Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 205–215. Pergamon Press. Printed in Great Britain

TRANSFORMATION OF [^{14}C]-PREGNENOLONE AND PRODUCTION OF CORTICOSTEROIDS BY ADRENAL GLANDS FROM RATS BEARING A TRANSPLANTABLE MAMMOTROPIC PITUITARY TUMOR

ALEJANDRO F. de NICOLA, VILMA DAHL and SUSANA KAPLAN
Laboratorio de Esteroides, Instituto de Biologia y Medicina Experimental, and Servicio de Endocrinologia del Hospital de Niños, Buenos Aires, Argentina.

(*Received* 16 *August* 1972)

SUMMARY

Rats with transplants of Furth's mammotropic pituitary tumor (MtT-F4) have high plasma levels of ACTH, growth hormone and prolactin. Adrenals from normal rats and rats bearing the tumor for 2, 3 and 5 weeks were incubated with [^{14}C]-pregnenolone in a time-course study. MtT-F4 rats showed an inhibition in the conversion to aldosterone and 18-hydroxycorticosterone, with an increase in steroids produced mainly in the zona fasciculata-reticularis: corticosterone, deoxycorticosterone and 18-hydroxydeoxycorticosterone. Measurement of steroids in adrenal venous blood showed decreased aldosterone secretion ($P < 0{\cdot}001$ vs. controls at the 3rd and 5th weeks of the tumor growth) and an increase in corticosterone and deoxycorticosterone secretion at the 5th week ($P < 0{\cdot}001$ and $P < 0{\cdot}05$ vs controls, respectively). A proportional higher increment in deoxycorticosterone secretion at the 5th week was found. Determination of mitochondrial 11β-hydroxylation of deoxycorticosterone showed higher basal activity in MtT-F4 adrenals than in controls ($P < 0{\cdot}001$); however, Krebs cycle intermediates at 10 mM stimulated hydroxylation better in normal than in MtT-F4 mitochondria. The reaction was stimulated better in MtT-F4 than in controls by β-hydroxybutyrate and α-glycerophosphate. Thus, rats bearing the MtT-F4 showed (1) functional inhibition of the zona glomerulosa with concomitant hyperfunction of the fasciculata-reticularis, and (2) abnormal regulation of the mechanisms supporting 11β-hydroxylation. These changes may be due to the high levels of ACTH circulating in these animals.

INTRODUCTION

RECENT publications have dealt with the action of ACTH in promoting a change in the activity of 11β-hydroxylase, an enzyme present in adrenocortical mitochondria which converts deoxycorticosterone (DOC) into corticosterone. Some authors have reported an increased activity after ACTH was added to *in vitro* systems [1] or injected acutely into animals [2, 3]. Others, have found decreased 11β-hydroxylation of steroid substrates under conditions of chronic ACTH hypersecretion, such as adrenal regeneration or rats bearing a mammotropic, ACTH-secreting tumor [4, 5].

We have carried out a series of *in vitro* and *in vivo* experiments to further elucidate the hydroxylating capacity of the rat adrenal cortex under the influence of an ACTH, prolactin and growth hormone secreting tumor, Furth's MtT-F4 [6, 7]. The results suggest that sustained ACTH action on the adrenal results in decreased function of the zona glomerulosa and increased function of the inner

*Career Investigator, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

zones of the cortex. Preliminary evidence for a different regulation of the 11β-hydroxylating enzyme system in mitochondria from control and MtT-F4 rats is also presented.

MATERIALS AND METHODS

Fischer rats bearing the MtT-F4 tumor, as well as normal male and female rats of the same strain were obtained through the courtesy of Dr. R. W. Bates, National Institutes of Health, Bethesda, Maryland, U.S.A. The tumor was transplanted by suspending the cells in sterile 0·9% w/v saline and injecting the suspension into both hind legs subcutaneously, as described by Wherry *et al.* [8]. With this procedure, a tumor take of 100% was obtained.

For *in vitro* experiments, female rats were decapitated and the adrenals taken out, cleaned of fat and surrounding tissue and weighed. Control adrenals were quartered; the huge adrenals from tumor-bearing rats were divided into pieces of the same size as the quarters from the controls. Approximately 100 mg of tissue, obtained from 5 controls or 2 rats bearing the tumor for either 2, 3 or 5 weeks, was pre-incubated in 5 ml of Krebs–Ringer bicarbonate glucose buffer, for 45 min at 37°C under an atmosphere of 95% O_2–5% CO_2. The tissue was then transferred to beakers containing [4-^{14}C]-pregnenolone* (S.A. 50 mCi/mmol) 850,000 d.p.m./beaker. A time-course study of steroid synthesis was performed as described previously [9]. At 5, 15, 30, 60 and 120 min of incubation, aliquots of the medium were removed and replaced with fresh Krebs–Ringer without substrate; in the calculation of the conversion of pregnenolone into metabolites, this dilution factor was calculated according to Vinson [10]. To the withdrawn aliquots, pure standards of aldosterone, 18-hydroxydeoxycorticosterone (18-OH-DOC), corticosterone and DOC were added for correction of procedural losses. Steroids were extracted 3 times with dichloromethane and the extracts were chromatographed in the toluene-propylene glycol system for 20 h; the effluents were collected and chromatographed in the Bush A system. The areas in the toluene-propylene glycol chromatogram with $R_{corticosterone}$ 0·1, 0·3, 0·5 and 1·0, corresponding in mobility to 18-hydroxycorticosterone (18-OH-B), aldosterone, 18-OH-DOC and corticosterone, and the area in the Bush A system corresponding to DOC were eluted and the steroids characterized as described previously [11]. Briefly, 18-OH-B aldosterone and 18-OH-DOC were oxidized to the corresponding lactones and the derivatives isolated by chromatography; corticosterone and DOC were purified by acetylation and rechromatography. Quantitation was performed by liquid scintillation counting, and the radioactivity present in steroid products was corrected to 100% recovery by estimating the losses in the carriers added after incubation.

For *in vivo* experiments, the animals were anaesthetized with Nembutal ip (4 mg/100 g) and adrenal venous blood was collected by the technique of Vogt [12] in extraction tubes containing tracer amounts of [^{14}C]-labelled aldosterone, corticosterone, 18-OH-DOC, and DOC. After paper chromatography in the toluene-propylene glycol system, the first three compounds were measured by double isotope fluorimetry and the Porter-Silber methods, respectively

*Trivial names of steroids refer to the following compounds: 18-hydroxycorticosterone (18-OH-B) = 11β,20,21-trihydroxy-18,20-epoxy-4-pregnen-3-one; 18-hydroxydeoxycorticosterone (18-OH-DOC) = 20,21-dihydroxy-18,20-epoxy-4-pregnen-3-one; pregnenolone = 3β-hydroxy-5-pregnen-20-one.

[9]. DOC secretion was measured in some normal and MtT-F4 rats as follows: the overflow from the toluene-propylene glycol system was collected and chromatographed in the Bush A system; after elution from these chromatograms, DOC was measured by reduction of tetrazolium [9]. The results are expressed as μg of steroid secreted/2 adrenals/100 g body wt./h, as in previous studies [13].

For the assay of the 11β-hydroxylase activity, about 200 mg of adrenal tissue from control or MtT-F4 rats were homogenized in cold 0·25 M sucrose and the homogenates were centrifuged at 900 *g* for 10 min in an International Equipment centrifuge. Mitochondria were isolated from the 900 *g* supernatant by centrifugation at 5000 *g* for 15 min, as described by Péron and McCarthy [14], washed once in 0·25 M sucrose and resedimented at the same speed. The pellets were suspended in 50 mM Tris-HCl buffer and 0·2 ml aliquots added to incubation tubes containing the reaction mixture of Kowal [1]: 50 mM Tris-HCl buffer pH 7·4, 5 mM $MgCl_2$, 0·1% BSA and 50 μg of DOC in a total volume of 1 ml. The reaction was started by the addition of 0·2–0·3 mg of mitochondrial protein, and incubated for 10 min in air at 37°C with shaking. The resulting corticosterone was extracted with dichloromethane and measured by fluorimetry [9], and the results were expressed as μg of corticosterone formed/mg protein/10 min. With this procedure, zero time values for corticosterone were not higher than the blank. There was no measurable corticosterone bound to non-incubated normal or MtT-F4 adrenal mitochondria, nor was there any corticosterone formed during a 10 min incubation of mitochondria in the absence of DOC. The concentration of Krebs cycle intermediates and metabolites added to the medium was 10 mM. Protein was measured by the method of Lowry *et al.* [15].

All [^{14}C]-labelled steroids were purchased from New England Nuclear (Mass., U.S.A.), with the exception of [^{14}C]-18-OH-DOC, which was prepared biosynthetically [9]. The sodium salts of succinic, malic, pyruvic, α-ketoglutaric, isocitric, α-glycerophosphoric and β-hydroxybutyric acids were obtained from Sigma (St. Louis, Mo. U.S.A.).

RESULTS

1. *Transformation of* [^{14}C]-*pregnenolone*

In tumor-bearing rats, the adrenal weight/100 g of body wt., shows an almost linear increase from the 2nd to the 10th week after implantation. In our experience, however, most of the implanted animals died after 6 weeks.

Time-course incubations with pregnenolone were performed with adrenal tissue from normal female rats and from females bearing the MtT-F4 for 2, 3 and 5 weeks (Figs. 1–3). In control rats, corticosterone was the main product from pregnenolone after 30 min of incubation; in the control experiments shown in Figs. 2 and 3, DOC was second in prevalence to corticosterone, whereas in the control shown in Fig. 1, 18-OH-DOC was synthetized in larger quantities than DOC after 30 min of incubation. The course of incorporation of radioactivity into aldosterone and 18-OH-B was similar, and almost linear with time, in the three control experiments.

In order to perform a statistical analysis of the data shown in Figs. 1–3, the control values for each steroid were pooled and compared with the pooled data from rats bearing the MtT-F4. The mean d.p.m. $\times 10^{-3}$ and standard error for each steroid and p values for differences between the two groups are shown in Table 1. In rats bearing the tumor, the biosynthesis of aldosterone was sig-

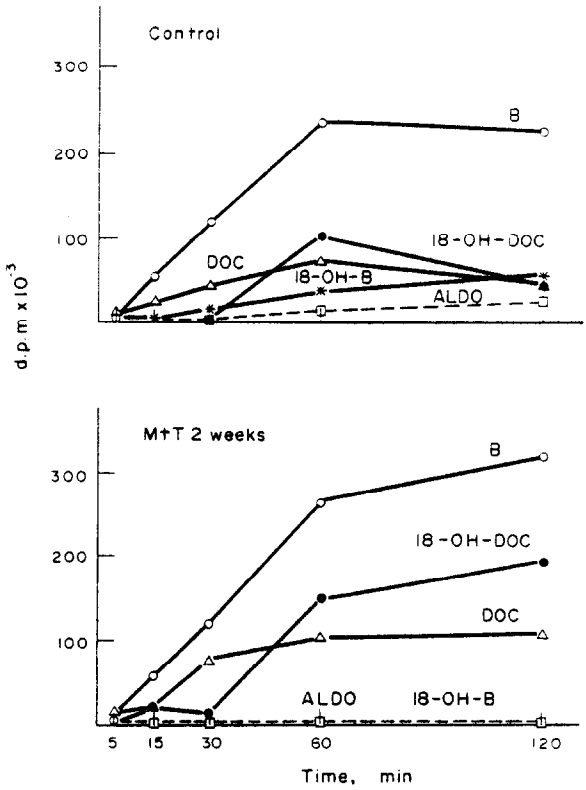

Fig. 1. Transformation of [^{14}C]-pregnenolone by adrenals from control rats (upper graph) and from rats bearing the MtT-F4 for 2 weeks (lower graph). One hundred mg tissue from either group was pre-incubated for 45 min and then incubated with labelled pregnenolone (850.000 d.p.m.) in Krebs–Ringer-bicarbonate-glucose buffer at 37°C under 95% O_2–5% CO_2; at the times specified in the abscissa, aliquots were removed from the medium and the steroids extracted and purified by derivative formation and chromatography. Dpm in the measured metabolites (ordinate) were corrected to 100% recovery by estimating the losses in non-radioactive carriers added after incubation. B = corticosterone; DOC = deoxycorticosterone; ALDO = aldosterone; 18-OH-DOC = 18-hydroxydeoxycorticosterone; 18-OH-B = 18-hydroxycorticosterone.

nificantly inhibited at 15, 30 and 120 min of incubation; 18-OH-B was decreased at 120 min. There was a concomitant significant increase at 120 min of compounds originating mainly, although not exclusively, in the zona fasciculata-reticularis: corticosterone, 18-OH-DOC and DOC. Besides, DOC in MtT was higher than in controls at 15 and 30 min, and 18-OH-DOC also increased significantly at 60 min of incubation with [^{14}C]-pregnenolone.

2. *Production of corticosteroids*

The results obtained after determination of steroids in adrenal venous blood of normal female rats and rats bearing the MtT-F4 for 3, 5, 7 and 9 1/2 weeks are shown in Fig. 4. The mean secretion rates of corticosterone, 18-OH-DOC and aldosterone in controls ($45{\cdot}8 \pm 5{\cdot}3$, $27{\cdot}7 \pm 6{\cdot}0$ and $0{\cdot}55 \pm 0{\cdot}21$ μg/2 adrenals/100 g body wt./h) were in the range of values obtained previously for female Wistar rats: $40{\cdot}1 \pm 6{\cdot}1$, $20{\cdot}3 \pm 1{\cdot}8$ and $0{\cdot}50 \pm 0{\cdot}05$ μg, respectively [9].

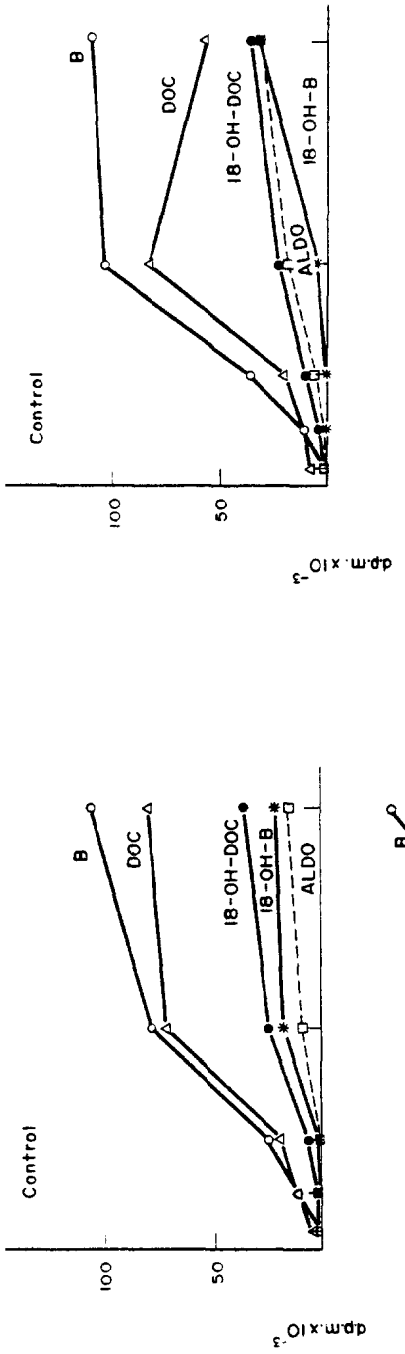

Fig. 2. Transformation of [14C]-pregnenolone by adrenals from control rats (upper graph) and from rats bearing the MtT-F4 for 3 weeks (lower graph). Incubation conditions and abbreviations as in Fig. 1.

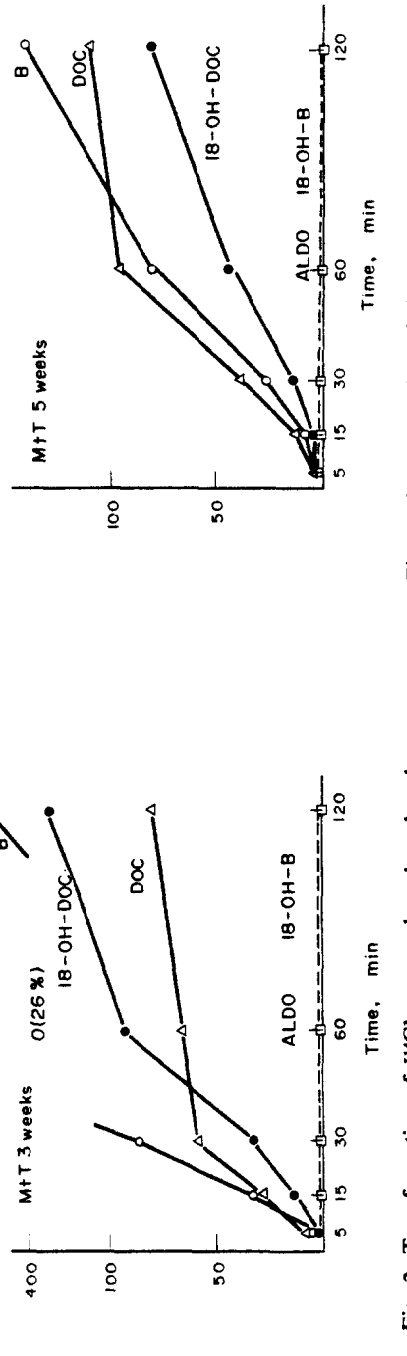

Fig. 3. Transformation of [14C]-pregnenolone by adrenals from control rats (upper graph) and from rats bearing the MtT-F4 for 5 weeks (lower graph). Incubation conditions and abbreviations as in Fig. 1.

Table 1. Statistical evaluation of the data from Figs. 1–3

Minutes of incubation with pregnenolone C^{14}	DOC		Corticosterone		18-OH-DOC		Aldosterone		18-OH-B	
	Control	MtT	Control	MtT	Control	MtT	Control	MtT	Control	MtT
5	6·13 ± 1·46	9·47 ± 1·24	3·04 ± 1·73	3·30 ± 0·67	0·96 ± 0·67	0·50 ± 0·34	0·65 ± 0·34	0·23 ± 0·15	0·19 ± 0·17	0·14 ± 0·10
15	15·78 ± 1·63	29·21 ± 2·56[2]	25·28 ± 4·59	33·13 ± 4·27	2·98 ± 0·78	12·27 ± 4·09	2·57 ± 0·45	0·98 ± 0·67[1]	2·27 ± 1·51	0·13 ± 0·15
30	31·64 ± 2·08	56·33 ± 3·57[3]	59·45 ± 9·14	76·87 ± 8·45	6·34 ± 2·15	19·16 ± 6·70	5·08 ± 1·70	0·700 ± 0·69	7·76 ± 7·19	0·32 ± 0·16
60	76·03 ± 3·64	87·96 ± 3·56	138·35 ± 15·8	188·28 ± 17·4	48·28 ± 8·04	93·21 ± 9·14[1]	13·99 ± 2·69	0·88 ± 0·51[3]	21·2 ± 10·45	1·69 ± 1·03
120	60·67 ± 3·21	97·01 ± 3·32	147·55 ± 12·8	293·91 ± 25·8[3]	38·50 ± 3·59	133·65 ± 9·89[4]	25·39 ± 4·73	1·13 ± 0·49[3]	36·88 ± 9·96	3·78 ± 2·04[1]

[1] $P < 0·05$; [2] $P < 0·02$; [3] $P < 0·01$; [4] $P < 0·001$, for differences between control rats and MtT-F4 rats. Data expressed as d.p.m. $\times 10^{-3}$ (mean ± SD).

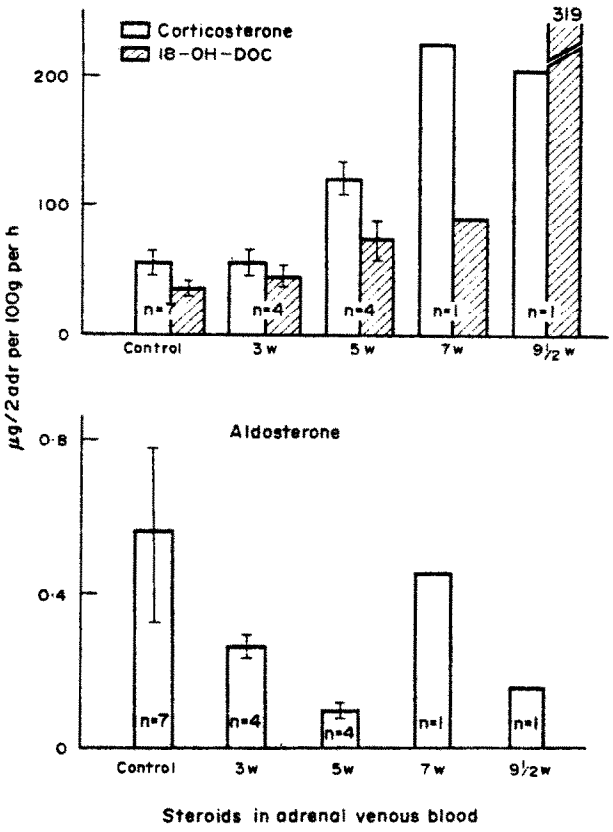

Fig. 4. Secretion of corticosteroids: Adrenal venous blood was collected from normal rats and rats bearing the MtT-F4 for the times indicated on the abscissa. The secretion rates (μg/2 adrenals/100 g body weight/h) of corticosterone, 18-OH-DOC (18-hydroxy-deoxycorticosterone) and aldosterone were determined after measurement of steroid levels by fluorimetry, Porter–Silber and double isotope methods, respectively.

Under the stress of laparotomy and hemorrhage for adrenal vein blood collection, the secretion rates of corticosterone and 18-OH-DOC were not greater than normal after 3 weeks of tumor implantation; however, corticosterone secretion was twice the control value after 5 weeks of tumor ($P < 0{\cdot}001$). The secretion of 18-OH-DOC at 5 weeks amounted to $55{\cdot}6 \pm 15{\cdot}6$ μg but the difference from controls was not significant ($P < 0{\cdot}1 > 0{\cdot}05$). There was a clear indication of an increase in both corticosterone and 18-OH-DOC in two rats studied after 7 and 9½ weeks of tumor growth; the increase in 18-OH-DOC levels in the last animal reversed the ratio corticosterone to 18-OH-DOC in adrenal venous blood.

Aldosterone secretion was markedly decreased after transplantation of the MtT-F4 ($P < 0{\cdot}001$ vs controls at 3 and 5 weeks), which indicated an impairment of the function of the zona glomerulosa. In the rats in which DOC was measured, there was almost a ten-fold elevation in animals bearing the tumor for 5 weeks: controls $14{\cdot}37 \pm 0{\cdot}45$ ($n = 5$), MtT $122{\cdot}68 \pm 40{\cdot}9$ ($n = 4$) μg/2 adrenals/100 g body wt./h, $P < 0{\cdot}05$.

3. *Role of mitochondria in the synthesis of corticosterone*

11β-Hydroxylation was studied by estimating the metabolism of DOC to corticosterone in mitochondrial preparations; the results of the enzyme assay, in μg corticosterone formed/mg mitochondrial protein/10 min, are presented in Table 2. The values obtained with male and female rats bearing the tumor for 3–6 weeks were pooled, since they were remarkably similar. Mitochondria incubated without Krebs cycle intermediates converted DOC to corticosterone, indicating that in our preparation the endogenous supply of substrates or reduced pyridine nucleotides was enough to support 11β-hydroxylation. This basal enzyme activity, that is, mitochondria incubated in the buffer mixture with DOC alone, was about 4-fold greater in tumor bearing rats; the increase was observed not only in the standard 10 min incubation but also at 5, 20 and 40 min. A basal enzyme activity was also obtained in MtT-F4 which was substantially higher than in controls when the amounts of mitochondrial protein added, increased from 60 to 600 μg per tube.

Table 2. 11β-Hydroxylation of deoxycorticosterone in adrenal mitochondria from control and MtT-F4 bearing rats, with and without the addition of Krebs cycle intermediates and metabolites to the medium

	μg corticosterone formed/mg protein/10 min		
additions	control	MtT-F4	*P*
—	1·00 ± 0·18 (10)	3·66 ± 0·55 (11)	< 0·001
succinate	3·57 ± 0·50 (6)	3·78 ± 0·39 (7)	NS
malate	12·13 ± 3·52 (6)	4·23 ± 0·52 (7)	< 0·05
pyruvate	12·46 ± 1·61 (4)	7·30 ± 0·72 (5)	< 0·02
isocitrate	23·53 ± 2·64 (6)	6·10 ± 1·79 (6)	< 0·001
α-ketoglutarate	38·11 ± 2·81 (7)	8·32 ± 1·47 (8)	< 0·001
α-glycerophosphate	2·20 ± 0·67 (4)	7·00 ± 1·27 (5)	< 0·02
β-hydroxybutyrate	4·81 ± 0·77 (4)	7·26 ± 0·38 (5)	< 0·02

The assay system for 11β-hydroxylation consisted of 50 mM Tris buffer pH 7·4, 5 mM $MgCl_2$, 0·1% BSA, 50 μg deoxycorticosterone and 0·2–0·3 mg mitochondrial protein, in a total volume of 1 ml. After incubation for 10 min at 37°C in air, the resulting corticosterone was extracted with dichloromethane and measured by fluorimetry. When added, the concentration of Krebs cycle intermediates and metabolites was 10 mM. Results are expressed as mean + SD; figures in brackets refer to number of observations.

In normal mitochondria, Krebs cycle intermediates and substrates were effective in supporting 11β-hydroxylation: the most active was α-ketoglutarate ($P < 0·001$ vs basal activity) and the least active was α-glycerophosphate ($P < 0·05$). On the other hand, in MtT-F4 mitochondria, neither succinate, nor malate or isocitrate, stimulated the hydroxylation of DOC significantly above basal levels. Higher rates were observed with α-ketoglutarate and pyruvate ($P < 0·01$ vs basal activity) but they were still below the levels achieved by control mitochondria. Cytoplasmic metabolites, like β-hydroxybutyrate and α-glycerophosphate, stimulated the reaction well: here the difference between controls and MtT-F4's was similar to the different rate of enzyme activity seen under basal conditions.

DISCUSSION

In rats bearing a mammotropic pituitary tumor, the plasma levels of ACTH, growth hormone and prolactin were found to be 6,000, 30 and 50 times that of normal plasma[7]. We have attempted to study in these chronically transplanted animals the effects that pharmacological levels of circulating ACTH have on the adrenal cortex. Recent publications have disclosed that in addition to acting upon the conversion of cholesterol to pregnenolone[16], ACTH also modifies the biosynthetic pathway after pregnenolone or progesterone[1–3, 17, 18].

Our experiments with the MtT-F4 seem to indicate that the function of the zona glomerulosa is greatly impaired, as revealed by decreased conversion of pregnenolone to aldosterone and 18-OH-B and lowered aldosterone secretion in adrenal venous blood. The inhibition of aldosterone and 18-OH-B synthesis was a consistent finding in the three incubations with MtT-F4 adrenals. A probable explanation for this effect would be that the high level of DOC that is generated suppress or by-pass the need for the zona glomerulosa to produce aldosterone.

The increase in the conversion of exogenous pregnenolone to corticosterone, 18-OH-DOC and DOC was also a constant finding in tissue from MtT-F4 rats. The secretion of corticosterone, 18-OH-DOC and DOC was elevated after prolonged implantation of the tumor. An imbalance in the secretory components from the zona fasciculata-reticularis existed in MtT-F4 rats, in the sense that at the 5th week we found a proportionally higher increase in the secretion rate of DOC. The finding of an extremely high output of 18-OH-DOC in a rat transplanted 9½ weeks before collection of blood, allows the study of the biological role of 18-OH-DOC in the rat.

Previous studies of adrenal steroid biosynthesis by the enlarged adrenals of rats with the MtT-F4 have shown an almost exclusive yield of 18-OH-B from progesterone[19] and recently, Brownie *et al.*[5] reported that in adrenal homogenates, progesterone yielded DOC but little corticosterone and 18-OH-DOC. The differences between our *in vitro* results and those of Brownie *et al.* and of Francois *et al.* might be explained by the quality of the substrate used. Whereas these workers incubated the adrenals with progesterone, we used pregnenolone. Although a pathway not requiring an obligatory route from pregnenolone → 5-pregnene-3,20-dione → progesterone might be operative in MtT adrenals, more definite studies are needed to establish this possibility.

In normal rat adrenal mitochondria, Krebs cycle intermediates efficiently stimulate steroid hydroxylations[14]. Under situations of high and chronic ACTH stimulation, as in rats bearing a MtT-F4 tumor, adrenal mitochondria showed an increase in basal enzyme activity, but a decrease in substrate-supported activity. These data corroborate the results of Brownie *et al.*[5], in that the hydroxylation of DOC to corticosterone stimulated by isocitrate is reduced in MtT adrenals. The enhanced basal activity found by us in the adrenals of MtT rats, as well as the increase in corticosterone synthesis from pregnenolone found with adrenal sections might be explained by increased intramitochondrial levels of Krebs cycle intermediates or reduced pyridine nucleotides, induced by ACTH.

β-Hydroxybutyrate, a ketonic body, and α-glycerophosphate, derived from fat hydrolysis, supported 11β-hydroxylation better in MtT adrenals than in controls, which suggests some connection between fat degradation and steroid synthesis. In this respect, it has been shown that ACTH activates adrenal lipase

activity[20]. The small effect of Krebs cycle intermediates in MtT-F4 adrenal mitochondria points out a defect in the transfer of reducing equivalents from the respiratory chain, or from NADH, to the P 450 chain. In turn, the P 450 chain itself might be defective, as suggested by Brownie *et al.*[5]. Hydroxylations in these abnormal mitochondria might still be stimulated by the so-called "shuttle mechanisms", composed of the β-hydroxybutyrate-acetoacetate and the α-glycerophosphate-dihydroxyacetone couples, which generate intramitochondrial NADH[21].

The fact that the enlarged adrenals from MtT-F4 rats secreted only twice as much corticosterone as the controls, in the presence of almost a ten-fold increase in DOC in adrenal venous blood, could indicate that this tissue has a partial deficiency in the 11β-hydroxylation. Alternatively, it is also possible that ACTH has a direct effect on cytoplasmic hydroxylations, for example the microsomal hydroxylation of progesterone at C-21 to DOC. McKerns[22] has presented evidence that ACTH stimulates glucose-6-phophate dehydrogenase, which increases NADPH, the cofactor for steroid hydroxylations. Considering the fact that mitochondria are impermeable to cytoplasmic NADPH, the excess cofactor may be used for the only extramitochondrial hydroxylation known to occur in the rat adrenal: the 21 hydroxylation*. Nevertheless, although mitochondria may be impermeable to NADPH, there are shuttle mechanisms: in the malate shuttle[23], cytoplasmic malic enzyme uses NADPH and pyruvate to generate malate, which rapidly penetrates the mitochondrial membranes. It is not known yet to what extent this pathway operates in MtT-F4 adrenal mitochondria.

ACKNOWLEDGEMENTS

We are deeply grateful to Dr. R. W. Bates for providing the MtT-F4 tumor and the Fischer rats. This work was supported by grants from the Consejo Nacional de Investigaciones Cientificas y Técnicas (No. 4182/69), Fundación Lucio Cherny, and Universidad de Buenos Aires. Miss S. Kaplan is the holder of a Predoctoral Fellowship from the Instituto Nacional de Farmacología y Bromatología. The technical assistance of Miss Julia Elena Lynch is gratefully acknowledged.

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*Such a mechanism might also explain the results of another study in which we demonstrated that small doses of SU-4885 and SU-5236 administered *in vivo* to rats stimulated the secretion of DOC without reducing the secretion of corticosterone or 18-OH-DOC[9].

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