[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Pituitary Hormones. IV. The Preparation of Potent Concentrates of Corticotropin and Corticotropin-B

BY ALVA W. BAZEMORE, JOHN W. RICHTER, DONALD E. AYER, JAMES FINNERTY, NORMAN G. BRINK AND KARL FOLKERS

RECEIVED SEPTEMBER 8, 1952

Procedures are described for the preparation in good yields of corticotropin and corticotropin-B concentrates at activity levels of approximately 80 u./mg. Whole frozen hog pituitary glands gave corticotropin-B preparations by a process consisting of acetone defatting, extraction with a methanol–acetic acid mixture, purification with oxycellulose and pepsin digestion. Commercial acid–acetone powder was processed by oxycellulose treatment and pepsin digestion to yield similarly purified concentrates of corticotropin-B.

The purification of corticotropin and of corticotropin-B, a pepsin digestion product of corticotropin, by adsorption and countercurrent distribution techniques was reported.[1] Details of the preparation, purification and properties of these adrenocorticotropically active substances are presented here and in the following two papers.[2,3]

More than two decades ago, it was shown that the anterior lobe of the pituitary gland contained a hormone which acted upon the adrenal cortex.[4] Numerous procedures were devised for the extraction of this adrenocorticotropically active principle, and in 1943 two groups of workers announced the isolation from hog[5] and sheep[6] pituitary glands of a potent preparation, a protein of molecular weight about 20,000, which was considered to be the pure hormone. The physiological functions of these preparations concerned the stimulation of the adrenal cortex, leading for example to increased excretion of glycogenic corticoids, 11-oxy-steroids, 17-ketosteroids and ascorbic acid. The decrease in adrenal ascorbic acid upon administration of the hormone became the basis of the now widely used assay method of Sayers, Sayers and Woodbury.[7] The important use of adrenocorticotropic preparations in human therapy is now well known.[8] It has also been firmly established that pepsin digestion does not destroy the adrenocorticotropic activity of the hormone.[9-11]

Of the various terms which have been used to designate the adrenocorticotropic hormone, corticotropin has become accepted in this country[12] and will be employed here. When corticotropin preparations are digested with pepsin, the resulting active product is chemically different from corticotropin. The name corticotropin-B has been used for this highly adrenocorticotropically active product obtained from the pepsin digest of corticotropin.[1]

Of the numerous procedures which have been described for the extraction of corticotropin from pituitary tissue, the two which have been most useful have been extractions with acetone and hydrochloric acid and with glacial acetic acid. The acid–acetone procedure was first applied by Lyons in 1937,[13] and has been widely used and improved upon by other workers.[14,15] Recently, extraction of acetone-dried anterior lobes of porcine pituitary glands by glacial acetic acid has been described.[16] The extraction is carried out at 70°, after which the glacial acetic acid solution is treated with acetone containing sodium chloride for the precipitation of material containing other hormones; corticotropin is then precipitated by the addition of ether. This extraction procedure is easy to carry out, gives a high recovery of corticotropic activity and, when anterior lobes are used, yields a product about twice as active as Armour Standard La-1-A and which is satisfactory for clinical use. Material apparently contaminated with pressor and oxytocic components and of somewhat lower adrenocorticotropic activity was obtained when this procedure was applied to whole porcine glands, rather than to the anterior lobes only.

The activity of corticotropin extracts has been increased by a variety of fractionation methods. These have included ultrafiltration,[17] countercurrent distribution,[16] chromatography[16] and ion-exchange treatment.[18] However, the procedure of most outstanding utility is the oxycellulose method of Astwood, Raben, Payne and Grady.[19] In this process, crude corticotropin (2 u./mg.) was treated in a batchwise manner with oxycellulose (10% car-

(1) N. G. Brink, F. A. Kuehl, Jr., J. W. Richter, A. W. Bazemore, M. A. P. Meisinger, D. E. Ayer and K. Folkers, THIS JOURNAL, **74**, 2120 (1952).

(2) J. W. Richter, D. E. Ayer, A. W. Bazemore, G. E. Boxer, V. C. Jelinek, N. G. Brink and K. Folkers, *ibid.*, **75**, 1952 (1953).

(3) F. A. Kuehl, Jr., M. A. P. Meisinger, G. E. Boxer, V. C. Jelinek, N. G. Brink and K. Folkers, *ibid.*, **75**, 1955 (1953).

(4) P. E. Smith, *J. Am. Med. Assoc.*, **88**, 158 (1926); *Am. J. Anat.*, **45**, 205 (1930).

(5) G. Sayers, A. White and C. N. H. Long, *J. Biol. Chem.*, **149**, 425 (1943).

(6) C. H. Li, H. M. Evans and M. E. Simpson, *ibid.*, **149**, 413 (1949).

(7) M. A. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1949).

(8) *Cf.* "Proceedings of the First Clinical ACTH Conference" and "Proceedings of the Second Clinical ACTH Conference," Vol. II, The Blakiston Co., Philadelphia, Penna., 1950 and 1951.

(9) C. H. Li, *Josiah Macy, Jr., Foundation, Conference on Metabolic Aspects of Convalescence*, **17**, 114 (1948).

(10) N. G. Brink, M. A. P. Meisinger and K. Folkers, THIS JOURNAL, **72**, 1040 (1950).

(11) J. B. Lesh, J. D. Fisher, I. M. Bunding, J. J. Kocsis, L. J. Walaszek, W. F. White and E. E. Hays, *Science*, **112**, 43 (1950).

(12) *J. Am. Med. Assoc.*, **147**, 326 (1951).

(13) W. R. Lyons, *Proc. Soc. Exptl. Biol. Med.*, **35**, 645 (1937).

(14) M. Reiss and I. D. K. Halkerston, *J. Pharm. Pharmacol.*, **2**, 236 (1950).

(15) E. L. Smith, G. Sayers, B. N. Ghosh and D. M. Woodbury, *Proc. Soc. Exptl. Biol. Med.*, **79**, 27 (1952).

(16) (a) E. B. Astwood, A. P. Cleroux, R. W. Payne and M. S. Raben, *Bull. N. E. Med. Center*, **12**, 2 (1950); (b) R. W. Payne, M. S. Raben and E. B. Astwood, *J. Biol. Chem.*, **187**, 719 (1950).

(17) B. Cortis-Jones, A. C. Crooke, A. A. Henly, P. Morris and C. J. O. Morris, *Biochem. J.*, **46**, 173 (1950).

(18) H. B. F. Dixon, Stanford Moore, M. P. Stack-Dunne and F. G. Young, *Nature*, **168**, 1044 (1951).

(19) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1950).

boxyl content) in dilute acetic acid solution. The activity was then eluted with dilute hydrochloric acid and the purified corticotropin concentrate recovered in 80% yield at a potency of about 80 u./mg.

The isolation of adrenocorticotropically active material from hog[5] and sheep[6] pituitary glands has been mentioned. Beef pituitary glands possess activity[20]; and the adrenocorticotropic activity of whale pituitaries has been demonstrated[21] and the whole gland used as a commercial source of an ACTH preparation.

The investigations recorded here and in the accompanying two papers[2,3] have been carried out on material from porcine sources, either whole frozen hog pituitary glands or a commercial acid--acetone extract.[22] We wish to describe here our experiences with the extractions of the whole glands, with pepsin digestion, and with the preliminary purification of the products studied with oxycellulose. Further purification, mainly by use of the ion-exchange resin IRC-50 and by countercurrent distribution, is reported in the following two papers.

By the introduction of some modifications of the published glacial acetic acid extraction procedure,[16] a corticotropin preparation suitable for further purification has been obtained from whole porcine pituitary glands. The fresh frozen whole glands were ground with acetone to remove water and fat, and then washed with methanol to remove other inert material as indicated in Table I. After the defatted glandular material was extracted with a 40% solution of acetic acid in methanol, crude corticotropin was precipitated directly from the methanol–acetic acid solution by the addition of ether. In this way, defatted glandular material of activity about 0.5 u./mg. has given a product of activity averaging 3 u./mg., with a nearly quantitative recovery of adrenocorticotropic activity. The use of ratios of methanol to acetic acid substantially different from that described led to lower yields and to products of lower activity. Some pertinent results are summarized in Table I.

TABLE I

EXTRACTION OF DEFATTED PITUITARY GLANDS[a]

	Solvent	Yield, g.	Activity, u./mg.	Recovery of activity, %
1	Methanol	2.5	<1.5	...
2	20% acetic acid in methanol	13.0	1.5	50
3	30% acetic acid in methanol	11.5	3	86
4	33 1/3% acetic acid in methanol	10.2	2.8	72
5	40% acetic acid in methanol	13.0	3	98
6	50% acetic acid in methanol	24.5	2	100
7	Acetic acid	22.6	2	100

[a] Extractions were carried out upon 100-g. portions of defatted porcine pituitary tissue having an activity of 0.4 u./mg. The yields represent the total precipitate obtained by the addition of ether to the extraction solution.

As may be seen from the table, extraction with pure acetic acid did give an essentially quantitative recovery of the activity, but at a lower level than was obtained when methanol–acetic acid mixtures were used. We have observed in repeating the published procedure[16] involving extraction with glacial acetic acid, that the preliminary precipitate obtained with acetone and sodium chloride and ordinarily discarded, was of essentially the same adrenocorticotropic activity as the subsequent ether precipitate. The modified extraction and isolation procedures described here avoid this loss.

Results of experiments on the purification with oxycellulose of corticotropin concentrates prepared by the methanolic acetic acid extraction procedure have closely paralleled those reported by Astwood, Raben, Payne and Grady[19]; and with this starting material, oxycellulose purification followed by pepsin digestion has constituted an excellent method for the preparation of corticotropin-B concentrates at an average activity level of about 80 u./mg. in yields ranging from 30 to 70%. Best results were obtained when 8% by weight of oxycellulose was used with the methanolic acetic acid extract.

In the pepsin digestion of corticotropin preparations of activity about 3 to 4 u./mg., a 24-hour reaction period with granular pepsin (Merck) gave optimum results. Extension of the digestion time to 48 hours produced no obvious change. These conditions also appeared to be optimal for the digestion of the more potent corticotropin fractions (60 to 100 u./mg.) purified by the use of oxycellulose. However, when crystalline pepsin (Armour Co.) was substituted for the granular enzyme, substantial losses of activity occurred.

Equally satisfactory results were obtained from commercial acid–acetone powder by the oxycellulose purification techniques; pepsin digestion was applied either before or after the oxycellulose step. When the oxycellulose method was applied to pepsin digests of commercial acid–acetone powder, the highest activities (70 to 80 u./mg.) resulted from the use of an amount of oxycellulose equal to about 50% of that of the crude hormone concentrate. It was necessary to employ an equal weight of oxycellulose when commercial acid–acetone powder was used without prior pepsin digestion. In this case, corticotropin concentrates ranging in activity from 60 to 100 u./mg. have been obtained in yields which varied from 50 to nearly 100%.

It should be noted that the examples presented in the Experimental section represent typical *good* preparations. Not infrequently, the activities and yields were lower than those described. These occasional bad results may be explained on a basis of inferior starting materials, inadvertent inactivation or misleading assay values.

At present, the most satisfactory method for obtaining corticotropin-B concentrates of about 60 to 100 u./mg. involves treating either the methanolic acetic acid extract of whole pituitary glands or the commercial acid–acetone powder with the proper amount of oxycellulose, and following this step by pepsin digestion and isolation of the fraction soluble in 5% trichloroacetic acid.

Experimental

A. Defatting of Fresh Frozen Glands.—One and one-half pounds of whole fresh frozen hog pituitary glands was

(20) E. B. Astwood and R. Tyslowitz, *Federation Proc.*, 1, 24 (1942).

(21) H. Holterman, *Farmakoterapi*, 1, 4 (1951).

(22) "Acthar-A," Armour Co., Chicago.

allowed to thaw partially while covered with acetone. The glands were then transferred, a small amount at a time, to a Waring blendor, covered with acetone and ground. When all the glands had been ground, the mixture was transferred to a beaker and stirred with two liters of acetone for two hours. After the solid had settled, the liquid was decanted. The solid was washed with two more two-liter portions of acetone, then with three two-liter portions of methanol. After a final washing with ether, the solid was allowed to dry in the air. The weight was 100 g., and the assay was 0.5 u./mg.[23]

B. **Extraction of Defatted Glands with Methanolic Acetic Acid.**—One hundred grams of defatted glands was placed in a 2-liter, three-necked flask fitted with mechanical stirrer, reflux condenser open to the atmosphere through a drierite tube, and heating mantle. To the flask was added 900 ml. of methanol, and then, with stirring, 600 ml. of glacial acetic acid, and the mixture was refluxed (*ca.* 75°) for two hours. At the end of this time, the contents were allowed to cool to room temperature and to settle. The mixture was centrifuged and the decantate collected. The residue was washed with a 40% solution of acetic acid in methanol and the washings were added to the collected decantate. The decantate was diluted with an equal volume of ether. After standing in the cold room the mixture was filtered, and the residue was washed with ether and dried. The weight of crude corticotropin was 12 g. and its activity was 3 u./mg. In other experiments, the products have given assay values of from 2 to 4 u./mg.

C. **Purification of Methanol-Acetic Acid Extract on Oxycellulose.**—Twelve grams of the precipitate obtained from the methanol–acetic acid extract (3 u./mg.) was stirred with 500 ml. of 0.1 *N* acetic acid. The mixture was centrifuged and the insoluble residue discarded. One gram of oxycellulose (10% carboxyl)[24] was washed with water, 1 *N* hydrochloric acid, water and 0.1 *N* acetic acid. The oxycellulose was then added to the solution and the mixture was stirred for 30 hours. At the end of this time, the oxycellulose was collected by centrifugation, and washed with 0.1 *N* acetic acid until the washings gave a negative biuret test. The oxycellulose was stirred for one hour with 30 ml. of 0.1 *N* hydrochloric acid and then filtered. This procedure was repeated and the hydrochloric acid eluates were collected and lyophilized. The weight of the corticotropin concentrate was 300 mg. and the potency, 85 u./mg. The recovery of activity was 70%.

D. **Pepsin Digestion of Oxycellulose-purified Methanolic Acetic Acid Extract.**—Two grams of the corticotropin (80 u./mg.) prepared by the oxycellulose purification of the methanol–acetic acid extract was taken up in 100 ml. of water and the solution was adjusted to *p*H 2.5. To the solution was added 8 mg. of pepsin (Merck granular), and the solution was allowed to stand for 24 hours at 38°. It was then heated to 95° for 15 minutes to inactivate the pepsin. After the solution had cooled to room temperature, 11.1 ml. of a 50% solution of trichloroacetic acid (1 ml. contained 0.5 g. of trichloroacetic acid) was added. The resulting mixture was centrifuged and the precipitate discarded. The decantate was washed six times with equal volumes of ether to remove the trichloroacetic acid. The ether was removed at the aspirator, and the solution was lyophilized. The yield of corticotropin-B concentrate was 1.7 g. of material of assay value, 80 u./mg. The recovery of activity was 85%.

E. **Pepsin Digestion of Commercial Acid–Acetone Powder.**—Forty-one grams of acid–acetone powder[22] of activity 3.5 u./mg. was taken up in one liter of water and the solution was adjusted to *p*H 2.5 with hydrochloric acid. To the solution was added 160 mg. of pepsin (Merck granular), and the mixture was stirred at 38° for 22 hours. The *p*H had risen to 3.1 by the end of 19 hours and then remained constant. After 22 hours, the mixture was heated to 95° for 15 minutes, then cooled to room temperature. Sixty grams of trichloroacetic acid in 200 ml. of water was added with stirring, and the mixture was centrifuged. The insoluble residue was discarded. The decantate was extracted five times with equal volumes of ether to remove the trichloroacetic acid. The ether was removed on the aspirator and the resulting solution was lyophilized. The crude corticotropin-B preparation weighed 23 g. and had an activity of 6 u./mg. The recovery of activity was 96%.

F. **Oxycellulose Purification of Pepsin-Digested Acid–Acetone Powder.**—A supernatant from the trichloroacetic acid precipitation treatment of pepsin-digested commercial acid–acetone powder was freed of ether and used directly in the oxycellulose step. To 1 l. of this solution, containing 25 to 30 g. of corticotropin-B concentrate at an activity of about 6 u./mg. was added 65 ml. of glacial acetic acid to make the concentration of acetic acid about 0.1 *N*. After the addition of 7 g. of oxycellulose, the mixture was stirred for 24 hours at room temperature. The oxycellulose was collected on a filter and washed five times with 100-ml. portions of 0.1 *N* acetic acid. The acetic acid eluates were saved. The oxycellulose was covered with 30 ml. of 0.1 *N* hydrochloric acid and the mixture stirred occasionally; after one-half hour the liquid was removed by filtration. Six such extractions were carried out, after which the *p*H of the combined hydrochloric acid extracts was adjusted to 3.0 by treatment with Amberlite IRA-400 (carbonate cycle). Lyophilization afforded 1.1 g. of a product of assay value 80 u./mg. The yield was thus about 50%. Treatment of the 0.1 *N* acetic acid filtrate described above with 7 g. of oxycellulose gave, after the same processing, 0.9 g. of material of potency 70 u./mg., representing a recovery of activity of about 40%. Thus, an almost quantitative over-all recovery of activity at the 70 to 80 u./mg. level was achieved.

G. **Purification of Commercial Acid–Acetone Powder on Oxycellulose.**—A 25-g. portion of commercial acid–acetone powder[22] having an activity of about 4.5 u./mg. was added to 1 l. of 0.1 *N* acetic acid and the mixture was stirred with 25 g. of oxycellulose for 20 hours. The oxycellulose was collected on a filter and thoroughly washed with 0.1 *N* acetic acid. It was slurried into 0.1 *N* hydrochloric acid with occasional stirring on the sintered glass filter over a half-hour period. After filtration, the oxycellulose was eluted twice more with 0.1 *N* hydrochloric acid. The combined filtrates were adjusted to *p*H 3.0 with IRA-400 (carbonate cycle) and lyophilized to give 973 mg. of a corticotropin concentrate of activity 100 u./mg. The recovery of activity was almost 90%.

H. **Pepsin Digestion of Corticotropin Prepared by Oxycellulose Purification of Commercial Acid–Acetone Powder.**—When 1 g. of a 100-u./mg. concentrate of corticotropin which had been purified by the oxycellulose treatment described above was digested with pepsin by the procedure described above, there was obtained 0.88 g. of a corticotropin-B concentrate active at 100 u./mg.

Acknowledgment.—The authors of this and of the following papers[2,3] are indebted to their associates in the Merck Institute for Therapeutic Research for the adrenocorticotropic assays reported.

RAHWAY, NEW JERSEY

(23) Adrenocorticotropic assays reported here and in the accompanying papers were carried out by a modification of the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury. A fuller description will be found in the article by N. G. Brink, F. A. Kuehl, Jr., M. A. P. Meisinger, M. N. Bishop and K. Folkers, THIS JOURNAL, **74**, 480 (1952). Values are reported in terms of U.S.P. corticotropin units, although a secondary house standard was used during most of this work. Because of the broad variations in the assay, all values should be regarded as approximate.

(24) The authors are indebted to Dr. R. W. Miller of Tennessee Eastman Co. for the gift of this material.