BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 1163—1172 (1970)

Synthesis of Corticotropin Peptides. XI. Synthesis and Biological Properties of [1-β-Alanine]-ACTH(1-18)-Octadecapeptide Amide

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(Received September 24, 1969)

The synthesis is described of an octadecapeptide amide, β-alanyl-tyrosyl-seryl-methionyl-glutamylhistidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide, corresponding to the first eighteen amino acid residues of corticotropin except for the amino end which has been substituted by a β-alanine for the serine in native hormone. The adrenalstimulating activities of the octadecapeptide amide are compared with those of the corresponding 1-serine or 1-glycine octadecapeptide amide to show that the 1-β-alanine peptide is remarkably more active than the latter when assayed by *in vitro*, subcutaneous and intamuscular approaches, and that the activities of the former are maintained for a longer period of time than those of the latter. The 1-β-alanine peptide as a lipotropic agent is also shown to be more active than the synthetic analogs and a native corticotropin on rat adipose tissue. The differences among these octadecapeptide analogs in the biological properties are discussed in terms of their susceptibility toward the action of aminopeptidase.

Syntheses of a large number of highly active corticotropin (ACTH) peptides have been reported from various laboratories. Among these studies it is of special interest that the amino end can be

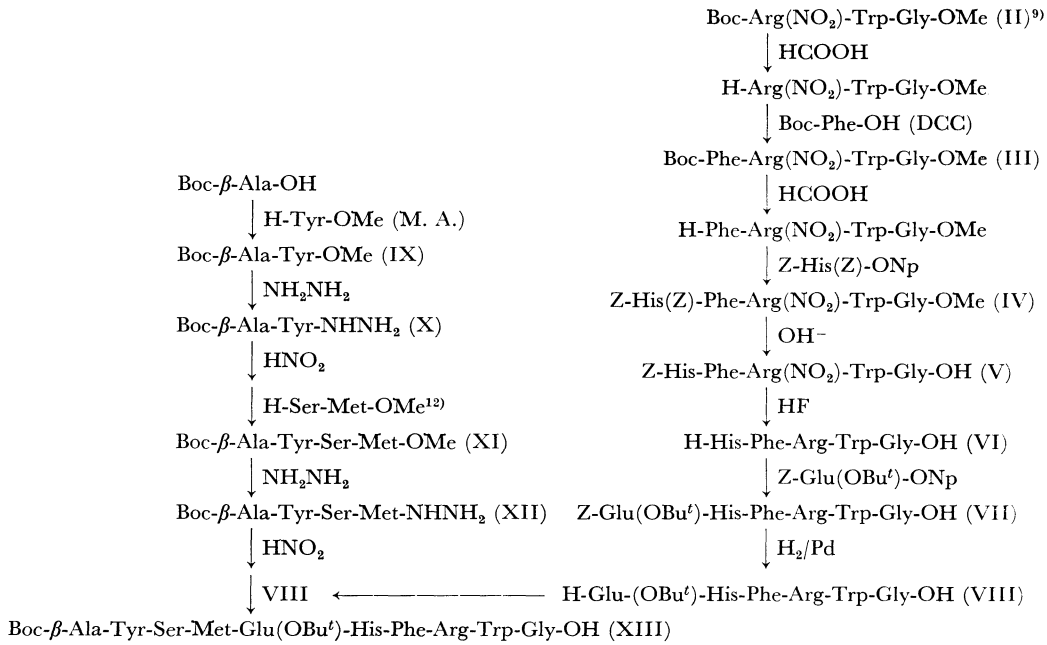

Fig. 1. Procedure for the synthesis of decapeptide intermediate.
Z, benzyloxycarbonyl; Boc, *t*-butyloxycarbonyl; Me, methyl; Bu^t, *t*-butyl; ONp, *p*-nitrophenoxy; M.A., mixed acid anhydride method; DCC, *N,N'*-dicyclohexylcarbodiimide method.

replaced by D-serine[1,2] or D-alanine[2] for the native L-serine in corticotropin peptides resulting in a marked increase in the biological activities. Since hormones generally require the cellular organization to exert their biological properties, there must be a number of factors affecting the observed level of activities. It seems, however, most likely that the apparent increase in activities observed with the 1-D-serine and 1-D-alanine analogs of corticotropin peptides is a consequence of the decreased susceptibility of peptides toward the action of aminopeptidase owing to the presence of D-amino acid at the amino end. This is also suggested by the fact that the replacement by glycine of the amino-terminal serine in porcine corticotropin,[3] ACTH-(1-18)-NH_2[4] and ACTH(1-23)-NH_2[5] has made no remarkable change in the adrenal-stimulating activity. If this is correct, such an amino acid, which forms amide bonds resisting the aminopeptidase action, should as well improve the biological properties of a corticotropin peptide as do the D-amino acids, when it is substituted for the amino terminal residue of peptide. In order to confirm this of view, the synthesis of an octadecapeptide amide, β-alanyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine amide (I), [β-Ala^1]-ACTH(1-18)-NH_2,[6] corresponding to the first eighteen amino acid residues of corticotropin with the exception of amino end was undertaken. β-Alanine is one choice, because it is known to form acid amides which are not hydrolyzed by hog kidney leucine aminopeptidase.[7] Biological properties of I were then compared with those of the corresponding 1-L-serine and 1-glycine

R-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-
1 2 3 4 5 6 7 8 9 10 11 12 13
Gly-Lys-Lys-Arg-Arg-NH_2
14 15 16 17 18

ACTH(1-18)-NH_2 (Ia): R=NH_2-CH(CH_2OH)-CO-

[Gly^1]-ACTH(1-18)-NH_2 (Ib): R=NH_2-CH_2-CO-

[β-Ala^1]-ACTH(1-18)-NH_2(I): R=NH_2-CH_2-CH_2-CO-

1) R. A. Boissonnas, St. Guttmann and J. Pless, *Experientia*, **22**, 526 (1966).

2) H. Kappeler, B. Riniker, W. Rittel, P. Desaulles, R. Maier, B. Schär and M. Staehelin, "Peptides" Proceedings of the 8th European Peptide Symposium, ed. by H. C. Beyerman, North-Holland Publishing Co., Amsterdam (1967), p. 214.

3) H. E. Lebovitz and F. L. Engel, *Endocrinol.*, **73**, 573 (1963).

4) H. Otsuka, M. Shin, Y. Kinomura and K. Inouye, This Bulletin, **43**, 196 (1970).

5) R. Geiger, K. Sturm, G. Vogel and W. Siedel, *Z. Naturforsch.*, **19b**, 858 (1964).

6) All amino acid residues are of the L-configuration, unless otherwise indicated. The abbreviated designation of amino acids, peptides and their derivatives accords with the proposal of the IUPAC-IUB Commission on Biochemical Nomenclature.

7) D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).

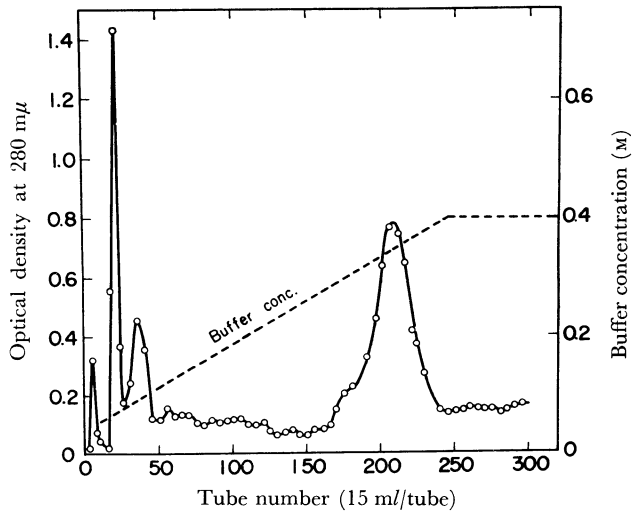

Fig. 2. Chromatography of synthetic octadecapeptide amide. Material: crude [β-Ala[1]]-ACTH(1-18)-NH_2 (I), 0.513 g. Column: carboxymethyl cellulose (Serva, 0.6 meq/g), 2.7×12 cm. Buffer: ammonium acetate (pH 6.84). Flow rate: 6 ml/min.

analogs, ACTH(1-18)-NH_2 (Ia) and [Gly[1]]-ACTH-(1-18)-NH_2 (Ib), whose syntheses were reported previously.[4,8]

A synthetic procedure of the decapeptide intermediate corresponding to the positions 1—10 of I is shown in Fig. 1. The procedure involves a new synthesis of H-Glu(OBu[t])-His-Phe-Arg-Trp-Gly-OH (VIII),[9] in which *t*-butyloxycarbonyl (Boc) groups were removed from Boc-Arg(NO_2)-Trp-Gly-OMe (II) and Boc-Phe-Arg(NO_2)-Trp-Gly-OMe (III) by treatment with formic acid[10] instead of trifluoroacetic acid formerly used,[9] and the conversion of Z-His-Phe-Arg(NO_2)-Trp-Gly-OH (V) into a pentapeptide VI was carried out in anhydrous hydrogen fluoride.[11] In these deprotection steps it was found that no detectable decomposition of the tryptophan residue occurred. The coupling of Z-Glu(OBu[t])-ONp with VI led to the formation of a protected hexapeptide (VII), which was readily converted into VIII by catalytic hydrogenolysis. Boc-β-Ala-Tyr-Ser-Met-$NHNH_2$ (XII) was synthesized by the same procedure as that described for Boc-Gly-Tyr-Ser-Met-$NHNH_2$,[12] except that a mixed acid anhydride method was used to prepare Boc-β-Ala-Tyr-OMe (IX). The azide derived from XII was then coupled with VIII to give a decapeptide Boc-β-Ala-Tyr-Ser-Met-Glu-(OBu[t])-His-Phe-Arg-Trp-Gly-OH (XIII).

In the final coupling step leading to the formation of a protected octadecapeptide amide, Boc-β-Ala-Tyr-Ser-Met-Glu(OBu[t])-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2(XV), compound XIII was esterified with *N*-hydroxysuccinimide by the carbodiimide method[13] and the resulting active ester, which was isolated, was allowed to react with a partially protected octapeptide amide (acetate), H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 (XIV),[14] in the presence of triethylamine. The product, without further purification, was treated with 90% trifluoroacetic acid to remove all the protecting groups. The deprotection has been found to be achieved by the anhydrous hydrogen fluoride treatment;[15] formic acid may also be useful for the same purpose. The crude octadecapeptide amide (I, trifluoroacetate) was converted by treatemnt with Amberlite CG-400 (acetate) into the acetate form, which was, after having been incubated with mercaptoethanol, submitted to chromatography on a carboxymethyl cellulose (CMC) column using an ammonium acetate buffer (pH 6.8) with a linear concentration gradient. The results of chromatography are shown in Fig. 2. An over-all yield of peptide I for the final coupling step was 54%.

8) H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, *J. Biochem.* (Tokyo), **58**, 512 (1965).
9) K. Inouye, This Bulletin, **38**, 1148 (1965).
10) B. Halpern and D. E. Nitecki, *Tetrahedron Lett.*, **31**, 3031 (1967).
11) S. Sakakibara and Y. Shimonishi, This Bulletin, **38**, 1412 (1965); S. Sakakibara, Y. Shimonishi, M. Okada and Y. Kishida, "Peptides" Proceedings of the 8th European Peptide Symposium, ed. by H. C. Beyerman, North-Holland Publishing Co., Amsterdam (1967), p. 44.
12) H. Otsuka, K. Inouye, F. Shinozaki and M. Kanayama, This Bulletin, **39**, 1171 (1966).
13) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964); *ibid.*, **85**, 3039 (1963).
14) H. Otsuka, K. Inouye, M. Kanayama and F. Shinozaki, This Bulletin, **39**, 882 (1966).
15) H. Otsuka and M. Shin, unpublished.

TABLE 1. ADRENAL-STIMULATING ACTIVITIES OF ACTH AND RELATED SYNTHETIC OCTADECAPEPTIDE AMIDES*

Method	Administration route	Peptide Ia	Ib	I	α_s-ACTH
Adrenal ascorbic acid depletion[19]	subscutaneous	45.4	25.6 (3)	135 (2)	120
In vitro steroidogenesis[20]	*in vitro*	13.7 (3)	20.8 (3)	237 (3)	120
In vivo steroidogenesis[21]	intravenous	92.0 (9)	167 (11)	125—285 (4)	100—180
In vivo steroidogenesis in:					
dexamethasone-Nembutal-mouse[22]	intravenous	139	70—168 (2)	259 (2)	—
hypophysecotomized rat peripheral blood	intramuscular	—	35.4 (4)	124	—

* The activities are expressed in USP units/mg, relative to the Third USP Corticotropin Reference Standard. The figures in parentheses are the number of independent assays. Ia: ACTH(1-18)-NH_2, Ib:[Gly^1]ACTH(1-18)-NH_2, I:[β-Ala^1]-ACTH(1-18)-NH_2, and α_s-ACTH: native sheep ACTH

A rechromatography on CMC afforded in an 86% recovery the pure material of I, which was found to be homogeneous to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in paper chromatography and paper electrophoresis. Quantitative amino acid analysis[16] showed that an acid hydrolysate of the product contained individual amino acids in the ratios expected by theory with the exception of tryptophan. The tryptophan-tyrosine ratio of intact I was determined spectrophotometrically to be unity.[17]

The susceptibility of β-Ala^1-octadecapeptide amide (I) toward the action of hog kidney leucine aminopeptidase (LAP) is compared in Fig. 3 with those of the corresponding Ser^1-analog (Ia) and Gly^1-analog (Ib). A commercial LAP preparation was found to hydrolyze [β-Ala^1]-ACTH(1-10)-OH and I, but not β-Ala-Tyr-Ser-Met-$NHNH_2$. Since there was no β-alanine detected in the enzymic digests on an amino acid analyzer,[16] the observed hydrolysis may be due to the presence of an endopeptidase in the LAP preparation. This apparent susceptibility of β-alanyl peptides to LAP was completely lost when the enzyme preparation had been treated with diisopropyl fluorophosphate (DFP). By the DFP-treated LAP, Ser^1-peptide (Ia) was hydrolyzed faster than Gly^1-peptide (Ib) and no hydrolysis was observed with β-Ala^1-peptide (I) within the error of measurements (Fig. 3). The results confirm and extend the finding of Spackman *et al.*[7] that hog kidney LAP does not split β-alanyl amide bonds.

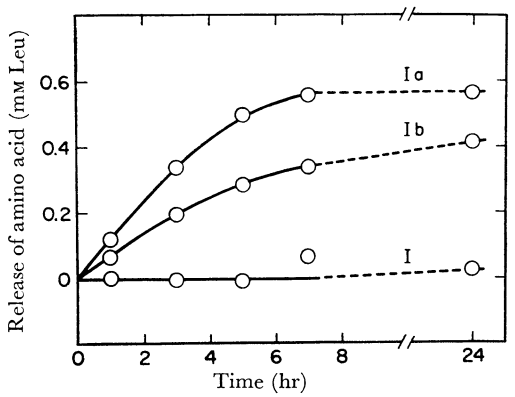

Fig. 3. Action of DFP-treated leucine aminopeptidase on synthetic octadecapeptide amides. Enzyme: DFP-treated hog kidney leucine aminopeptidase, 1.95 units/ml (see Experimental section for the definition of unit). Substrates: I, [β-Ala^1]-ACTH(1-18)-NH_2; Ia, ACTH(1-18)-NH_2; Ib, [Gly^1]-ACTH(1-18)-NH_2; concentration, 0.04 mM. Reactions were carried out in 0.02M Tris buffer (pH 8.3) at 37°C.

The adrenal-stimulating activities of β-Ala^1-octadecapeptide amide (I) were compared with those of the synthetic analogs (Ia and Ib) and sheep corticotropin (α_s-ACTH)[18] by five different assay procedures: 1) adrenal ascorbic acid depletion,[19] 2) *in vitro* steroidogenesis,[20] 3) *in vivo* steroidogenesis,[21] 4) *in vivo* steroidogenesis in the dexamethasone-pentobarbital-blocked mouse[22] and 5) steroidogenesis in the hypophysectomized rat by the intramuscular administration. The results are summarized in

16) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

17) G. H. Beaven and E. R. Holiday, *Advan. Protein Chem.*, **7**, 319 (1952); T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

18) We thank Professor Choh Hao Li, Hormone Research Laboratory, University of California, San Francisco, California, for his generous supply of the pure preparation of native hormone.

19) The United States Pharmacopeia, XVII, 147 (1965).

20) M. Saffran and A. V. Schally, *Endocrinol.*, **56**, 523 (1955).

21) H. S. Lipscomb and D. H. Nelson, *ibid.*, **71**, 13 (1962).

22) A. Tanaka and M. Nakamura, "Integrative Mechanism of Neuroendocrine System," Hokkaido University Medical Library Series, Vol. 1, 49 (1968).

Table 1, in which some of the data for peptides Ia and Ib are revised by the present assays from those reported previously.[4] As shown in Table 1, peptide I is highly active in any respect to the adrenal-stimulating properties. The *in vivo* steroidogenic activity of I is in the same order of magnitude as those of Ia, Ib and α_s-ACTH, when administered by intravenous injection. However, peptide I exhibited a strikingly higher activity than those of Ia and Ib, when assayed by *in vitro* steroidogenesis and adrenal ascorbic acid depletion methods. It is noteworthy that the potency ratios of subcutaneous or *in vitro vs.* intravenous administration are 1—0.5 for peptide I, whereas the ratios for peptides Ia and Ib are 1/7—1/8. Peptide I is, in this respect, much closer than Ia and Ib to the native hormone (α_s-ACTH) which gives the ratio of unity. Figure 4 shows that the β-Ala^1-peptide (I) can remain active for a longer period of time than Gly^1-peptide (Ib), when administered by intramuscular injection; the steroidogenic activity is expressed in terms of the 11-hydroxycorticosteroid (11-OHCS) level in plasma.

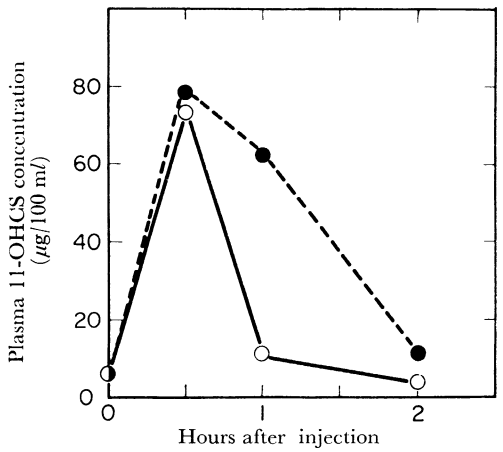

Fig. 4. Time course of synthetic octadecapeptide amides in steroidogenesis.
○—○, [Gly^1]-ACTH(1-18)-NH_2 (Ib); ●---●, [β-Ala^1]-ACTH(1-18)-NH_2 (I). Peptide (5 μg/rat) was administered into the thigh muscle of hypophysectomized rat and 11-OHCS concentration in blood was measured. See Experimental section for details.

TABLE 2. *In vitro* LIPOTROPIC ACTIVITY OF ACTH AND RELATED SYNTHETIC OCTADECAPTIDE AMIDES[23]

	Minimal effective dose (10^{-6} mg/50 mg tissue)			
	Ia	Ib	I	α_s-ACTH
Rat adipose tissue	3.0	6.3	0.62	6.0
Rabbit adipose tissue	0.004	0.35	0.065	7.1
Rat/rabbit ratio, approx.	750	20	10	1

Ia: ACTH(1-18)-NH_2, Ib: [Gly^1]-ACTH(1-18)-NH_2, I: [β-Ala^1]-ACTH(1-18)-NH_2, and α_s-ACTH: native sheep ACTH.

Peptide I also exhibits a high lipotropic activity, which is compared in Table 2 with the activities of synthetic peptides Ia and Ib, and a native hormone (α_s-ACTH).[23] β-Ala^1-peptide (I) was roughly ten times more active than Ia, Ib and α_s-ACTH, when assayed in the rat. A lesser amount of I can exert the activity in the rabbit as compared to that in the rat. This is the case with the other peptides as well. However, it is to be noted that the ratio of the minimal effective dose in rat fat *vs.* that in rabbit fat is smaller for I than those for Ia and Ib. Figures 5A and B show the inactivation patterns of I and Ib as lipotropic agents in the fresh plasma and in the buffer containing rat muscle fragments, respectively. There was no significant difference between I and Ib in the case of plasma,

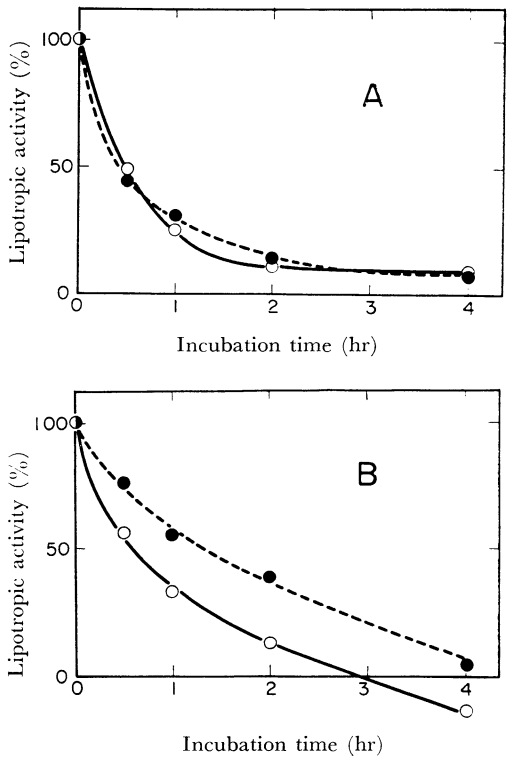

Fig. 5. Inactivation of the lipotropic activity of synthetic octadecapeptide amides.
A: by plasma. B: by tissue. ○—○, [Gly^1]-ACTH(1-18)-NH_2 (Ib) 1 μg/fat pad; ●---●, [β-Ala^1]-ACTH(1-18)-NH_2 (I) 0.1 μg/fat pad. Incubations were carried out at 37°C in the plasma of anesthetized rat in A and in the buffer containing rat muscle fragments in B. See Experimental section for details.

23) A. Tanaka, B. T. Pickering and C. H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962).

whereas the inactivation of I was markedly delayed compared with that of Ib in the case of tissue.

The results described above clearly demonstrate that the introduction of β-alanine into the amino terminal position of ACTH(1-18)-octadecapeptide amide causes not only remarkable enhancement but also prolonged duration of the adrenocorticotropic and the lipotropic activities of the peptide. Whether a similar effect as that produced by β-alanine is expected by other amino fatty acids, such as aminobutyric acids and aminovaleric acids, remains to be examined. In any case, one can not exclude the possibility that the distance between the terminal amino group and the center of active site of the hormone peptide may become another important factor which affects the activities. Since no direct comparison has been made between the β-Ala^1-peptide and its corresponding D-Ala^1-analog in the present studies, it is not clear whether the former differes from the latter in the activity levels, solely owing to the fact that the terminal amino group of the former is located one methylene unit away from the regular position.

The present results also indicate that the remarkable changes in activities, which are produced when the amino terminal residue of octadecapeptide amide (Ia or Ib) is replaced by a β-alanine, are quite smilar in characteristics to those which were reported to occur when the relpacement was made by a D-amino acid for the amino end of the corticotropin peptides.[1,2,24] On the other hand, it is known that the adrenocorticotropic activity of corticotropin peptides is little affected by the replacement by glycine[3-5] and even by the deletion[5] of the amino terminal residue. The present results as well as the other observations support the view that the increased biological activities and their prolonged duration, which were found with the analogs bearing a D-amino acid[1,2] or a β-alanine at the amino end, are the consequence of the diminished susceptibility of peptides toward the action of intracellular aminopeptidase. By analogy, the findings that the peptide amides[8,25] and the peptide terminating with a prolinol[26] are more active than the corresponding acids and the proline amide analog, respectively, would be partly explained by the fact that an amide group at the carboxyl end protects the peptide from the action of carboxypeptidase, and by assuming that a prolinol residue assures the resistance of peptide toward the prolidase action.

As has been pointed out above, the potency ratio of subcutaneous *vs.* intravenous administration in the assay for adrenocorticotropic activity changes from 1/8—1/7 up to 0.5—1, when the amino end of octadecapeptide amide (Ia or Ib) is replaced by a β-alanine, approaching the ratio of α_s-ACTH (Table 1). This probably means that the difference in *s.c./i.v.* ratio between the synthetic peptide (Ia or Ib) and the native hormone mostly depends on the inactivation rate of peptide in the tissue, where an aminopeptidase must act predominantly on the peptide. In blood the inactivation seems to involve another type of enzyme, by which both the synthetic and the native peptides are attacked fairly equally. An enzyme inactivating thenative hormone in blod has been suggested to be an endopeptidase.[27] However, the above view remains to be elucidated by comparing the inactivation patterns of the synthetic peptides (I, and Ia or Ib) and the native hormone. Such work is in progress in this laboratory.

Experimental

All melting points were uncorrected.

***t*-Butyloxycarbonyl-β-alanine.** β-Alanine (8.91 g, 0.1 mol) was dissolved in N sodium hydroxide (100 m*l*), and sodium bicarbonate (21 g) and dioxane (70 m*l*) were added. To this was slowly added a solution of *t*-butyl azidoformate (11.2 g, 0.12 mol) in dioxane (30 m*l*) and the mixture was stirred at 50°C for 42 hr. After removal of dioxane by evaporation *in vacuo*, the residue was acidified at 0°C with ice-cold 4N hydrochloric acid to pH 2 and the desired product was extracted with cold ethyl acetate. The extracts were combined, dried over sodium sulfate and evaporated *in vacuo* to give a sirupy residue which crystallized from ethyl acetate-petroleum ether; yield 12.7 g (67%), mp 77—78°C. Recrystallization from the same solvent did not alter the melting point.

Found: C, 50.99; H, 8.06; N, 7.47%. Calcd for $C_8H_{15}NO_4$: C, 50.78; H, 7.99; N, 7.40%.

***t*-Butyloxycarbonyl-β-alanyl-tyrosine Methyl Ester (IX).** To a solution of *t*-butyloxycarbonyl-β-alanine (1.89 g, 0.01 mol) and tri-*n*-butylamine (2.04 g, 0.01 mol) in anhydrous tetrahydrofuran was added dropwise ethyl chloroformate (1.19 g, 0.01 mol) at an ice-salt bath temperature and the mixture was stirred for 10 min, and a suspension of tyrosine methyl ester (1.95 g, 0.01 mol) in tetrahydrofuran was then introduced. The reaction mixture was stirred at the above temperature for 30 min and at room temperature for 3 hr. After removal of the solvent by evaporation *in vacuo* the residue was dissolved in ethyl acetate. The solution was washed with ice-cold N hydrochloric acid, 5% sodium bicarbonate and water, dried over sodium

24) W. Rittel, Advan. Extl. Med. Biol., Vol. 2, "Pharmacology of Hormonal Polypeptides and Proteins" ed. by N. Back *et al.*, Plenum Press, New York (1968), p. 35.

25) J. Ramachandran, D. Chung and C. H. Li, *J. Amer. Chem. Soc.*, **87**, 2696 (1965).

26) W. Oelofsen and C. H. Li, *ibid.*, **88**, 4252 (1966).

27) a) G. Pincus, T. F. Hopkins and O. Hecter, *Arch. Biochem.*, **37**, 408 (1952); b) E. B. Astwood, M. S. Raben and R. W. Payne, *Recent Progress in Hormone Research*, **7**, 1 (1952); c) H. H. Tomizawa and R. H. Williams, *J. Biol. Chem.*, **217**, 685 (1955); d) W. F. White and A. M. Gross, *J. Amer. Chem. Soc.*, **79**, 1141 (1957).

sulfate and evaporated *in vacuo* to afford a sirupy residue, which crystallized upon addition of ether; yield 2.99 g (82%), mp 139—141°C. Recrystallization from methanol-ether afforded the product with mp 141—142°C, $[\alpha]_D^{23}$ +8.2±0.5° (*c* 1.020, methanol).

Found: C, 59.03; H, 7.12; N, 7.63%. Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65%.

***t*-Butyloxycarbonyl-β-alanyl-tyrosine Hydrazide (X).** To a solution of IX (2.56 g) in ethanol (25 m*l*) was added hydrazine hydrate (1.7 m*l*) and the mixture was kept at room temperature for 5 hr. Crystals which separated were, after having been kept in a refrigerator overnight, filtered off, washed with cold ethanol and ether and dried; yield 2.54 g (99%), mp 210—212°C. These crystals were recrystallized from water in an 85% recovery, mp 214—215°C, $[\alpha]_D^{23}$ +3.6±0.6° (*c* 0.978, 50% acetic acid).

Found: C, 55.74; H, 7.11; N, 15.30%. Calcd for $C_{17}H_{16}N_4O_5$: C, 55.72; H, 7.15; N, 15.29%.

***t*-Butyloxycarbonyl-β-alanyl-tyrosyl-seryl-methionine Methyl Ester (XI).** Compound X (1.10 g, 3 mmol) was dissolved in ice-cold N hydrochloric acid (7.5 m*l*) and to this was added 2M sodium nitrite (1.6 m*l*) at an ice-salt bath temperature. The azide which separated was extracted with ice-cold ether. The ethereal extracts were combined, washed with ice-cold M sodium bicarbonate, dried over sodium sulfate and evaporated *in vacuo*. The residue was dissolved in ice-cold acetonitrile together with seryl-methionine methyl ester (0.76 g, 3 mmol)[14] and the mixture was kept at 4°C for 2 days. The solvent was removed by evaporation *in vacuo* and the residue was dissolved in a mixture of ethyl acetate and water. The organic phase was washed successively with ice-cold N hydrochloric acid, 5% sodium bicarbonate and water, dried and evaporated *in vacuo* to yield a gelationous residue, which was twice reprecipitated from ethyl acetate; yield 1.04 g (60%), mp 121—123°C, $[\alpha]_D^{23}$ −13.0±0.6° (*c* 0.997, methanol).

Found: C, 52.94; H, 6.85; N, 9.65; S, 5.48%. Calcd for $C_{26}H_{40}N_4O_9S$: C, 52.41; H, 6.90; N, 9.58; S, 5.48%.

***t*-Butyloxycarbonyl-β-alanyl-tyrosyl-seryl-methionine Hydrazide (XII).** To a solution of XI (0.97 g) in dimethylformamide (6 m*l*) was added hydrazine hydrate (0.5 m*l*) and the mixture was kept at 4°C for 2 days. Addition of ethyl acetate yielded gelatinous precipitates, which were washed with cold ethyl acetate and dried *in vacuo* (1.01 g). The product cyrstallized upon heating with water; yield 0.85 g (88%), mp 187—188°C, $[\alpha]_D^{23}$ −20.1±0.5° (*c* 1.346, 50% methanol).

Found: C, 49.72; H, 7.04; N, 14.50; S, 5.15%. Calcd for $C_{25}H_{40}N_6O_8S\cdot H_2O$: C, 49.82; H, 7.02; N, 13.94; S, 5.32%.

Nitroarginyl-tryptophyl-glycine Methyl Ester Formate. *t*-Butyloxycarbonyl-nitroarginyl-tryptophyl-glycine methyl ester (II, 5.77 g)[28] was dissolved in formic acid (98—100%, 50 m*l*) and the solution was allowed to stand for 3.5 hr. The formic acid was removed by evaporation at 40—45°C and the residue was dissolved in water. The solution was washed with ether and lyophilized; yield 4.76 g (91%), mp 107—111°C, $[\alpha]_D^{23}$ +13.0±0.5° (*c* 2.062, methanol).

Found: C, 47.25; H, 6.03; N, 20.90%. Calcd for $C_{20}H_{28}N_8O_6\cdot HCOOH\cdot \frac{1}{2}H_2O$: C, 47.45; H, 5.88; N, 21.08%.

***t*-Butyloxycarbonyl-phenylalanyl-nitroarginyl-tryptophyl-glycine Methyl Ester (III).** This compound was synthesized in exactly the same manner as described previously, except for using the tripeptide ester formate obtained above instead of the trifluoroacetate.[9] Mp 176—177°C, $[\alpha]_D^{23}$ −20.4±0.2° (*c* 2.040, methanol); lit,[9] mp 172—173°C, $[\alpha]_D^{25}$ −20.1±1° (*c* 2.054, methanol).

Found: C, 56.34; H, 6.22; N, 17.58%. Calcd for $C_{34}H_{45}N_9O_9$: C, 56.42; H, 6.27; N, 17.42%.

Phenylalanyl-nitroarginyl-tryptophyl-glycine Methyl Ester Formate. Compound III (1.81 g) was dissolved in formic acid (98—100%, 18 m*l*) and the solution was kept at room temperature for 3.5 hr. After removal of the formic acid the residue was crystallized from *n*-butanol. The crystalline product was filtered off, washed with ether and dried *in vacuo*; yield 1.71 g (92%), mp 124—125.5°C, $[\alpha]_D^{23}$ −9.0±0.5° (*c* 0.977, methanol).

Found: C, 53.00; H, 6.10; N, 17.79%. Calcd for $C_{29}H_{37}N_9O_7\cdot HCOOH\cdot \frac{1}{2}C_4H_9OH$: C, 54.38; H, 6.28; N, 17.84%.

***N*$^{\alpha}$, *N*$^{\mathrm{Im}}$-Dibenzyloxycarbonyl-histidyl-phenylalanylnitroarginyl-tryptophyl-glycine Methyl Ester (IV).** This compound was synthesized in exactly the same manner as described previously, except for using the tetrapeptide ester formate obtained above instead of the corresponding trifluoroacetate,[9] in a 75% yield; $[\alpha]_D^{27}$ −22.3±0.3° (*c* 2.084, dimethylformamide). Lit,[9] $[\alpha]_D^{25.5}$ −21.5±0.5° (*c* 2.429, dimethylformamide).

Found: C, 58.63; H, 5.70; N, 15.89%. Calcd for $C_{51}H_{56}N_{12}O_{12}\cdot H_2O$: C, 58.50; H, 5.58; N, 16.05%.

Histidyl-phenylalanyl-arginyl-tryptophyl-glycine Monoacetate (VI). Benzyloxycarbonyl-histidyl-phenylalanyl-nitroarginyl-tryptophyl-glycine (V, 0.72 g)[9] and anisole (0.72 m*l*) were dissolved in anhydrous hydrogen fluoride (7—8 m*l*) at a dry-ice-acetone bath temperature and the mixture was stirred at 0°C for 30 min. After removal of hydrogen fluoride by evaporation the residue was dried over sodium hydroxide pellets overnight *in vacuo*. The residue was dissolved in water (20 m*l*) and the solution was washed thoroughly with ethyl acetate and then passed through a small column of Amberlite CG-400 (acetate form). The column was washed with portions of water. The aqueous effluents were combined and lyophilized; yield 0.64 g (98.5%), $\lambda_{max}^{0.1N\ HCl}$=279 mμ ($E_{1cm}^{1\%}$ 66.2), 288 mμ ($E_{1cm}^{1\%}$ 53.5) and $\lambda_{max}^{0.1N\ NaOH}$=280 mμ ($E_{1cm}^{1\%}$ 65.0), 288 mμ ($E_{1cm}^{1\%}$ 54.5). Paper chromatography (*n*-butanol : acetic acid : pyridine : water=30 : 6 : 20 : 24 by volume, BAPW, as solvent) showed the presence of one major component (R_f=0.47—0.53) and two traces, all of which were reactive to ninhydrin, Pauly and Ehrlich reagents; only the major one was reactive to Sakaguchi reagent. The product was used in the subsequent coupling reaction without further purification.

Benzyloxycarbonyl-γ-*t*-butyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine Monoacetate (VII). A solution of compound VI (0.83 g, 1 mmol) and triethylamine (0.28 m*l*, 2 mmol) in 90% dimethyl-

28) In a previous communication compound II was reported as amorphous solid with mp 112—114°C.[9] Later it was obtained in a crystalline state from methanol, mp 189—190°C, $[\alpha]_D^{23}$ −13.6±0.5° (*c* 1.026, dimethylformamide). Found: C, 51.94; H, 6.23; N, 19.26%. Calcd for $C_{25}H_{36}N_8O_8$: C, 52.07; H, 6.29; N, 19.53%.

formamide (10 ml) was stirred at 0°C and to this was added benzyloxycarbonyl-γ-*t*-butyl-glutamic acid *p*-nitrophenyl ester (0.46 g, 1 mmol),[29] and the mixture was stirred at 4°C overnight. An additional quantity (0.46 g) of the active ester was introduced and the mixture was kept at 4°C until the peptide VI became indetectable in thin-layer chromatography (BAPW as solvent). The reaction mixture was then added dropwise into ice-cold ethyl acetate (100 ml) to afford gelatinous precipitates, which were filtered off, washed with ethyl acetate and dried *in vacuo*. The product was reprecipitated from acid-water to give the desired hexapeptide (VII); yield 0.84 g (70%), $[\alpha]_D^{23}$ −26.6±0.5° (*c* 1.377, 50% acetic acid). Lit,[30] $[\alpha]_D$ not given.

Found: C, 52.86; H, 6.74; N, 13.98%. Calcd for $C_{51}H_{64}N_{12}O_{11}\cdot CH_3COOH\cdot 7H_2O$: C, 52.73; H, 6.85; N, 13.92%.

γ-*t*-Butyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine Monoacetate (VIII). Compound VII (0.265 g) in 50% acetic acid (10 ml) was hydrogenolyzed in the presence of palladium black for 2.5 hr. After the catalyst had been removed by filtration the filtrate was evaporated *in vacuo* at a bath temperature of 45—50°C. The residue was lyophilized from acetic acid and dried over sodium hydroxide pellets *in vacuo*; yield 0.22 g. The product behaved as a single component in paper chromatography (*n*-butanol: acetic acid : water=4 : 1 : 2 by volume, BAW, as solvent).

Found: C, 49.46; H, 6.77; N, 15.49%. Calcd for $C_{43}H_{58}N_{12}O_9\cdot CH_3COOH\cdot 8H_2O$: C, 49.53; H, 7.21; N, 15.40%.

***t*-Butyloxycarbonyl-β-alanyl-tyrosyl-seryl-methionyl-γ-*t*-butyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl glycine (XIII).** To a solution of XII (0.45 g, 0.75 mmol) in 90% dimethylformamide (4.5 ml), which had been chilled in an ice-salt bath, were added ice-cold N hydrochloric acid (2.0 ml) and ice-cold M sodium nitrite (0.83 ml) and the mixture was stirred for 4 min. Ice-cold saturated sodium chloride (20 ml) and ice-cold ethyl acetate were then introduced. The aqueous layer which separated was extracted twice with ethyl acetate. The organic solutions were combined, washed with cold 5% sodium bicarbonate, dried over sodium sulfate and added to a solution of VIII (0.50 g, 0.5 mmol) and triethylamine (0.21 ml, 1.5 mmol) in dimethylformamide (15 ml) and the mixture was evaporated at a bath temperature of 10—15°C *in vacuo* to remove ethyl acetate. The resulting clear solution was kept at 4°C overnight. An additional quantity of the azide (prepared from 0.23 g of XII as has been described above) was introduced and the reaction mixture was kept at 4°C for one more day. It was then added dropwise into ice-cold ethyl acetate (200 ml) to afford amorphous precipitates, which were filtered off, washed with ethyl acetate and dried *in vacuo*; yield 0.69 g (94%). Reprecipitation from dimethylformamide-methanol (2 : 5) gave the pure decapeptide derivative (XIII) in a 66 per cent recovery; $[\alpha]_D^{23}$ −19.4± 0.7° (*c* 0.882, dimethylformamide). The product behaved as a single component (R_f=0.54—0.57) in thin-layer chromatography (dimethylformamide : ethyl acetate : acetic acid=15 : 10 : 2 by volume as solvent).

Found: C, 51.62; H, 6.66; N, 14.06; S, 2.64%. Calcd for $C_{68}H_{94}N_{16}O_{17}S\cdot 8H_2O$: C, 51.57; H, 7.00; N, 14.15; S, 2.02%.

β-Alanyl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginine Amide, [β-Ala[1]]-ACTH(1-18)-NH$_2$ (I). To a solution of compound XIII (0.37 g, 0.25 mmol) in dimethylformamide (10 ml) was added ice-cold N hydrochloric acid (0.25 ml) at 0°C and the solution was immediately added to ice-cold ethyl acetate-ether (1 : 1, 200 ml). The resulting precipitates were filtered off, washed with ether and dried *in vacuo*; 0.36 g. These precipitates were dissolved in dimethylformamide (5 ml) and to this were added *N*-hydroxysuccinimide (0.115 g, 1 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (0.21 g, 1 mmol) successively and the mixture was stood at 4°C overnight. After removal of the urea which separated, the filtrate was introduced into ice-cold ethyl acetate-ether (1 : 1, 200 ml). The precipitates were filtered off, washed with ether and dried *in vacuo* to give the decapeptide active ester; 0.39 g.

The triacetate of N^{ε}-t-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-N^{ε}-*t*-butyloxycarbonyl-lysyl-arginyl-arginine amide (0.23 g, 0.155 mmol)[14] was dissolved in dimethylformamide (3 ml) and triethylamine (0.24 ml) was added. To this was then added a solution of the decapeptide active ester obtained above (0.39 g, 0.24 mmol) in dimethylformamide and the reaction mixture (total volume *ca.* 5 ml) was allowed to stand at 4°C for 60 hr. The crude protected octadecapeptide was obtained as amorphous solid when the reaction mixture was introduced into cold ethyl acetate (100 ml); yield 0.55 g.

The protected peptide obtained above (0.55 g) was dissolved, for deprotection, in 90% trifluoroacetic acid (6 ml) and the solution was kept at room temperature for 60 min, after which the solvent was removed by evaporation *in vacuo*. The residue was dissolved in water and the solution was passed through a column (2.2×7 cm) of Amberlite CG-400 (acetate form); the column was washed with portions of water. The aqueous solutions were combined and concentrated to 10—15 ml *in vacuo*. To the concentrate was added M mercaptoethanol (1 ml) and the mixture was incubated at 37°C overnight and lyophilized. The crude deblocked peptide (0.51 g) thus obtained was subejcted, for purification, to chromatography on a column (2.7×12 cm) of carboxymethyl cellulose (CMC, Serva, 0.6 meq/g) using an ammonium acetate buffer (pH 6.8, 4000 ml) with a linear concentration gradient of 0.05—0.4M.[31] The results of chromatography are shown in Fig. 2. The fractions (15 ml/tube) corresponding to a main peak (tubes 166—245) were combined and the bulk of the solvent was removed *in vacuo*. The residue was lyophilized repeatedly to constant weight to give the partially purified I; 0.22 g (54%). The product (0.2 g) was chromatographed, for further purification, on a column (2.2×18 cm) of CMC (Serva, 0.6 meq/g) using an

29) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman and N. Yanaihara, *J. Amer. Chem. Soc.*, **87**, 611 (1965).

30) R. Geiger, K. Sturm and W. Siedel, *Chem. Ber.*, **96**, 1080 (1963).

31) A buffer with a particular concentration, for example 0.05M, was prepared by adding the reagent grade aqueous ammonia (28%, w/w) to 0.05N acetic acid until the desired pH was obtained.

ammonium acetate buffer (pH 6.8, 2000 m*l*) with a linear cocentration gradient of 0.05—0.8M. The fractions (7.5 m*l*/tube) corresponding to a main peak (tubes 125—160) were combined, concentrated and lyophilized to obtain the pure peptide I; 0.17 g, $[\alpha]_D^{27}$ $-54.0 \pm 1.8°$ (*c* 0.515, 0.1N acetic acid). $\lambda_{max}^{0.1N\ HCl}$=280 m$\mu$ ($E_{1cm}^{1\%}$ 23.0), $\lambda_{shoulder}^{0.1N\ HCl}$=288.5 m$\mu$ ($E_{1cm}^{1\%}$ 17.5) and $\lambda_{max}^{0.1N\ NaOH}$= 281.5 m$\mu$ ($E_{1cm}^{1\%}$ 24.9), 288 mμ ($E_{1cm}^{1\%}$ 24.3). The peptide behaved as a single component to ninhydrin, Pauly, Ehrlich, Sakaguchi and methionine (PtI_6'') reagents in paper chromatography (BAPW as solvent) and in paper electrophoresis (600 V/36 cm, in 2N acetic acid). Amino acid ratios in acid hydrolysate:[16] Ser 0.75, Glu 0.90, Pro 0.91, Gly 1.89, Val 1.00, Met 0.96, Tyr 1.01, Phe 0.97, β-Ala 1.00, Lys 2.83, His 0.92, Arg 2.82, Trp 0.55, NH_3 1.42. The Trp/Tyr ratio in intact I was determined spectrophotometrically to be 1.16.[17]

Enzymic Studies. A commercial preparation of hog kidney leucine aminopeptidase (LAP) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U. S. A.; LAP-C 7HB.[32]

The enzyme preparation (a suspension in 75% saturated ammonium sulfate) was dialyzed against 0.005M trishydroxymethyl aminomethane (Tris) buffer (pH 8.0, 0.005M $MgCl_2$) at 4°C before use. A DEP-treated LAP was obtained in the following way: the LAP suspension (5 mg enzyme) was diluted five fold with 0.005M Tris buffer and to this was added 0.08 m*l* of M diisopropyl fluorophosphate (DFP) in isopropyl alcohol, and the mixture was incubated at 37°C for 45 min and then dialyzed against 0.005M Tris buffer at 4°C. The LAP activity of the enzyme solutions was determined at pH 8.3 and 25°C according to Tuppy *et al.*[33] with some modifications, using L-leucine *p*-nitroanilide (LNA) as substrate. One LAP unit was temporarily defined as the amount of enzyme that produced, in the present assay procedure,[34] an increase in absorbance at 400 mμ of 0.001 per minute above the blank, using 1-cm cells in a Hitachi photoelectric spectrophotometer.

The syntheses of ACTH(1-18)-NH_2 (Ia) and [Gly^1]-ACTH(1-18)-NH_2, (Ib), used as substrates, were described previously.[4,8] [β-Ala^1]-ACTH(1-4)-$NHNH_2$ and [β-Ala^1]-ACTH(1-10)-OH as substrates were prepared from compounds XIV and XIII, respectively, by the treatment with formic acid.[10]

For the measurements of the cleavage of synthetic substrate by LAP, a mixture comprising one part of 0.1 mM substrate (in water), one part of 0.05M Tris buffer (pH 8.3) and a half part of enzyme (4.88 units/m*l*, LNA as substrate) was incubated at 37°C. From the incubation mixture 0.5-m*l* samples were withdrawn and immediately mixed with 0.5 m*l* of ninhydrin reagent[35] and the mixture was then heated in a boiling water bath for 15 min and chilled; after appropriate dilution with 50% ethanol, the absorbance at 570 mμ was determined in a Hitachi spectrophotometer. For each run, control incubation mixtures without substrate or without enzyme were analyzed. In the ninhydrin reaction a standard solution of L-leucine was also checked every time as a reference. The results of the enzymic cleavage of peptides, I, Ia, and Ib, by DFP-treated LAP are shown in Fig. 3, where the release of amino acids is expressed in terms of the leucine concentration.

Characterization of Biological Properties. *1) Adrenal-stimulating Activity.* Assays for adrenal-stimulating activities were carried out according to five different procedures. The adrenal ascorbic acid depleting activity in the hypophysectomized rat was assayed by the method of United States Pharmacopeia, XVII.[19] The *in vitro* steroidogenic activity was assayed by the Saffran and Schally method.[20] The *in vivo* steroidogenic activity by the intravenous administration to hypophysectomized rat was assayed by the method of Lipscomb and Nelson[21] with a minor modification.[36] The *in vivo* steroidogenic activity was also assayed in the dexamethasone-pentobarbital-primed mouse.[22] In addition, the steroidogenic activity by the intramuscular administration to hypophysectomized rat was determined, where a preparation (0.05 m*l*/rat) was injected into the thigh muscle and a blood sample was collected from the abdominal aorta 30 min after the injection. Throughout the experiments, the Third USP Corticotropin Reference Standard was used as a standard and the production of 11-hydroxycorticosteroids (11-OHCS) was determined by the fluorophotometric method of Peterson.[37] For each assay method several determinations were performed and the data obtained independently were submitted to statistical treatment by the Sheps and Moore procedure.[38] The results of these assays are summarized in Table 1.

2) Time Course of Steroidogenesis. The change in steroidogenic activity of the peptides I and Ib, administered by intramuscular injection, was followed: 5-μg/rat samples (1 mg peptide/m*l*) were injected into the thigh muscle of the hypophysectomized rats and blood samples were collected from the abdominal aorta in appropriate time intervals starting 30 min after the injection. The plasma 11-OHCS level was determined fluorophotometrically.[37] The results are shown in Fig. 4.

3) Lipotropic Activity. Assays were carried out according to the method described by Tanaka *et al.*[23] with the rat epididymal and the rabbit perirenal adipose tissues. The increase of nonesterified fatty acid concentration in both medium and tissue is the parameter. The results are summarized in Table 2, in which the activity is expressed in terms of the minimal effective dose per 50 mg itssue.

32) The manufacturer denotes that the preparation has an activity of 150 units/mg (L-leucine amide as substrate) and is free from the activities toward benzoyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester or tosyl-L-arginine methyl ester.

33) H. Tuppy, U. Wiesbauer and E. Wintersberger, *Z. Physiol. Chem.*, **329**, 278 (1962).

34) A 10-m*l* assay medium is composed of 0.625 mM substrate solution (4 m*l*), 0.1M phosphate buffer (pH 8.3, 4 m*l*) and an enzyme test solution (2 m*l*), which has been prepared from a stock solution by appropriate dilution with 0.005M Tris buffer (pH 8.0, 0.005M $MgCl_2$). With a single run in this assay medium four consecutive determinations are usually possible in appropriate time intervals.

35) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

36) A. Tanaka and C. H. Li, *Endocrinol. Japonica*, **13**, 180 (1966).

37) R. E. Peterson, *J. Biol. Chem.*, **225**, 25 (1957).

38) M. C. Sheps and E. A. Moore, *J. Pharmacol. Extl. Therap.*, **128**, 99 (1960).

4) Inactivation of Lipotropic Activity by Plasma and by Tissue. A peptide sample was dissolved in the fresh plasma of anesthetized rat and in the Krebs-Ringer bicarbonate buffer containing bovine serum albumin, thigh muscle fragments of rat and glucose. These mixtures were then incubated at 37°C and the aliquots taken from the incubation mixtures were assayed for the lipotropic activity by the above method at 0.5, 1, 2 and 4 hr of incubation time. The results are shown in Fig. 5A and 5B.

Grateful acknowledgement is made to Miss Keiko Namba, Mr. Kunio Watanabe (synthesis), Miss Kazuko Kondo (enzymic work), Mr. Kunihiro Odaguchi, Mr. Takayoshi Furukawa and Mr. Kuniyoshi Nakayama (biological assays) for able technical assistance. The authors also thank Dr. Mitsuo Ebata and staff for amino acid analysis, Dr. Kaoru Kuriyama and staff for optical rotation measurements, and Dr. Satoshi Mizukami and staff for elemental microanalysis.