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EFFECT OF ACTH ON THE TOTAL PREGNENOLONE CONTENT OF THE ADRENAL GLAND AND ITS SUBCELLULAR DISTRIBUTION

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SUMMARY

In the dog hypophysectomy caused a fall and close arterial infusion of ACTH resulted in a rise in the pregnenolone content of the adrenal gland. A study of the distribution of steroids in subcellular fractions of the dog adrenal gland showed that the largest total quantities (and concentrations per mg protein) were present in the mitochondria. The mitochondrial pregnenolone content was increased by ACTH. These findings do not support the hypothesis that end product inhibition by pregnenolone of its synthesis from cholesterol in the mitochondria plays a key role in the regulation of adrenal steroid synthesis.

INTRODUCTION

Pregnenolone is the first steroid with 21 carbon atoms which is synthesized in the adrenal gland from cholesterol and thus occupies a key position in the synthesis of the corticosteroids. The amount of pregnenolone present in the adrenal gland is very small; 5–10 nmol/g gland were found in the unstressed rat adrenal [1] which is about equivalent to the total amount of C-21 steroids secreted in 30 s. Therefore any stimuli which result in an increased secretion of corticosteroids must first of all stimulate pregnenolone synthesis.

Several years ago Koritz and Hall[2] suggested that pregnenolone controls the rate of its own synthesis from cholesterol inside the adrenal mitochondrion through end product inhibition. Thus an increase in the mitochondrial pregnenolone concentration would inhibit pregnenolone synthesis and consequently the synthesis of all major corticosteroids. It was suggested that ACTH relieved this block by increasing the permeability of the mitochondrial membrane to pregnenolone [2], allowing a decrease in mitochondrial pregnenolone content to occur, with consequent relief of the inhibition of pregnenolone synthesis.

In previous experiments we have observed that pregnenolone is also secreted by the adrenal gland of the dog, the pig [3, 4] and the rat [5] at rates which are approximately equivalent to 5% of the total corticosteroids secreted. Hypophysectomy has been found to decrease [3, 6] and ACTH to increase [6] adrenal pregnenolone secretion. This latter observation could be interpreted as a sign of the suggested increase in mitochondrial permeability to pregnenolone caused by ACTH.

The theory of Koritz and Hall was derived from experiments in which the conversion of [7α-^{3}H]-cholesterol to [7α-^{3}H]-pregnenolone was studied on extracts from an acetone-dried powder of beef adrenal mitochondria. If this end product inhibition occurs also in the intact adrenal gland, then, it was argued, the exposure of the gland to ACTH should result in a decrease of its pregnenolone content [7]. Contrary to this expectation Holzbauer and Newport[1] found in rats that stress caused a 2–3-fold rise in the pregnenolone content of the rat adrenal gland. Evidence against an inhibition of cholesterol side chain cleavage by accumulated pregnenolone was also obtained by Farese[8] on rat adrenal sections incubated in the presence of cyanoketone. Furthermore, experiments on isolated rat adrenal mitochondria have shown that ACTH does not alter the percentage of pregnenolone passing through the mitochondrial membrane [9]. On the other hand, Urquhart, Krall and Li[10] were able to design a dynamic model of steroid synthesis using the data of Koritz and Hall[2] (with some modifications) which seemed sufficient to account for the dynamics of ACTH-stimulated cortisol secretion.

To further investigate the validity of the theory, experiments were carried out in which the effect of intra-arterial infusion of small quantities of ACTH close to the adrenal gland on the adrenal content of pregnenolone and on steroid secretion was studied in hypophysectomized dogs. Furthermore the subcellular distribution of pregnenolone in intact dog adrenal tissue was studied which was incubated in the absence and presence of ACTH.

MATERIALS AND METHODS

In vivo *adrenal perfusion experiments*

Adult mongrel dogs of both sexes (15–38 kg) were anaesthetized with sodium pentobarbitone and hypophysectomized by the transbuccal method. The left

adrenal gland was then prepared for perfusion *in situ* with the dog's own blood, by a previously published method [11]. Two types of experiment were carried out. In the first type, an adrenal venous blood sample was collected for 10 min and then a small piece of adrenal tissue (150 mg) removed from the upper pole of the left adrenal gland. Five min later the infusion of ACTH (Parke–Davis, partially purified, 16–17 U/mg, dissolved in 0·9% sodium chloride containing 0·1% of bovine albumin and kept in a polypropylene beaker at 4°C for 2–3 h) was started into the arterial supply to the adrenal glands. The rate was adjusted to maintain the concentration of ACTH in the blood reaching the gland at 5 μU/ml. This concentration is equivalent to the resting values for ACTH which we found in undisturbed, non-hypophysectomized pigs. When the ACTH infusion had proceeded for 25 min a second adrenal venous blood sample was collected for 10 min and the rest of the left adrenal gland removed while the ACTH infusion continued.

In the second type of experiment, the ACTH infusion was started 30 min before the collection of the first sample, continued during the collection (10 min) of the first sample and whilst a piece of the left adrenal gland was removed; then the infusion was stopped and 30 min later, a second adrenal blood sample was collected and then the rest of the gland removed. This was done in order to see whether any accumulation of pregnenolone would occur when the stimulus ceased.

Effect of acute hypophysectomy on adrenal pregnenolone content

Six puppies (7·5–10 kg, less than 6 months old) were anaesthetized with sodium pentobarbitone. The sphenoid bone was then drilled away to expose the dura covering the pituitary gland. At the same time the abdomen was opened and the left adrenal gland taken out. Immediately thereafter, the pituitary gland was removed, and 30, 60 or 120 min later, the right adrenal gland taken.

Effect of ACTH on the subcellular distribution of pregnenolone in the adrenal gland

Both adrenal glands from three male dogs (15–27 kg) were rapidly removed and each gland divided into four pieces. Two pieces of the left gland were combined with two pieces of the right gland, the pieces were once more bisected and put into two flasks containing 10 ml of continuously oxygenated Krebs–Ringer with bicarbonate and glucose. Ten minutes later the Ringer solution was exchanged and the glands preincubated for 1 h at 37°C in a shaking incubation bath. After 1 h the incubation medium was again renewed. To one sample 0·25 mg of ACTH (Cortrosyn, Organon, synthetic ACTH, amino acids 1 24, 100 U/mg) were added, the second served as control. Incubation was continued for another hour. The gland pieces were then removed, rinsed, homogenized in 0·32M sucrose and the following subcellular fractions isolated by gradient centrifugation: P_1: nuclear fraction; P_2: mitochondrial fraction; P_3: microsomal fraction; S_3: high speed supernatant, and L: a "lipid" fraction. The procedures have been described in detail previously [12, 13].

Chemical procedures

Whole blood samples were extracted with ethyl acetate after dilution with one volume of water and addition of small quantities of [4-^{14}C]-pregnenolone, [4-^{14}C]-progesterone and [4-^{14}C]-cortisol in order to allow correction for losses. In the experiments in which the steroid content of whole adrenals was estimated, the gland tissue was homogenized, extracted in a mixture of water and ethyl acetate (approx. 1:5) and the radioactively labelled steroids added to the extracts.

The extracts were purified as described previously [4], the individual steroids separated by paper chromatography in two systems and quantitatively estimated by gas liquid chromatography [4]. The results were corrected for losses. Cortisol was measured in eluates of the corresponding regions of the first paper chromatogram by its reaction with blue tetrazolium.

The subcellular fractions were suspended in water in order to lyse the particles and the suspensions were extracted with ethyl acetate. Their steroid content was analyzed as described for the blood samples. For the estimation of "free" cholesterol, 5% of each fraction was removed and a tracer amount of [^{14}C]-cholesterol added. It was applied directly to paper and developed in the E_1 system of Eberlein and Bongiovanni[14] in which cholesterol travels with the solvent front. The steroid was then estimated by g.l.c. using 11β-OH-progesterone as internal standard. In order to assess the contamination of a given fraction with mitochondria, the activity of monoamine oxidase (EC 1.4.3.4., MAO), an enzyme which is restricted to the outer mitochondrial membrane [15] was estimated in small portions of each fraction taken before the ethyl acetate extraction. MAO activity was measured using kynuramine [16], tyramine [17] and dopamine as substrates. The protein content of the fractions was measured by the method of Lowry, Rosebrough, Farr and Randall[18]. For the final calculations it was assumed that all the pregnenolone found in the mitochondrial (P_2) fraction was actually associated with mitochondrial material. The results of all three MAO assays were taken into account. The results for the distribution of pregnenolone and cholesterol were corrected accordingly.

RESULTS

In vivo *adrenal perfusion experiments*

The mean values of the results obtained on *in vivo* perfused adrenal glands in hypophysectomized dogs are given in Table 1. In the experiments of group I, ACTH caused an increase in cortisol secretion

Table 1. Effect of ACTH on adrenal pregnenolone content and secretion

Experiment No. and mean b.wt.(kg)	Protocol of experiment	Time (min)	Mean steroid values ** Adrenal tissue Pregnenolone content (μg/g)	Adrenal venous blood Secretion rates (μg/g adrenal/min) Pregnenolone	Cortisol
I	Hypophysectomy	0	-	-	-
	Adrenal blood collection (S_1)	205-215	-	0.096 ± 0.028	0.8 ± 0.40
18.2 ± 1.1	Adrenal piece removed	225	0.54 ± 0.09	-	-
	ACTH infusion*	230-275	-	-	-
	Adrenal blood collection (S_2)	255-265	-	1.048 ± 0.295	2.1 ± 0.04
	Rest of adrenal removed	270	2.19 ± 0.48	-	-
II	Hypophysectomy	0	-	-	-
	Adrenal blood collection (S_1)	195-198	-	-	1.55 ± 0.37
26.6 ± 4.3	ACTH infusion started*	200	-	-	-
	Adrenal blood collection (S_2)	230-240	-	0.073 ± 0.022	3.10 ± 0.38
	Adrenal piece removed	245	0.66 ± 0.27	-	-
	ACTH infusion stopped	247	-	-	-
	Adrenal blood collection (S_3)	277-290	-	0.011 ± 0.003	1.19 ± 0.75
	Rest of adrenal removed	295	0.26 ± 0.07	-	-

* ACTH was infused as a solution containing 5 μu/ml at a rate of 5-6 ml/min.

** Mean values ± standard error of the mean (n = 4 in all instances).

which was accompanied by an increase in the pregnenolone content of the adrenal glands. In the second group cortisol secretion in response to ACTH was 50% faster than in group I whereas the gland content of pregnenolone was much smaller. Fifty minutes after cessation of the ACTH infusion it was about 60% lower than during the infusion. Thus, no accumulation of pregnenolone in the adrenal glands occurred after ACTH stimulation was stopped.

Effect of hypophysectomy on adrenal pregnenolone content

In six puppies the pregnenolone concentration of the left adrenal gland was measured after the animals had been exposed to the stress of cranial surgery and laparotomy, and that of the right adrenal gland at different time intervals after hypophysectomy. The adrenal pregnenolone concentration of each puppy showed a drop of more than 60% after hypophysectomy. This was accompanied by a similar fall in the adrenal progesterone concentration (Table 2).

Effect of ACTH on the subcellular distribution of pregnenolone

Figure 1 and Table 3 show the results of experiments in which pieces of dog adrenal glands were incubated in the presence and absence of ACTH. They were subsequently homogenized in 0·32 M sucrose and the subcellular fractions separated. When expressed as percentage of the total amount of pregnenolone present in the glands the largest quantities were found in the mitochondrial fraction with the exception of Experiment I, control gland (Table 3) in which the lipid fraction contained somewhat more. In all three experiments ACTH caused a rise in the pregnenolone concentrations of the mitochondrial fraction (see Fig. 1). In Experiments II and III there were also small increases in microsomal pregnenolone. In Experiments I and II it was increased about 7-fold, in Experiment III 1·5-fold. There was also a large increase in the total gland content of pregnenolone in all three cases. The amount of cortisol present in the gland tissue and incubation medium was increased by

Table 2. Effect of hypophysectomy on the adrenal concentrations of pregnenolone and progesterone. (Left gland removed 30–60 min after injection of sodium pentobarbitone and consequent exposure of the pituitary gland; right gland removed 30–120 min after hypophysectomy)

Puppy No.* sex and body weight	Time between hypophysectomy and removal of right adrenal gland (min)	Steroid content Pregnenolone μg/g tissue L. gland (control)	R. gland (after hypophysectomy)	Change %	Progesterone μg/g tissue L. gland (control)	R. gland (after hypophysectomy)	Change %
3; ♀ 7.5 kg	30	1.73	0.60	-65	2.65	0.31	-88
6; ♀ 8.0 kg	30	1.10	0.32	-71	4.31	2.92	-33
1; ♀ 8.8 kg	60	1.72	0.35	-80	6.00	0.30	-95
5; ♂ 9.0 kg	60	1.57	0.24	-85	5.23	0.75	-86
2; ♀ 10.0 kg	120	1.00	0.39	-61	3.74	0.18	-95
4; ♂ 7.8 kg	120	2.09	0.80	-62	lost	lost	-

* The numbers correspond to the sequence in which the experiments were carried out.

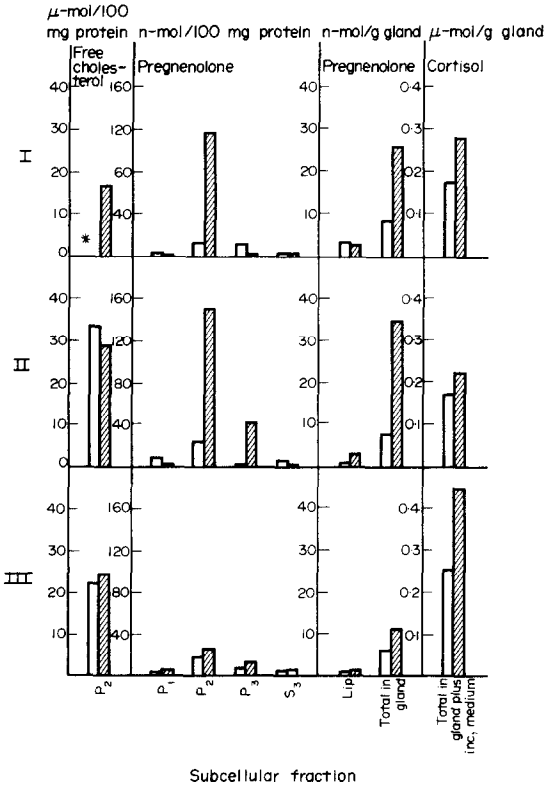

Fig. 1. Subcellular distribution of pregnenolone in dog adrenal glands incubated *in vitro* in the absence □ and presence of ACTH ■. Glands homogenized in 0·32 M sucrose. P_1: nuclear fraction (900 *g*, 5 min); P_2: mitochondrial fraction (12,000 *g*, 30 min); P_3 microsomal fraction (100,000 *g*, 60 min); S_3: high speed supernatant. Lip.: combined lipid layers; inc. medium: incubation medium. *: Lost.

ACTH by 64% in Experiment I, by 29% in Experiment II and by 77% in Experiment III. Figure 1 shows that the quantities of free cholesterol present in the mitochondrial fraction before ACTH stimulation can be about 1000 times larger than those of pregnenolone.

DISCUSSION

End product inhibition by pregnenolone of the side chain cleavage of cholesterol was widely accepted as being an important factor in steroid biosynthesis and although no longer considered as such by most steroid biochemists is often quoted by other workers. It was therefore felt that a reinvestigation of the problem under more physiological conditions might be purposeful.

The present experiments have demonstrated that ACTH in physiological quantities causes a rise in the pregnenolone content of the adrenal gland of hypophysectomized dogs and removing of the pituitary gland causes a fall. On *in vitro* incubated dog adrenal sections it was furthermore observed that the largest percentage of pregnenolone is present in the mitochondria and that ACTH can cause a large increase in mitochondrial pregnenolone. These findings in combination with previous *in vivo* [1] and *in vitro* [8, 9] observations make it unlikely that ACTH acts by increasing the permeability of the mitochondrial membrane to pregnenolone thus relieving cholesterol side chain cleavage from end product inhibition.

The theory of "end product inhibition by pregnenolone" was derived from experiments in which an extract of acetone dried powder of bovine adrenocortical mitochondria was incubated in the presence of 7-[^{3}H]-cholesterol and non-labelled pregnenolone at concentrations between 25 and 500 μg/100 mg dry powder. An inhibition of about 50% was achieved with 250 μg pregnenolone/100 mg dry powder [2]. We found however in the non-stimulated, intact dog adrenal gland only 0·5–2·5 μg pregnenolone/g gland and in the mitochondria about 3·5 μg pregnenolone/100 mg protein. In the ACTH-stimulated glands the

Table 3. Distribution of pregnenolone between the different subcellular fractions of dog adrenal homogenates. (Total amount of pregnenolone present in each fraction expressed as a percentage of the amount of pregnenolone contained in the whole gland)

		Fraction:				
		P_1 (%)	P_2 (%)	P_3 (%)	S_3 (%)	Li (%)
I	(C	4.5	26.7	16.7	14.5	37.5
	(A	<1.0	86.0	0.5	3.6	9.4
II	(C	20.6	54.8	0.4	15.7	8.5
	(A	<1.0	82.5	8.7	<1.0	8.7
III	(C	5.6	52.9	10.4	19.8	11.3
	(A	9.2	55.0	13.4	13.1	9.2
Dog '9'	L	<1.0	47.2	32.6	8.0	12.2
	R	3.7	57.3	25.4	6.3	7.4

Experiments I-III: glands cut into 8 pieces each and incubated in vitro for 2 h before homogenization. C: controls. A: incubated in the presence of ACTH.

Dog '9': glands homogenized immediately after removal from animal. L: left gland. R: right gland.

P_1: nuclear fraction; P_2: mitochondrial fraction; P_3: microsomal fraction; S_3: high speed supernatant; Li: lipids.

highest pregnenolone content found was 10 μg/g gland and 50 μg/100 mg protein in the mitochondria. There is also a discrepancy in the ratio between non-esterified cholesterol and pregnenolone between the experiments on extracts of dried mitochondria and the situation in the adrenal gland. We found in the freshly separated mitochondrial fractions a ratio of free cholesterol to pregnenolone between 140 and 1400 to 1. In the experiments with the dried mitochondrial powder the cholesterol concentration was equal to or even less than that of pregnenolone [2]. These major quantitiative differences between the intact gland and the acetone extracts of the gland may account for the differences in the results.

As pregnenolone is not stored in the adrenal gland, any increase in corticosteroid secretion must be preceded by an increase in pregnenolone synthesis. The increase in the pregnenolone content of the mitochondria after ACTH is the result of a slightly faster rate of pregnenolone synthesis than of pregnenolone metabolism and secretion of unconverted pregnenolone. Sometimes the difference between these rates is small and only little pregnenolone accumulates in the mitochondria, for example, Experiment III, Fig. 1.

As more pregnenolone is used in an ACTH stimulated gland, more has to pass through the mitochondrial membrane to reach the sites of its metabolism. Whether a modification of the physico-chemical properties of this membrane is required to allow the increased passage and whether ACTH causes this modification cannot be decided from the data available. If it were so, it would only constitute a minor factor in the mechanism of action by which ACTH stimulates adrenal steroid synthesis.

From all the data available at present it must be concluded that the main site of action of ACTH has to be sought in the chain of events which induce cholesterol side chain cleavage.

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