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Increased Responsivity of Adrenals from Reserpine-Treated or Stressed Rats to Steroidogenic Activity of ACTH. (28894)

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In an attempt to determine whether catecholamines serve a permissive role for the steroidogenic action of adrenocorticotrophic hormone (ACTH) in the adrenal cortex, as has been reported for the lipolytic action of ACTH on the fat pad(1), adrenals from reserpine-treated rats were compared to glands from untreated rats with respect to their responsivity *in vitro* to steroidogenic agents. No evidence for a permissive role for catecholamines was obtained in the adrenal system, even when glands from resperine-treated rats were preincubated with acetylcholine and reserpine in an attempt further to deplete residual catecholamines from the adrenals(2).

However, an interesting difference in response to *in vitro* stimulation by ACTH, cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) or reduced triphosphopyridine nucleotide (NADPH) was shown by adrenals from reserpine-treated rats compared to those from untreated rats. The adrenals of the former group showed a considerably greater production of corticosteroids than those from untreated animals, in response to ACTH or cyclic adenosine 3′,5′-monophosphate (3′,5′-AMP), without change in the steroidogenic response to reduced triphosphopyridine nucleotide (NADPH). This reserpine effect appears to result from non-specific stress activation of adrenals *in vivo*, since injections of formaldehyde or exogenous ACTH reproduced the pattern of *in vitro* response observed following reserpine administration. The results of these investigations are presented here and discussed in connection with current concepts of the mechanism of action of ACTH on the adrenal cortex.

Experimental. Male Sprague-Dawley rats (135-150 g) were used throughout. Groups of animals were treated with one of the following regimens:

Reserpine (Serpasil, Ciba): administered in 2 daily injections for 2 days, 0.1 ml (0.25) mg, intraperitoneally on the first day and 0.05 ml (0.125 mg) intramuscularly on the second day.

When used *in vitro,* reserpine (Mann Research Lab., Inc., New York) was added to the incubation medium as a suspension in KRB to give a final concentration of 1 mg/ml.

ACTH: corticotrophin carboxymethylcellulose (Duracton, Nordic Biochemicals, Inc.); given for 2 days in 3 subcutaneous injections of 0.1 ml (4 U.S.P. units), once on the first day and twice on the second day. For *in vitro* incubations, a pure peptide from sheep pituitaries (α^s-ACTH, obtained from Dr. C. H. Li) was employed.

Formalin stress: a 10% solution of formaldehyde was given subcutaneously for 2 days, 0.1 ml on the first day and 2 injections of 0.2 ml each on the second day.

The response to *in vitro* steroidogenic agents by adrenals from the treated groups was compared with responses obtained in adrenals of untreated rats, which received no injections and were handled as little as possible.

TABLE I. Steroidogenic Action of ACTH, 3′, 5′-AMP and NADPH on Adrenal Bisects from Rats Treated with Reserpine, ACTH or Formaldehyde.

In vitro stimulus	*In vivo* treatment			
	None	Reserpine	ACTH	Formalin
	% increase in corticosteroid production			
ACTH (250 mU/ml)	136 ± 14 (12)*	268 ± 12 (23)	—	—
3′, 5′-AMP (15 mM)	184 ± 13 (14)	334 ± 14 (21)	305 ± 24 (7)	350 ± 23 (3)
Glucose-6-P (3 mg/ml) + NADP (2 mg/ml)	142 ± 16 (12)	168 ± 9 (20)	102 ± 11 (8)	160 ± 48 (3)

* Values are means ± S.E. No. of determinations in parentheses.

In all cases adrenals were removed on the third day under pentobarbital anesthesia 18 hours after the last injection. The glands were freed of fat, bisected and preincubated for one hour at 37° in 2 ml Krebs-Ringer bicarbonate (KRB) in the presence of 0.2% glucose(3) with 95% O_2-5% CO_2 as gas phase, 4 adrenal bisects being used per incubation vessel.

At the end of the preincubation period the tissue was transferred to 2 ml of fresh KRB (without glucose) followed by addition of: α^s-ACTH (500 mU in 0.05 ml 0.01 N HCl); 3′,5′-AMP (Sigma, 30 μM in 0.15 ml KRB, neutralized with NaOH); or glucose-6-phosphate (6 mg in 0.05 ml KRB) plus NADP (4 mg in 0.05 ml KRB neutralized with NaOH) added and a second incubation carried out for 3 hours. At the end of the second incubation the tissue was removed and the medium extracted twice with 4 ml methylene chloride, the solvent evaporated in a stream of nitrogen at 40°C, and corticosteroids in the extract estimated as blue tetrazolium chromogens by the procedure of Mader and Buck(4).

Results. Treatment with reserpine for 2 days resulted in an increase in adrenal weight from a control level of 28.6 ± 0.49 mg (71 rats) to 35.7 ± 0.25 mg (227 rats). The corticosteroid output of preincubated adrenal bisects incubated for 3 hours in the absence of a steroidogenic agent was 22.4 μg/100 mg adrenal tissue for the untreated group and 18.4 μg/100 mg for the reserpine-treated rats.

Addition of ACTH, 3′,5′-AMP or glucose-6-phosphate plus NADP to the incubation medium in concentrations sufficient to induce maximal steroid production in glands from untreated rats(5,6,7) resulted in the steroidogenic responses shown in Table I. It will be seen that the percent increase in corticosteroid output over that found in non-stimulated glands due to addition of either ACTH or 3′,5′-AMP is considerably greater in the reserpine-treated group than in the untreated. In contrast, the steroidogenic action of glucose-6-phosphate plus NADP is essentially the same in both groups of rats.

To determine whether the augmented response to ACTH and 3′,5′-AMP seen in the reserpine-treated group was a specific response to reserpine or merely the result of a stress induced release of endogenous ACTH, groups of rats were treated with a commercial long-acting preparation of ACTH (total dose 12 units) for 2 days or stressed by subcutaneous injection of 10% formaldehyde solution. The response of adrenal bisects from such animals to stimulation by 3′,5′-AMP or glucose-6-phosphate plus NADP is also shown in Table I, where it will be seen that the response to 3′,5′-AMP and NADPH is similar to that found in the reserpine-treated group.

Since the treatment with reserpine may not have completely depleted the adrenal of catecholamines, adrenal bisects from reserpine-treated rats were preincubated for one hour in 2 ml KRB (0.2% glucose) in the presence of reserpine (1 mg/ml) and acetylcholine in an attempt to deplete further residual catecholamines. Acetylcholine chloride was added at 15-minute intervals throughout the preincubation period, a total of four 0.5 μM (0.05 ml) additions. The adrenal bisects were then transferred to 2 ml of fresh KRB and incubated for a further 3 hours with and without addition of the steroidogenic agents as in the previous experiments. The *in vitro*

TABLE II. Effect of *in vitro* Pretreatment with Acetylcholine and Reserpine on Response of Adrenals from Reserpine-Treated Rats to ACTH, 3′, 5′-AMP and NADPH.

Stimulation	% increase in corticosteroid production	
	Without *in vitro* treatment	With *in vitro* treatment
ACTH (250 mU/ml)	281 ± 19 (7)*	302 ± 24 (8)
3′, 5′-AMP (15 mM)	339 ± 18 (7)	327 ± 32 (8)
Glucose-6-P (3 mg /ml) + NADP (2 mg/ml)	149 ± 17 (7)	200 ± 17 (7)

* Values are means ± S.E. No. of determinations in parentheses.

treatment with reserpine and acetylcholine did not prevent the steroidogenic action of either ACTH or 3′,5′-AMP (Table II).

Discussion. We were unable to demonstrate a permissive role for catecholamines in the steroidogenic action of ACTH on the adrenal cortex, as has been shown for ACTH effects on the epididymal fat pad(1). Despite an attempt to deplete residual catecholamines from the adrenals of reserpine-treated rats by additional *in vitro* treatment with acetylcholine and reserpine, the complete absence of catecholamines from the system was not definitely established. Accordingly the possibility that catecholamines in trace amounts may yet play a role in ACTH action upon the rat adrenals cannot be definitely excluded.

Independent of this uncertainty, the present evidence demonstrates that the adrenals of reserpine-treated rats exhibit an augmented steroidogenic response to stimulation by ACTH or 3′,5′-AMP. This effect appears to be the result of a non-specific stress reaction, since similar results are obtained when either ACTH or formalin is administered to intact rats for 2 days prior to removal of the adrenals. Increased *in vitro* responsivity to ACTH has been previously reported by Stark *et al* (8) who used adrenals from rats treated with ACTH for longer periods of time ranging from 5 to 14 days. Our results, which show that response to 3′,5′-AMP is also increased after a 2-day stress period, extend the findings of Stark *et al* and strongly suggest that adrenal response to stress involves activation of a rate-limiting step subsequent to 3′,5′-AMP generation.

The present results also show that ACTH action involves activation of systems other than those solely concerned with the generation of NADPH. The fact that stressed glands failed to show increased responsivity when presented with saturating amounts of glucose-6-phosphate and NADP indicates that in such glands ACTH (and 3′,5′-AMP) also have an effect to either (a) increase the availability of cholesterol (and other precursors) to the enzymes of the steroidogenic sequence; or (b) activate a "key" steroidogenic enzyme; or (c) increase the translocation of cofactor (NADPH) from intracellular sites where it is formed to the sites of enzymatic synthesis of corticosteroids. These possibilities have been previously discussed with regard to the locus of ACTH action in adrenal steroidogenesis(5,9,10,11,12). The finding that the adrenal response to saturation with NADPH is at a similar level in adrenals from both stressed and non-stressed rats suggests that the activities of the rate-limiting enzymes involved in transformation of cholesterol to corticosteroids have probably not been altered by previous stress treatment. There is no basis for assuming that cholesterol availability is increased in adrenals of stressed rats, but the possibility that a precursor other than cholesterol may be more available cannot be excluded(5,13). Similarly, the possibility that stress influences the cell so that translocations of cofactors and substrates are facilitated must be considered (12). Although our data do not clarify the precise loci of ACTH action, they lend support to the idea that the steroidogenic activity of ACTH involves a multiplicity of intracellular sites.

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Further Studies on Fluoride Absorption.* (28895)

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Fluoride absorption has been studied by several different approaches including isotope studies with labeled fluoride(1-3), metabolism studies utilizing urinary and fecal fluoride levels in comparison to skeletal retention values(4-7), and by using isolated intestinal segments in experimental animals(8-11). Earlier studies in our laboratories employing the latter technique confirmed other reports as to the rapid rate of fluoride absorption and further indicated that, in the rat, fluoride is absorbed not only in the stomach but also in the duodenum and small intestine. The present studies were conducted using the isolated intestinal segment technique to determine what effect anesthesia, starvation, ascorbic acid, and different inorganic ions have upon rate of fluoride absorption in the rat.

Experimental. Series I was designed to measure the rate of fluoride absorption from the entire length of the gastrointestinal tract of the albino rat. A total of 60 male albino Sprague-Dawley strain rats, ranging in weight from 70 to 90 g, were divided equally into 10 groups. The animals were starved for 30 hours before administration of fluoride. Each animal was given 1.0 mg of aqueous fluoride (as sodium fluoride) by means of a stomach tube. At 10 different periods following fluoride administration the animals were sacrificed and the entire gastrointestinal tract removed by the technique previously described(11). The tracts were placed in silica dishes, covered with exactly 1.0 g of fluoride-free calcium oxide, slurried with redistilled fluoride-free water, evaporated to dryness under infra-red lamps, and ashed in a muffle furnace at 600°C for 6 hours. Aliquots of the weighed ash were then analyzed for fluoride by perchloric acid distillation and titration of the distillate with thorium nitrate as described previously(12).

Series II was designed to study the effect of anesthesia upon rate of fluoride absorption. Eighteen female Sprague-Dawley strain albino rats weighing between 100 and 130 g were divided equally by weight into 3 groups. All of the animals were starved 24 hours prior to the study and were then given 1.0 mg of fluoride (as sodium fluoride) using stomach tube techniques. Animals in Group 1 were anesthetized with ether while those in Group 2 were anesthetized with sodium nembutal. Animals in Group 3 served as controls and were not anesthetized after fluoride administration. After a 2-hour absorption period, all animals were sacrificed and their entire gastrointestinal tracts ligated at the esophagus and rectum, and fluoride content determined as previously described.

Series III was designed to measure the effect of starvation upon fluoride absorption. Twenty Sprague-Dawley strain albino rats weighing between 70 and 90 g were divided equally into 2 groups. Group 1 was starved for 24 hours prior to administration of fluoride while Group 2 received a low-fluoride (0.8 μg/g) stock corn diet and distilled water *ad libitum*. Immediately after administration of 1.0 mg of aqueous fluoride (as sodium

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