experiment in which essential fatty acids were also available.

In attempting to account for the disparity in the results in the two laboratories, one might conjecture that the deficiency symptoms produced by MacKay and Barnes were primarily those of pyridoxine lack. No evidence was presented by them that the symptoms would respond to the administration of biotin. The method of producing the manifestations over so prolonged a period would further leave their interpretation open to question. It has been the experience of several laboratories(7,8) that the sex and initial weight of the rats placed on egg white rations are very important factors in the development of the characteristic egg white syndrome. Male rats show earlier and more marked manifestations than females. Gradations in depletion may also be seen between groups started at 35, 45 and 55 g initial weight respectively; the larger the animals, the more resistant they are to the onset of the characteristic signs of depletion. Hence, it may be significant that the rats of MacKay and Barnes were 40-day-old females with an initial body weight of about 70 g, whereas the rats in the present study were 21-day-old males with an average body weight of 37 g. Inasmuch as the source of pyridoxine in the basal ration of MacKay and Barnes was not the pure vitamin but 5% of yeast, the degree of its availability was not established. The exact requirement would also be conditioned by the amino acids furnished by the basal ration.

Summary. Parenteral doses of pyridoxine failed to cure or to delay the onset of the egg white syndrome in rats on a ration which included the essential fatty acids. The assumption by others, therefore, that ". . . pyridoxine and essential fatty acids act mutually to enhance or replace biotin," is not supported by these experiments with the rat.

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Adrenocorticotrophic Hormone: Stability Studies.* (19260)

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The effects of hydrogen ion concentration, ionic strength and temperature upon the biological activity of adrenocorticotrophic hormone (ACTH) have been investigated in order to define conditions which may be expected to produce little or no inactivation of the hormone. Stability data are obviously of considerable practical importance for the successful isolation of the hormone from pituitary, for the evaluation of its potency by bioassay, and for the preparation of solutions suitable for clinical use. In addition, conditions which inactivate pituitary contaminants and which have little or no effect upon ACTH activity can be applied to the biological puri-

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TABLE I. Stability Studies on ACTH Crude Extracts of Pituitary. Single-Dose Assays. All extracts, 1 g of dry pituitary per 100 ml of extraction fluid.

Exp. No.	Preparation and treatment, hog pit.	Dose in μg	Individual responses*	Avg response	Estimated change in activity, %
1	Extracted with .9% NaCl-.01 N NaOH at 4°C for 10 min	5	143, 93, 123, 99, 102, 125, 100	112	
	Extracted with water at room temp., 3 hr	50	49, -13, -17, -58, 18, 24	1	>-98
2	Extracted with .9% NaCl-.01 N NaOH at 4°C for 10 min	5	116, 101, 118, 86, 195	123	
	Extracted with water at pH 7 at room temp. for 10 min—stored at 4°C, 24 hr	5	-4, 28, 0, 4, -16, -6	1	>-90
	Extracted with water at pH 8 at room temp. for 10 min—stored at 4°C, 24 hr	5	-17, 9, -9, -46, -1, -27	-15	>-90
3	Extracted with .9% NaCl-.01 N NaOH at room temp., 30 min	5	104, 129, 143	125	
	Extracted with water at room temp., 30 min	7.5	-5, 23, -8, -11, 47	9	>-90
4	Extracted with .9% NaCl-.01 N NaOH at room temp., 10 min	5	107, 150, 96, 109, 105, 74, 62	100	
	Same for 30 min	5	87, 94, 80, 95, 30, 14, 119	74	ca-50
	" " 180 min	5	11, 46, 34, -27, 26, 10, 2	15	>-80
5	Extracted with .9% NaCl-.01 N NaOH at 4°C, 10 min	5	103, 191, 160, 136, 79, 191	143	
	Extracted at pH 1 (HCl) at room temp., 10 min—stored 24 hr at room temp.	5	180, 177, 148, 111, 143, 166	154	No sign. change
	Extracted at pH .1 (HCl) at room temp., 10 min—stored 24 hr at room temp.	5	111, 149, 142, 77, 120	120	0 to -50
	Extracted at pH .1 (HCl) at 85°C, 10 min	5	139, 83, 108, 109	110	0 to -50
6	Extracted with .9% NaCl-.01 N HCl, room temp., 10 min	10	103, 198, 166, 120, 124, 116	138	
		2.5	90, 106, 105, 94, 83	96	
	Same extract stored 35 days at 4°C	5	119, 144, 102, 96, 158, 130	125	No sign. change

* Concentration of ascorbic acid in left adrenal minus concentration in right adrenal.

fication of the hormone.

Activation of biological activity must be considered as at least theoretically possible. Li(1) has claimed that potency is enhanced by exposure of a solution of ACTH in weak acid to high temperature. This claim has been subjected to experimental test since it has important implications for practical problems of isolation and bioassay and for theoretical considerations of the chemical nature of the hormone.

Bioassay technic. ACTH activity was determined by the adrenal ascorbic acid-depletion technic in hypophysectomized rats as described by Sayers *et al.*(2). Estimates of potency, based on single-dose assays, are frequently in error by a factor of 2; in a few instances they may be in error by a factor of 4. Single-dose assays have an important role in preliminary screening of potency. For example, complete or nearly complete inactivation of ACTH activity can be correctly evaluated by single-dose assay technic. However, the determination of relatively small changes in activity requires the application of multidose technic. Four to 8 rats at each of 2 or 3 dose levels are employed for standard and for unknown. The estimate of potency with standard error of the estimate is calculated by standard technics(2).

Stability studies. (1) *Crude extracts of pituitary.* The ACTH activity of fresh pituitary remains unchanged for a number of hours after removal of the gland from the animal; the temperature may vary from 4°C to 37°C. However, as soon as cell structure is destroyed, for example, by homogenization, activity rapidly disappears. Fresh frozen pitui-

TABLE II. Stability Studies on Purified Preparations of ACTH. Single-Dose Assays. Concentration of ACTH in solution—20 μg per ml.

Exp. No.	Preparation and treatment, ACTH	Dose in μg	Individual responses*	Avg response	Estimated change in activity, %
7	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—assayed immediately	2	137, 102, 87, 191, 86	121	
	Sol. in water at pH 7—stored at room temp., 24 hr	10	30, 20, 0, 21	18	>-95
		2	5, 2, 22, 33	16	
	Sol. in water at pH 7—stored at 4°C, 24 hr	10	83, 36, 38, 29, 37	45	ca-95
		2	38, 9, 28, 10	21	
8	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—assayed immediately	1	82, 92, 83, 48	76	
	Sol. in HCl at pH 1—stored at 4°C, 24 hr	1	20, 129, 97, 57, 52	71	No sign. change
9	Sayers *et al.*(3). Sol. in .9% NaCl-.01 N NaOH—stored at 4°C, 24 hr	1	126, 118, 168, 92	126	
	Sol. in HCl at pH 1—stored at 4°C, 24 hr	1	129, 172, 80, 131, 86	120	No sign. change

* Concentration of ascorbic acid in left adrenal minus concentration in right adrenal.

tary rapidly loses ACTH activity when thawed even when the temperature does not rise above 4°C. It appears that ACTH and enzymes which destroy ACTH occupy different loci in the adenohypophyseal cell. Dry pituitary, suitable for the isolation of ACTH, can be prepared from fresh tissue by desiccating the glands in acetone or by lyophilization. Once frozen, the glands should not be allowed to thaw. If fresh frozen tissue is used as starting material for isolation of ACTH, it should be ground in the presence of an extraction medium such as acid acetone in which ACTH activity is stable.

The results of stability studies on crude extracts of porcine pituitary are presented in Table I (Exp. 1 to 6). A 0.9% sodium chloride solution, made to 0.01 N with sodium hydroxide, extracts ACTH activity from pituitary (Exp. 1 to 5). The activity of such an extract is relatively stable at 4°C. However, at room temperature, activity is almost entirely lost after 3 hours (Exp. 4). Sodium chloride exerts a stabilizing effect on purified preparations of ACTH at pH 7.0 or higher. There are no definitive experiments to prove the point, but it is most probable that sodium chloride also exerts the same stabilizing influence on ACTH activity in crude extracts.

Aqueous extracts of pituitary lose ACTH activity very rapidly at room temperature (Exp. 1, 2, 3). ACTH is very stable at pH 1.0 at room temperature (Exp. 5) and at 4°C (Exp. 6). Exposure of ACTH to pH 0.1 for 24 hours at room temperature or for 10 minutes at 80°C may have induced a slight loss in activity although the single dose assays are not accurate enough for definite conclusions on this point. From a practical point of view these stability studies indicate that isolation procedures should be conducted at a low pH. Activity may be expected to be lost rapidly at neutral or alkaline reaction at room temperature; low temperatures diminish but do not prevent loss of activity at high pH values.

(2) *Purified preparations.* Stability studies on preparations of ACTH made by the method of Sayers *et al.*(3) and by the lithium procedure(4) are presented in Table II (Exp. 7 to 9, single-dose assays) and Table III (Exp. 10 to 19, multidose assays).

ACTH is relatively stable in a 0.9% sodium chloride solution made to 0.01 N with NaOH (Exp. 7, 8, 9). In water at pH 7.0 (Exp. 7, 11) and at pH 13 activity is rapidly lost. At high pH values sodium chloride exerts a stabilizing influence upon ACTH activity (Exp. 13, 14). ACTH is stable at pH 1.0 at 4°C (Exp. 8, 9), at room temperature (Exp. 12) and at 100°C (Exp. 15, 16). The resistance of the hormone to destruction by strong acid at 100°C is remarkable (Exp. 17, 18); a 30-minute exposure at boiling tem-

TABLE III. Stability Studies on Purified Preparation of ACTH. Multidose Assays. Standard in solution at pH 1 (HCl).

Exp. No.	Preparation and treatment, ACTH	Conc. ACTH per ml	Estimated change in activity in %, ± stand. error
10	Sayers *et al.*(3). Veronal buffer pH 8.4 at room temp., 24 hr	10 μg	>−90
11	Sayers *et al.*(3). Acetate buffer pH 4 at room temp., 20 hr	10	−15 ±24
	Veronal buffer pH 8.3 at room temp., 20 hr	10	>−90
	Water pH 7.1 at room temp., 20 hr	10	>−90
12	Sayers *et al.*(3). Phosphate buffer pH 7.3 at room temp., 20 hr	10	−27 ±24
	pH 1 (HCl) at room temp., 20 hr	10	−15 ±28
13	Sayers *et al.*(3). pH 12.9 (NaOH) at room temp., 20 hr	10	>−90
	pH 12.6 (NaOH)—.9% NaCl at room temp., 20 hr	10	−82 ±9
14	Sayers *et al.*(3). .9% NaCl at pH 7.2 at room temp., 20 hr		−57 ±21
15	(Lithium). pH 1 (HCl) boiled one hr	20 mg	±13 ±22
16	Sayers *et al.*(3). pH 1 (HCl) boiled one hr	10 μg	−25 ±18
17	(Lithium). 1 M HCl boiled 30 min	20 mg	−37 ±21
18	(Lithium). 1 M HCl boiled 30 min	20	−39 ±9
	Lyophilization	20	+4 ±18
19	(Lithium). Lyophilization.	20	+2 ±17

perature of a solution of the hormone in 1.0 M hydrochloric acid destroys only 40% of activity. Although ACTH is stable in acetate buffer at pH 4.0 (Exp. 11) and in phosphate buffer at pH 7.3 (Exp. 12), it is unstable in veronal buffer at pH 8.4 (Exp. 10, 11).

It is of considerable practical importance to know that lyophilization has no effect upon ACTH activity (Exp. 18, 19). The pH of the medium was adjusted to 4.5 with sodium hydroxide before lyophilization.

Purified preparations of ACTH made by the technic of Sayers *et al.*(3) are precipitated from solution by 10% trichloracetic acid, by 10% tannic acid and by 1% tungstic acid. Single dose assays indicate that the precipitates are biologically active although no exact estimates of potency have been made. Crooke *et al.*(5) have also demonstrated that trichloracetic acid precipitates ACTH activity under certain conditions.

Discussion. The demonstrated stability of ACTH in acid provides a rational basis for the use of acetic acid(6,7) or weak hydrochloric acid(8) for the extraction of ACTH from pituitary. The rapid loss of ACTH activity from neutral aqueous extracts of pituitary suggests that the hormone undergoes enzymatic destruction. It is likely that the pituitary enzymes responsible for inactivation of the hormone are destroyed or inhibited by acid extraction media. The instability of purified ACTH at pH 7 or higher may in part be a result of proteolytic destruction. Indeed, it has recently been found that aqueous extracts of pituitary contain active proteinases (9). However, that instability can not be due entirely to proteinase activity is indicated by recent experiments in this laboratory(10) in which a purified preparation of ACTH was found to be unstable in water following acid heat treatment in 1.0 M hydrochloric acid at 100°C. It is unlikely that pituitary proteinase would survive such treatment. The mechanism by which sodium chloride inhibits ACTH inactivation at neutral or weakly alkaline reaction is unknown.

Weak hydrochloric acid is the medium of choice for the bioassay of ACTH. We use 0.01 N hydrochloric acid routinely in preparing solutions for ACTH assay. This strength of acid exerts no detectable toxic action in the test animal.

The present studies fail to confirm the claim of Li(1) that ACTH activity can be enhanced by exposure of a dilute acid solution to heat. The discrepancy may be due to the fact that Li employed sheep whereas we employed hog ACTH; the strength of acid was not identical. Actually, the results of the single-dose assays presented by Li are not in disagreement with the assay results of this report. It is in the evaluation of the data that the disagreement arises. The error of estimate of the single-dose assays does not indicate that significant activation occurred. Likewise, the

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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