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Selective Acetylation of the Terminal Amino Group of Corticotrophin

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The amino acid sequence in α -melanocyte-stimulating hormone (α -MSH) is identical with that of the *N*-terminal tridecapeptide in corticotrophin (I) (Harris & Lerner, 1957; Harris, 1959). α -MSH (II) differs from the latter in having an *N*-acetyl substituent on its *N*-terminal serine, and an amide group on its *C*-terminal valine.

terminal serine in corticotrophin, which is essential for its adrenocorticotrophic activity, may also be one of the factors which inhibit the potential melanocyte-stimulating activity of the corticotrophin molecule. It was therefore of interest to determine whether selective *N*-acetylation of the *N*-terminal serine residue in corticotrophin would

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Ser.Tyr.Ser.Met.Glu.His.Phe Arg.Try.Gly.Lys.Pro.Val.Gly...Phe
 (I)

N-Acetyl Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.Lys.Pro.(Val.NH₂)
 (II)

This common sequence, part of which is also found in β -melanocyte-stimulating hormone (β -MSH), provides an explanation in terms of chemical structure for the intrinsic melanocyte-stimulating activity of corticotrophin reported by Bell (1954) and Dixon (1956*a*). However, although it contains the entire amino acid sequence of α -MSH within its structure, corticotrophin possesses melanocyte-stimulating activity of only 1% (or less) of that of α -MSH, on a weight basis.

Harris (1958, 1959) suggested that the free *N*-

diminish its corticotrophic activity and also enhance its melanocyte-stimulating activity.

The *N*-terminal serine of corticotrophin cannot be selectively *N*-acetylated by the usual methods of acylation, since the ϵ -amino groups of the four lysine residues in the molecule would also be acetylated under the same conditions.

In their account of the synthesis of α -MSH peptide fragments, Guttman & Boissonnas (1958) reported that treatment of the protected pentapeptide benzyloxycarbonylSer.Tyr.Ser.Met. α -methyl- γ -benzylGlu with a solution of hydrogen bromide in acetic acid, to remove the benzyloxy-

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carbonyl group, resulted in *O*-acetylation of both serine residues. On adjustment of the pH above 7, *N*-acetylSer.Tyr.*O*-acetylSer.Met. α -methyl- γ -benzylGlu was obtained, as a result of an *O* $\rightarrow$ *N* acyl shift. The preparation of *O*-acetylserine by a similar reaction, with hydrochloric acid in acetic acid, was reported by Sheehan, Goodman & Hess (1956).

O-Acyl substituents of hydroxyamino acids, which are stable under acid conditions, are easily hydrolysed on exposure to alkali, provided that the amino function is not free to participate in the acyl-migration reaction (Synge, 1939).

These observations provide the basis for a method of selective *N*-acetylation of the *N*-terminal serine in corticotrophin, through the following sequence of reactions:

Corticotrophin A_1 (Dixon & Stack-Dunne, 1955) was used as a starting material. Conditions of alkali exposure were chosen which produced the *O* $\rightarrow$ *N* acyl shift on the serine in position 1, the hydrolysis of the *O*-acetyl group on the serine in position 3, and the removal of amide ammonia to convert corticotrophin A_1 into A_2 in a single step.

The resulting modified corticotrophin, referred to below as *N*-acetylcorticotrophin A_2 , has been purified by ion-exchange chromatography, characterized and assayed for melanocyte-stimulating and corticotrophic activity.

EXPERIMENTAL

Materials

Corticotrophin A_1 . This was prepared by the method of Dixon & Stack-Dunne (1955) with minor modifications. Frozen pig-pituitary glands were used in place of acetone-dried glands so as to prevent the conversion of corticotrophin A_1 into A_2 . Ion-exchange chromatography was performed with 0.25% of chlorbutol (1:1:1-trichloro-2-methylpropan-2-ol) in the place of phenol as a bacteriostat in the buffer so that polypeptide could be detected by its absorption of ultraviolet radiation. The product was obtained from the phosphate buffer solution by the desalting method of Dixon (1959) described below.

Corticotrophin A_2 . This was prepared by alkaline treatment of corticotrophin A_1 (Dixon & Stack-Dunne, 1955; Li, Geschwind, Dixon, Levy & Harris, 1955; Shepherd, Howard *et al.* 1956) with the conditions described below for the treatment of *O*-acetylated corticotrophin A_1 . The product was isolated after ion-exchange chromatography.

α -Melanocyte-stimulating hormone. This was required to compare the *N*-terminal fragments released by trypsin digestion of α -MSH and *N*-acetylcorticotrophin A_2 . It was

prepared by one of us (J.-P.W.), using a melanocyte-stimulating hormone (MSH)-concentrate (Armour, lot D 14083-4) as starting material. The first step in the purification was essentially the method described by Steelman, Andersen & McGregor (1959).

MSH-concentrate (2.5 g.) was twice extracted with 0.01 *M*-acetic acid (25 ml.). The insoluble material (20–23% by weight) was removed by centrifuging, and the clear supernatant adjusted to pH 6.5 with aq. NH_3 . After removal of the precipitate, the supernatant (containing 65–70% by weight of the starting material) was placed on a column of carboxymethylcellulose (0.5 *m*-equiv./g.) equilibrated with 0.01 *M*-ammonium acetate, pH 6.5. β -MSH was not retained under these conditions. After washing with 0.01 *M*-buffer, the α -MSH was eluted with 0.1 *M*-ammonium acetate buffer, pH 6.5. The peak material was desalted on Amberlite IRC-50 (Dixon, 1959), freeze-dried and reappplied on carboxymethylcellulose equilibrated with 0.01 *M*-ammonium acetate, pH 6.5. It was eluted with a

gradient of 0.1 *M*-ammonium acetate (pH 6.5), by using a mixing chamber of 250 ml.

Although Steelman *et al.* (1959) reported that a similar procedure gave highly pure α -MSH, our product was heterogeneous and was purified further by preparative paper chromatography, with the solvent system butan-1-ol-acetic acid-water-pyridine (30:6:24:20, by vol.) described by Waley & Watson (1953) (butan-1-ol was purified by the method of Clarke, Robinson & Smith, 1927). It was applied on Whatman no. 3 paper (pre-washed with 33% acetic acid) at a concentration of 1 mg./cm., for an ascending run of 10 hr. α -MSH, which migrated as the fastest-moving component (R_f 0.69), was recovered by elution with 5% acetic acid, followed by freeze-drying of the eluate.

β -Melanocyte-stimulating hormone. This was prepared from pig pituitary glands by ion-exchange chromatography (Dixon, 1960).

Hydrogen bromide in acetic acid. For the requirements of the reaction with corticotrophin, it was essential to prepare this reagent free from all traces of bromine.

Bromine was added dropwise into a filter-flask containing anhydrous tetralin, through a separating funnel whose stem reached below the surface of the liquid. The HBr gas generated was passed successively through a wash-bottle containing tetralin, a tube filled with copper turnings and a second wash-bottle containing tetralin, to remove the last traces of bromine. The gas was finally absorbed into acetic acid in a wash-bottle covered with black paper, externally cooled to compensate for warming during absorption, and protected from moisture by means of a drying tube containing CaCl_2 . Polyethylene tubing was used for all connexions.

The rate of flow of HBr was regulated by gentle heating of the flask containing tetralin, and absorption continued until a concentration of approximately 3.5 *N*-HBr was reached. Samples of this colourless and slightly viscous solution were removed to determine the concentration of

HBr by titration, and the remainder diluted with acetic acid and redistilled thioglycolic acid to a final concentration of 2.8N-HBr and 5% (by vol.) of thioglycolic acid. This reagent was immediately used for reaction with corticotrophin.

On standing, the reagent developed a yellow colour within a few hours, which increased progressively. A new batch was prepared for each acetylation reaction.

Ammonium bicarbonate buffer (pH 10.0). This was prepared by mixing 0.2N-NH₄HCO₃ (100 ml.) with aq. 0.8N-NH₃ solution (100 ml.).

Methods

Treatment of corticotrophin A₁ with hydrogen bromide in acetic acid. A freshly prepared solution (1.5 ml.) of 2.8N-HBr in acetic acid containing 5% of thioglycolic acid was added to corticotrophin A₁ (12.0 mg.; approx. 2.5 μM) in a small, glass-stoppered test tube covered with dark paper. Complete dissolution was achieved by shaking gently by hand for about 30 min. After a total reaction time of 75 min. at 25°, the tube was rapidly connected to a water pump to remove most of the HBr, and the material immediately precipitated by addition of anhydrous, peroxide-free ether (10 ml.). After centrifuging and removal of the supernatant, the white precipitate was resuspended in ether (10 ml.), the suspension centrifuged, and the supernatant discarded. The ether wash was repeated twice more to ensure complete removal of thioglycolic acid. The precipitate was freed from last traces of ether *in vacuo*, dissolved in water (1.5 ml.) and freeze-dried. The recovery of *O*-acetylated corticotrophin A₁ was quantitative (14.0 mg.; approx. 2.5 μM, accounting for the addition of 9 moles of HBr and 2 acetyl groups).

Alkali-exposure of O-acetylated corticotrophin A₁. (a) Aq. NH₃ soln. (1.0M; pH 11.5; 1 ml.) was added to *O*-acetylated corticotrophin A₁ (10.0 mg.) in a small glass-stoppered test tube, and the resulting clear solution held at room temperature for 6 hr. The material was then recovered quantitatively by freeze-drying, and used directly for purification by ion-exchange chromatography on Amberlite IRC-50.

(b) Ammonium bicarbonate buffer at pH 10 (1.5 ml.) was added to *O*-acetylated corticotrophin A₁ (14.0 mg.), and the clear solution held at room temperature for 12 hr. The material was recovered and treated further as described in (a).

Ion-exchange chromatography on Amberlite IRC-50. The details were those described by Hirs, Moore & Stein (1953) and Dixon & Stack-Dunne (1955). The stock solution of buffer used for the isolation of corticotrophin A₁ had a composition of 0.1M-Na₂HPO₄, 0.1M-NaH₂PO₄ and 0.25% (w/v) of chlorbutol. The buffer used for chromatography of the product of acetylation was a dilution of the stock buffer to three-quarters of its strength ([Na⁺], 0.225N). All columns were equilibrated before use so that the effluent possessed the same pH value as the influent within 0.01 unit.

Isolation of products after chromatography (desalting). The method of Dixon (1959) was used. The solution was acidified by the addition of 5% (v/v) acetic acid and run through a small column of the acid form of finely ground carboxylic resin Amberlite IRC-50, which adsorbed the product. The resin was washed with 5% acetic acid until the effluent was salt-free, and the product displaced with 50% acetic acid.

It was obtained in the dry state by diluting the 50% acetic acid effluent with water and drying while frozen. The other operations were carried out in the cold (4°).

N- and C-Terminal amino acid analyses. *N*- and *C*-Terminal group analyses were carried out on samples of corticotrophin A₁, corticotrophin A₂ and *N*-acetylcorticotrophin A₂ in a series of parallel experiments.

The *N*-terminal group was determined on samples of 2.5 mg. (approx. 0.5 μM) by the phenyl isothiocyanate method (Edman, 1950) according to the paper-strip procedure described by Harris & Li (1954) for sheep corticotrophin and by Harris & Roos (1959) for β-MSH.

The *C*-terminal analyses were performed by digesting samples of 0.6 mg. (approx. 0.12 μM) with carboxypeptidase, according to the method described by Fraenkel-Conrat, Harris & Levy (1955).

Trypsin digestion of corticotrophin A₂, N-acetylcorticotrophin A₂ and α-melanocyte-stimulating hormone. Corticotrophin A₂ (2.5 mg.; approx. 0.5 μM) in 5 mm-2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer at pH 7.8 (2.5 ml.) was treated with 10 μl. of enzyme solution [Boehringer trypsin: 0.3% soln. in 0.02N-HCl which had been heated to 37° for 24 hr. at 1% concentration in 0.06N-HCl to reduce chymotryptic contamination (cf. Northrop & Kunitz, 1936; Redfield & Anfinsen, 1956); enzyme-substrate ratio, 1:85]. The stoppered solution was kept at 25° for 3 hr., then acidified to pH 4 and freeze-dried.

N-Acetylcorticotrophin A₂ was digested in a similar manner. It was relatively insoluble at pH 7.8, but a clear solution was obtained within 1 hr. after the addition of enzyme. The solution was kept at 25° for 4 hr.

α-MSH (0.8 mg.; approx. 0.5 μM) in 5 mm-tris-HCl buffer at pH 7.8 (1 ml.) was treated with 3 μl. of the enzyme solution (enzyme-substrate ratio, 1:90). After 3 hr. at 25°, the solution was acidified to pH 4 and freeze-dried.

Separation of the peptide fragments derived from trypsin digestion. Each digest (from approx. 0.5 μM of substrate) was applied on Whatman no. 1 paper as a narrow, 8 cm. long line, and was submitted to high-voltage ionophoresis (Michl, 1951) in pyridine acetate buffer at pH 6.5 (pyridine-acetic acid-water, 10:0.4:90, by vol.) for 75 min. at 2000 v.

The peptide bands were located by staining narrow guide strips with ninhydrin and with tyrosine-, histidine- and arginine-specific reagents (see Block, Durrum & Zweig, 1955). From each paper, the only band containing both tyrosine and histidine and corresponding to the *N*-terminal octapeptide sequence Ser.Tyr.Ser.Met.Glu.His.Phe.Arg was eluted with 5% acetic acid. The three solutions were evaporated to dryness, and the recovered materials were used for further characterization of the *N*-terminal octapeptide from each digest.

Biological assays. All biological assays were performed on twice-chromatographed *N*-acetylcorticotrophin A₂. Corticotrophic activity was measured by adrenal ascorbic acid depletion of hypophysectomized rats by the method of Sayers, Sayers & Woodbury (1948).

MSH-activity was assayed in the illuminated intact frogs, according to the method of Landgrebe & Waring (1950). Assays on the isolated frog skin were performed by Dr Margaret R. Wright and Dr Aaron B. Lerner, using the method of Shizume, Lerner & Fitzpatrick (1954).

RESULTS

Chromatographic patterns

The products of the sequence of reactions described separated on chromatography into components as shown in Figs. 1 and 3. Of the ultraviolet-absorbing material applied on the column, 34–45% was recovered in the main peak, whose characterization as *N*-acetylcorticotrophin A_2 is described below.

On rechromatography of the main peak (*N*-acetylcorticotrophin A_2), 87% of the absorption applied (91% of the absorption recovered from the column) emerged in the main peak (Fig. 2).

Experiments were performed in which a lower pH was used for the last stage of reaction. When *O*-acetylated corticotrophin A_1 (14.0 mg.) treated with ammonium bicarbonate buffer at pH 10 (1.5 ml.) for 12 hr. was chromatographed, a fairly similar pattern was obtained (Fig. 3).

Fig. 1. Chromatography of the reaction products obtained by alkaline treatment (0.1 *N*- NH_3 soln., pH 11.5, for 6 hr.) of *O*-acetylated corticotrophin A_1 . A column (41.5 cm. $\times$ 0.6 cm.²) of carboxylic resin Amberlite IRC-50 (XE-64) was equilibrated and developed with a buffer containing 0.075 *M*- NaH_2PO_4 and 0.075 *M*- Na_2HPO_4 ; load, 7.3 mg. The figures under the peaks express the yields with respect to the total ultraviolet absorption applied to the column. The total recovery was 70%.

Fig. 2. Rechromatography of *N*-acetylcorticotrophin A_2 . A column (41.3 cm. $\times$ 0.6 cm.²) of carboxylic resin was equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 4.6 mg. Of the ultraviolet absorption applied, 87% (91% of the absorption recovered) emerged in the main peak.

Alkaline treatment at a still lower pH [pH 9.0, 0.1 *M*-sodium bicarbonate buffer for 60 hr., used by Shepherd, Howard *et al.* (1956) for removing amide ammonia from β -corticotrophin] resulted in a higher yield of the peak having a retention volume of 150–160 ml./100 ml. of resin bed.

Further exposure to alkali (0.1 *M*-ammonium bicarbonate buffer at pH 10 for 10 hr.) of the peak at 150–160 ml./100 ml. resin bed produced *N*-acetylcorticotrophin A_2 , as shown by rechromatography. Both its position on the chromatogram and its conversion into *N*-acetylcorticotrophin A_2 on further treatment with alkali suggest that this material is *NO*-diacetylcorticotrophin A_2 . This further conversion increases the possible yield of *N*-acetylcorticotrophin A_2 to 50% of the u.v.-absorbing material applied (65% of that emerging). The rest of the absorption recovered is accounted for partly by the unacetylated corticotrophin A_2 and mainly by the peak at 60–70 ml./100 ml. of resin bed.

In attempts to identify the material from the peak at 60–70 ml./100 ml. of resin bed, separate samples were rechromatographed after the following treatments: (i) further exposure to alkali (ammonium bicarbonate buffer, 0.1 *M*, pH 10 for 12 hr.); (ii) reduction with thioglycolic acid (0.2 *M*, pH 3.5, 70° for 8 hr.) (Dedman, Farmer & Morris, 1955, 1957; Dixon, 1955, 1956*a*). Both treatments failed to produce *N*-acetylcorticotrophin A_2 as shown by rechromatography. No further attempt was made to characterize this material.

Corticotrophin A_1 emerged, in the system used, as a peak with retention volume of about 500 ml./100 ml. of resin bed. After treatment of corticotrophin A_1 with ammonia solution (*M* for 6 hr.), the result shown in Fig. 4 was obtained. The main peak at about 280 ml./100 ml. of resin bed is corticotrophin A_2 . Unfortunately, minor components emerged with the same retention volume as

Fig. 3. Chromatography of the reaction products obtained by alkaline treatment (ammonium bicarbonate buffer, pH 10, for 12 hr.) of *O*-acetylated corticotrophin A_1 . A column (45.5 cm. $\times$ 0.6 cm.²) of carboxylic resin equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 7.7 mg. The figures under the peaks express the yields with respect to the total ultraviolet absorption applied to the column. The total recovery was 78%.

N-acetylcorticotrophin A_2 . The nature of these minor components has not been established. (Some of the corticotrophin A_1 used for these preparations has since been found to contain chromatographically less-retarded components which were produced during desalting when a particular batch of acetic acid was used. The impurities in the corticotrophin A_2 may be related to such components.) Their existence means that the *N*-acetylcorticotrophin A_2 peak may be slightly contaminated with biologically-active unacetylated derivatives of corticotrophin. If, however, these minor components still possess an *N*-terminal serine residue, after the acetylation reaction they should be largely replaced by the corresponding *N*-acetyl derivatives, which should be less retarded in the chromatographic system than *N*-acetylcorticotrophin A_2 , so that the degree of contamination should be small. These *N*-acetyl derivatives may make up part of the peak observed at 60–70 ml./

100 ml. of resin bed. There is chromatographic evidence that the material of this peak is heterogeneous.

Characterization of *N*-acetylcorticotrophin A_2

Ultraviolet-absorption spectrum. The ultraviolet-absorption spectra of desalted corticotrophin A_1 and *N*-acetylcorticotrophin A_2 were compared for solutions of 1.05 mg. in 3.0 ml. of 2% acetic acid (Fig. 5), with a Beckman Automatic Recorder. The extinction reading at the absorption maximum (278 m μ) was identical for the two compounds, corresponding to a value of $E_{1\text{ cm.}}^{1\%}$ of 16.0.

***N*- and *C*-Terminal group analyses.** No free *N*-terminal group was detected when the phenylisothiocyanate method was applied to *N*-acetylcorticotrophin A_2 . Control experiments with corticotrophins A_1 and A_2 yielded an average of 0.65 mole of serine as *N*-terminal amino acid.

There was no indication of modification of the *C*-terminal end of the molecule when the amino acids released by carboxypeptidase digestion of *N*-acetylcorticotrophin A_2 and corticotrophins A_1 and A_2 were compared. Paper chromatography in the solvent system butanol-acetic acid-water (4:1:5, by vol.), followed by development with ninhydrin, revealed the presence of phenylalanine and two weaker spots of glutamic acid and leucine, with comparable intensities in all three samples.

Trypsin digestion. When corticotrophin is digested with the enzyme trypsin, the *N*-terminal octapeptide Ser. Tyr. Ser. Met. Glu. His. Phe. Arg is released (Bell, 1954). Under similar conditions, the corresponding *N*-acetyl fragment is released from α -MSH (Harris, 1959).

Trypsin digestion was used to compare the *N*-terminal octapeptide released from *N*-acetylcorticotrophin A_2 with those from corticotrophin A_2 and α -MSH. Fig. 6 shows the pattern obtained when digests of the three substrates were submitted to paper ionophoresis in pyridine acetate buffer at pH 6.5, in a similar run. Ionophoresis at this pH provided the best means of resolving the *N*-terminal octapeptide from the other basic and acidic fragments released during trypsin digestion of each substrate.

Peptide A (from corticotrophin A_2) was slightly basic at pH 6.5, compatible with the structure Ser. Tyr. Ser. Met. Glu. His. Phe. Arg. It gave a brown spot with ninhydrin and in addition responded positively to the tyrosine, histidine and arginine colour-tests.

No bands of corresponding mobility were observed with the digests of *N*-acetylcorticotrophin A_2 and α -MSH. Instead, a band of a slightly acidic substance, compatible with the structure *N*-acetylSer. Tyr. Ser. Met. Glu. His. Phe. Arg, appeared with both digests (peptides B and C re-

Fig. 4. Chromatography of the reaction products obtained by alkaline treatment (M-NH₃ soln. for 6 hr.) of corticotrophin A_1 . A column (44.5 cm. $\times$ 0.6 cm.²) of carboxylic resin was equilibrated and developed with sodium phosphate buffer as in Fig. 1; load, 6.6 mg. Of the ultraviolet absorption applied, 55% emerged in the corticotrophin A_2 peak. The total recovery was 87%.

Fig. 5. Ultraviolet-absorption spectra of corticotrophin A_1 and *N*-acetylcorticotrophin A_2 . —, Corticotrophin A_1 ; ---, *N*-acetylcorticotrophin A_2 . Concentration: 1.05 mg. in 3.0 ml. of 2% (v/v) acetic acid solution. $E_{1\text{ cm.}}$ at 278 m μ , 0.550.

spectively). These bands gave no colour with ninhydrin, but responded positively to the tyrosine, histidine and arginine colour-tests.

Apart from the difference in bands A and B, the trypsin digests of corticotrophin A₂ and N-acetylcorticotrophin A₂ were resolved into an identical pattern on ionophoresis at pH 6.5.

Further characterization of bands A, B and C was achieved by comparing the R_F of the three peptides by chromatography on paper, in the solvent system butanol-acetic acid-water (4:1:5, by vol.). On development with the tyrosine reagent, peptide B (from N-acetylcorticotrophin A₂) gave an intense spot with an R_F of 0.43 and a minor spot with an R_F of 0.26 which was identified as the corresponding methionine sulfoxide peptide. Peptides containing methionine normally separated into two components when chromatographed in the solvent system butanol-acetic acid-water (4:1:5, by vol.), as a result of partial oxidation of the methionine into methionine sulfoxide during operations (see Harris & Roos, 1959). Peptide C (from α-MSH) gave an identical pattern, whereas peptide A (from corticotrophin A₂) revealed the same pair of spots at much lower R_F values (0.17 and 0.09 respectively), as expected from the absence of the N-terminal acetyl group.

Finally, peptides A, B and C were analysed for amino acid composition. Samples were hydrolysed with 5.7N-HCl for 16 hr., and the amino acids identified by paper chromatography in the solvent system butanol-acetic acid-water (4:1:5, by vol.),

and by paper ionophoresis in 2.5% formic acid at pH 2.1. All three samples were found to contain the expected amino acids (Ser, Tyr, Ser, Met, MetSO, Glu, His, Phe and Arg).

Chromatography on paper. Samples of desalted N-acetylcorticotrophin A₂ and corticotrophin A₂ (0.25 mg. of each) were applied on Whatman no. 1 paper for chromatography in the solvent system butanol-acetic acid-water-pyridine (30:6:24:20, by vol.) described by Waley & Watson (1953). Corticotrophin A₂ migrated as a slightly elongated spot with an R_F of 0.35, while N-acetylcorticotrophin A₂ moved as a single, fairly compact spot with an R_F of 0.50. This high value agrees with the presence of an N-terminal acetyl group.

Biological assays

Corticotrophin assays. Table 1 shows that N-acetylcorticotrophin A₂ possessed less than 10% of the activity of corticotrophin A₁. The slight activity found (about 2%) could be intrinsic, but could also be due to slight contamination with non-acetylated products, such as those with the same retention volume produced by the action of alkali on corticotrophin A₁ (Fig. 4).

Melanocyte-stimulation assay. (a) In the intact frog N-acetylcorticotrophin A₂ did not show increased activity compared with corticotrophin A₁ if the melanocyte index was read up to 1 hr. after injection, and was probably less active at 0.5 hr. (Table 2). But its action was prolonged, so that if the comparison was made later (6 or 18 hr.) it had

Fig. 6. Ionophoresis diagrams of products of tryptic hydrolysis of corticotrophin A₂, N-acetylcorticotrophin A₂ and α-MSH. Ionophoresis was carried out in pyridine acetate buffer (pH 6.5) at 35–40 v/cm. for 75 min.

Table 1. ACTH assay of N-acetylcorticotrophin A₂: depletion of ascorbic acid in hypophysectomized rats

The estimate of the standard deviation of the responses was 16 (9 degrees of freedom) and it was 15 if another 6 rats (another 4 degrees of freedom) which were used in the same assay for another test were also included. The intravenous injection was 0.25 ml./100 g. rat weight. Corticotrophins A₁ and A₂ do not differ significantly in ACTH activity (Dixon & Stack-Dunne, 1955; Shepherd, Howard *et al.* 1956).

Substance	Dose (μmg./ml.)	No. of rats	Mean change of adrenal concn. of ascorbic acid (mg./100 g. of tissue)
Corticotrophin A ₁	40	4	-49
N-Acetylcorticotrophin A ₂	4000	3	-82
	400	3	-1
	40	3	+16

Table 2. *Melanocyte-stimulating assay of N-acetylcorticotrophin A₂ on intact frogs*

Substance	Dose (μ mg.)	No. of frogs	Mean melanocyte index					Mean difference from initial melanocyte index			
			Before injection	After injection				$\frac{1}{2}$ hr.	1 hr.	6 hr.	18 hr.
				$\frac{1}{2}$ hr.	1 hr.	6 hr.	18 hr.				
<i>N</i> -Acetylcorticotrophin A ₂	500	4	1.7	3.0	3.9	4.6	3.4	1.3	1.9	2.9	1.7
	150	3	1.7	2.8	3.6	2.4	—	1.1	1.7	0.7	—
Corticotrophin A ₁ *	5000	4	1.4	3.4	3.9	3.5	1.9†	2.0	2.5	2.1	0.5
	1500	3	1.5	3.3	3.9	3.3	—	1.8	2.4	1.8	—
	500	3	1.8	3.5	3.7	2.6	—	1.7	1.9	0.8	—
β -Melanocyte-stimulating hormone	15	4	1.4	3.8	3.9	1.4	—	2.3	2.4	-0.1	—
	5	3	1.6	3.3	3.8	1.3	—	1.7	2.2	-0.3	—
Standard deviation ...			0.24	0.35	0.47	0.63	0.50				

* Corticotrophins A₁ and A₂ are reported to have the same MSH activity (Shepherd, Howard *et al.* 1956).

† One frog (index 1.6) dead; mean of remainder, 2.0.

more than 10 times the activity of corticotrophin A₁. In contrast, β -MSH showed much higher activity than corticotrophin A₁ mainly at short time intervals after injection, about 100 times the activity at 1 hr., but much less than 33 times at 6 hr. The data (particularly those at 6 hr.) suggest that the potency ratio obtained depended on the dose used. This type of 'invalid' assay may be expected when substances of different properties are compared.

(b) Two samples of *N*-acetylcorticotrophin A₂ assayed in isolated frog skin had activity of 410 and 440 units/ μ g. respectively. The corresponding values for corticotrophin A₁ were 50 and 75. The activities ascribed to α -MSH and β -MSH by the same assay method are about 25 000 and 10 000 units/ μ g. respectively.

N-Acetylcorticotrophin A₂ thus appears to be 5–10 times as active as corticotrophin A₁ when assayed on isolated frog skin. While the melanocyte-stimulating activity of the latter is less than 0.5% that of α -MSH, that of *N*-acetylcorticotrophin A₂ is increased to approx. 2%, when compared on a weight basis (or to 5% when compared on a molar basis).

DISCUSSION

The results reported suggest that selective acetylation of the α -amino group of the *N*-terminal serine residue of corticotrophin has been achieved under the conditions described.

The large fall in corticotrophin (ACTH) activity found on acetylation, together with the similar falls after other reactions of the same serine residue (White, 1955; Shepherd, Willson *et al.* 1956; Dixon, 1956*b*; Geschwind & Li, 1957), supports the view that the unsubstituted *N*-terminal serine residue is an essential constituent of a highly active ACTH molecule. As we do not know whether the slight ACTH activity found in the *N*-acetylcorti-

cotrophin A₂ is intrinsic or due to contamination, we cannot tell if any activity is possessed when the group is blocked. If possessed, such activity could be due either to a low activity at the site of action or to a low degree of deacetylation in the animal body.

In the introduction we posed the question whether *N*-acetylcorticotrophin A₂ has greater MSH activity than has corticotrophin A₁. While there is no simple answer to this question, we can say that no spectacular increase of MSH activity has been observed. The results of the assay with isolated frog skin indicate that *N*-acetylcorticotrophin A₂ possesses about 2% of the activity of α -MSH on a weight basis (or about 5% on a molar basis) compared with about 0.3% on a weight basis for corticotrophin A₁, while in the assay with intact frogs a potentiation of the same order of magnitude is apparent, but only at the longer times after injection. The high activity of β -MSH in comparison with corticotrophin is, in contrast, most marked shortly after injection and much less at longer intervals. Since some of the methods for assessing MSH action demonstrate increased activity, the idea that the free amino group inhibits this activity is supported. Anfinsen (1959) quotes Boissonnas as finding that synthetic α -MSH has about seventy times the activity of the corresponding peptide without the acetyl group.

The low order of MSH activity in *N*-acetylcorticotrophin A₂, which possesses the complete sequence of α -MSH, suggests that the structural features of the part of the corticotrophin molecule that is outside this sequence (e.g. chain length, configuration and charge distribution) seem to inhibit the potential MSH activity of the *N*-acetylcorticotrophin A₂. The diminution of activity which accompanies the replacement of the glutamyl residue of pig β -MSH by the seryl residue of ox β -MSH (Li, 1957) may be a similar inhibition of potential activity, since the locus concerned is

outside the sequence common to MSH-active substances to which activity is presumably due.

Assay methods are often invalid in the sense that the potency ratio which they yield depends on the doses of material tested. Furthermore the potency ratio may depend on the conditions of the test. For MSH activity different potency ratios may be shown under different conditions (e.g. Lock, 1952). In particular, many preparations show a prolongation of action when treated with alkali (cf. Landgrebe & Mitchell, 1954, and review by Li, 1957), so that such materials would show a higher potency against a standard if the assessment were made long after injection. From our data it appears that even the potency ratio between natural corticotrophin and β -MSH depends on the time after injection at which the effects are assessed and may also depend on the doses used. This emphasizes the necessity for specifying the conditions of assay when quoting any figure for a potency ratio.

The use of the isolated frog skin for the determination of MSH activity does not avoid this indeterminacy of potency ratio, since the treatment of some materials with alkali leads to a prolongation of action (Dr A. B. Lerner, personal communication). All the differences cannot, therefore, be due to differences in rates of absorption and excretion of the injected substances by the whole frog.

Indeterminacy of potency ratio is to be expected where substances of greatly different chemical properties exhibit a biological effect, because a series of reactions of different specificities may occur in the production of the effect. This is apart from the other invalidities in the assay of impure substances produced by interference by the impurities. The indeterminacy in testing pure substances may occur with corticotrophic potency, though the evidence is less clear than that for MSH activity. Only substances of similar molecular size possess ACTH activity, and this may explain why a unique value for the potency ratio is often obtained. Certainly the addition of other substances or peptic digestion of its molecules can prevent corticotrophin from showing its action if it is injected subcutaneously, in contrast with the full action shown by the same preparations injected intravenously. Hence assays with subcutaneous injection, e.g. that of United States Pharmacopeia XV, which are designed not to determine the amount of hormone present but to predict the effect that a preparation will have when administered intramuscularly or subcutaneously (Thompson & Fisher, 1953), give potency ratios very different from the original ascorbic acid-depletion test with intravenous injection (Sayers *et al.* 1948). The assay with subcutaneous injection allows other substances (e.g. proteolytic enzymes) to interact

with the corticotrophin after injection to about the same extent that they may be expected to interact after intramuscular injection into man, and so gives a better measure of the effect likely to be obtained clinically than the test with intravenous injection, designed to measure the amount of corticotrophin present. The result of this test can be affected by adding unrelated protein (Brown, Moyer, Davies & Cox, 1955). The observation by Fortier (1958) that crude pituitary extracts from different species show different rates of absorption by adrenal tissue, as judged by its subsequent production of steroids, may mean that even pure substances would show different corticotrophic potency ratios according to the conditions of testing.

The indeterminacy of potency ratio emphasizes the difficulties of ascribing complex biological activities to particular features of molecular structure. It should be noted particularly for MSH activity, both because the variations in potency ratio can be large and because opportunities exist for studying a series of compounds of natural, chemically-modified and synthetic origins which possess MSH activity.

SUMMARY

1. The serine residues of corticotrophin A_1 were *O*-acetylated by treatment with acetic acid containing hydrogen bromide. Subsequent treatment with mild alkali moved one acetyl group to the terminal amino group, hydrolysed the other and liberated amide ammonia. The product (*N*-acetylcorticotrophin A_2) has been purified by ion-exchange chromatography and characterized.

2. *N*-Acetylcorticotrophin A_2 possesses less than 10% of the corticotrophic activity of corticotrophin A_1 . This result supports suggestions that a free amino group on the *N*-terminal serine residue is essential for high corticotrophic activity.

3. *N*-Acetylcorticotrophin A_2 possesses 5–10 times the melanocyte-stimulating activity of corticotrophin A_1 when assayed on isolated frog skin.

4. In the intact frog *N*-acetylcorticotrophin A_2 possesses no more melanocyte-stimulating activity than corticotrophin A_1 if the response is assessed up to 1 hr. after injection, but more than ten times the activity if the response is assayed after 6 hr. β -Melanocyte-stimulating hormone possesses about 100 times the melanocyte-stimulating activity of corticotrophin A_1 if the response is assessed 1 hr. after injection into the frog, but much less if the assessment is made later.

5. Comparison of the results of melanocyte-stimulating assays with chemical structure suggests that the potential activity of the sequence

of α -melanocyte-stimulating hormone in *N*-acetyl-corticotrophin A_2 is inhibited by other structural features of its molecule.

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The Preparation and Purification of Tritium-Labelled Hexoestrol of Very High Specific Activity on the 5 mg. Scale

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Tritium can be introduced into organic compounds by catalyst-induced exchange with ionic tritium, which is usually contained in the solvent (water, acetic acid, sulphuric acid, etc.) in which the reaction takes place, by radiation-induced exchange with tritium gas (Wilzbach, 1957), or by

catalytic addition of tritium to unsaturated compounds. Exchange with ionic tritium suffers from the disadvantages that the high temperatures required may cause decomposition (Bloch & Rittenberg, 1943), that specific activities are limited by that obtainable in the solvent, which