleaved from the resin, purified by affinity and conventional chromatography, characterized for structure and purity, and assayed as a substrate for the nuclear histone deacetylase.

The synthesis began with 5 g of chloromethyl-copoly(styrene-1%-divinylbenzene)

resin (0.24 mmol Cl/g). The polymer was esterified by mixing with 2.4 mmol of the cesium salt of Boc-leucine in 40 ml of DMF at 50°C for 14 hr (*39*). Amino acid analysis and picrate titration (*40*) showed 0.24 mmol of leucine/g. Thus, quaternary ammonium sites and excess chloromethyl sites on the resin were avoided. The Boc-Leu-resin (2.48 g) was placed in the reaction vessel of a Beckman Model 990 solid phase synthesizer and subjected to a synthetic protocol that included deprotection, neutralization, double coupling, acetylation, and picrate monitoring following deprotection and each of the

TABLE 1

THE PROGRAM[a] FOR AUTOMATED SOLID PHASE SYNTHESIS AND MONITORING OF DIACETYLATED HISTONE H4-(1–37)

Step	Reagent	Operation	Vol (ml)	Number	Mix time (min)
1	CH_2Cl_2	Deprotection	50	5	1
2	TFA (50% in CH_2Cl_2)		40	1	1
3	TFA (50% in CH_2Cl_2)		40	1	30[b]
4	CH_2Cl_2		50	5	1
5	*i*-PrOH		40	2	1
6	CH_2Cl_2		50	2	1
7	*i*-PrOH		40	2	1
8	CH_2Cl_2	Monitor blank	50	6	1
9	DIEA (5% in CH_2Cl_2)	Neutralization	40	3	2
10	CH_2Cl_2		50	5	1
11	Picric Acid (0.05 *M*)[c]	Monitor deprotection	40	2	5
12	DMF (5% in CH_2Cl_2)		40	2	1
13	CH_2Cl_2		50	5	1
14	DIEA (5% in CH_2Cl_2)		40	3	2
15	CH_2Cl_2		50	4	1
16	*i*-PrOH		40	3	1
17	CH_2Cl_2		50	5	1
18	Boc-Amino acid in CH_2Cl_2	Coupling	9	1	0
19	CH_2Cl_2 rinse		4.3	1	5
20	DCC in CH_2Cl_2		7	1	1
21	CH_2Cl_2 rinse		4.3	1	30
22	CH_2Cl_2		50	5	1
23	*i*-PrOH		40	2	1
24	CH_2Cl_2		50	2	1
25	*i*-PrOH		40	2	1
26	CH_2Cl_2	Monitor blank	50	6	1
27–35	Repeat steps 9–17	Monitor coupling			
36–44	Repeat steps 18–26	Second coupling			
45–53	Repeat steps 9–17	Monitor 2nd coupling			
54	Ac-Imidazole in CH_2Cl_2	Acetylation	30	1	30
55–59	Repeat steps 22–26				
60–68	Repeat steps 9–17	Monitor acetylation			

[a] Begin with 2.48 g Boc-Leu-Resin (0.687 mEquiv).

[b] Beginning with deprotection 22 (Lys^{16}) the time of TFA treatment was reduced to 12 min.

[c] Beginning with coupling 19 (His^{18}) imidazolium picrate (0.1 *M*) was used.

coupling and acetylation steps (Table 1). The Boc group was used for N^{α}-protection for all amino acids. Side chain protection was: Ser(Bzl), Thr(Bzl), Asp(OBzl), Arg(Tos), Cys(MeOBzl), Lys(2,4-Cl_2Z), His(Dnp), Asn(Mbh), Gln(Mbh). For lysine-12 the side chain was protected with N^{ϵ}[^{14}C]Ac and for lysine-16 with N^{ϵ}[^{3}H]Ac. *N*-Acetylimidazole (10 mg/ml in CH_2Cl_2) was the terminating reagent. The standard coupling conditions involved the addition of Boc-amino acids (3 Equiv compared to the number of free amino groups) in 9 ml of CH_2Cl_2 to the peptide-resin, followed by a 4.3-ml rinse with CH_2Cl_2. After 5 min, dicyclohexylcarbodiimide (3 Equiv) in 7 ml of CH_2Cl_2 was added and mixed for 1 min. Finally, a 4.3-ml rinse with CH_2Cl_2 was added, and the coupling reaction was continued for 30 min. The coupling volume was 10 ml/g of polystyrene-DVB resin. Because of their poor solubility in CH_2Cl_2, Boc-Arg(Tos), Boc-Asn(Mbh), and Boc-Gln(Mbh) were dissolved in 2.3 ml of DMF and diluted with 6.7 ml of CH_2Cl_2. Boc-Lys([^{3}H]Ac) (3.55×10^6 dpm/μmol) and Boc-Lys([^{14}C]Ac) (1.55×10^6 dpm/μmol) were coupled in 1.5 Equiv and followed by a second coupling with non-radioactive Boc-Lys(Ac). The total incorporation of [^{3}H] was 113.5 μmol and of [^{14}C] was 103.5 μmol; the final specific activities were [^{3}H] 3.27×10^6 dpm/μmol and [^{14}C] 1.39×10^6 dpm/μmol. In this synthesis the N^{α}-amino group of Ser^{1} was not acetylated.

The first 21 residues were deprotected with 50% (v/v) TFA/CH_2Cl_2 for a 1-min prewash followed by a 30-min reaction, and the remaining residues were deprotected for only 1 min plus 12 min. Neutralization was brought about with 5% diisopropylethylamine (three times for 2 min each). These washes were collected and served as the blank for the picrate monitoring procedure.

Picrate Monitoring of the Synthesis

The picrate method of Gisin (*40*) for measuring resin-bound free amino groups has been automated by Hodges and Merrifield (*25*). The apparatus was incorporated into the Beckman 990 synthesizer so that the monitoring program became a subprogram of the synthesizer and could be actuated at appropriate places in the synthetic cycle. The sensitivity, precision, and accuracy of this nondestructive method were evaluated in detail on several small model peptides (*25*). The present synthesis is the first test of the procedure on a longer and more complex peptide. The synthesis was monitored after each coupling and termination step, and after many of the deprotection steps.

For the monitoring of the first 19 residues, the reagent was 0.05 *M* picric acid in CH_2Cl_2; but after the introduction of His (Dnp) at the 19th coupling (residue 18 of histone H4 sequence), it was necessary to monitor with imidazolium picrate to avoid partial titration of the imidazole (*25*). In some instances, when indicated by the monitoring, additional couplings were carried out.

The monitoring for unreacted amino groups following the coupling reactions led to an unexpected finding which has subsequently become better understood. As shown in Fig. 2, curve A, there was a continual and appreciable rise in the background picrate value as the peptide chain continued to grow. After 7 steps it had reached 39 μmol, or 6.8% of the initial value of peptide chains on the resin. Beyond that point there was considerable fluctuation, and values as high as 80 μmol were obtained. We have now found (*41*) that this was an artifact not related to the number of unreacted amino groups, but to the conditions of the automated picrate monitoring, i.e., failure to remove completely the excess, nonspecifically adsorbed picrate following the initial treatment with picric acid or

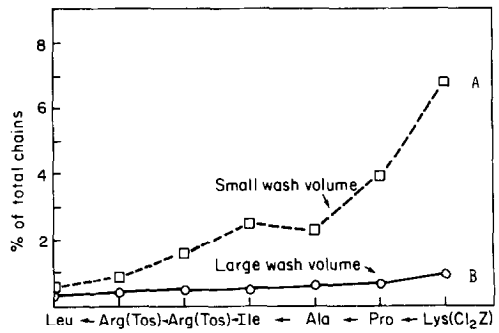

FIG. 2. Picrate monitoring of the coupling reaction. After equilibration with 0.05 *M* picric acid, the resin was washed (A), with 5% DMF in CH_2Cl_2 twice and with CH_2Cl_2 four times at a ratio of 16 ml/g resin; (B) with 5% DMF in CH_2Cl_2 six times at a ratio of 40 ml/g resin.

imidazolium picrate. Two requirements are now known that were not appreciated at the time the method was first worked out: (i) It is very important to use an adequate volume to weight ratio during the wash. Each of the six washes should be with at least 40 ml solvent per gram of resin. (ii) The washes should be with 5% DIEA in CH_2Cl_2. When these modifications were made, the monitoring data were quite satisfactory (Fig. 2, curve A). In spite of the deficiency in the monitoring program, the data were very useful in guiding the synthesis of the H4-(1–37) peptide. We relied not on the absolute level of the picrate value but rather on its constancy after 1, 2, or more couplings of the same Boc-amino acid or after the acetylimidazole termination step. Thus, the couplings were repeated until two consecutive picrate titrations were in acceptable agreement with one another. The average difference between the final two couplings in the 36 steps of this synthesis was 2.2 μmol.

The results of the monitoring of free amino groups after the deprotection step are

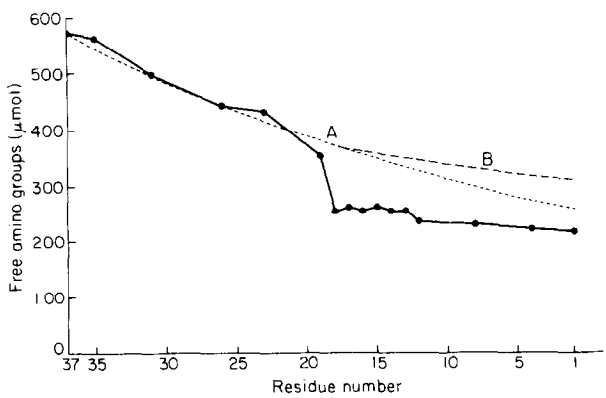

FIG. 3. Picrate monitoring after deprotection. Deprotection with 50% TFA-CH_2Cl_2 was for 1 + 30 min for the first 21 synthetic cycles (residues 37 through 17) and for 1 + 12 min for the last 16 cycles (residues 16 through 1). The heavy line connects the experimental points. The dotted line (A) shows the remaining amino groups calculated for a uniform loss of 2.2% per synthetic cycle (residues 37 through 17), and the dashed line (B) is calculated for a loss of 1% (residues 16 through 1). The reagent was picric acid for residues 37–19 and imidazolium picrate for residues 18–1. The graph has been corrected for the removal of 90 μmol of peptide-resin after the coupling of Arg^{23}.

shown in Fig. 3. This curve gives a measure of the growing peptide chains at each cycle of the synthesis. It was found that the available amino groups decreased at a rather uniform rate of approximately 2.2% per cycle when the deprotection was with 50% TFA/CH_2Cl_2 for 1 + 30 min. Because of this large loss, the time of deprotection was reduced to 1 + 12 min beginning with deprotection 22 (Lys^{16}), and it was then observed that the loss was reduced to approximately 1% per cycle. This loss of amino groups has at least two causes: (i) acidolysis of the benzyl ester anchoring bond, with a resulting loss of peptide chains from the resin, and (ii) acylation of growing chains to give terminated chains. The proportion of the two reactions could be estimated for the last half of the synthesis where radioactive acetyl-lysine residues were present in the peptide. By following the amount of both ^{14}C and ^{3}H in the filtrates of each deprotection reaction a value of 0.7 ± 0.2% chain loss was calculated. The additional 0.3% loss of amino groups per step indicated by the picrate titration was interpreted to be due to chain termination. A small part of this was a result of intentional termination by acetylimidazole and the major part is now known to be due to trifluoroacetylation by a mechanism not understood at the time this synthesis was carried out (*42*). Additional syntheses of histone fragments are now under way using the newly developed aminoacyloxymethylphenylacetamidomethyl-resin (Pam-Resin) (*43*, *44*), which has been shown to reduce chain loss to 1% of the values observed here and at the same time to reduce trifluoroacetylation to low levels (*42*). In addition to these uniform losses at every step, a major termination occurred after coupling of Boc-His(Dnp) at residue 18. This is thought to have been due to an inadvertant contamination of the reagent by trace amounts of acetic acid. We conclude, therefore, that this stepwise synthesis led to a total loss of approximately 37% of the initial peptide chains by acidolysis and to the acetylation or trifluoroacetylation of approximately 25% of the original chains. On the other hand, very few deletion peptides were expected to be present. Because of the presence of this large amount of terminated chains, a purification method capable of distinguishing between growing chains and terminated chains was needed. The Cys-Met affinity purification method was designed and developed specifically for that purpose.

Affinity Purification

This purification method is based on the addition of a readily removable functional group onto those peptide chains that are still growing at the end of the synthesis. After cleavage from the resin support, the derivatized peptides are specifically bound to a chromatographic column while all underivatized terminated peptides will pass through unretained. Elution of the peptide and removal of the affinity group then regenerates the purified product. The scheme devised for affinity purification of H4-(1–37) (*38*) involved the addition of Boc-Met and then Boc-Cys(MeOBzl) by a simple continuation of the DCC coupling procedure that was used to prepare the resin-bound 1–37 sequence. Cleavage with HF then gave Cys-Met-H4-(1–37), free of protecting groups and containing a unique sulfhydryl group on the desired peptide chains, together with a mixture of acylated peptides and any peptide chains that were inaccessible to the Met and Cys reagents. The free sulfhydryl then served as the specific affinity group, which could be covalently bound to an organomercurial agarose column while the terminated peptides passed through unretarded. The Cys-Met-peptide was then eluted with excess cysteine. Finally the affinity reagent was specifically cleaved with cyanogen bromide

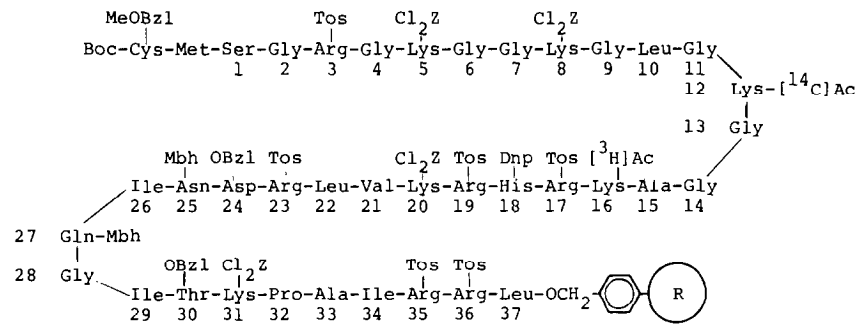

FIG. 4. The fully protected, affinity labeled histone H4-(1–37) peptide-resin.

(*45*) and the liberated H4-(1–37) peptide was desalted by gel filtration. This strategy does not, of course, remove deletion or modified peptides that are still growing at the end of the solid phase assembly of the peptide chain or which may be formed during the HF cleavage reaction. These by-products must be avoided or removed by independent procedures. The Cys-Met strategy was successful for H4-(1–37) because no other cysteine or methionine residues were present in the peptide. Had they been present, a modified protection scheme would have been necessary or a more general procedure such as the newly developed Sulfmoc method (*46*) would have been needed.

The fully protected Boc-Cys(MeOBzl)-Met-H4-(1–37) peptide-resin (Fig. 4) weighed 3.0 g and was deprotected in two steps. First the dinitrophenyl group was removed from histidine by thiolysis (*47–49*). A 1.0-g sample was stirred with 10 ml of a 0.2 *M* solution of thiophenol in DMF for 30 min, and the extent of the deprotection was determined by the absorbance of the 2,4-dinitrophenyl phenyl sulfide product at 337 nm (*49*). The data agreed with the amount of histidine subsequently found by hydrolysis and amino acid analysis. The peptide-resin was then placed in a Diaflon reaction vessel of the HF cleavage apparatus and dried *in vacuo*. Anisole (1 ml) and methionine (65 mg) were added as scavengers and 10 ml of HF was collected at −78°C. The temperature was raised to −25°C for 30 min and then to 0°C for an additional 45 min. After evaporation, the residue was extracted three times with ether, and finally the peptide was extracted four times with 8-ml portions of 1 *M* acetic acid. The filtrate contained 8.6×10^7 cpm of $^3H + {}^{14}C$. The solution was lyophilized, redissolved in 6 ml of 1 *M* acetic acid, and chromatographed on a Bio-Gel P-2 column (2.5 × 100 cm) in 1 *M* acetic acid. The asymmetric peak at the void volume containing 8.6×10^7 cpm was lyophilized (yield, 291 mg).

To assure that all of the cysteine residue was in the reduced sulfhydryl form before affinity purification, part of the crude Cys-Met-peptide (5.8×10^7 cpm) was dissolved in 4 ml of a pH 8.9 buffer containing 6 *M* guanidine HCl, 0.5 *M* Tris, and 2 m*M* EDTA, and flushed with oxygen-free nitrogen. The peptide was then stirred for 10 hr with 200 mg (150 Equiv) of dithiothreitol. Fresh dithiothreitol (50 mg) was added, and stirring was continued at 40°C for 6 hr. Gel filtration on Bio-Gel P-2 in 1% acetic acid gave three uv-absorbing peaks eluting ahead of the salt peak. Most of the radiolabel was present in the first peak. The reduced peptide was dissolved in 4.5 ml of a nitrogen-flushed buffer (0.05 *M* NaOAc, 0.1 *M* KCl, pH 5) and applied to a 1.5 × 23-cm column of organomercurial-agarose (36 μmol of Hg by titration with Ellman's reagent). Elution

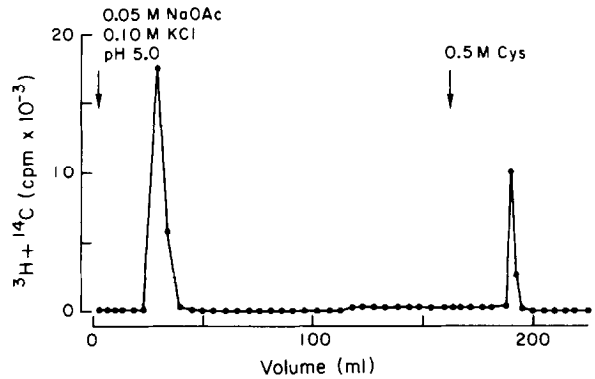

FIG. 5. Affinity purification of Cys-Met-[Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}] histone H4-(1–37) on an organomercurial-agarose column.

with the same buffer for 15 hr at 16 ml/hr removed the nonthiol-containing peptides, which accounted for about 70% of the radiolabel. Subsequent elution for 6 hr at 10.4 ml/hr with 0.5 *M* cysteine displaced the Cys-Met-peptide (1.8×10^7 cpm total count), as shown in Fig. 5. The Cys-Met peptide fraction was desalted and lyophilized; the Cys-Met affinity group was removed by treatment with cyanogen bromide (160 mg) in 6.5 ml of 70% aqueous formic acid for 11 hr. Fresh CNBr (105 mg) was added, and stirring was continued for 10 hr. Filtration on Bio-Gel P-2 in 1% acetic acid now gave a single radioactive peak. In order to remove any Cys-Met-peptide that had failed to cleave with cyanogen bromide, part of this product (2.9×10^6 cpm) was reduced with dithiothreitol and reapplied to the organomercurial-agarose column. Elution with the pH 5 NaOAc-KCl buffer gave an early peak (2.5×10^6 cpm, 87%) of histone H4-(1–37). Subsequent elution with 0.5 *M* cysteine gave uncleaved Cys-Met-H4-(1–37) (0.4×10^6 cpm, 13%). If desired, this fraction can be recycled to add to the final yield of purified peptide.

Ion Exchange Chromatography

The CNBr cleavage product, which had been freed of uncleaved Cys-Met-peptide by passing through the organomercurial-agarose column, was desalted on Bio-Gel P2 and lyophilized. A portion was dissolved in 2 ml of pH 5 0.01 *M* NaOAc containing 0.01 *M* NaCl and applied to a 0.9 × 50-cm column of carboxymethyl cellulose (Cellex-CM) that had been preequilibrated in the same buffer. The column was eluted with 150 ml of the buffer and then with a linear gradient consisting of 375 ml of the same buffer in the mixing chamber and 375 ml of pH 5 0.01 *M* NaOAc containing 0.50 *M* NaCl in the addition chamber. The main fraction between 675 and 711 ml was pooled, gel filtered, and rechromatographed on the Cellex-CM column with the same NaCl gradient. A single symmetrical radioactive component was found, with a peak at tube 135 (686 ml) (Fig. 6). The final specific activities of this purified histone H4-(1–37) peptide were 1.39 $\times 10^6$ dpm ^{14}C/μmol and 3.17×10^6 dpm ^{3}H/μmol based on total counts and amino acid analyses. The mole ratio ^{14}C/^{3}H was 1.03 based on the specific activity of the individual isotopes incorporated. Samples of this product were used for amino acid analyses, gel electrophoresis, and deacetylase assays.

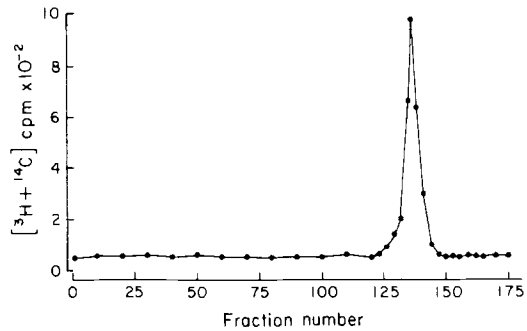

FIG. 6. Ion exchange chromatogram of diacetylated histone H4-(1–37) on a 0.9 × 50-cm carboxymethylcellulose column in a NaCl gradient at pH 5.0.

Amino Acid Analyses

The results of quantitative amino acid analyses of peptide samples taken at selected stages of the purification are shown in Table 2. The details of the analyses are described in the Methods section. The crude peptide after HF cleavage was known to contain large amounts of terminated chains and, therefore, to contain more of the early residues and fewer of the residues coupled late in the synthesis. This inhomogeneity is reflected in the amino acid analysis. In particular, the single threonine was the 8th residue from the

TABLE 2

AMINO ACID ANALYSIS OF SYNTHETIC HISTONE H4-(1–37)

Amino acid	After HF	Retained on 1st affinity column	Not retained on 2nd affinity column	Purified peptide after CMC column	Theory
Lys	2.4	5.4	6.1	5.91	6
His	0.5	0.8	0.8	0.90	1
Arg	4.2	5.3	5.9	6.20	6
Asp	1.9	2.0	2.0	2.02	2
Thr[a]	1.0	1.0	1.1	1.01	1
Ser[a]	0.2	1.0	0.9	1.05	1
Glu	1.0	1.1	1.2	1.11	1
Pro	1.1	1.0	1.0	1.01	1
Gly	2.7	8.0	8.5	8.90	9
Ala	1.3	1.9	2.1	2.08	2
Val	0.7	0.9	0.9	1.01	1
Ile	2.9	3.1	3.2	2.92	3
Leu	2.1	2.9	3.1	3.10	3
Cys[b]	0	0.9	0.0	0.00	0
Met[b]	0	0.9	0.0	0.01	0
$\frac{\text{Thr}}{\text{Ser}}$	6.0	1.0	1.1	0.96	1

[a] Threonine and serine values were increased 5 and 8%, respectively, to compensate for destruction during acid hydrolysis in 6 *M* HCl at 110°C, 18 hr.

[b] Present only in the affinity-labeled peptide. These values were obtained on samples oxidized with performic acid (*26*) before hydrolysis.

carboxyl end where the synthesis began, and the single serine, *N*-terminal residue 37, was the last to be added. In the crude preparation the Thr/Ser ratio was 6.0, showing that many of the chains did not continue to grow to the end. However, the ratio was very close to 1.0 after the removal of terminated chains by affinity chromatography and final purification by ion exchange chromatography. In addition, the final preparation gave quite satisfactory analyses of all other residues and was free of Cys and Met, which were removed after the affinity purification.

Polyacrylamide Gel Electrophoresis of Diacetylated Histone H4-(1–37)

Electrophoresis was performed on 30% gels as described in the Methods section. Figure 7 shows the gels of the peptides obtained after CNBr cleavage of the affinity purified peptide and after the carboxymethyl-cellulose column. The large amounts of terminated peptides (Fig. 5) were absent after affinity purification, and the remaining small contaminant was removed by the ion exchange column. The purified histone H4-(1–37) peptide was quite homogeneous by this very discriminating gel system.

FIG. 7. Polyacrylamide gel electrophoresis of diacetylated histone H4-(1–37). The peptide was dissolved in 10 *M* urea–2 *N* HOAc at 1 mg/ml and 10 μg was applied to Gel C (30% acrylamide) in a 0.6 × 25-cm tube. Run at constant 1.5 mA for 20 hr and stained with amido black. (1) Peptide after affinity purification and CNBr deprotection, (2) peptide after ion exchange chromatography, 1.0 mg/ml, (3) peptide after ion exchange chromatography, 0.2 mg/ml.

Enzymatic Deacetylation of Histone Fragments

A. The assay. The enzyme assays in the early stages of these studies made use of the EtOAc extraction procedure (*13*, *14*) for separating the released acetate. It was found that the apparent maximum release of acetyl groups from total calf thymus histone was approximately 50%. This low value has now been traced to systematic losses of acetic acid during the extraction. The new chromatographic method for separation of released acetic acid from the histone has proven to be much better. Under the same conditions, the maximum release of acetyl groups from total histone deacetylation was approximately 90%. Figure 8 shows data from a typical enzyme assay by the new method. It can be seen that the intact radiolabeled histone (first peak) is readily separated from the radiolabeled acetate (second peak). After 60 min nearly all of the [^{3}H]acetyl-histone was replaced by [^{3}H]acetate. The early comparative data are still valid, however, because they were always calculated relative to a histone control.

B. Native histone H4-(1–102) and H4-(1–84). It has already been shown (*16*) that the deacetylase releases [^{3}H]acetate at the same rate from equimolar solutions of the intact acetylated histone H4 and the 84-residue fragment derived from it. Therefore, the *C*-terminal 18 residues are not necessary for recognition by the enzyme.

C. Small substrates. It has been shown that synthetic Ala-Lys([^{14}C]Ac)-Arg-His-Arg-Lys-Val, representing residues 15–21 of H4 (*16*), and natural Leu-Gly-

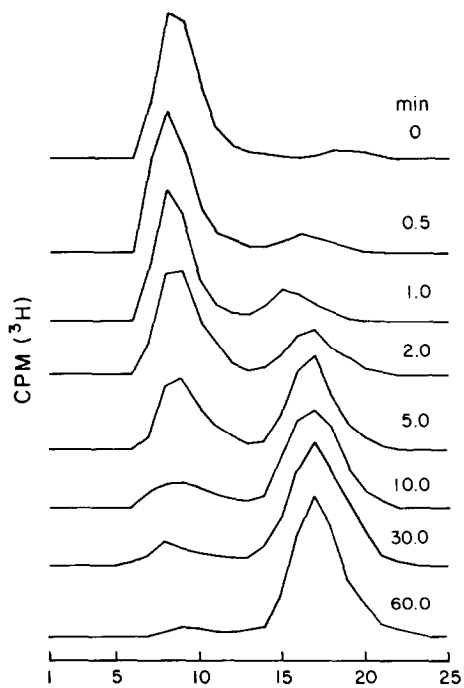

FIG. 8. Assay for enzymatic deacetylation of total calf thymus histone. Aliquots of the substrate were incubated with purified histone deacetylase for the indicated periods. The mixtures were separated by passage through Bio-Gel P-2 columns (0.7 × 20 cm). Aliquots of the collected tubes were counted for radioactivity. Peaks centered at tube 9 represent radiolabeled histone. Peaks centered at tube 17 represent released radiolabeled acetate.

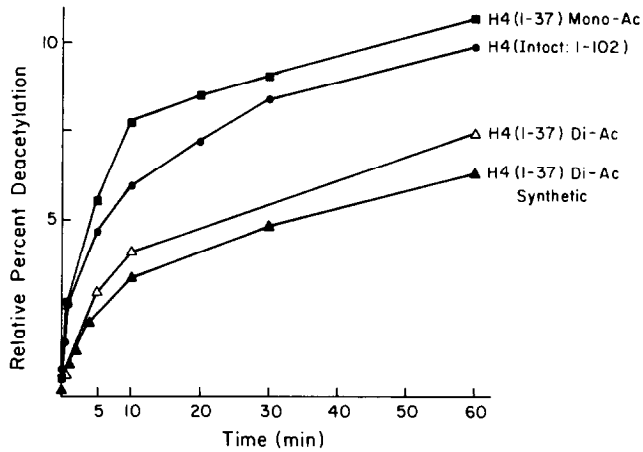

FIG. 9. A comparison of the rates of enzymatic deacetylation of natural and synthetic histone fractions.

Lys([^{3}H]Ac)-Gly-Gly-Ala-Lys([^{3}H]Ac)-Arg-His-Arg-Lys-Val, representing residues 10–21 (*15*), are not deacetylated by the histone deacetylase.

D. Mono- and diacetylated histone H4-(1–37) peptides. Preliminary data on the relative rates of deacetylation of the 37-residue fragments of H4 are summarized in Fig. 9. The peptides were incubated with the enzyme at 37°C, pH 6.8, in a 50:1 mole ratio, and the percent deacetylation was calculated from the released radioactivity. The observed rates for the natural and synthetic diacetylated H4-(1–37) peptides were essentially equal, showing that the absence of an N^{α}-acetyl group in the synthetic peptide did not have a significant effect on the ability of the peptide to serve as a substrate. The natural monoacetylated [Lys([^{3}H]Ac)^{16}] H4-(1–37) was deacetylated at approximately twice the rate of the diacetylated peptide, and the intact histone H4,

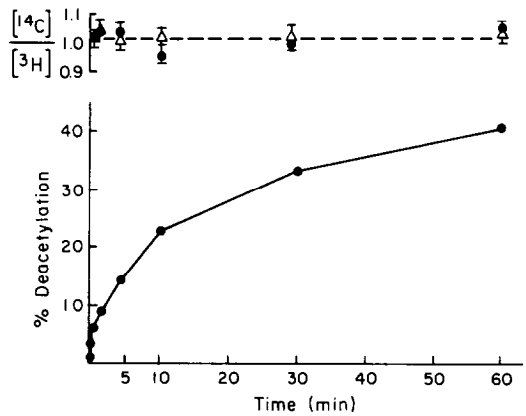

FIG. 10. Enzymatic deacetylation of synthetic [Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}]histone H4-(1–37). The percent deacetylation in the lower curve is the sum of the release of both labels. The upper curve is the mole ratio of the two labels. After incubation for various times the mixtures were separated on Bio-Gel P-2 into peptide and acetate fractions. The labels were then separated by combustion and quantitated in the scintillation counter. ● is the ^{14}C/^{3}H mole ratio in the acetyl groups remaining on the peptide; ▲ is the ^{14}C/^{3}H mole ratio in the acetate released by the deacetylase.

which was 90% monoacetylated at lysine-16, was deacetylated at a rate only slightly slower than the monoacetylated H4-(1–37) fragment.

Since both of the acetyl groups of the natural diacetylated H4-(1–37) fragment were labeled with [^{3}H], is was neither possible to distinguish between them nor to obtain data on the question of whether they are enzymatically removed in a sequential or random manner. For this reason the synthesis of the selectively labeled diacetyl peptide was undertaken. The highly purified synthetic [Lys([^{14}C]Ac)^{12}, Lys([^{3}H]Ac)^{16}]H4-(1–37) peptide was incubated with the purified deacetylase and at various times the reaction was stopped and the released acetate was separated from the remaining peptide on the P-2 column. The two fractions were then combusted, and the [^{3}H]H_2O and [^{14}C]CO_2 were separated on the sample oxidizer and counted separately. From the known specific activity of each isotope in the synthetic peptide, the mole ratio of ^{14}C/^{3}H could be calculated both from the amounts of label in the released acetate and in the remaining peptide. Figure 10 shows that this ratio was 1.03 $\pm$ 0.03 in both fractions throughout the time course of the experiment. The curve for percent deacetylation therefore represents the rate of release of either acetyl group.

The Mechanism of Deacetylation

Two possible routes of deacetylation of the synthetic diacetyl H4-(1–37) peptide are illustrated in Fig. 11. The enzyme could react in a random way with either acetyllysine-12 or acetyllysine-16 at equivalent but limiting rates to give a mixture of the monoacetyl-16 or monoacetyl-12 peptides, followed by further deacetylation at equivalent rates to the unsubstituted H4-(1–37) peptide. Alternatively, there may be an ordered sequence of events, either routes *a* or *b* of Fig. 11, in which only acetyl-12 or only acetyl-16 of the diacetylated peptide can serve as the initial substrate and release the corresponding monoacetyl peptide in a rate limiting step, to be followed by a second, very fast, enzymatic reaction with release of the second acetyl group. By either mechanism the observed appearance of ^{14}C-acetyl and ^{3}H-acetate would be equal.

In the experiments just described it was found that monoacetyl-lysine-16 was removed no more than twice as fast as the first acetyl group from the diacetylated molecule. Therefore route *b* would not give the observed equimolar release of acetate throughout the experiment. Since the rate of deacetylation of monoacetyl-lysine-12 is

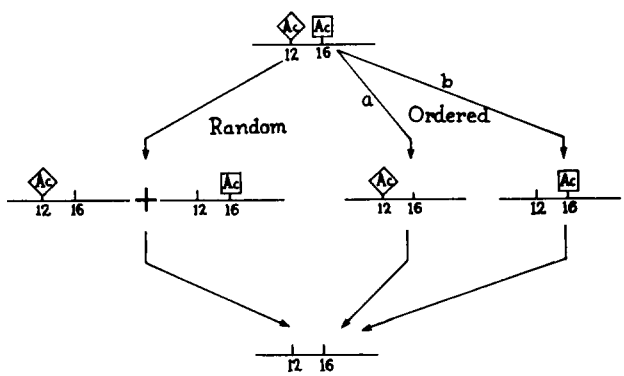

FIG. 11. Possible routes for the deacetylation of dual-labeled diacetylated histone H4-(1–37) synthetic peptide.

not yet known, route *a* remains a possibility. It is also possible that once the substrate is bound to the enzyme both acetyl groups can be released before the complex dissociates. In that case, the hydrolysis of one or the other of the acetyls could be much faster than it is when added as the monoacetyl peptide. If this were the case, neither route *a* or *b* is ruled out. Since there is a large excess of substrate (~50 Equiv), there must be a turnover of enzyme during the experiment. Therefore, mechanisms whereby the enzyme-bound substrate is not released after cleavage of one or both acetyl groups appear to be eliminated. Possible intramolecular acetyl transfer reactions have not yet been examined.

Work is now under way to obtain the monoacetyl-lysine-12 peptide and to compare its rate of deacetylation with the mono-16. Further kinetic analyses of the reactions are also required and, most importantly, a careful product analysis over the entire course of the enzymatic reaction is necessary. It is clear that selective radiolabeling by chemical synthesis will continue to be a most effective way to study the mechanism of deacetylation of the histones.

In addition to the findings on histone deacetylation, this synthesis has been very valuable from the point of view of solid phase peptide synthesis because it has provided a rigorous test of the picrate monitoring technique and has led to significant improvements in the method (*41*), it has led directly to the development of two new affinity purification methods (*38*, *46*), and it has led to an elucidation of the principal mechanism of chain termination by trifluoroacetylation and to ways in which this side reaction can be eliminated (*42*).

REFERENCES

1. F. M. JOHNSON AND V. G. ALLFREY, "Biochemical Actions of Hormones" (G. Litwack, Ed.), Vol. 5, pp. 1–53. Academic Press, New York, 1978.
2. E. L. GERSHEY, G. VIDALI, AND V. G. ALLFREY, *J. Biol. Chem.* **243**, 5018 (1968).
3. W. K. PAIK AND S. KIM, *J. Biol. Chem.* **245**, 6010 (1970).
4. E. L. GERSHEY, G. W. HASLETT, G. VIDALI, AND V. G. ALLFREY, *J. Biol. Chem.* **244**, 4871 (1969).
5. T. A. LANGAN AND P. HOHMANN, "Chromosomal Proteins and Their Role in the Regulation of Gene Expression" (G. S. Stein and L. J. Kleinsmith, Eds.), p. 113. Academic Press, New York, 1975.
6. V. G. ALLFREY, R. FAULKNER, AND A. E. MIRSKY, *Proc. Natl. Acad. Sci. USA* **51**, 786 (1964).
7. V. G. ALLFREY, "Chromatin and Chromosome Structure" (H. J. Li and R. Eckardt, Eds.), p. 167. Academic Press, New York, 1977.
8. D. M. P. PHILLIPS, *Biochem. J.* **87**, 258 (1963).
9. A. RUIZ-CARRILLO, L. J. WANGH, AND V. G. ALLFREY, *Science* **190**, 117 (1975).
10. P. R. LIBBY, *Biochem. Biophys. Res. Commun.* **32**, 59 (1968).
11. D. GALLWITZ, *Biochem. Biophys. Res. Commun.* **32**, 117 (1968).
12. S. C. BONDY, S. ROBERTS, AND S. MORELOS, *Biochem. J.* **119**, 665 (1970).
13. A. INOUE AND D. FUJIMOTO, *Biochem. Biophys. Res. Commun.* **36**, 146 (1969).
14. G. VIDALI, L. C. BOFFA, AND V. G. ALLFREY, *J. Biol. Chem.* **247**, 7365 (1972).
15. K. HORIUCHI AND D. FUJIMOTO, *J. Biochem. (Tokyo)* **73**, 117 (1973).
16. D. E. KRIEGER, R. B. LEVINE, R. B. MERRIFIELD, G. VIDALI, AND V. G. ALLFREY, *J. Biol. Chem.* **247**, 3863 (1972).
17. R. J. DELANGE, D. M. FAMBROUGH, E. L. SMITH, AND J. BONNER, *J. Biol. Chem.* **244**, 319 (1969).
18. M. T. SUNG AND G. H. DIXON, *Proc. Natl. Acad. Sci. USA* **67**, 1616 (1970).
19. Y. OGAWA, G. QUAGLIAROTTI, J. JORDAN, C. W. TAYLOR, W. C. STARBUCK, AND H. BUSCH, *J. Biol. Chem.* **244**, 4387 (1969).

20. W. KÖNIG AND R. GEIGER, *Chem. Ber.* **103**, 2041 (1970).
21. R. S. HODGES AND R. B. MERRIFIELD, *J. Biol. Chem.* **250**, 1231 (1975).
22. B. W. ERICKSON AND R. B. MERRIFIELD, *J. Amer. Chem. Soc.* **95**, 3757 (1973).
23. P. CUATRECASAS, *J. Biol. Chem.* **245**, 3059 (1970).
24. A. RUIZ-CARRILLO, "Methods in Enzymology" Vol. 37, (W. B. Jakoby and M. Wilchek, Eds.), Vol. 37, pp. 547–552. Academic Press, New York, 1974.
25. R. S. HODGES AND R. B. MERRIFIELD, *Anal. Biochem.* **65**, 241 (1975).
26. S. MOORE, *J. Biol. Chem.* **238**, 235 (1963).
27. K. LÜBKE AND E. SCHROEDER, *Ann. Chem.* **692**, 237 (1966).
28. G. VIDALI, E. L. GERSHEY, AND V. G. ALLFREY, *J. Biol. Chem.* **243**, 6361 (1968).
29. E. L. BÖHM, W. N. STRICKLAND, M. STRICKLAND, B. H. THWAITS, D. R. VAN DER WESTHUZEN, AND C. VON HOLT, *FEBS Lett.* **34**, 217 (1973).
30. L. WANGH, A. RUIZ-CARILLO, AND V. G. ALLFREY, *Arch. Biochem. Biophys.* **150**, 44 (1972).
31. S. PANYIM AND R. CHALKLEY, *Arch. Biochem. Biophys.* **130**, 337 (1969).
32. S. PANYIM AND R. CHALKLEY, *Biochemistry* **8**, 3972 (1969).
33. P. LEWIS, E. M. BRADBURY, AND C. CRANE-ROBINSON, *Biochemistry* **14**, 3391 (1975).
34. R. B. MERRIFIELD, *J. Amer. Chem. Soc.* **85**, 2149 (1963).
35. R. B. MERRIFIELD, *Advan. Enzymol.* **32**, 221)1969).
36. B. W. ERICKSON AND R. B. MERRIFIELD, "The Proteins" (H. Neurath and R. H. Hill, Eds.), 3rd ed., Vol. 2, pp. 255–527. Academic Press, New York, 1976.
37. B. GUTTE AND R. B. MERRIFIELD, *J. Biol. Chem.* **246**, 1922 (1971).
38. D. E. KRIEGER, B. W. ERICKSON, AND R. B. MERRIFIELD, *Proc. Natl. Acad. Sci. USA* **73**, 3160 (1976).
39. B. GISIN, *Helv. Chim. Acta* **56**, 1476 (1973).
40. B. GISIN, *Anal. Chim. Acta* **58**, 248 (1972).
41. R. B. MERRIFIELD, manuscript in preparation.
42. S. B. H. KENT, A. R. MITCHELL, M. ENGELHARD, AND R. B. MERRIFIELD, *Proc. Natl. Acad. Sci. USA* **76**, 2180 (1979).
43. A. R. MITCHELL, B. W. ERICKSON, M. N. RYABTSEV, R. S. HODGES, AND R. B. MERRIFIELD, *J. Amer. Chem. Soc.* **98**, 7357 (1976).
44. A. R. MITCHELL, S. B. H. KENT, M. ENGELHARD, AND R. B. MERRIFIELD, *J. Org. Chem.* **43**, 2845 (1978).
45. E. GROSS AND B. WITKOP, *J. Biol. Chem.* **237**, 1856 (1962).
46. R. B. MERRIFIELD AND A. E. BACH, *J. Org. Chem.* **43**, 4808 (1978).
47. S. SHALTIEL, *Biochem. Biophys. Res. Commun.* **29**, 178 (1967).
48. J. M. STEWART, M. KNIGHT, A. C. M. PAIVA, AND T. PAIVA, "Progress in Peptide Research" (S. Lande, Ed.), pp. 59–64. Gordon and Breach, New York, 1972.
49. M. C. LIN, B. GUTTE, D. G. CALDI, S. MOORE, AND R. B. MERRIFIELD, *J. Biol. Chem.* **247**, 4768 (1972).