AN ATTEMPT TO EVALUATE THE STEROIDOGENIC ACTIVITY OF SYNTHETIC ADRENOCORTICOTROPIC PEPTIDES: THE CIRCULATION METHOD*

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SYNOPSIS

Apparent differences between the time curves (as determined by a method designated as the circulation method) of corticosterone concentration in the adrenal venous blood in hypophysectomized rats followed by the administration of natural ACTH and synthetic adrenocorticotrophic peptides were demonstrated. When the areas under the curves obtained for 2 or 3 different doses of various adrenocorticotrophic preparations were assumed as the responses, parallel dose-response regression lines were obtained for all the peptides tested. The relative potencies computed from this dose-response relationship somewhat differed from those by the established methods. It is suggested that the circulation method may be useful in evaluating the adrenal-stimulating potency of various preparations for therapeutic purposes.

Because of recent progress in peptide chemistry it is possible to achieve the synthesis of a number of active polypeptides possessing adrenocorticotrophic (ACTH) activity. Obvious discrepancies have been noted, however, among the relative potencies of these peptides and that of natural ACTH when assayed by different techniques. The present work is an attempt to obtain more accurate relative potencies by a method *in vivo* which can approximate the actual potencies of the preparations, either native or synthetic, in the circulating system.

EXPERIMENTAL

α_s -ACTH was isolated from sheep pituitary glands and purified by the method reported previously (Li *et al.*, 1955; Pickering *et al.*, 1963). The International Standard for ACTH, established in 1962, was obtained from the National Institute for Medical Research, London. adrenocorticotrophic peptides corresponding to various NH_2 -terminal amino acid sequences Synthetic of ACLH were as follows (Li *et al.*, 1960; Li *et al.*, 1964a and b):

α^{1-19} -ACTH: H-Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.
Lys.Pro.Val.Gly.Lys.Lys.Arg.Arg.Pro-OH

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α^{1-17} -ACTH: H-Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.Gly.

Lys.Pro.Val.Gly.Lys.Lys.Arg-OH

$\alpha^{(1-10)+(15-19)}$ -ACTH: H-Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.

Try.Gly.Lys.Lys.Arg.Arg.Pro-OH

The circulation procedure

Male rats of the Long-Evans strain, weighing 400~550 g, are hypophysectomized by the intra-aural method (Tanaka, 1955) 24 hrs. before assay. The animals are placed under Nembutal anesthesia, and a clear polyethylene tubing (Cannula B; Clay-Adams, Intramedic PE 60, 0.030" inside diameter, 0.048" outside diameter, and 5" long) is connected with the left femoral vein. A heparin solution (200 units/0.2 ml/rat) is injected through this cannula. After the laparotomy, the left renal vein is ligated at 2 points, at the confluence with the inferior vena cava and at the hilus of the kidney. The capillary veins which flow into the renal and adrenal veins are closed with a cautery. Sometimes the internal spermatic vein flows directly into the renal vein; in this case, another ligature is necessary to avoid this flow. A second tubing (Cannula A; 5" long) is inserted into the renal vein between the two ligatures and fixed. Both cannulas must be filled with saline solution before they are connected with the vein. When the free ends of both cannulas are connected together with a metal tube (the authors used a #22 gauge injection needle for this purpose), the flow of adrenal venous blood passing through the cannulas can be seen (Fig. 1).

CIRCULATION METHOD

Fig. 1. Schematic drawing of the circulation method

Adrenal venous blood in the amount of 0.1 ml is collected gently from Cannula A, with a 0.5 ml syringe containing 0.3 ml of saline and fitted with a #22 gauge injection needle. A

solution of hormone in saline (0.1 ml per 100 g body weight) is administered into the femoral vein through Cannula B. The cannulas are then connected together again to allow the blood to circulate. Before the hormone administration and at intervals of 10 mins. thereafter, 0.1 ml of saline containing 25 units of heparin is supplied through Cannula B in the same manner to prevent depression of blood pressure.

The blood that is collected is poured into a small centrifuge tube and centrifuged for 5 mins. at 3,000 r.p.m. Supernatant fluid (0.25 ml corresponding to 0.625 ml of blood) is used for the determination of corticosterone by the fluorometric technique (Peterson, 1957). The response is expressed in terms of $\mu\text{g/ml}$ of adrenal venous blood.

After the curve showing blood corticosterone concentration as a function of time is drawn (Fig. 2), the area under the curve is measured with a planimeter. When these areas corresponding to 2 or 3 different doses of peptides are compared with those of standard ACTH, the relative potency is then calculated in accordance with the statistical method indicated in the U.S. Pharmacopoeia (1960).

The direct cannulation method

All the peptides were also assayed by a slight modification of the "jugular" method of Lipscomb and Nelson (1962). Male rats weighing 200 to 250 g were used 24 hrs. after hypophysectomy. Under Nembutal anesthesia, the ventral wall was opened transversely at the level of the kidney. Several capillary veins flowing into the left adrenal vein are closed by a cautery. Heparin (200 units/rat) is administered into the femoral vein. Exactly 5 mins. after the hormone injection (0.1 ml/100 g) into the jugular vein, a clear polyethylene cannula (10" in length and equipped with a #22 gauge injection needle at one end) is inserted directly into the adrenal vein. The adrenal venous blood dripping through the cannula is collected in a graduated microcentrifuge tube for a period of 5 mins. from the 5th to the 10th min. after the hormone injection. When the bleeding is ended, the left adrenal is removed and weighed. The corticosterone content in the blood sample is determined by the method described above. Response is expressed as $\mu\text{g}/100 \text{ mg adrenal}/5 \text{ min.}$

RESULTS

Time-response relationship of various peptides

Typical curves of blood corticosterone as a function of time were obtained for dosages greater than 1 milliunit per 100 g body weight of Standard ACTH (Fig. 2). Similar patterns were observed for doses of sheep α -ACTH ranging from 0.01 to 1 μg (Fig. 3).

The patterns of the production of corticosteroids as a function of time in the case of the synthetic peptides differ from those in natural ACTH. The blood corticosterone concentration following the administration of the nonadecapeptide (Fig. 4) and the pentadecapeptide (Fig. 6), which possess the same amino acid residues, Lys. Lys. Arg. Arg. Pro at their C-termini, was not as high, but a significant concentration was maintained for a much longer period of time than in the case of natural ACTH, suggesting that the activities of both synthetic peptides are of longer duration than the native hormone. On the other hand, the heptadecapeptide (Fig. 5) gave a high corticosteroid concentration but for a considerably shorter duration.

Fig. 2. Corticosterone level in adrenal venous blood following intravenous injection of International Standard ACTH.

Fig. 3. Corticosterone level in adrenal venous blood following intravenous injection of the sheep ACTH (α_s -ACTH).

Fig. 4. Corticosterone level in adrenal venous blood following intravenous injection of nonadecapeptide, α^{1-19} -ACTH.

Fig. 5. Corticosterone level in adrenal venous blood following intravenous injection of heptadecapeptide, α^{1-17} -ACTH.

Fig. 6. Corticosterone level in adrenal venous blood following intravenous injection of pentadecapeptide, $\alpha^{(1-10)+(15-19)}$ -ACTH.

Relative potencies as estimated by the circulation method and the direct cannulation method

The areas calculated from the time-response curves corrected for the basal level of corticosterone in the pre-injection blood samples are listed in Table 1. The average area for each dose of the various preparations was plotted on semi-logarithm section paper (Fig. 7). Since the log-dose regression lines for the peptides and for α_s -ACTH were parallel with that of standard ACTH, the relative potencies could be calculated. Table 1 also gives the potencies with the

Fig. 7. Dose-area (steroidogenesis) relationship of various peptides.

Table 1. *In vivo* steroidogenic activities of various peptides
by the "circulation method"

Peptide	Dose (per 100 g B.W.)		No. of Rats	Area (mean $\pm$ S.E.)	Relative potency Units/mg	Precision index λ
Standard*	50	mU	4	165.3 $\pm$ 13.0		
	10		3	85.7 $\pm$ 12.4		
	1		3	5.0 $\pm$ 3.1		
α 1-39-ACTH**	1	μ g	4	191.0 $\pm$ 16.6	112.3	0.331
	0.1		3	99.3 $\pm$ 3.2		
	0.05		1	65		
	0.01		3	14.0 $\pm$ 6.4		
α 1-19-ACTH	1	μ g	3	150.1 $\pm$ 10.8	40.5	0.208
	0.1		3	58.3 $\pm$ 7.2		
α 1-17-ACTH	100	μ g	3	123.2 $\pm$ 14.1	0.23	0.241
	10		3	36.6 $\pm$ 4.5		
	1		1	16.1 $\pm$ 4.5		
α (1-10)+(15-19)-ACTH	100	μ g	3	153.0 $\pm$ 7.6	0.19	0.351
	30		3	72.8 $\pm$ 11.6		
	10		3	14.9 $\pm$ 4.8		

* International Standard for Corticotropin, 1962.

**Purified sheep ACTH (α s-ACTH).Table 2. *In vivo* steroidogenic activities of various peptides
by the "direct cannulation method"

Peptide	Dose (per 100 g B.W.)		No. of rats	Corticosterone secretion (μ g/5 min/100 mg adrenal)	Relative potency Units/mg	Precision index λ
Standard*	1	mU	8	18.58 $\pm$ 2.14		
	0.25		8	8.12 $\pm$ 0.79		
	0.0625		7	2.70 $\pm$ 0.81		
α 1-39-ACTH**	0.02	μ g	2	25.47	113.1	0.301
	0.01		7	19.11 $\pm$ 2.05		
	0.005		4	13.52 $\pm$ 2.18		
	0.0025		3	9.34 $\pm$ 1.31		
α 1-19-ACTH	0.025	μ g	4	23.22 $\pm$ 3.41	77.8	0.379
	0.0061		4	23.22 $\pm$ 3.50		
	0.0015		4	2.33 $\pm$ 0.20		
α 1-17-ACTH	0.2	μ g	4	10.83 $\pm$ 1.01	1.7	0.232
	0.05		4	2.53 $\pm$ 0.64		
	0.0125		3	0.81 $\pm$ 0.20		
α (1-10)+(15-19)-ACTH	5	μ g	4	13.94 $\pm$ 2.48	0.11	0.237
	1.25		4	3.29 $\pm$ 0.64		
	0.313		4	0.52 $\pm$ 0.09		
Saline			9	4.48 $\pm$ 0.03		

* International Standard for Corticotropin, 1962.

**Purified sheep ACTH (α s-ACTH).

precision index (λ) of the preparations tested. Table 2 gives the results obtained by the direct cannulation method. The minimal effective dose for this method was about 0.05 milliunits. When the potencies obtained by both methods are compared, it is noted that the potencies of α_s -ACTH and the synthetic pentadecapeptide are almost equal by both methods, whereas those of the nonadecapeptide and the heptadecapeptide differed by each method. The potency of the nonadecapeptide obtained by the circulation method was about 1/2 that obtained by the direct cannulation method, and the potency of the heptadecapeptide by the circulation method was only 1/5 of the potency obtained by the direct cannulation method.

DISCUSSION

The potency of a hormonal preparation involves not only the degree of response but also the length of time that a significant response is maintained. It has been found that the activities of the various synthetic peptides, as well as of naturally-occurring ACTH, have different periods of duration. For example, the nonadecapeptide (Fig. 4) corresponding to the first 19 amino acid residues of α_s -ACTH and the pentadecapeptide (Fig. 6) corresponding to an altered sequence of the α_s -ACTH structure maintained an elevated corticosterone concentration in adrenal venous blood for a longer period than α_s -ACTH (Fig. 3) or the International Standard preparation (Fig. 2). On the other hand, the concentration of corticosterone in blood following the administration of a heptadecapeptide (Fig. 5) corresponding to the first 17 amino acid residues of α -ACTH was elevated and then returned to the initial level in a much shorter period of time than was the case after administration of the native hormone (Fig. 3).

Although these differences in the actions of the various synthetic peptides and of α_s -ACTH and International Standard ACTH have now been demonstrated, the various assay methods in existence for estimating adrenocorticotrophic activity were designed under the assumption that all adrenocorticotrophic hormones act in the same manner. Thus, comparative assay by these methods sometimes gives dose response curves that are not parallel with each other or with that of International Standard ACTH. For a more accurate evaluation of the activity of the various synthetic peptides prepared in this laboratory, we have applied a method which attempts to measure the total response of a hormone, in terms of duration as well as of intensity of response. By the circulation method, response regression lines were obtained for all the preparations tested.

The importance of the basicity of the amino acid residues in positions 15 to 18 for the steroidogenic activity of ACTH has recently been demonstrated (Ramachandran *et al.*, 1965). This basic core may involve the binding of hormone to receptor site. The long duration of the action of the nonadecapeptide and the altered pentadecapeptide, and the shorter duration of action of the heptadecapeptide, may be explained by this concept, since both the former are more strongly basic than the native hormone, whereas the basicity of the latter is considerably lower than the other synthetic peptides.

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