SENSITIVE ASSAY METHOD FOR ACTH USING AN INCREASE IN SECRETION RATE OF ADRENAL VENOUS CORTICOSTERONE IN HYPO- PHYSECTOMIZED RATS OF WISTAR STRAIN

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For determination of ACTH in biological fluid the assay method to be used must be highly sensitive as well as specific to this trophic hormone. LIPSCOMB and NELSON described an excellent method to detect a minor amount of ACTH by measuring corticosterone concentration in adrenal venous blood of hypophysectomized rats¹⁾. GIRARD and VANCE modified this method and reported that the sensitivity could be further increased by injecting the material into the abdominal aorta at the level of the adrenal artery and one assay rat would be injected several doses of standard and unknown making it possible to calculate the potency of unknown in one rat²⁾. However, the difference of strain and condition in which animals are raised may likely result in different response patterns to exogenous ACTH even if an assay method of identical design is used. The present paper is dealt with the study on the pattern of adrenal corticosterone secretion following intravenous administrations of ACTH U. S. P. and also describes a sensitive method for ACTH using female rats of Wistar strain which are the most commonly used in Japan.

MATERIALS AND METHODS

Animals used in the study were Wistar strain female rats weighing from 150 g. to 250 g. Hypophysectomy was performed under anesthesia with sodium hexobarbitum (Oltopan sodium, Dai-Nippon Pharm. Co.) 10 mg./100 g. i. p. The pituitary gland was removed from the external auditory canal by means of a 18 gauge thin wall needle attached to a 2 ml.-syringe containing about 1 ml. of water³⁾. Assay was carried out from 2 to 6 hours after hypophysectomy. The rat was anesthetized with Nembutal, 4 mg./100 g. i. p., and underwent laparotomy to clearly expose the left renal vein and

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FIG. 1. Method of cannulation.

its adjacent vessels. As illustrated in FIG. 1, a polyethylene tubing (Intramedic Polyethylene Tubing, PE 50, CLAY-ADAMS) filled with heparine solution ($\times 1000$) was inserted into the left renal vein toward the vena cava and fixed in place by fastening the catheter to the renal vein and also sutured to the abdominal wall covering the left kidney. The renal vein was securely tied at 2 sites near the vena cava and the left kidney as to lead the adrenal venous blood into the catheter. Another tubing was introduced into the vena cava at the level of the left renal vein and advanced until the end entered the right atrium. The tubing was sutured to the abdominal wall above the pubis to avoid dislodging. The free end was connected with the catheter inserted into the adrenal vein by means of a short PE 100 polyethylene tubing as a connector allowing the adrenal venous blood freely flow into the vena cava. The left ovarian vein was cauterized, but small veins emerging the left adrenal vein were left intact. Heparine solution ($\times 1000$) filled in the tubings previously, approximately 0.3 ml., was usually sufficient to prevent blood clotting, but, if necessary, another 0.1-0.2 ml. heparine solution was given into the rat. To collect the adrenal venous blood, the junction of tubings was disconnected and the free end of the catheter inserted in the renal vein was introduced in a graduated centrifuge tube. A 10 ml. syringe containing 50% polyvinyl pyrrolidone (Plasgen, Nanrin Pharmaceut. Ltd.) in 5% glucose solution was connected to the free end of another catheter. The plasma expander was injected into the vena cava to compensate the blood loss and also to regulate the adrenal venous flow. In the preliminary experiments in which several collections of adrenal venous blood were made over a long period, hypophysectomized rats' blood diluted 2 times with 5% glucose solution was infused in place of the artificial plasma expander.

Adrenal venous blood of the assay rat was usually collected over a 10-minute control period otherwise indicated. Then, standard ACTH solution or 2 ml. of the blood to be assayed was infused into the vena cava through the polyethylene tubing cannulated over 30 seconds during which the adrenal venous blood was allowed to freely flow out, but the amount of blood lost was negligibly small, usually less than 0.2 ml..

At the 15th minutes after the beginning of injection of the sample, the second collection of adrenal venous blood was made over a 10-minute period, e.g. from 15 to 25 minutes after the injection of sample. The volume of blood collected was adjusted as to be the same as that collected during the control period, being approximately 2 ml. per 10 minutes, by controlling the infusion rate of the plasma expander.

The blood volume was measured as accurately as possible and a small portion of the blood was determined for its hematocrit by means of microhematocrit centrifuge to calculate the plasma volume. The rest of the blood was centrifuged at 2500 rpm for 20 minutes and aliquot 0.3 ml. plasma separated was determined for corticosterone content by fluorometry according to the semimicromethod described by GUILLEMIN et al⁴⁾.

Pooled plasma obtained from adrenalectomized rats served for plasma blank to correct the residual fluorescent. The rate of corticosterone secretion was calculated and expressed in $\mu\text{g./min.}$. The secretion rate during the control period was subtracted from that during the second blood collection and the net increase in the secretion rate was used to represent ACTH activity of the test sample.

Completeness of hypophysectomy in the assay rat was macroscopically examined at autopsy. ACTH used was ACTH U.S.P.. For its diluent, 0.01 N HCl/0.9% saline/0.2% gelatin was employed to prevent adsorption of the hormone by glass wall⁵⁾.

RESULTS AND DISCUSSION

Preliminary experiments. Although maximum increase in secretion rate of adrenal corticosterone in hypophysectomized rats was reported to be seen from 5 to 10 minutes after injection of relatively a small amount of ACTH^{2,6,7)}, confirmation of this response pattern in the animals used in our laboratories may be necessary to establish an adequate method for ACTH assay in our hand.

Following the collection of adrenal venous blood over a 10-minute control period, 0.05 mU. of ACTH was injected over 30 seconds into the vena cava. The time of start of ACTH injection was set as 0 time.

The second and third blood collections were made for 2 10-minute periods from the 5th to 15th and from the 20th to 30th minute respectively. At the 40th minute time 0.2 mU. of ACTH was injected. Blood collections were further performed from the 45th to 55th and from the 60th to 70th minute periods. As illustrated in Fig. 2, corticosterone secretion rate during the second blood collection after ACTH injection was always higher than that during the first.

In other words, the maximum increase in corticosterone secretion was not seen between 5 and 15 minutes after ACTH injection in the range of dose used. The response in adrenal corticosterone secretion following ACTH in the rats employed occurred in more sluggish manner than that reported elsewhere^{2,6,7)}.

Since variation in steroidogenic responses to exogenous ACTH between assay animals may be larger than that in an individual one, it seems prefer-

FIG. 2. Changes in adrenal venous corticosterone secretion rate after injection of ACTH U.S.P. in hypophysectomized rats.

able to determine the potency of unknown sample against the standard in one assay animal. GIRARD et al. suggested that this method could be applied in ACTH assay measuring adrenal corticosterone secretion rate in a hypophysectomized rat²³. One hundredth mU. of ACTH was injected 3 times at intervals of 40 minutes at the 0, 40th and 80th minute time. Blood collections in this experiment were made over a 5-minute period at the -20~-15, 15~20, 35~40,

FIG. 3. Changes in adrenal venous corticosterone secretion rate following repeated injections of ACTH U.S.P. in 3 different hypophysectomized rats.

55~60, 75~80, 95~100 and 115~120th minute time. As illustrated in FIG. 3, the rat-1 showed good regular responses following every ACTH injection. The values 40 minutes after ACTH returned to low level but slightly higher than that of control period. The rat-2 showed also regular responses which were, however, much smaller than those seen in the rat-1. The 40-minute values were lower than the 20-minute one, but still remained high. The rat-3 showed very irregular response to ACTH. Twenty minutes after the first injection, the corticosterone secretion rate increased, but the 40-minute value was still very high. The second injection of ACTH at this time induced no response at all. This rat showed again a good response following the third injection of ACTH. The result infers that rats do not always show an identical response to repeated injections of ACTH, but a good response may be obtained 20 minutes after the first injection of ACTH.

Log-dose-response curve. Since the fore-going results indicated that estimation of ACTH by injecting unknown and standard into one assay rat was impossible to perform in our laboratories, a net increase in corticosterone secretion rate by one ACTH injection was decided to use as the response parameter. The second blood collection was made over a 10-minute period from the 15th to 25th minute after the injection of standard or unknown. If the plasma volume obtained during the control and the second sampling period was same, the calculation for obtaining a net increase in corticosterone secretion due to ACTH injection would eliminate the error due to residual fluores-

FIG. 4. ACTH standard dose response curve. Net increase in adrenal venous corticosterone secretion rate following ACTH injection was used as the index. Each point represents the mean value of 7 observations at each dosage level except at zero (9 observations). Vertical lines indicate the standard error of mean.

cence, if an assumption is correct that the residual fluorescence remains unchanged during the assay.

The dose-response curve was shown in FIG. 4. A linear relationship was obtained between the dose levels from 0.02 mU. to 0.64 mU. with a precision index (λ) of 0.284 using 7 rats at each dose. The increase in corticosterone secretion rate after injection of 0.02 mU. ACTH U.S.P. was significantly higher than that after injection of the vehicle ($P < 0.01$, $t = 0.303$, $n = 14$).

In our hand, a linear relationship was seen between log dose and the response, while in the experiments reported elsewhere between log dose and log response. Although no satisfactory explanation can be made at present, more sluggish steroidogenic response and blood collection for a longer period might

be able to account for the difference.

Parallelism between the standard and the blood obtained from stressed rats. Rats were given synthetic lysine vasopressin (SANDOZ Ltd.) intraperitoneally in a dose of 400 mU./100 g. to induce endogenous ACTH release. Ten minutes

FIG. 5. Net increase in adrenal venous corticosterone secretion rate following ACTH U.S.P. and the blood obtained from stressed rats at 2 dosage levels respectively.

TABLE 1.
Factorial analysis of two-dose assay for ACTH shown in Fig. 5.

	Factorial coefficients				Divisor	Sum of products	Variance
Variance due to	S ₁	S ₂	U ₁	U ₂	4f	S(xTd)	S ² (xTd)/16
(a) Standard vs. unknown	-1	-1	+1	+1	16	-110=Ta	756.25=D ²
(b) Combined slope	-1	+1	-1	+1	16	1504=Tb	141376=B ²
(c) Lack of parallelism	+1	-1	-1	+1	16	188=Tab	2209
Dose total Td	321	979	172	1018			2211.9=s ²

For the t test of Tab, $t=1.003$, D. F. in $s^2=9$.

after the injection the animals were sacrificed by decapitation and the blood obtained from the trunks was pooled. Two and 0.5 ml. of the blood were simultaneously infused into assay rats for determination for ACTH using 4 rats at each dosage level. The parallelism was tested between the dose-response curves obtained at 2 dosage levels of the blood and the standard, 0.04 and 0.16 mU. of ACTH U.S.P. (FIG. 5).

The analysis of a two dose factorial assay (TABLE 1) performed according to BLISS⁸⁾ revealed that the dose-response curves for the standard and the unknown were parallel within the variation to be expected from the experimental error.

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REFERENCES

- 1) LIPSCOMB, H.S. AND NELSON, D.H. Measurement of corticosterone in rat anrenal venous plasma as a bioassay for ACTH. *Fed. Proc.*, **18**: 373, 1959.
- 2) GIRARD, F. AND VANCE, V.K. Modification of Lipscomb-Nelson bioassay for ACTH. *Endocrinology*, **71**: 325, 1962.
- 3) TANAKA, A. A simple method of hypophysectomy on rats. *Shionogi Kenkyusho Nempo*, **5**: 678, 1955.
- 4) GUILLEMIN, R., CLAYTON, G.W. LIPSCOMB, H.S. AND SMITH, J.D. Fluorometric measurement of rat plasma and adrenal corticosterone concentration. *J. Lab. Clin. Med.*, **53**: 830, 1959.
- 5) LIPSCOMB, H.S. AND NELSON, D.H. A sensitive biologic assay for ACTH. *Endocrinology*, **71**: 13, 1962.
- 6) LIPSCOMB, H.S. AND NELSON, D.H. Dynamic changes in ascorbic acid and corticosteroids in adrenal vein blood after ADTH. *Endocrinology*, **66**: 144, 1960.
- 7) LIDDLE, G.W. ISLAND, D. AND MEADOR, C.K. Normal and abnormal regulation of corticotropin secretion in man, in "Recent Prog. in Hormone Res.", ed. by Pincus, G. Academic Press, New York, **18**: 125, 1962.
- 8) BLISS, C.I. The Statistics of Bioassay, Academic Press, New York, 1952.