Biological Characterization of a New and Highly Potent Synthetic Analogue of Corticotrophin

During the last decade research on adrenocorticotrophic hormone has developed very successfully. The chemical structure of this hormone which has 39 amino acids in a straight chain was elucidated in 1954 by BELL[1,2]. In 1963 SCHWYZER[3] succeeded in synthesizing this peptide which, given subcutaneously, showed a biological activity of 115 IU/mg[4] in the classical adrenal ascorbic acid depletion test. During the past few years many attempts have been made to localize the particular sequence of amino acids within the whole molecule responsible for biological activity, i.e. stimulation of adrenal steroid synthesis and secretion. By studying fragments, obtained both by hydrolysis of highly purified natural hormone and by synthesis, the discovery was made that only the first *N*-terminal 19–24 amino acids are necessary to obtain activity in the range of that of the natural hormone. β^{1-24} corticotrophin with 106 IU/mg, was most potent[5,6]. Many peptides with a slightly modified amino acid sequence have also been synthesized, but none showed an activity superior to that of the natural hormone.

Three modifications of the natural sequence were introduced for the following reasons in the synthesis by BOISSONNAS, GUTTMANN, and PLESS[7] of the peptide D-serine[1]-norleucine[4]-valinamide[25]-β-1-25-corticotrophin. (a) In order to increase the resistance against degradation by aminopeptidases, D-serine was substituted for L-serine at the *N*-terminal. (b) To increase the resistance against degradation by carboxypeptidases, valinamide was substituted for asparagine in position 25. (c) Since it is known that the oxidation of methionine to methionine-sulphoxide results in inactivation[8], methionine in position 4 was substituted by its isologue norleucine.

The aim of this study was the biological characterization of this pentacosapeptide, named DW-75.

Methods. The design of the 3 methods of standardization described below was a 6-point-assay with randomized distribution of animals and tissue samples. In each test, the pentacosapeptide was compared directly with the 3rd International Working Standard[9]. Statistical analysis, i.e. calculation of the activity including fiducial limits, was performed as recommended by the British Pharmacopoeia 1963[10]. All activities were expressed in International Units (IU) per mg peptide (free base).

Adrenal ascorbic acid depletion in vivo (SAYERS test s.c.)[11]. The assay was performed as laid down in the British Pharmacopoeia 1963 and U.S.P. 17. Only male rats weighing 140–160 g, hypophysectomized 24 h previously, were used. The peptide and standard were administered subcutaneously in 3 logarithmically spaced doses in the range 100–1000 milli-U/100 g body weight. Exactly 3 h later the adrenals were removed under ether anaesthesia, and their ascorbic acid content estimated using the method described by the British Pharmacopoeia 1963[10]. In each single assay, 6–8 animals per dose were used, making a total of 40 animals per dose in the complete standardization of this peptide.

Corticosterone release in vitro. The estimation of activity based on the in vitro release of corticosterone from quartered rat adrenals was performed by the method originally described by SAFFRAN and SCHALLY[12]. Logarithmically spaced doses (IU/ml) of the peptide and of the standard were added to freshly dissected adrenals of male rats and incubated for 1 h in a modified Krebs-Ringer solution at 37 °C in a Dubnoff shaker. Corticosterone was extracted from the medium by methylenechloride and, after having been transferred to sulphuric acid, measured fluorometrically with an Amino-Bowman-Spectrophotofluorometer[13]. In each assay the adrenals of 12 rats were used.

Lipolytic activity in vitro. The mobilization of free fatty acids (FFA) from adipose tissue by the pentacosapeptide was quantitatively determined. Freshly dissected rat epididymal fat pads were weighed and incubated in modified Krebs-Ringer solution at 37 °C in a Dubnoff shaker. After renewal of the medium, 3 logarithmically spaced doses of the peptide and of the standard (IU/ml) were added, proceeding for a further 60 min. The liberated FFA were extracted and estimated by titration[14]. Palmitinic acid was used as reference standard and the values

[1] P. H. BELL, J. Am. chem. Soc. *76*, 5565 (1954).
[2] R. G. SHEPHERD, S. D. WILLSON, K. S. HOWARD, P. H. BELL, D. S. DAVIES, S. B. DAVIS, E. A. EIGNER, and N. E. SHAKESPEARE, J. Am. chem. Soc. *78*, 5066 (1956).
[3] R. SCHWYZER and P. SIEBER, Nature *199*, 172 (1963).
[4] P. BARTHE, P. A. DESAULLES, B. SCHÄR, and M. STAEHELIN, Nature *202*, 908 (1966).
[5] H. KAPPELER and R. SCHWYZER, Helv. chim. Acta *44*, 1136 (1961).
[6] W. SCHULER, B. SCHÄR, and P. DESAULLES, Schweiz. med. Wschr. *93*, 1027 (1963).
[7] R. A. BOISSONNAS, ST. GUTTMANN, and J. PLESS, Experientia *22*, 526 (1966).
[8] M. L. DEDMAN, T. H. FARMER, and C. J. O. R. MORRIS, Biochem. J. *78*, 348 (1961).
[9] D. R. BANGHAM, M. V. MUSSET, and M. P. STACK-DUNNE, Bull. Org. mond. Santé *27*, 395 (1962).
[10] *British Pharmacopoeia* (Pharmaceutical Press, London 1963).
[11] M. A. SAYERS, G. SAYERS, and L. A. WOODBURY, Endocrinology *42*, 379 (1948).
[12] M. SAFFRAN and A. V. SCHALLY, Endocrinology *56*, 523 (1955).
[13] W. DOEPFNER, E. STÜRMER, and B. BERDE, Endocrinology *72*, 897 (1963).
[14] V. P. DOLE, J. clin. Invest. *35*, 150 (1956).

	1 4 24 25 39	Adrenal ascorbic acid depletion assay s.c. Activity IU/mg
β-1-39-corticotrophin	Ser-Tyr-Ser-Met- -Pro-Asp- -Phe	115
β-1-24-corticotrophin	Ser-Tyr-Ser-Met- -Pro	106
DW-75	D-Ser-Tyr-Ser-Nle- -Pro-Val -NH_2	625

Differences in amino acid sequence and biological activity of synthetic β^{1-39}-, β^{1-24}- and D-Ser[1]-Nle[4]-(Val-NH_2)[25]-β^{1-25}-corticotrophin (DW-75).

obtained were calculated in μEq/g/h. In order to obtain comparative values, the potency of the peptide was calculated and expressed also in IU.

Results and discussion. The pentacosapeptide described above had an activity of 625.0 IU/mg (F.L. 82.2–121.6%) as estimated by the ascorbic acid depletion assay s.c. This value is remarkable when compared with the values obtained for synthetic β^{1-39} and β^{1-24} corticotrophin. The activities of these peptides and their structural differences are shown in the Figure. This new synthetic peptide is shown to be almost 6 times more potent than β-corticotrophin itself[4] and its β^{1-24} sequence[6].

Estimation of corticosterone release in vitro is used as a direct criterion of adrenal stimulation. Lipolytic activity in vitro is an inherent extraadrenal effect of corticotrophin.

The activity of the pentacosapeptide as determined by these 2 methods is given in the Table.

The value obtained in the corticosterone test was almost twice that obtained in the lipolytic activity test.

Detailed studies of the pentacosapeptide have been made to assess its capacity to stimulate corticosteroid secretion in vivo. Details of these studies will be published separately. It may be briefly mentioned that in these studies, the pentacosapeptide by different routes of administration was again shown to be superior to natural hormone.

ACTH activity in vitro of D-serine[1]-norleucine[4]-valinamide[25]-β-1-25-corticotrophin

Methods	Potency IU/mg peptide	Fiducial limits in % ($P = 0.05$)
Corticosterone release in vitro (SAFFRAN and SCHALLY)[12]	275.0	78.4–127.6
Lipolytic activity in vitro	148.0	80.8–123.7

The outstanding activity of this peptide induced us to test its efficacy in humans. Since the ascorbic acid depletion assay, recommended by most Pharmacopoeias, is generally employed for the standardization of ACTH used therapeutically, it was of great importance to ascertain how far the potency of the new peptide estimated by the ascorbic acid depletion test is related to human dosage.

3 healthy male volunteers received by i.v. injection on alternate days 25 and 50 IU of the new peptide (corresponding to 40 and 80 μg respectively) and the same dosage of a commercial corticotrophin preparation. The subsequent urinary excretion of 17-hydroxycorticosteroids and 17-ketosteroids was estimated and taken as a criterion of the effect. In these preliminary studies, calculating the values as a 4-point assay, the following figures were obtained: 17-hydroxycorticosteroids 840 IU/mg and 17-ketosteroids 580 IU/mg. These figures confirm the high potency of this peptide in humans. A detailed clinical study employing a wider dosage range with frequent blood steroid estimations has been performed by JENNY et al.[15].

The above data clearly demonstrate the remarkable activity of D-serine[1]-norleucine[4]-valinamide[25]-β-1-25-corticotrophin in both animal and human experiments.

Zusammenfassung. Ein ACTH-Analogon: D-Ser[1]-Nle[4]-(Val-NH_2)[25]-β-1-25-Corticotrophin wurde tierexperimentell sowie orientierend humanpharmakologisch untersucht. Das Pentacosapeptid (DW-75) zeigt auf Grund der Substitution von 3 Aminosäuren eine gegenüber Peptiden mit natürlicher Sequenz beachtlich erhöhte ACTH-Aktivität.

W. DOEPFNER

Biological and Medical Research Division, Sandoz Ltd., Basel (Switzerland), May 13, 1966.

[15] M. JENNY, A. F. MULLER, and R. S. MACH, Experientia *22*, 528 (1966).

The Adrenocorticotropic Action of a New Synthetic Pentacosapeptide

Since the description in 1954 by BELL et al.[1,2] of the structural formula of adrenocorticotropic hormone (ACTH), various polypeptides with corticotropic action have been synthesized[3–8]. The polypeptides made up of 19–25 amino acids have an effect comparable to that of an ACTH extracted from pituitaries, are well tolerated and do not appear to exert an antigenic effect[9–16]. BOISSONNAS et al.[17] have recently synthesized a pentacosapeptide with 3 modifications of the usual sequence of naturally occurring ACTH-D-serine[1]-norleucine[4]-valinamide[25]-β-1-25-corticotropin (DW 75). The pharmacological studies of DOEPFNER[18] have shown that this polypeptide has an adrenocorticotropic effect in vitro and in vivo in the rat. This study was undertaken to show that this polypeptide has also an adrenocorticotropic action in man.

Methods. DW 75[19] was given by a single i.v. or i.m. or s.c. injection in doses of 5, 10, 25 and 125 U (corresponding to 8, 16, 40, 200 μg of peptide) at 8 a.m. in patients with no evidence of endocrinopathy or of cardiac, hepatic or renal failure. 4–6 patients were used at each dose level. In order to eliminate the endogenous secretion of ACTH, all patients received 2 mg of dexamethasone orally 8 h before beginning the test, and a further 2 mg were given at the time of the DW 75 injection. Blood was taken for plasma steroid estimation immediately prior to the injection and after 1, 2 and 4 h. The plasma steroids were measured by the method of PETERSON et al.[20].

Results. In 52 control subjects who had received no dexamethasone, the plasma steroid level at 8 a.m. was 15.6 ± 4.6 μg/100 ml. In patients receiving dexamethasone, the level was 2.7 ± 2.2 μg/100 ml at 8 a.m., and this level remained practically constant throughout the day without diurnal variation.

The injections of DW 75, administered i.v., i.m. and s.c., were well tolerated. The changes in steroid levels obtained are shown in the Table and in the Figure. The results are the arithmetic mean with standard deviation of a group of 4–6 patients. The number of patients in each