standard multidose technic of bioassay with appropriate experimental design and statistical analysis indicates that weak acid at high temperature induces no significant change in ACTH activity. The single-dose assay is useful for preliminary screening or for detecting relatively large changes in biological activity. However, failure to recognize the error of estimate of single-dose assays can be responsible for incorrect conclusions.

Summary. 1. ACTH activity disappears rapidly from neutral aqueous, 0.9% sodium chloride, or basic extracts of pituitary. Activity is stable in weak hydrochloric acid extracts of the gland. ACTH prepared by the acid-acetone technic(3) or a modification of same (4) is unstable in water; sodium phosphate and sodium chloride exert a stabilizing influence on biological activity. Purified ACTH is stable in weak hydrochloric acid solution. 2. Under the experimental conditions employed we have been unable to confirm the claim of Li that ACTH is "activated" by heating in weak hydrochloric acid solution.

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Adrenocorticotrophic Hormone: Preparation of Material with a High Degree of Biological Purity.* (19261)

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A simple technic has been devised for the isolation of adrenocorticotrophic hormone (ACTH) in good yield. Acid heat treatment of this product results in a preparation with a high degree of biological purity. The product, although chemically inhomogenous, will be of assistance to the clinician and the physiologist interested in determining the therapeutic, toxic and metabolic actions of ACTH rendered free from other biologically active pituitary hormones.

Technic for bioassay of ACTH. The preparations were assayed for ACTH activity by the adrenal ascorbic acid-depletion technic in hypophysectomized rats(1). Potency is expressed as U.S.P. units per mg. One U.S.P. unit equals one International Unit which is the activity of one mg of the International Standard Powder (La-1-A).

The lithium method for isolation of ACTH from pituitary. General principles of the technic. ACTH is soluble and stable in a 1:4 mixture of water and acetone at pH 1.5 whereas 80% of total pituitary solids are insoluble in acid acetone. Extraction of pituitary with this medium effects a 5-fold increase in ACTH activity. At pH 5.6 in the presence of salt, ACTH remains in solution whereas inactive material is precipitated; an additional 2-fold increase in ACTH activity is accomplished by this step. ACTH is recovered by increasing the concentration of acetone in the super-

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CHART 1. Lithium Technic for the Isolation of ACTH. 200 g dried, finely ground hog pituitary tissue—40000 U.S.P. units ACTH activity extracted 3 times with a total of 10000 ml of 80% acetone at pH 1.4 at room temperature.

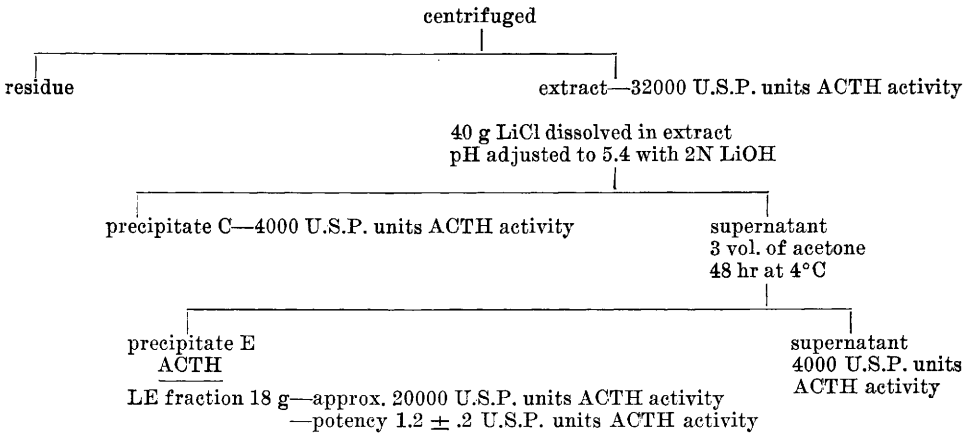

TABLE I. Potency and Yield of ACTH Isolated by the Lithium Technic.

No.	Starting material, g dried pituitary	Total ACTH activity in starting material, U.S.P. units × 1000	Wt LE fraction, mg	Potency of LE fraction, U.S.P. units ACTH activity/mg*	Total ACTH activity recovered in LE fraction, U.S.P. units*
LE-1	10, hog	2	938	.65 to 2	600 to 1900
2	10, hog	2	650	.5 to 2	300 to 1300
3	10, hog	2	900	1 to 1.4	900 to 1300
4	200, hog	40	18000	1 to 1.4	18000 to 23000
6	50, hog	10	2900	.75 to 1.5	2000 to 4500
7	50, beef	2.5 to 5	1500	.75 to 1.5	1000 to 2500

* The limits given in these columns reflect the uncertainty of the assays.

natant to 98%. Both sodium chloride and lithium chloride promote solubility of ACTH in 80% acetone at pH 5.6. However, the lithium salt is preferred since, in contrast to sodium chloride, it is soluble in 95% acetone.

Details of the technic. The following description is for 200 g of acetone-dried or lyophilized, finely ground, porcine pituitary powder. However, any convenient amount of tissue may be employed; the required quantities of the various reagents are proportional to the weight of starting material. Eight liters of acetone (C.P.) are mixed with 2 liters of 0.1 N hydrochloric acid. Four liters of this acid acetone are stirred with 200 g of pituitary tissue for 10 minutes; the pH is adjusted to 1.3 to 1.5 by the dropwise addition of concentrated hydrochloric acid. The mixture is stirred 45 minutes and centrifuged. The extract is collected and the residue is stirred with an additional 4 liters of acid acetone for 30 minutes and centrifuged The residue is extracted a third time with 2 liters of acid acetone for 15 minutes. The 3 extracts are combined, 40 g of lithium chloride are added and dissolved, and the pH is adjusted to 5.4 by the dropwise addition of 2 N lithium hydroxide. A flocculant precipitate forms. An hour later the precipitate is removed on a fluted filter paper. Three volumes of acetone are added to the supernatant, the mixture is stirred and allowed to stand in the refrigerator for 48 or more hours. A gummy precipitate forms and the supernatant liquid is easily siphoned off. The precipitate may be collected and dried by repeatedly washing in acetone or it may be dissolved in water and lyophilized. Chart 1 is a flow sheet of the procedure. Table I presents the yields and potencies of the ACTH preparations which have been obtained by the lithium procedure when applied to both porcine and bovine glands. The yield is approx-

imately 50% of the total ACTH activity in the pituitary powder; the potency is approximately one U.S.P. unit of ACTH activity per mg.

Biological purification of ACTH by acid heat treatment. Effect of acid heat treatment on ACTH activity. A 2% solution of LE-5 in 1.0 hydrochloric acid was exposed to boiling temperature for 30 minutes. Approximately 40% of the ACTH activity was destroyed by such treatment. (See Exp. 18 and 19, Table III of the accompanying paper) (2). It is apparent that ACTH is remarkably stable to acid heat treatment as compared to other adenohypophyseal hormones and posterior pituitary principles. This characteristic of the hormone is the basis of a simple technic for the preparation of biologically pure ACTH. As will be shown below, a 30-minute exposure to 100°C in 1.0 HCl destroys posterior pituitary antidiuretic principle and growth, gonadotrophic and thyrotrophic hormones. Acid heat treatment applied to the LE fraction produces an ACTH preparation with a very high degree of biological purity. Even in the largest doses employed clinically no responses characteristic of posterior pituitary principles or growth, gonadotrophic and thyrotrophic hormones may be expected to occur.

Effect of acid heat treatment on posterior pituitary antidiuretic principle. Antidiuretic activity was measured by inhibition of water diuresis in the rat. The technic employed was that described by Goodman and Gilman(3). Fig. 1 illustrates the results of an experiment designed to determine the effect of acid strength on the destruction of antidiuretic

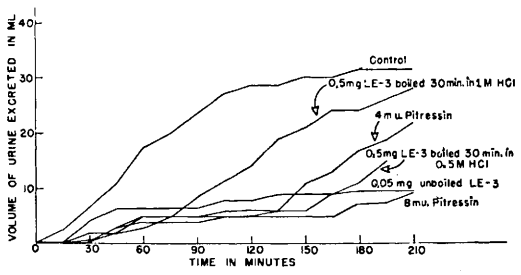

FIG. 1. Effect of strength of acid on degree of inactivation of antidiuretic activity in ACTH preparation.

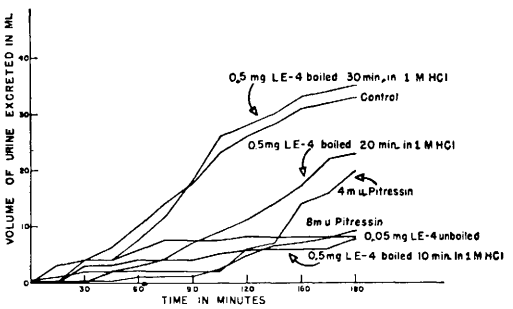

FIG. 2. Effect of duration of heating on degree of inactivation of antidiuretic activity in ACTH preparation.

activity. Solutions of LE-3 (2.0% solids) in 1.0 M HCl and in 0.5 M HCl were placed in a boiling water bath for 30 minutes. Untreated LE-3 in a dose of 0.05 mg and 8 milliunits of Pitressin induced a maximum or nearly maximum inhibition of a water diuresis. Boiling in 0.5 M HCl resulted in a loss of antidiuretic activity. The response induced by 0.5 mg of LE-3, boiled 30 minutes in 0.5 M HCl, was greater than that induced by 4 milliunits and less than that induced by 8 milliunits of Pitressin. This treatment may be estimated to destroy about 90% of posterior pituitary antidiuretic activity. After boiling in 1 M HCl, LE-3 produced a response less than that of 4 milliunits of Pitressin. This treatment is estimated to destroy 98% of antidiuretic activity.

Fig. 2 illustrates the effect of various times of exposure to heat upon posterior pituitary antidiuretic activity. A 10-minute exposure to boiling temperature in 1.0 M HCl had no detectable effect upon antidiuretic activity. A 20-minute exposure resulted in considerable loss (greater than 95% destruction). LE-3, exposed 30 minutes to heat, exhibited no antidiuretic activity; more than 98% of antidiuretic activity was destroyed, a result which confirms the experiment presented in Fig. 1. Each U.S.P. unit of ACTH activity of LE-3 exposed to boiling temperature for 30 minutes in 1.0 M HCl is associated with approximately 0.004 unit of posterior pituitary activity.

Effect of acid heat treatment on growth, gonadotrophic and thyrotrophic hormones. ACTH interferes with the response of the

TABLE II. Growth, Gonadotrophic and Thyrotrophic Activities of LE-5BL and Hog Pituitary Powder. LE-5BL—total dose 50 U.S.P. ACTH units. Pituitary powder—total dose 50 U.S.P. ACTH units. Hypophysectomized adrenalectomized rats—given .9% sodium chloride solution to drink and .1 mg cortisone acetate daily.

	Body wt, g	Testes wt, mg	Seminal vesicles wt, mg	Prostate wt, mg	I^{131} uptake by thyroid % injected dose
Male					
Hypox controls	—16 ± 3	1224 ± 44	52 ± 3.8	14 ± 1.2	1.40 ± .10
LE-5BL	—22 ± 4	1207 ± 56	54 ± 3.5	16 ± .8	1.75 ± .53
Pit. powder	+ 8 ± 6	2954 ± 68	425 ± 14.4	178 ± 7	13.05 ± .69
			Ovarian wt, mg	Uterus wt, mg	
Female					
Hypox controls	—10 ± 3		33 ± 3.1	74 ± 9.1	2.44 ± .33
LE-5BL	—14 ± 3		30 ± 1.5	86 ± 4.5	2.68 ± .19
Pit. powder	+ 8 ± 2		118 ± 8.4	251 ± 13.9	11.41 ± .91

hypophysectomized animal to growth(4) and to thyrotrophic hormone(5). Furthermore, increased secretory activity of the adrenal cortex interferes with the response of the secondary sex organs to sex steroids(6,7). Hence, an evaluation of the degree of contamination of ACTH preparations with growth, gonadotrophic and thyrotrophic hormones must be conducted in hypophysectomized adrenalectomized rats.

Hypophysectomized adrenalectomized rats were given 1.0% sodium chloride solution to drink and injected with 0.1 mg of cortisone daily.† Hypophysectomy was followed immediately by adrenalectomy. Injections were started 24 hours after operation. The total dose was divided into 30 equal aliquots injected thrice daily for 10 days. The animals were sacrificed the morning after the last injection. The gonads and accessories were weighed. The uptake of I^{131} by the thyroid was measured by a technic described by Ghosh *et al.*(8). The animals were weighed daily during the injection period. Table II presents the results of assay of LE-5BL (30-minute exposure to boiling temperature in 1.0 M HCl) for growth, thyrotrophic and gonadotrophic hormones. The relatively enormous total dose of 50 U.S.P. ACTH units of LE-5BL induced no growth, no increase in weight of the secondary sex organs and no increase in I^{131} uptake by the thyroid, whereas a total dose of 50 U.S.P. ACTH units of dried porcine pituitary induced definite responses characteristic of growth, gonadotrophic and thyrotrophic hormones. The sensitivity of the hypophysectomized adrenalectomized rat may be judged from the observation that a combination of 0.6 mg of a potent growth hormone preparation‡ plus 0.2 mg of thyrotrophin§ plus 5 International Units of pregnant mares' serum produced very definite responses as measured by the 3 indices described above. LE-5BL has a very high degree of biological purity.

Discussion. The lithium method is a simple, inexpensive technic for the preparation of ACTH in high yield. Only three steps are involved in contrast to the large number of manipulations in the method of Sayers *et al.* (9) and of Li *et al.*(10). The low yields of the last two procedures are in large measure due to inactivation as a result of exposure to neutral or alkaline reaction, to losses on dialysis and to incomplete precipitation of ACTH activity by adjustment of pH or salt concentration. The lithium method compares favorably with the glacial acetic acid technic of Payne *et al.*(11).

Exposure of a solution of ACTH in 1.0 M hydrochloric acid to high temperature effectively eliminates posterior pituitary, growth,

† The technic has recently been modified. Hypophysectomized adrenalectomized rats are now maintained with one 15-mg pellet of desoxycorticosterone acetate and given water to drink.

‡ Kindly supplied by Dr. Irby Bundy of Armour Laboratories.

§ Parke-Davis Rx 099802, kindly supplied by Mr. L. W. Donaldson of Parke-Davis and Co.

gonadotrophic and thyrotrophic contaminations. It is likely that acid heat treatment also reduces antigenic activity of the preparation. Undoubtedly, less drastic treatment than that employed in the present studies would still reduce contamination to a clinically acceptable level; this would result in less destruction of ACTH activity and give higher yields. The exact conditions to be employed must await the establishment of limits of contamination of clinically useful preparations of ACTH.

The lithium technic provides starting material suitable for further purification studies on ACTH. LE fractions, obtained from hog and beef pituitary, when processed by the oxycellulose technic of Astwood *et al.*(12), had ACTH activity equal to 10 to 20 U.S.P. units per mg.

Both porcine and bovine pituitary may be employed for the preparation of ACTH. Porcine glands are a potent source of ACTH; each mg of dry porcine pituitary has about 0.2 U.S.P. unit of ACTH activity. In our experience, bovine glands are less potent and more variable in activity; the potency of bovine pituitary has varied from 0.01 to 0.1 U.S.P. unit per mg. Purification of pituitary of sheep has not yet been tried. The one sample of glands of this species which we have assayed by a single-dose test indicated the potency to be about one-quarter that of porcine glands.

The preparations of ACTH obtained by the lithium technic are electrophoretically inhomogenous. Since there appears to be no correlation between biological potency and electrophoretic homogeneity(13,14), no great significance may be attached to this fact.

Summary. A technic has been described for the preparation of ACTH in good yield and in potency acceptable for clinical use. Exposure of a solution of the product in 100°C for 30 minutes results in a preparation with a high degree of biological purity. Hypophysectomized adrenalectomized rats have been employed for the bioassay of growth, thyrotrophic and gonadotrophic hormones which may contaminate ACTH.

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