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A Component of Substance P Active in Releasing ACTH.* (22554)

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Substance P is a potent smooth muscle stimulating factor first described by von Euler and Gaddum(1), and since studied by other investigators(2-7). The precise function of P is not clear, nor has the substance been well characterized chemically, although it is regarded by some as a complex polypeptide(1,5,8). The typical action of P upon smooth muscle, *e.g.*, the guinea pig ileum, is induction of a slow contraction starting after a short latent period. This effect is not abolished by atropine, antihistaminics or ganglionic blocking agents(4). Similarities in activity between the acid hydrolysate of P and that of vasopressin (Pitressin, Parke, Davis and Co.), now definitely known to contain a component which releases adrenocorticotropin, induced the writers to test P for comparable releasing activity.

Methods. Substance P,† prepared from the intestine of the horse, was tested for ACTH-releasing activity using the *in vitro* technic of Saffran *et al.*(9,10) and the Sayers adrenal ascorbic acid depletion test on 120-140 g male rats hypophysectomized by the parapharyngeal approach for 18-24 hours. Two minor modifications of the Saffran method were introduced in these experiments: (1) the rats from which the pituitaries were removed for incubation were gentled for several days before use and then quickly guillotined without anesthesia; (2) arterenol was not added to the incubation fluid since it served no useful purpose.

Results. Substance P was hydrolyzed by boiling 1.5 hours in 2.2 N HCl, using the method previously described for hydrolysis of vasopressin(11,12). The results of the tests, shown in Table IA, demonstrate that both hydrolyzed and nonhydrolyzed P exhibit marked ACTH-releasing activity amounting to -106 ± 11.5 and -110 ± 12.3 mg % adrenal ascorbic acid depletion. Thus acid hydrolysis does not seem to destroy the activity of P. Equivalent amounts of incubation fluid from the Warburg flasks containing control pituitary glands and given I.V. to 18 hypophysectomized rats induced negligible ascorbic acid decline. Nonhydrolyzed P was also tested for its *in vivo* ACTH-releasing action on intact rats whose ACTH output had been inhibited by subcutaneous injections of 10 mg DCA plus 10 mg of the free alcohol of hydrocortisone 18 hours previously. P was administered I.V., to 6 rats in a dose of 0.25 mg one hour before removal of the second adrenal for the ascorbic acid test. The results were essentially negative, but should be repeated when purer preparations are available. Since P is destroyed by strong acid at boiling temperature, whereas the hydrolysate retains its ACTH-releasing activity, it seems evident that the releasing agent is not the same substance designated as P by von Euler, Gaddum and others, but is quite a different entity.§ To avoid confusion in terminology the ACTH-releasing component of the P complex will be referred to hereafter simply as P_1 until it is more precisely characterized chemically.

* Part of the expenses of this investigation was defrayed by Sharp and Dohme, Division of Merck and Co., West Point, Pa., and Ciba Pharmaceutical Products Co., Summit, N. J.

† We are indebted to Drs. D. A. McGinty and J. J. Pfiffner of Parke, Davis and Co. for generous supplies of Pitressin and Substance P.

§ Chromatographic fractions (obtained from Merck & Co.) also show separation of releasing agent and P.

TABLE I. Adrenocorticotropin Releasing Substances.

	Amt material used in Sayers assay	No. of rats	Ascorbic acid depletion, mg %*
A. Release of ACTH from anterior pituitaries *in vitro* by Substance P, 0.67 mg P in 0.5 cc incubation fluid/Warburg flask			
		Hypophysectomized	
P—Nonhydrolyzed	.20 ml Warburg fluid	17	−110 ± 12.3†
Control	*Idem*	11	− 17 ± 7.3
P—Hydrolyzed	"	7	−106 ± 11.5†
Control	"	7	− 19 ± 13.6
Pitressin—Hydrolyzed	"	37	−111 ± 18.6†
Control	"	43	− 23 ± 6.6
B. Ineffectiveness of Substance P when infused I.V. in hypophysectomized rats			
P—Nonhydrolyzed	5.35 mg/rat	5	+ 22 ± 19.2

* Mean ± stand. error. † Differs significantly from controls ($P < 0.01$).

Substance P (containing P_1) is without effect on the adrenals of animals lacking pituitary glands when infused I.V., and differs in this respect from pitressin, since the latter when administered I.V., in very large doses (300 units) to hypophysectomized rats is toxic and apparently is contaminated with sufficient ACTH to markedly deplete adrenal ascorbic acid. In our experience such massive dosage induced a sharp decline in ascorbic acid amounting to −128 ± 3.9 mg %. Nonhydrolyzed P, rich in P_1 factor, does not exhibit strong pressor activity, hence is devoid of toxicity and can be infused I.V. into hypophysectomized rats in large doses. Five animals whose pituitary glands had been extirpated 18-24 hours earlier, were given 5.35 mg of P by vein. No signs of toxicity appeared nor was the adrenal ascorbic acid depleted (Table IB). Incubation of 0.67 mg of this same material in a Warburg flask containing pituitary glands released ACTH which greatly decreased the adrenal ascorbic acid of hypophysectomized rats when the incubation fluid was infused.

Experiments on the Guinea Pig Ileum. The suggestion has been made(5) that Substance P may consist of more than one polypeptide. The following experiment indicates that there are probably 3 factors in P, each possessing different pharmacological properties. Hydrolysis with 2.2 N HCl destroys the agent which stimulates the slow increase in tone of the isolated guinea pig ileum but does not inactivate the ACTH-releasing factor in P. Hydrolysis also frees a material which exhibits physiological properties identical with those of the histamine-like substance obtained by similar hydrolysis of pitressin(11,12). Thus nonhydrolyzed P when tested on the ileum, induces a slow increase in tone (Fig. 1), which is not antagonized by pyribenzamine or atropine. The hydrolysate given in the same dosage evokes an altogether different response, indistinguishable from that elicited by histamine and which is abolished by pyribenzamine (Fig. 1). Since both hydrolyzed and nonhydrolyzed P are ACTH releasers, the striking difference in the gut response to acid and non-acid treated material affords additional evidence that P itself is not the re-

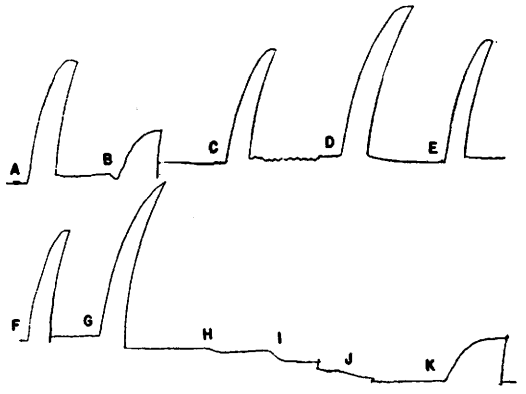

FIG. 1. Isolated ileum of guinea pig. Doses given per cc of bath fluid. A, C, E, F: Histamine base 0.015 γ/cc. B and K: Substance P, 67 γ/cc. D and G: Hydrolyzed Substance P, 67 γ/cc. H: Pyribenzamine 0.5 γ/cc. I: Hydrolyzed Substance P, 67 γ/cc. J: Histamine base 0.015 γ/cc. K: Nonhydrolyzed Substance P.

leasing agent. This is an uncharacterized substance tentatively designated here as P_1. To date the ACTH-releasing factor in the hydrolysate of P has not been separated from the histamine-like component. However, it is probable that this latter substance is not concerned with ACTH release because a similar, if not identical, pharmacologically active agent occurring in pitressin hydrolysates can be eliminated (unpublished data) from ACTH-releasing fractions without loss of potency by the latter. This separation is readily accomplished by distribution in the Craig Counter Current apparatus using a suitable solvent system.

The hydrolysate of P was tested on the blood pressure of the etherized-atropinized cat and the results compared with those elicited by histamine and 5-hydroxytryptamine. The vasodepressor action of P-hydrolysate was less evident than that shown by equal amounts of hydrolyzed pitressin(11). The fall in blood pressure was comparable to that evoked by histamine but quite unlike that induced by 5-hydroxytryptamine (Fig. 2, A-D).

Discussion. The data presented by Guillemin‡ and the present writers‡ render it probable that the ACTH-releasing agent now definitely known to be associated with both Substance P and commercial vasopressin, either as a contaminant or possibly a fragment of an originally larger protein molecule, are one and the same entity. This offers interesting possibilities in view of the widespread distribution of P (including P_1), since it is especially prevalent in the nervous system, both central and peripheral. According to Pernow(4), Amin *et al.*(7) and others, P occurs in the hypothalamus in greater concentration than in most other tissues. The fact that P (P_1) releases ACTH is significant, since it has been suggested(4,6,7) that this physiologically potent substance may be the chemical transmitter liberated by the first sensory neurones. Such an hypothesis if definitely established would perhaps afford a rational explanation for the extremely rapid release of ACTH under conditions of stress.

‡ Reported at Annual Meeting of Fed. Soc. for Exp. Biol. and Med., April 1956, Atlantic City, N. J.

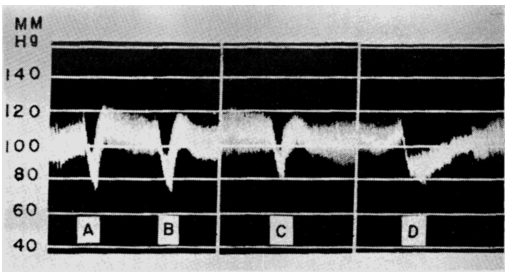

FIG. 2. Cat, 2.4 kg, atropinized, etherized, carotid blood pressure. A: Histamine base 0.6 γ/kg I.V. B: Histamine base 1.8 γ/kg I.V. C: Hydrolyzed Substance P, 10.4 mg/kg I.V. D: 5-hydroxytryptamine 20 γ/kg I.V.

Although the exact chemical nature of the active ACTH-releasing component of P and pitressin is unknown, evidence exists that they are probably identical and can be separated as distinct entities from (1) pitressin; (2) Substance P, and (3) the histamine-like material which makes its appearance in acid hydrolysates of both P and pitressin.

Summary. Substance P, prepared from horse's intestine, is rich in a component which is highly active in releasing ACTH from pituitary glands *in vitro*. This factor is not P itself but is an accompanying material of unknown nature which can be separated from P by strong acid hydrolysis and shown to contain all of the ACTH-releasing activity. The hydrolysate also contains a potent histamine-like substance the activity of which, unlike that of P, is readily abolished by pyribenzamine. The releasing factor, provisionally designated as P_1, is apparently identical with the ACTH-releasing agent known to occur in commercial pitressin.

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Note on Calcium Ion Requirements for Threone Activity.* (22555)

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The conversion of prothrombin to thrombin occurs in several ways. Two possibilities may be represented by the following equations:

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin}\ \text{Thromboplastin}]{Ca^{++}} \text{Thrombin}$$

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin}\ \text{Threone}]{Ca^{++}} \text{Thrombin}$$

In the first type of activation, thromboplastin of tissue origin is involved and it is known that a certain optimum calcium concentration best supports the reaction(s). This optimum is found to be about the same irrespective of whether whole blood or plasma is clotting (1,2) or whether purified prothrombin is being activated(3).

In the type of activation represented by the second equation, platelets are involved instead of thromboplastin; for, threone activity consists of the simultaneous presence, in suitable proportions, of platelet cofactor I from the plasma and platelet factor 3 from the platelets(4). This may be represented as follows: Platelet factor 3 + Platelet Cofactor I = Threone. In the present study we measured the calcium ion requirements for threone activity.

Methods. Purified prothrombin was prepared from bovine sources(5,6,7) and assayed by a modification of the 2-stage technic(8), and thrombin activity was measured as described by Seegers and Smith(9). Platelet suspensions were prepared as described previously(10); and in this study, it was found that they could also be replaced with purified platelet factor 3 obtained and assayed quantitatively for its activity as described(11). Platelet cofactor I concentrates were obtained from bovine sources and assayed according to procedures we hope to describe in detail in a later communication. Our preparations of platelet cofactor I were free of autoprothrombin II activity. The following reaction mixture was composed: (a) Purified prothrombin, 2400 u/ml; (b) platelet homogenates or purified platelet factor 3, 75 u/ml; (c) Platelet cofactor I concentrate, 65 u/ml; and, (d) $CaCl_2$ dissolved in imidazole buffer pH 7.25. The latter was the only variable. The reaction mixture was placed in a water bath at 28°C, and periodically samples were removed for the quantitative determination of thrombin activity. The results are presented on Fig. 1.

Results. In our study of the calcium ion requirements for the conversion of prothrombin to thrombin with threone, we found that there is no sharp optimum concentration. Once a certain minimum calcium ion concentration is supplied, the rate of prothrombin activation and the yield of thrombin is the same from 0.009 M to 0.04 M concentration (Fig. 1). With higher concentration, calcium is inhibitory. The wide range of calcium concentration most suitable for threone

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