

Table 1. Acetylation of des- $N^{\alpha 1}$ -Ac- α -MSH and related peptides by an S15 preparation

Exp. no.	Incubation	[3 H] Acetate incorporated (pmol)
1	S15	8
2	S15 + des- $N^{\alpha 1}$ -Ac- α -MSH	92
3	S15 preincubated with ZnSO ₄ + des- $N^{\alpha 1}$ -Ac- α -MSH	4.5
4	S15 + $N^{\epsilon 11}$ -Ac- α -MSH	15
5	S15 + α -MSH	17
6	S15 + des- $N^{\alpha 1}$ -Ac- $N^{\epsilon 11}$ -Ac- α -MSH	37
7	S15 + des- $N^{\alpha 1}$ -Ac- $N^{\epsilon 11}$ -Msc- α -MSH	36.5
8	S15 + NH ₂ -terminal tetrapeptide	10.5
9	S15 + NH ₂ -terminal octapeptide	27.4
10	S15 + NH ₂ -terminal decapeptide	28.10
11	S15 + NH ₂ -terminal peptide of ACTH (residue 1-24)	80.7

Details of the incubation and assay are described in *Materials and Methods*. The incubation mixture contained 3 mg of S15 preparation, 108 nmol of substrate, and 0.5 μ Ci of [3 H]acetylCoA at pH 7.4. After 15 min at 37° the reaction was stopped by adding ZnSO₄ up to a concentration of 6 mM. Excess [3 H]acetylCoA was removed by adsorption onto a weakly basic ion exchanger. The [3 H]acetate incorporated into protein was then measured. No corrections have been made for a possible loss of substrate during its purification.

incubated at 37° for 2 hr. The clear solution was lyophilized and the residue subjected to high voltage paper electrophoresis. Chymotryptic peptides of des- $N^{\alpha 1}$ -Ac- α -MSH and $N^{\epsilon 11}$ -Ac- α -MSH were run in parallel on the same sheet of paper. The

dried electropherograms were cut into 1-cm strips and the radioactivity of each strip was measured in a Packard Tricarb 2450 Liquid Scintillation Counter. The chymotryptic peptides of the unlabeled substrates des- $N^{\alpha 1}$ -Ac- α -MSH and $N^{\epsilon 11}$ -Ac- α -MSH were detected by UV fluorescence, staining with ninhydrin, or specific staining for tyrosine (12).

Analysis of the Total Enzymatic Digest of Labeled Substrates. The incubated and labeled substrates were lyophilized and suspended in 0.4 ml of 0.1 M NH₄HCO₃, pH 8.9, and digested by successive addition of chymotrypsin, Pronase, and carboxypeptidase A, B, and C (100 μ g of each enzyme) during a 12-hr period at room temperature. The enzymatic digest was fractionated on a small column of Dowex 50 H⁺ (0.5 \times 7 cm). The nonadsorbed material was eluted with water to obtain the "acidic" fraction, which contained NH₂-terminally blocked amino acids and peptides. All the adsorbed material was eluted with 0.5 M NH₄OH to obtain the "basic" fraction containing the free amino acids and peptides. The eluates were lyophilized and analyzed by high voltage electrophoresis and paper chromatography. Reference [14 C]acetyl-amino acids were prepared by treatment of 1 mmol of the amino acid with 1 mmol of [14 C]acetic anhydride in boiling acetic acid (13).

Protein Determination. Proteins were estimated according to Lowry *et al.* (14), with bovine serum albumin as a standard.

RESULTS

Acetylation of des- $N^{\alpha 1}$ -Ac- α -MSH by a lens extract: Specificity of the acetylation reaction

The different substrates were incubated with an S15 preparation and labeled acetylcoenzyme A at 37° as described in *Ma-*

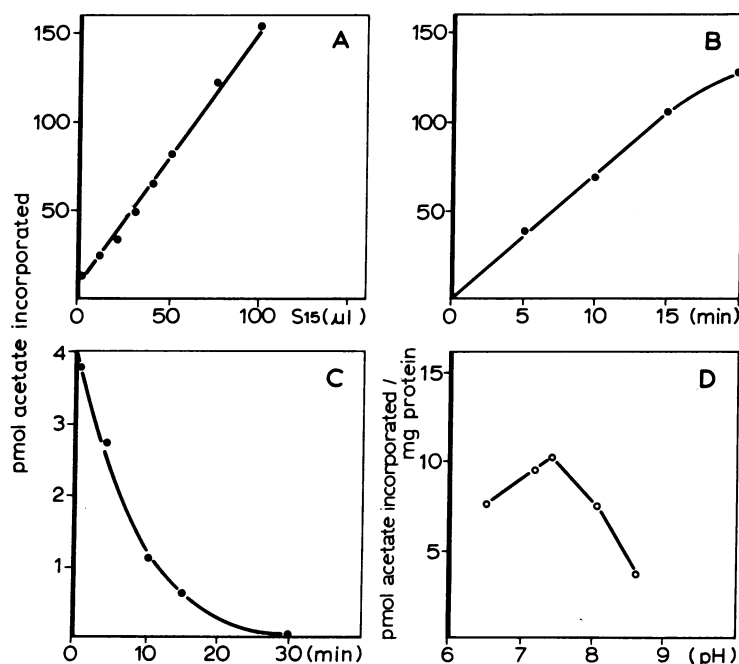


FIG. 1. Characteristics of the acetylation reaction. Details of incubation are described in *Materials and Methods*. The difference in [3 H]acetate incorporation obtained with an S15 preparation incubated in the presence and absence of substrate is considered to be due to enzymatic acetylation. (A) Effect of supernatant concentration. Different amounts of the S15 preparation (89 μ g of protein per μ l) were incubated with [3 H]acetylCoA and des- $N^{\alpha 1}$ -Ac- α -MSH at 37° for 15 min, and the amount of incorporated acetate was measured. (B) Kinetics of the reaction. 160 μ l of S15 preparation (89 μ g of protein per μ l) were incubated. At different time intervals an aliquot fraction of 40 μ l was removed and the reaction was stopped by adding ZnSO₄ up to a concentration of 6 mM. (C) Heat inactivation. The S15 preparation (4 μ g/ μ l) was incubated at 55°. At different time intervals aliquots were removed and chilled in melting ice. The extent of acetylation of des- $N^{\alpha 1}$ -Ac- α -MSH by these heat-treated S15 preparations was measured after an additional incubation for 15 min at 37°. (D) Effect of pH. A lyophilized S15 preparation was dissolved in 50 mM Tris-HCl, 30 mM sucrose, 50 mM KCl, 30 mM MgOAc, at different pH values. The acetylation reaction was performed for 15 min at 37°.

terials and Methods. The presence of 50 mM KCl should minimize the nonenzymatic acetylation of ϵ -NH₂ groups of lysine (15).

Table 1 shows that a low amount of radioactivity is incorporated into proteins when the S15 preparation is incubated with [³H]acetylCoA in the absence of substrate (Exp. 1). This incorporation may be due to enzymatic and/or nonenzymatic acetylation of endogenous lens proteins. When des-*N*^{α1}-Ac- α -MSH is added to the incubation mixture (Exp. 2), the incorporation of [³H]acetate is 10 times higher. Preincubation of the S15 preparation with 6 mM ZnSO₄ (Exp. 3) greatly decreases the incorporation of labeled acetate. The data strongly suggest that des-*N*^{α1}-Ac- α -MSH is enzymatically acetylated by the lens extract.

On des-*N*^{α1}-Ac- α -MSH two potential sites for NH₂-acetylation are available: the free amino group of the NH₂-terminal serine and the ϵ -NH₂ group of lysine-11. In order to determine whether one or both of these residues become acetylated by the S15 preparation, we incubated des-*N*^{α1}-Ac- α -MSH, α -MSH, and *N*^{ε11}-Ac- α -MSH with an S15 preparation and [³H]acetyl-CoA. The amount of acetate incorporated when *N*^{ε11}-Ac- α -MSH is used as a substrate (Exp. 4) is of the same order as the incorporation of acetate in lens protein (Exp. 1) plus the nonenzymatic acetylation of des-*N*^{α1}-Ac- α -MSH (Exp. 3).

Exp. 5 shows that α -MSH also is not a substrate for the acetylating enzyme, indicating that the lens system does not promote acetylation of the ϵ -NH₂ group of lysine-11. However, des-*N*^{α1}-Ac-*N*^{ε11}-Ac- α -MSH (Exp. 6) and des-*N*^{α1}-Ac-*N*^{ε11}-Msc- α -MSH (Exp. 7), which both have free NH₂-termini but carry a blocking group on lysine-11, are acetylated by the S15 preparation, although to a lesser degree than the completely unblocked des-*N*^{α1}-Ac- α -MSH. Although the presence of the blocking group on residue 11 affects the process of NH₂-terminal acetylation (irrespective of the size of this blocking group), it appears that the acetylation reaction is specific for the α -NH₂ terminus of peptides.

In order to get some information about the minimum length required for the substrate to become acetylated, we tested the NH₂-terminal tetra-, octa-, and decapeptides of des-*N*^{α1}-Ac- α -MSH. As shown in Table 1 (Exps. 8, 9, and 10), the tetrapeptide cannot be acetylated by the S15 preparation, whereas the octa- and decapeptides become acetylated to some degree.

Furthermore, a longer peptide (consisting of the residues 1–24 of ACTH), of which the 13 NH₂-terminal residues are identical to des-*N*^{α1}-Ac- α -MSH, is as good a substrate as des-*N*^{α1}-Ac- α -MSH itself.

Attempts to acetylate histones in the S15 preparation gave negative results.

Some characteristics of the acetylation process are given by the experiments depicted in Fig. 1. Acetylation of des-*N*^{α1}-Ac- α -MSH is proportional to the amount of S15 added, at least up to 100 μ l (corresponding to about 9 mg of protein) (Fig. 1A). The reaction shows a linear increase for about 15 min (Fig. 1B); it is a thermolabile process (Fig. 1C), with an optimum pH near 7.4 (Fig. 1D).

Localization of the acetyl group incorporated in des-*N*^{α1}-Ac- α -MSH

The localization of the acetyl group(s) in incubated des-*N*^{α1}-Ac- α -MSH and related peptides was established by analysis of either labeled chymotryptic peptides or labeled acetyl amino acids obtained after extensive digestion with chymotrypsin, Pronase, and carboxypeptidases.

Characterization of Labeled Chymotryptic Peptides. Four

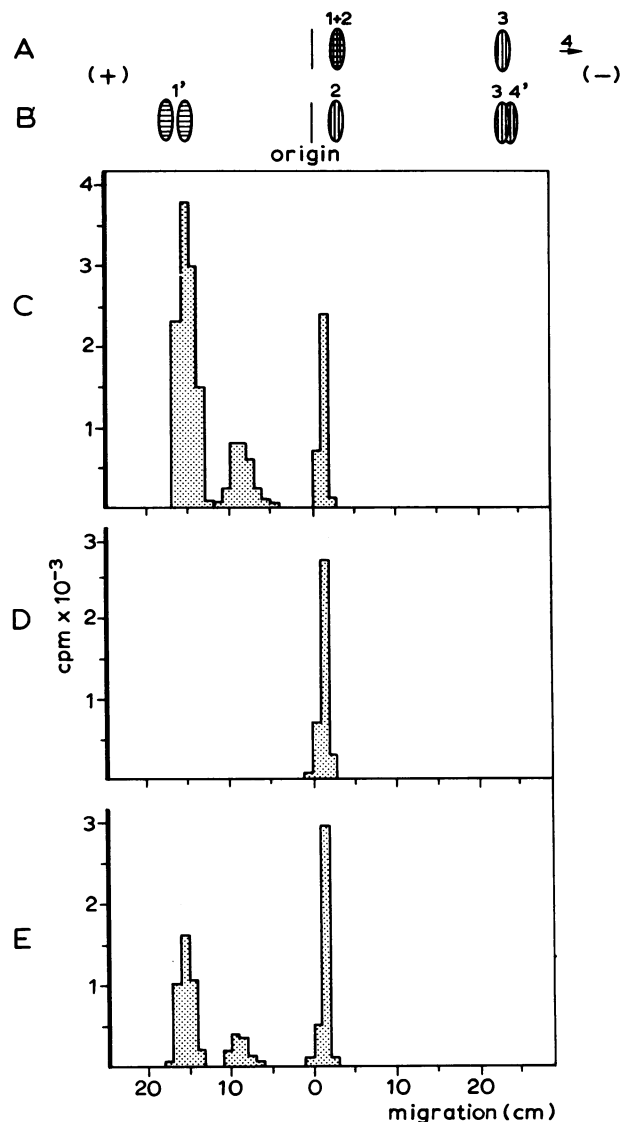


FIG. 2. High voltage paper electrophoresis of chymotryptic peptides of [¹⁴C]acetylated substrates. Electrophoresis was carried out for 105 min at 50 V/cm in pyridine-acetic acid-water (25:1:225 by volume), pH 6.5. A and B represent chymotryptic digests of synthetic des-*N*^{α1}-Ac- α -MSH and *N*^{ε11}-Ac- α -MSH, respectively. Vertical hatching indicates spots stained with ninhydrin; horizontal hatching indicates spots giving a positive tyrosine staining. C, D, and E represent the radioactivity profiles of the electrophoresed chymotryptic digests of des-*N*^{α1}-Ac- α -MSH, α -MSH, and des-*N*^{α1}-Ac-*N*^{ε11}-Ac- α -MSH, respectively, incubated with an S15 preparation and acetylCoA as described in *Materials and Methods* except that [¹⁴C] instead of [³H]acetylCoA was used as acetate donor.

chymotryptic peptides with different electrophoretic mobilities can be obtained from des-*N*^{α1}-Ac- α -MSH: (1) H-Ser-Tyr-OH; (2) H-Ser-Met-Glu-His-Phe-OH; (3) H-Arg-Trp-OH; and (4) H-Gly-Lys-Pro-Val-NH₂ (Fig. 2A). Peptide 4 migrated off the paper, but could be detected when the time of electrophoresis was shortened. Cleavage may occur between methionine and glutamic acid in peptide 2, but this will not change the electrophoretic pattern. When the α -NH₂ of serine-1 and the ϵ -NH₂ of lysine-11 are acetylated (*N*^{ε11}-Ac- α -MSH), the electrophoretic mobilities of peptides 1 and 4 become completely different (1' and 4' in Fig. 2B) and thus provide an appropriate tool to discriminate between α - and ϵ -NH₂-acetylation. In fact, chymotryptic digestion of *N*^{ε11}-Ac- α -MSH yields two negatively

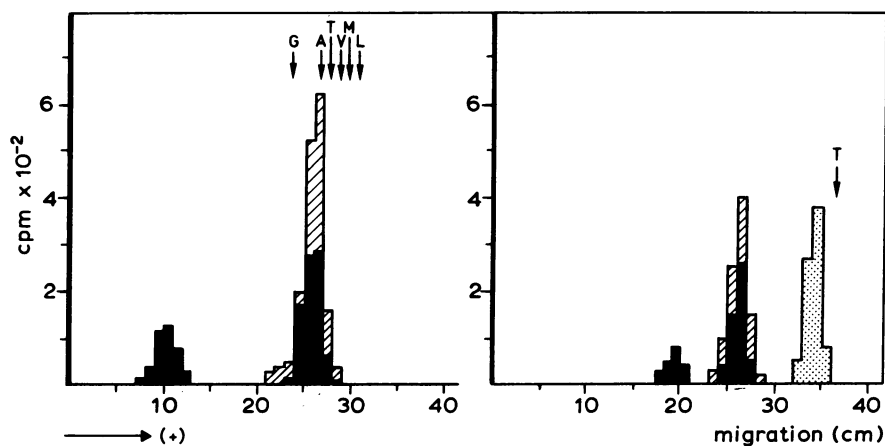


FIG. 3. Identification of acetyl amino acids after extensive digestion of [^3H]acetylated substrates. Des- $N^{\alpha 1}$ -Ac- α -MSH was incubated with an S15 preparation and [^3H]acetylCoA as described in *Materials and Methods*. The ^3H -labeled substrates were digested to their constituent amino acids. The enzymatic digest was then fractionated by chromatography on a Dowex 50 H^+ column. Thereafter, the "acidic" fraction was subjected to electrophoresis and chromatography. (Left) High voltage electrophoresis in pyridine-acetic acid-water (25:1:225 by volume), pH 6.5, at 50 V/cm for 90 min. The patterns of reference [^{14}C]acetylserine (hatched area) and of the total enzymatic digest of incubated des- $N^{\alpha 1}$ -Ac- α -MSH (black area) are shown. The positions of reference [^{14}C]acetyl derivatives of glycine (G), alanine (A), threonine (T), valine (V), methionine (M), and leucine (L) are indicated by arrows. (Right) Descending chromatography in butanol-acetic acid-water (12:3:5 by volume). The patterns of reference [^{14}C]acetylserine (hatched), of [^{14}C]acetylalanine (dotted), and of the total enzymatic digest of incubated des- $N^{\alpha 1}$ -Ac- α -MSH (black) are shown together with the position of [^{14}C]acetylthreonine (T).

charged peptides of equal intensity, which are both ninhydrin-negative and tyrosine-positive. The reason for this phenomenon and the question of which of the two peptides corresponds to genuine Ac-Ser-Tyr is still unsolved. However, the results shown in Fig. 2C and D confirm that in any case no acetylation of the ϵ - NH_2 group of lysine occurs. Both des- $N^{\alpha 1}$ -Ac- α -MSH (Fig. 2C) and α -MSH (Fig. 2D) have a free ϵ - NH_2 group available, but do not show any trace of labeling in the position of the chymotryptic peptide 4. When the incubation is performed in the presence of a substrate with a free NH_2 -terminus (Fig. 2C and E), two labeled peptides migrate towards the anode. Both must have a blocked NH_2 -terminus. The most slowly moving peptide might result from incomplete cleavage. The fastest moving peptide comigrates with the NH_2 -terminal chymotryptic peptide of synthetic $N^{\epsilon 11}$ -Ac- α -MSH. Because of the remaining ambiguity due to the two anodal chymotryptic peptides 1' of $N^{\epsilon 11}$ -Ac- α -MSH, it was necessary to unequivocally identify the acetylated amino acid. Comparison of Fig. 2C and E with Fig. 2D allows the conclusion that under the conditions of our experiments no constituent of the lens becomes acetylated that would coincide with acetyl-Ser-Tyr.

Characterization of Labeled Acetyl Amino Acids in the Incubated Substrate. The total enzymatic digests of incubated substrates were applied onto a small Dowex 50 H^+ column. About 95% of the radioactivity was recovered in the "acidic" fraction, which contains NH_2 -terminally blocked amino acids and peptides. After high voltage electrophoresis, the radioactivity was found in two positions (Fig. 3, left): the fast moving spot comigrates with standard [^{14}C]acetylalanine and [^{14}C]acetylserine; the slowly moving one has not been identified. [^{14}C]acetylalanine and [^{14}C]acetylserine can be resolved by chromatography. Fig. 3, right, clearly shows the presence of N -[^3H]acetylserine in the extensive enzymatic digest of incubated des- $N^{\alpha 1}$ -Ac- α -MSH. Again, a second peak of unknown identity is present. These results demonstrate that incubation of des- $N^{\alpha 1}$ -Ac- α -MSH with an S15 preparation results in NH_2 -acetylation of the substrate and that the reaction is specific for the NH_2 -terminal amino acid.

DISCUSSION

NH_2 -terminal acetylation of proteins has been reported to be a postinitiation event (5) and appears to be a rather common phenomenon in prokaryotic and eukaryotic cells (1, 2, 16, 17). It has been shown in this laboratory that, at least for lens proteins, acetylation takes place while the nascent chain is still on the ribosome (18). From these studies it appeared that the approximate length of the growing polypeptide chain is between 20 and 25 amino acid residues when NH_2 -terminal acetylation starts. This length would be required for a polypeptide to protrude from the ribosome. Furthermore, it could unequivocally be demonstrated that the translation product of the 14S α -crystallin messenger becomes acetylated in NH_2 -terminal position independent of the heterologous system that is used for translation (19-21). The latter observation strongly supports the idea that NH_2 -terminal acetylation of a protein is not limited to the cell species in which it normally occurs.

The results described in this paper demonstrate the enzymatic acetylation of a synthetic tridecapeptide, des- $N^{\alpha 1}$ -Ac- α -MSH, by a calf lens extract using [^3H]acetylcoenzyme A as acetate donor. Two potential sites for NH_2 -acetylation are available on the substrate: the α - NH_2 group of the NH_2 -terminal serine and the ϵ - NH_2 group of lysine-11. Our studies of the incorporation of labeled acetate into this substrate, which was modified by blocking one or both of the amino groups, indicated that acetylation took place preferentially at the α - NH_2 group of the terminal amino acid residue. Identification of the acetyl group after enzymatic digestion of the labeled substrate confirms the assumption that acetylation occurred at the NH_2 -terminal seryl residue and that no acetate is incorporated into the ϵ - NH_2 -lysyl group of residues.

The acetylation reaction shows an optimum pH around 7.4 and is a thermolabile process, which suggests enzymatic control of the reaction. This is sustained by the findings that nonenzymatic acetylation of proteins requires a more alkaline pH and is a thermostable process (22).

The minimum length required for the substrate to be acetylated seems to be between five and eight amino acid residues.

A maximum incorporation of acetate is obtained with des- $N^{\alpha 1}$ -Ac- α -MSH. Blocking the ϵ -NH₂ group of lysine-11 results in a decrease of NH₂-terminal acetylation, irrespective of the size of the blocking group (e.g., acetyl or methylsulfonyl-ethyloxycarbonyl). Furthermore, the NH₂-terminal tetracosapeptide of corticotropin (ACTH), of which the sequence of the first 13 residues is identical to that of des- $N^{\alpha 1}$ -Ac- α -MSH, seems to be as good a substrate as des- $N^{\alpha 1}$ -Ac- α -MSH itself. Native ACTH can also be acetylated to some extent in the bovine lens systems (unpublished results).

Earlier observations revealed that the translation product of lens α -crystallin messenger becomes NH₂-terminally acetylated in a variety of heterologous cell-free systems. Hence it is very likely that the acetylation of native α -MSH in the pituitary gland is brought about by an enzyme similar to the one present in the lens system. It therefore seems that the specificity of the enzyme is rather broad in view of the dissimilar NH₂-terminal sequences of α -MSH and α -crystallin. Amino acids that are involved in NH₂-terminal acetylation are, almost exclusively, alanine, glycine, serine, threonine, and methionine, although, on the other hand, proteins having such an NH₂-terminal amino acid are not necessarily acetylated. Comparison of the NH₂-terminal sequences of a series of α -NH₂-acetylated proteins does not provide obvious similarities which might account for the acetylation.

Enzymatic ϵ -NH₂ acetylation of lysyl residues has been proven to occur in a variety of proteins (3, 23, 24), but it has most extensively been studied in histones. Proposals suggesting the initiation of protein synthesis by acetylated amino acids as a general mechanism in eukaryotes (25) or as a special case for histones (26) have been refuted (27). Nevertheless, very recently this abandoned idea has been put forward again (28).

The present results provide an experimental approach to the problem of NH₂-terminal acetylation of eukaryotic proteins.

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