[7] A. BUZZELL AND J. M. STURTEVANT, *J. Am. Chem. Soc.*, 73 (1951) 2454.
[8] G. C. CHEESEMAN, *J. Dairy Research*, 29 (1962) 163.
[9] B. JIRGENSONS, T. IKENAKA AND V. GORGURAKI, *Makromol. Chem.*, 28 (1958) 96.
[10] H. A. MCKENZIE AND R. G. WAKE, *Australian J. Chem.*, 12 (1959) 734.
[11] J. GARNIER, G. MOCQUOT AND G. BRIGNON, *Compt. rend.*, 254 (1962) 372.
[12] R. J. PODOLSKY AND J. M. STURTEVANT, *J. Biol. Chem.*, 217 (1955) 603.

Received August 14th, 1962

Biochim. Biophys. Acta, 69 (1963) 169–171

SC 2183

The action of *S*-methylisothiourea on corticotropin

α-Melanocyte-stimulating hormone isolated from mammalian pituitary glands has been shown to be an *N*-acetyl tridecapeptide[1] amide in which the sequence of amino acids is identical to that of the N-terminal tridecapeptide portion of corticotropin[2]. However, in spite of this remarkable similarity in chemical structure corticotropin possesses less than 1 % of the melanocyte-stimulating potency of α-MSH.

Although corticotropin consists of 39 amino acid residues, its melanocyte-stimulating activity is undoubtedly associated with the sequence of amino acids which it possesses in common with the melanocyte-stimulating hormones (α-MSH and β-MSH)[3,4]. Within this common sequence the most obvious chemical difference between the two hormone molecules is that the α-NH_2 group of the N-terminal serine occurs as the *N*-acetyl derivative in α-MSH. Thus in corticotropin the $\begin{matrix}CH_2\text{–}CH\text{–}\\ |\quad\quad |\\ OH\quad NH_2\end{matrix}$ structure which is known to be essential for ACTH activity[5] may at the same time have an inhibitory effect on its potential melanocyte-stimulating activity.

In order to investigate this possibility, it was necessary to achieve the selective acetylation of the α-NH_2 group of the N-terminal serine in corticotropin. A possible approach to this problem would be to protect the ε-NH_2 groups of its four lysine residues by reaction with *S*-methylisothiourea and then to acetylate the resulting guanidyl derivative with acetic anhydride. Earlier studies with chymotrypsinogen[6], RNAase[7] and growth hormone[8] had indicated that under strongly alkaline conditions (pH 10.5–11.0), *O*-methylisourea reacts selectively with ε-NH_2 groups of lysine residues, or δ-NH_2 groups of ornithine residues[9], and that α-NH_2 groups do not react to any significant extent under these conditions.

When corticotropin was allowed to react with *S*-methylisothiourea the resulting guanidyl derivative was found not to possess a free α-NH_2 group, indicating that both α- and ε-NH_2 groups had reacted with the reagent. Although this unexpected development was clearly inimical to the original purpose, it was decided to study the reaction and to investigate the chemical structure of the resulting product. In this communication evidence is presented to demonstrate that the free α- and ε-NH_2 groups had reacted.

Corticotropin A_1 (5 mg) was dissolved in 0.5 ml ammonium hydroxide (pH 10.5) and allowed to react with excess of *S*-methylisothiourea (25 mg) at room temperature for 48 h. The pH was maintained at this value by intermittent additions of small

quantities of 1 N ammonium hydroxide, and shaken mechanically over this period of time. Simultaneously a control sample (5 mg) of corticotropin A_1 was treated with ammonium hydroxide alone (pH 10.5) and kept at this pH for 48 h. This was used for comparison with the modified hormone in subsequent experiments.

The sample treated with *S*-methylisothiourea had not only the ε-amino groups of lysine guanidinated, but also the α-amino group of N-terminal serine. After desalting the treated hormone on an Amberlite IRC-50 column, and eluting it with 50 % acetic acid by the technique elaborated by DIXON[10], the effluents were concentrated to a small volume and freeze-dried. The eluates from the column were followed and controlled by examination of the ultraviolet absorption at 280 mμ of the effluents. The yield of material obtained was of the order of 89–90 % of the theoretical. Evidence for the complete guanidination of the hormone was assembled from the following angles:

(a) Sealed-tube acid hydrolysis (6 N HCl at 105° for 24 h) of the guanidinated compound, followed by ionophoresis for 2 h at pH 3.5 and 2000 V of the hydrolysate revealed the disappearance of lysine residues with a concomitant increase of "arginine"-type residues (Sakaguchi reaction) homo-arginine having a lower mobility than lysine when submitted to ionophoresis at pH 6.5.

(b) The guanidinated hormone was treated with phenylisothiocyanate on paper by a modification of the Edman technique[11]. No phenylthiohydantoin of serine was produced as evidenced by the lack of the ultraviolet-absorption maximum at 268 mμ associated with phenylthiohydantoin of amino acids. Moreover, the product after elution from paper with ethanol – ether solvent (1:1, v/v) was submitted to chromatography with a control of serine phenylthiohydantoin in pyridine – *n*-heptane solvents (3:7, v/v) system. There was no spot corresponding to the applied control. However, when the control ACTH was submitted to the same procedure, it produced the phenylthiohydantoin of serine. The results indicated that the α-NH_2 group of the modified hormone had been blocked and consequently unable to react with phenylisothiocyanate.

(c) Enzymic degradation with chymotrypsin at 30° for periods upwards of 16 h produced 5 major fragments in accordance with results of earlier studies on ACTH[12]. One of the fragments was isolable from the others by ionophoresis at pH 6.5 (pyridine–acetate buffer), 1600 V for 1.5–2 h. On paper this fragment produced a very slight positive ninhydrin reaction and gave a red colour with α-nitroso-β-naphthol reagent for tyrosine. After elution from the paper with 5 % acetic acid, and then bringing to dryness, it gave a pink coloration with the modified Sakaguchi reagent of WEBER[13]. The fact that it gave a negative histidine test with Pauly's reagent (diazotised sulphanilic acid) indicates that this peptide fragment is not part of the heptapeptide core Ser–Tyr–Ser–Met–Glu–His–Phe– which might conceivably occur if the enzyme had merely attacked the molecule at the Phe–Arg bond (Fig. 1). These results would indicate that this fragment is a guanidyl derivative involving a tyrosine moiety, and this could only be the N-terminal dipeptide fragment Ser–Tyr which is known to be cleaved easily by chymotrypsin. Moreover, its mobility under the applied electrophoretic conditions suggests that it possesses slightly basic character which is to be expected if guanidination of the terminal serine had been achieved.

Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Try–Gly . . . . . Phe·OH[39]
↑ ↑ ↑

Fig. 1. Arrows indicate the main sites of hydrolysis of ACTH by chymotrypsin.

(d) An attempt to prepare *N*-acetyl corticotropin after treatment with *S*-methylisothiourea failed. By making use of an O → N acyl shift, WALLER AND DIXON[14] have prepared the *N*-acetyl derivative, which has increased melanocyte-stimulating activity.

At pH 6.5 both the guanidinated and untreated hormones had the same ionophoretic mobility, a result which is expected if the original α-amino group of N-terminal serine has an unusually high p*K* value. Replacement of the α-amino group by a guanidyl one ought to produce a more basic molecule, but in corticotropin this property would only be manifested at a higher pH value, that is within the pH range of ε-amino group of lysine.

GREENSTEIN[9] and HUGHES, SAROFF AND CARNEY[15] found that α-amino groups of proteins did not react with *O*-methylisourea, although the ε-amino groups of lysine did, from which it may be inferred that the reagent under analogously mild conditions was somewhat specific for ε-amino groups in proteins. This is supported by the work of ROCHE, MOURGUE AND BARET[16] who were unable to detect α-guanidino carboxylic acids in acid hydrolysates of proteins treated with *O*-methylisourea.

Kinetic studies of the reaction of corticotropin with fluorodinitrobenzene and phenylisothiocyanate[17] indicated that the α-amino group had an abnormally high p*K*, that is a p*K* closer to that of ε-amino group of lysine than to a "normal" α-amino group. This view is substantiated by the fact that corticotropin is so readily guanidinated by *S*-methylisothiourea a milder reagent than the *O*-methyl analogue[18].

The possibility that α-guanidino carboxylic acids may escape detection in acid hydrolysates by a conversion of the "creatine–creatinine" type was obviated by degrading the treated hormone with chymotrypsin whose specificity towards ACTH has been well authenticated[12]. The detection of one of the fragments as guanidylseryltyrosine shows conclusively that the α-amino group of the reactive N-terminal serine had undergone reaction with the reagent. Moreover, the fact that this fragment did not give a creatinine reaction with the reagent of BENEDICT AND BEHRE[19] confirms the observation that no transformation had taken place under the conditions used for its isolation.

The results suggest that some degree of caution should be exercised during the modification of proteins with this reagent, since, in large molecules of this kind, the α-amino residues may have p*K* values somewhat modified by their environment with other amino acids and therefore would behave altogether differently from "normal" α-amino groups. In corticotropin there is evidence that the α-amino group clearly possesses an unusually high p*K* which had caused it to react with *S*-methylisothiourea.

I am grateful to Dr. J. I. HARRIS and Dr. F. SANGER for helpful advice and discussion, to Dr. H. B. F. DIXON for samples of corticotropin A_1, and to Professor F. G. YOUNG for allowing me the facilities of his laboratories in Cambridge and to the Inter-University Council for a Fellowship to conduct this work.

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[1] J. I. HARRIS AND P. ROOS, *Nature*, 178 (1956) 90.
[2] C. H. LI, I. I. GESCHWIND, R. D. COLE, J. I. HARRIS AND J. S. DIXON, *Nature*, 176 (1955) 687.
[3] J. I. HARRIS AND A. B. LERNER, *Nature*, 179 (1957) 1346.

[4] J. I. HARRIS, *Nature*, 184 (1959) 167.
[5] H. B. F. DIXON, *Biochem. J.*, 62 (1956) 25P.
[6] C. H. CHERVENKA AND P. E. WILCOX, *J. Biol. Chem.*, 222 (1956) 635.
[7] P. J. VITTRAYATHIL AND F. M. RICHARDS, *J. Biol. Chem.*, 235 (1960) 1092.
[8] C. H. LI, *Advan. Protein Chem.*, 11 (1956) 102.
[9] J. P. GREENSTEIN, *J. Biol. Chem.*, 109 (1935) 541.
[10] H. B. F. DIXON, *Biochim. Biophys. Acta*, 34 (1959) 251.
[11] H. FRAENKEL-CONRAT, *J. Am. Chem. Soc.*, 76 (1954) 3606.
[12] W. F. WHITE AND W. A. LANDMANN, *J. Am. Chem. Soc.*, 77 (1955) 771.
[13] C. J. WEBER, *J. Biol. Chem.*, 86 (1930) 217.
[14] J. P. WALLER AND H. B. F. DIXON, *Biochem. J.*, 75 (1960) 320.
[15] W. L. HUGHES JR., H. A. SAROFF AND A. L. CARNEY, *J. Am. Chem. Soc.*, 71 (1949) 2376.
[16] J. ROCHE, M. MOURGUE AND R. BARET, *Bull. Soc. Chim. Biol.*, 36 (1955) 85.
[17] C. H. LI AND A. L. LEVY, *J. Biol. Chem.*, 213 (1955) 487.
[18] H. SCHUTTE, *Z. Physiol. Chem.*, 279 (1943) 59.
[19] S. R. BENEDICT AND J. A. BEHRE, *J. Biol. Chem.*, 114 (1936) 515.

Received August 30th, 1962

SC 2177

Acetylated intermediates of arginine synthesis in *Bacillus subtilis*

In *Escherichia coli*, ornithine (a precursor of arginine) is synthesized from glutamate via the following acetylated intermediates: *N*-acetylglutamate[1,2], *N*-acetyl-γ-glutamyl phosphate[3], *N*-acetylglutamic-γ-semialdehyde[1], and N^{α}-acetylornithine[1,4]. The formation of ornithine from N^{α}-acetylornithine is mediated, in this organism, by the hydrolytic enzyme, acetylornithinase[1,4,5]. In *Bacillus subtilis*, this enzyme has thus far not been detected, nor has an acetyltransferase such as reported for *Micrococcus glutamicus*[6]. It, therefore, was of interest to examine the path of arginine synthesis in *B. subtilis*.

Tracer experiments have now indicated that *B. subtilis* has a glutamic family (*cf.* VOGEL AND BONNER[7]) consisting of glutamate and its biosynthetic products, arginine and proline. Thus, Table I shows that [2-^{14}C]acetate labels these 3 amino acids at approximately equal specific activity, which differs from that of alanine and from that of threonine and lysine (both of which are derivatives of aspartate[9]). Qualitatively similar results were obtained in analogous experiments with [1-^{14}C]glutamate, [3-^{14}C]-

TABLE I

INCORPORATION OF TRACERS INTO PROTEIN AMINO ACIDS OF *B. subtilis*, ATCC 6051 (AS RELATIVE SPECIFIC RADIOACTIVITY ON MOLAR BASIS)

The organism was grown in a glucose–salts medium, supplemented with tracers (0.1 mg/ml; approx. 1 mC/mmole), as indicated. The isotope methods used were essentially those previously employed[8].

Tracer	*Glu*	*Arg*	*Pro*	*Ala*	*Thr*	*Lys*
[2-^{14}C]Acetate, sodium salt	100	81	104	9	45	42
DL-[2-^{14}C]Ornithine hydrochloride	100	1005	—	—	—	—