Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 78 DECEMBER, 1951 No. 3

SECTION MEETINGS

CLEVELAND	
Academy of Medicine	November 9, 1951
ILLINOIS	
University of Chicago	November 13, 1951
MINNESOTA	
Mayo Foundation	October 26, 1951
SOUTHWESTERN	
Oklahoma A. & M. College	November 9-10, 1951

Preparation of a Clinically Active Adrenocorticotropic Hormone (ACTH) in Good Yield from Sheep Pituitary Glands.* (19175)

CHOH HAO LI, GRANT W. LIDDLE, W. O. REINHARDT, AND L. L. BENNETT.

From the Department of Biochemistry, the Divisions of Anatomy and Physiology, University of California, Berkeley; the Metabolic Laboratory, and the Division of Medicine, University of California School of Medicine, San Francisco.

The isolation of adrenocorticotropic hormone (ACTH) from sheep and swine pituitary glands has already been described(1,2). It may be noted that the yields of the hormone prepared by these procedures are low. We wish to describe herewith a simple procedure for obtaining in much higher yield an adrenocorticotropic hormone (ACTH) preparation which is practically free from both anterior and posterior pituitary hormone contaminants, and is effective in clinical applications.

The following steps were performed at room

TABLE I. Bioassay of ACTH Fractions by the Ascorbic Acid Depletion Method.

Fraction	Dose, μg	No. of rats	Avg ascorbic acid depletion, mg/100 g adrenal	ACTH standard equivalent,* μg
Acid-acetone powder	5	10	63 ± 5†	.8
NaCl precipitate‡	5	35	126 ± 4	6.4
Final product after acid-heat treatment	2	16	111 ± 1	3.8

* These values represent ascorbic acid depleting activity in terms of standard ACTH prepared in this laboratory.

† Mean ± standard error.

‡ Obtained by NaCl precipitation between .06 and 1 saturation.

* This work is supported in part by the U. S. Public Health Service, the Lasker Foundation, the Armour Laboratories, the Merck Co., the Eli Lilly Co., and a state appropriation for Research in Arthritis.

temperature (22-24 C) except for the precipitation of acid-acetone extract which was carried out in a cold room (2-3°C).

Acid-acetone powder (AAP). One kilogram of frozen whole sheep pituitary glands was finely ground and extracted with 4.1 liters of acid-acetone solution† by vigorous stirring for one hour. The mixture was filtered and the residue was re-extracted with 2 liters of 80% acetone. After removal of the residue by filtration, the combined extracts were poured into 30 liters of cold acetone. The precipitate formed was dried in vacuum after repeated washing with acetone. The product is designated as "acid acetone powder" (AAP) and the yield from one kg of glands averages 35 g. *NaCl fractionation.* Twenty grams of AAP was next dissolved in 940 cc water and adjusted to pH 3.0. A saturated NaCl solution (60 cc) was added dropwise with constant stirring: the precipitate formed was centrifuged off and saved for the isolation of lactogenic hormone(3). The supernatant was brought to saturation with solid NaCl: the precipitate formed was separated and dissolved in 100 cc water and dialysed against running water until salt-free. The dialysed solution was frozen and dried in vacuum; yield, 3 g. *Acid-heat treatment.* The dry product (3 g) was next dissolved in 300 cc 0.2 M HCl, and the solution was kept in a boiling water bath for 60 minutes. After cooling, 1 M NaOH was added to pH 3.0. The slightly acidified solution was lyophilized; the dry solid is the final product.

From 1 kg of fresh sheep whole pituitary glands,‡ approximately 5 g§ of the final product may be obtained. Testing of the various discarded fractions by the ascorbic assay method provided evidence that the recovery of ACTH by this method is practically complete. As shown in Table I, the preparation has an ACTH potency, as estimated by the

† The acid-acetone solution was prepared by mixing 0.5 l of water, 4 l of acetone and 100 cc concentrated HCl.

‡ A similar ACTH preparation from swine pituitary glands may also be prepared by the same procedure.

§ This value was obtained by deduction of the amount of NaCl from the total solid.

TABLE II. Assay by Maintenance Test of Purified ACTH Preparation.*

Group (No. of rats)	Total dose,† mg	Body wt (range)		Avg organ wt (ranges)			
		Initial, g	Final, g	Adrenals (2), mg	Thymus, mg	Thyroids, mg	Testes, mg
Hypophysectomized controls (6)	0	52 (43-56)	60 (56-64)	6.3 (5-8)	93 (75-128)	5.6 (5-7)	84 (59-98)
Hypophysectomized treated with ACTH (7)	36	55 (46-60)	60 (53-67)	22.1 (19-24)	32 (22-68)	5 (3-8)	90 (72-119)
Normal controls (5)	0	50 (46-57)	147 (127-164)	24.2 (17-32)	324 (275-379)	13.4 (11-16)	1314 (1074-1492)

* Prepared by the procedure described in this paper.

† Male rats of the Long-Evans strain hypophysectomized at 21 days of age were used; intraperitoneal injections begun on the day of hypophysectomy and continued for a period of 21 days. The rats received two injections daily (.5 mg per inj. for the first 6 days, then 1 mg per inj. for 15 days); controls were inj. with saline. (Unpublished data of Ulrich, Reinhardt and Li, 1951).

TABLE III. Effect of Purified ACTH Preparation* Upon Circulating Eosinophiles, Fasting Blood Sugar, Urinary 17-Ketosteroids, Urinary Sodium, and Body Weight.

		Patient L. G.			Patient R. D.		
		Pre-treatment	ACTH	Post-treatment	Pre-treatment	ACTH	Post-treatment
Circulating eosinophiles, per cu mm	Mean	127	16	83	98	8	120
	Range	(93-160)	(12-22)	(50-103)	(78-115)	(7-9)	(93-135)
Fasting blood sugar, mg per 100 cc	Mean	77	92	78	68	87	58
	Range	(68-84)	(79-101)	(75-82)	(65-69)	(73-95)	(57-60)
Urinary 17-ketosteroids, mg per 24 hr	Mean	5.3	7.8	5.4	5.6	10.5	4.6
	Range	(2.3-7.3)	(6.1-9.1)	(4.6-6)	(3.9-7.6)	(6.8-14.4)	(3.6-5.2)
Urinary Na^+ mEq. per 24 hr	Mean	105	33	158	76	37	162
	Range	(82-133)	(13-80)	(34-300)	(48-100)	(14-72)	(49-250)
Body wt, kg		46.3-46.9 Max. range for 6 days	49.2 After 7 days Rx	46.8 3 days after withdrawal	73-73.4 Max. range for 6 days	75.6 After 6 days Rx	71.5 6 days after withdrawal

* Prepared by the procedure described in this paper.

ascorbic acid depletion method(4), equivalent to that of the hormone isolated by the procedure previously described(1). The product also exhibits activity in maintaining the adrenal weight of hypophysectomized rats, as is evident from the data in Table II. From the same table, it is clear that the preparation is free from gonad-stimulating, growth-promoting and thyrotropic activities as determined by target organ and body weight changes. The preparation is also substantially free from contaminating antidiuretic and pressor principles(5). No tests were made for lactogenic or oxytocic activity.

The therapeutic and metabolic effects of the preparation were investigated on 4 patients with active rheumatoid arthritis. The subjects were maintained on a carefully controlled metabolic balance regimen during treatment periods of from 6 to 12 days and appropriate control periods. The dietary intake of total calories, carbohydrate, fat, protein, and minerals was constant from day to day. Twenty-four hour urine collections were obtained, and stools were pooled for 6-day periods. Every 6 hours during treatment periods each patient received an intramuscular injection of this ACTH preparation in doses equivalent to 25 mg LA-I-A (Armour Standard) as assayed by the ascorbic-acid depletion method(4). The results which were obtained followed the patterns which have become familiar in the use of other ACTH preparations(6). In every case there occurred a significant remission of heat, pain, tenderness, limitation of motion, and soft-tissue swelling of involved joints. Those patients in whom body temperatures had been elevated became afebrile. All patients experienced an increased sense of well-being. This clinical improvement became apparent within 24 hours after treatment was started, and relapse began within 24 hours of the time treatment was withdrawn.

The clinical evidence that the ACTH preparation obtained by the procedure herein described contained a potent adrenocorticotropic principle was confirmed by laboratory tests. Treatment periods were characterized by sodium retention, water retention, weight gain, eosinopenia, an increase in urinary 17-ketosteroids, and a return of erythrocyte sedimentation rates toward normal. When the treatment was stopped these effects were promptly and completely reversed. Data from two of the cases which illustrate some of these points are presented in Table III. In every case, although over-all potassium balances were unaffected by treatment, a marked decrease in urinary excretion of both potassium and phosphate occurred during the first 24 hours following withdrawal of the treatment. No untoward effects were encountered. There was no evidence of local tissue irritation or of a systemic reaction to pyrogens. Treatment periods were not of sufficient duration to show whether prolonged use of this preparation would result in undesirable metabolic effects. Presumably it would not differ from other ACTH preparations in this respect.

Summary. A method is described for the preparation in high yield, from whole sheep pituitary glands, of purified ACTH, substantially free from growth, gonadotropic, thyrotropic, antidiuretic and pressor activities. Clinical and laboratory evidence indicates that ACTH prepared by this method is therapeutically and metabolically effective in patients with rheumatoid arthritis.

1. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, 1943, v149, 413.

2. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, v149, 425.

3. Li, C. H., Simpson, M. S., and Evans, H. M., *J. Biol. Chem.*, 1942, v146, 627.

4. Sayers, M. A., Sayers, G., and Woodbury, L. A., *Endocrinology*, 1948, v42, 379.

5. Reinhardt, W. O., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 836.

6. Thorn, G. W., Forsham, P. H., Frawley, T. F., Hill, S. R., Jr., Roche, M., Staehelin, D., and Wilson, D. L., *New England J. Medicine*, 1950, v242, 783.

Received October 31, 1951. P.S.E.B.M., 1951, v78.

Chromatographic Behavior of Adrenocorticotropic Peptides in Diatomaceous Earth and Starch Column.* (19176)

R. MENDENHALL† AND CHOH HAO LI.

From the Department of Biochemistry, University of California, Berkeley.

In a recent paper(1), we described the purification of adrenocorticotropically active peptides (ACTH peptides) in paper partition chromatography, displacement development analysis and carrier displacement chromatography. The present paper concerns the results obtained from starch and diatomaceous earth chromatographic columns. A preliminary account on the purification of ACTH peptides in starch has appeared(2).

Experimental. The ACTH peptide mixture was prepared from the pepsin digest of the sheep protein hormone by the method previously described(2,3). The adrenocorticotropic activity was estimated by the ascorbic acid depletion in the adrenals of hypophysectomized male rats(4).

The potato starch and diatomaceous earth‡ were commercial products. The columns were prepared and operated according to the procedure described by Stein and Moore(5,6). The effluent fractions (0.5 cc) were analyzed by the ninhydrin colorimetric method(7); alternate tubes were selectively pooled and dried out and the nitrogen content was determined by the micro-Kjeldahl technic.§ The column size was 0.9 x 30 cm and the rate of flow was maintained by a pressure head of 15 cm Hg. Some of the developing solvents were those suggested by Stein and Moore (5,6) for the separation of amino acids.

Results. Three developing solvents were employed for the *diatomaceous earth* column: Solvent A, ethanol: 0.9% NaCl = 1:1; Solvent B, butanol: glacial acetic acid: water = 63:10:25, and Solvent C, benzyl alcohol: butanol: water = 1:1:0.288 with an addition of 0.5% thiodiglycol. It may be noted in Table I that a large amount of ninhydrin-reacting material appears in Fractions No. 42-62, comprising 70% of the material applied in the column when Solvent A was used as the eluent. There are some small peaks after Fraction No. 115, indicating that a large number of components is present in the original peptide mixture. A recovery of 97% of the nitrogen was achieved. Using Solvent B as the developing solvent, a large

* This work is supported in part by grants from U. S. Public Health Service, the Armour Laboratories and the Merck Co.

† Cutter Laboratories Predoctorate Fellow, 1950-51.

‡ The diatomaceous earth, Dicalite 223-T, was a special acid washed material and obtained through the courtesy of Dr. G. W. Breger, Great Lake Carbon Corp., Walteria, Calif.

§ We wish to thank Harold Papkoff for the nitrogen determinations.