Corticotrophic Activity of Human Plasma Constituents.* (23648)

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The nature of corticotrophin (ACTH) as it occurs in the pituitary gland has been well studied by a variety of technics but little has been reported on the properties of this hormone as it occurs in the peripheral circulation of man, although its presence in minute amounts has been indicated(1). In this report the activity of fractions of human plasma prepared according to the method of Cohn and his associates(2,3) has been assayed for ACTH content by the bioassay technics of Nelson and Hume(4).

Methods and material. Human blood used in this study was collected into acid-citrate-dextrose (ACD) solution or through a cationic exchange resin column (Dowex 50). The plasma was separated from the red cells by centrifugation at 4000 rpm for 30 minutes at 2°C. Plasma pools from 5 to 10 donors were then subjected to fractionation with the cold ethanol methods 6 and 9(2,3). The following fractions with their major constituents as noted below were obtained: Fraction I–fibrinogen. Fraction II + III – γ-globulins and β-lipoproteins. Subfractions of II + III: III-0: β-lipoproteins: II + IIIw: –γ-globulins, prothrombin, plasminogen. Fraction IV–1: α-globulins, cholesterol. Fraction IV–4: α and β-globulins and β_1–metal binding globulins. Fraction V: albumin. The separated plasma fractions were dissolved in a minimum volume of 0.15 M sodium chloride and dialysed for 12 hours against 0.15 M sodium chloride at 2°C at pH 6.8 to 7.0. The samples were frozen until bioassayed. The bioassay was performed in hypophysectomized, anesthetized dogs in whom a catheter had been placed in the right lumbo-adrenal vein in order to make possible the timed, intermittent collection of adrenal venous blood (4). ACTH activity in plasma fractions administered intravenously during 2 minutes to these animals was compared with standard ACTH given in a similar manner. One minute following the injection of the ACTH or the unknown sample, the collection of adrenal venous blood was begun and was continued for exactly 10 minutes. The response of each intravenously administered fraction was evaluated in terms of the increase in 17-hydroxy-corticosteroid output by the dog adrenal over the control secretion when only the diluent saline was given. The potency and approximate 95% confidence limits of each fraction in which activity was found were calculated by the usual formulas(5) from the log-dose response curve of the increased output of steroids produced by the fraction sample and that produced by 1, 2, 5 and 8 milliunit doses of U.S.P. Standard ACTH. Because of the small quantities of ACTH present, it was not possible to construct a log-dose curve for each sample given, but values were obtained by comparison with the standard ACTH log-dose curve. Standards and unknowns were given to the same assay animal. This value has been expressed in the table as milliunits of ACTH/100 ml of reconstituted plasma; the equivalent volume of fractionated plasma injected has also been given.

Results. Studies have been performed on 3 types of human plasma: 1. plasma collected from normal human donors; 2. plasma collected from normal human donors to which U.S.P. ACTH was added *in vitro* one week following collection; 3. plasma obtained from patients and one normal donor following the intravenous infusion of ACTH (Upjohn).

Summary of the findings on 4 plasma pools collected from normal human donors in ACD or through resin is shown in Table I. ACTH activity was consistently found in Fraction

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TABLE I. ACTH Activity in Plasma Fractions from Pooled Blood of Normal Subjects.

Plasma fraction	Pool sample No.	ACTH activity present	Adrenal venous 17-OHCS, μg per 10 min. Control mean*	Exp.	ACTH, mu/100 ml whole plasma	95% confidence limits	Fraction Vol inj. (ml)	Fraction Equivalent in whole plasma (ml)
Precipitate I	3	0	22	16	0		16	150
	3	0	2	5	0		16	150
" II + III	1	+	11	52	.3	.06–1.9	20	300
	2	+	12	53	.7	.2 –2.6	25	160
	2	+	12	33	.9	.2 –3.6	12	100
	3	+	12	33	.3	.1 – .4	30	150
Subfraction II + IIIw	3	+	6	45	.9	.6 –1.3	33	150
" III-0	3	+	6	59	1.4	.8 –2.4	20	150
Precipitate IV-1	1	0	4	4	0		13	250
	1	0	2	4	0		13	125
	2	+	12	35	.6	.1 –2.5	20	150
	3	0	12	12	0		16	150
	4	+	1	25	.3	.08–1.0	10	150
" IV-4	3	+	6	33	.6	.4 – .9	19	150
	4	0	7	4	0		13	150
" V	1	0	9	18	0		20	190
	1	0	2	4	0		20	190
	2	0	17	17	0		20	200
	3	0	7	11	0		18	150

Plasma pools 1, 2 and 4 were from whole blood collected from 5-10 donors in ACD.

Plasma pool 3 was from whole blood collected from 5 donors through cationic exchange resins (Dowex-50).

* Mean of 2 or 3 collection periods following infusion of saline control.

II + III and occasionally in Fraction IV-1 or Fraction IV-4. Activity was present in both subfractions of II + III.

In the second group of experiments, 10 milliunits of ACTH were added to week-old plasma 12 hours before fractionation. Assay as shown in Table II revealed the presence of ACTH activity in Fraction II + III and also in Fraction I. The occasional contamination of Fraction I with whole plasma during the procedure could explain this result.

The assay of fractions obtained following the intravenous infusion of ACTH into 4 groups of patients gave results similar to those obtained in normal plasma except that the level of activity was higher (Table III). The plasma volume assayed from pool 2 was not large enough to give a significant increase in 17-hydroxycorticosteroid output as whole plasma or as fraction II + III. However, the larger volume given as subfractions II + IIIw and III-0 did have slight activity. In the ta-

TABLE II. ACTH Activity in Plasma Fractions following *In Vitro* Addition of 10 Milliunits of ACTH to 200 ml Pooled Plasma.†

Plasma fraction	ACTH activity present	Adrenal venous 17-OHCS, μg per 10 min. Control mean*	Exp.	ACTH, mu/100 ml whole plasma	95% confidence limits	Fraction Vol inj. (ml)	Fraction Equivalent in whole plasma (ml)
Precipitate I	+	1	32	1.0	.4–2.6	13	100
" II + III	+	1	21	.8	.3–2.05	25	100
" IV-1	—	1	2	0		20	100
" IV-4	—	1	5	0		20	100
" V	—	1	3	0		25	100

* Mean of 2 collection periods following infusion of saline control.

† U.S.P. standard. ACTH added to plasma 12 hr prior to fractionation.

TABLE III. ACTH Activity in Plasma Fractions from Pooled Blood of Subjects Infused with ACTH.

Plasma fraction	Pool sample No.	ACTH activity present	Adrenal venous 17-OHCS, μg per 10 min. Control mean*	Exp.	ACTH, mu/100 ml whole plasma	95% confidence limits	Fraction Vol inj. (ml)	Fraction Equivalent in whole plasma (ml)
Plasma control	1	+	3	143	48	3.7 -630	4	4
	2	0	6	10	0		5	5
	2	0	5	17	0		5	5
	3	+	4	41	18	7.8 - 41.05	7	7
	4	0	6	5	0		20	20
Precipitate I	4	0	6	4	0		7.5	100
" II + III	1	+	3	230	90	10.5 -780	5	10
	2	0	6	7	0		2	10
	2	0	6	12	0		5	20
	3	0	4	11	0		8	16
	4	±	6	13	1.0	.3 - 3.0	32	100
	4	+	7	29	1.1	.6 - 2.0	33	100
Subfraction II + IIIw	1	+	1	54	41	7.4 -231	10	20
	2	0	5	4	0		5	20
	2	+	6	54	8.6	2.4 - 31	13.5	18
	3	0	4	4	0		9	27
" III-0	1	±	1	16	8.1	.8 - 79	11	20
	2	0	5	8	0		5	20
	2	±	6	16	3.3	.8 - 14	17	19
	3	+	4	78	4.7	2.04- 10.8	18	40
Precipitate IV-1	4	0	6	1	0		25	100
" IV-4	4	0	6	13	0		23	100
" V	4	0	6	1	0		23	100

Plasma pool 1 collected from a patient at the end of an 8 hr infusion of 25 units ACTH.

Plasma pools 2 and 3 collected at the end of an 8 hr infusion of 25 units ACTH, 2 patients contributing to each pool.

Plasma pool 4 collected from a normal subject 15 min. following intrav. inj. of 25 units ACTH.

* Mean of 2 or 3 collection periods following infusion of saline control.

bles, the volume actually injected into the assay animal does not necessarily correlate with the equivalent of whole plasma from which it was obtained. This is due to some technical variations in the final concentration of the particular plasma fraction which had been obtained for assay.

Discussion. ACTH activity has been shown to occur in the γ-globulin and beta-lipoprotein fractions of normal human plasma (Fraction II + III). The activity found is low and is in approximate agreement with the amounts estimated to be present in normal plasma(1).

Fraction II + III is composed primarily of protein molecules of low solubility such as γ-globulins and β-lipoproteins. Since ACTH as isolated from the pituitary gland has been shown to be a small polypeptide of high solubility(6) there is the suggestion that ACTH as found in human plasma has different physical characteristics. If it exists as a polypeptide similar to that isolated from the pituitary, it most likely is present in plasma as a complex with a protein of a higher molecular weight. The occasional occurrence of activity in Fractions IV-1 or IV-4 may also indicate the existence of more than one ACTH complex with different solubility characteristics.

Studies in which commercial ACTH was added to human plasma *in vivo* and *in vitro* confirm the occurrence of activity primarily in Fraction II + III and suggest further the existence of ACTH in plasma as a protein-polypeptide complex.

It is of interest to note that ACTH activity has been found in the same fraction in which antidiuretic hormone, pituitary gonadotrophins(7) and insulin-like activity(8) have been found.

Summary. Fractions of human plasma prepared by methods 6 and 9 of Cohn and his associates have been assayed for ACTH activity by the procedure of Nelson and Hume. ACTH activity was found in Fraction II + III and occasionally in Fraction IV-1 and Fraction IV-4. Fractionation of human plasma to which ACTH had been added *in vivo* and *in vitro* gave similar results.

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Rapid Slide Precipitin Microreaction of Poliomyelitis Antigens and Antisera in Agar.* (23649)

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The precipitation of viral suspensions by specific antisera has been applied to plant viruses, and recently also to animal viruses. Belyavin(1) described the precipitation of influenza virus in aqueous suspension by means of specific antisera. Wilson Smith *et al.*(2) reported a flocculation reaction with polioviruses. Since their original method required large amounts of antigen, the same authors developed a micromethod of immuno-precipitation of polioviruses in aqueous medium in capillary tubes(3). Le Bouvier(4), as well as the present authors(5) independently described the use of the Ouchterlony method of double diffusion in an agar gel(6) for the study of the reaction between polioviruses and specific antisera. Since the usual technic in Petri dishes requires large quantities of antigen, a micromethod, described elsewhere(7) for the study of venoms and other antigens, was applied to the polioviruses.

Materials and methods. Routine microscopic slides, cleaned in alcohol, are used. Each slide is covered with 3 ml of a 1% hot agar solution. After solidification, 2 mm holes are punched in the agar with a special instrument. A fixed pattern of 6 holes disposed around a seventh, as shown in Fig. 1, proved to be very useful. The volume of each hole is approximately 4 cu. mm. *Agar solution.* A 2% solution of commercial, pure agar in distilled water is clarified by precipitation with egg albumin at 120°C. For use, this agar solution is melted and diluted to 1% in a M/7.5 phosphate buffer solution (Sørensen) and while very hot poured on the slides. The final pH should be 7.2. This concentration of agar was chosen in accord with Polson's data(8), because it is the minimal amount of agar giving a sufficiently solid sheet. *Antigens.* Cultures of KB cells in large flasks are massively infected with the respective type strains in use here (type 1 = strain No. 6,186 from Dr. Goffe; type 2 = strain MEF_1; type 3 = strain Saukett). In

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