J. steroid Biochem. Vol. 22, No. 2, pp. 249–256, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

THE ROLE OF ACTH IN DETERMINING THE METABOLIC PATHWAYS OF DEOXYCORTICOSTERONE BY NEWBORN RAT ADRENAL CELLS IN PRIMARY CULTURE

L. C. RAMIREZ,* P. BOURNOT and B. F. MAUME
Laboratoire de Biochimie des Interactions Cellulaires, Faculté des Sciences, Université de Dijon, B.P. 138, 21004 Dijon Cédex, France

(*Received* 4 *June* 1984)

Summary—The metabolism of deoxycorticosterone (DOC) by newborn rat adrenal cells in primary culture at various times after culture, with and without ACTH, was studied. After 5 days in culture before addition of ACTH, the main products of the metabolism of DOC were corticosterone and 18-hydroxy-11-deoxycorticosterone in a 2:1 ratio. Smaller amounts of 20α-dihydrocorticosterone and 18-hydroxycorticosterone were also found. No reduced metabolites of DOC were detected. Without ACTH the conversion of DOC to corticosterone and 18-hydroxyDOC declined rapidly. After 13 days in culture, this conversion accounted for only half the metabolites. The reductive metabolism of DOC which yields products reduced at 20α and/or 3α/β and 5α accounted for the other half. When ACTH (22 mU/ml) was added to the culture daily for several weeks, the primary metabolism of DOC remained that of 11β- and 18-hydroxylation yielding corticosterone and 18-hydroxyDOC. A minor reductive metabolism was found. Both cultures produced 6β-hydroxyDOC. These results demonstrate that ACTH is needed to maintain the efficiency of the 11β/18-hydroxylating system. They also show that ACTH controls the type of metabolism predominant in the rat adrenal cell and may be responsible for the balance between the biosynthesis of glucocorticoids and their reductive catabolism in the fasciculata zone of the adrenal gland.

INTRODUCTION

In vivo, in the adrenal gland, deoxycorticosterone (DOC)† is the immediate precursor of corticosterone and 18-hydroxy-11-deoxycorticosterone (18-hydroxyDOC). In primary monolayer cultures of newborn rat adrenocortical cells, developed in this laboratory [1] the main pathway in the biosynthesis of corticosterone and 18-hydroxyDOC is the 11β/18-hydroxylation of deoxycorticosterone, while a minor pathway goes through 11β- or 18-hydroxyprogesterone [2]. These cells in culture respond to ACTH with increased steroidogenesis from cholesterol supplied by the serum complemented medium [3–6]. The main steroids biosynthesized by ACTH stimulated cells are 11β- and 18-hydroxylated.

These results are in agreement with those of O'Hare and Neville[7] who have reported increased production of 11β- and 18-hydroxylated steroids in ACTH treated adult rat adrenocortical cells in culture and those of Salmenpera and Kahri[8] who reported the same in foetal rat adrenocortical tissue cultures. These previous studies suggest an increase in the activity of the 11β/18-hydroxylase system due to ACTH.

In order to study the regulation of the 11β/18-hydroxylating system in newborn rat fasciculata zone cells in culture, it was necessary to determine the different possible metabolic pathways of its principal substrate. Using deoxycorticosterone as added exogenous substrate, a quantitative analysis of its metabolites was undertaken. The effect of the age of the culture and of ACTH treatment on the metabolic pathways of DOC, corticosteroid biosynthesis and reductive catabolism are reported.

EXPERIMENTAL

Reagents

All solvents were pure for analysis quality (Merck, Darmstadt, F.R.G.). The following reagents were used: sodium borohydride from Merck, magnesium sulfate from Prolabo (Paris, France), *O*-methoxyamine hydrochloride from Pierce Chemical

*To whom correspondence should be addressed

†The following trivial names and abbreviations are used: deoxycorticosterone (DOC): 21-hydroxy-4-pregnene-3,20-dione; 11β-hydroxyprogesterone: 11β-hydroxy-4-pregnene-3,20-dione; 18-hydroxyprogesterone: 18-hydroxy-4-pregnene-3,20-dione; corticosterone (B): 11β,21-dihydroxy-4-pregnene-3,20-dione; 18-hydroxy-11-deoxycorticosterone (18-hydroxyDOC): 18,21-dihydroxy-4-pregnene-3,20-dione; 6β-hydroxyDOC: 6β,21-dihydroxy-4-pregnene-3,20-dione; 18-hydroxycorticosterone: 11β,18,21-trihydroxy-4-pregnene-3,20-dione; aldosterone: 11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al; 5α-dihydroDOC: 21-hydroxy-5α-pregnane-3,20-dione; 20α-dihydroDOC: 20α,21-dihydroxy-4-pregnen-3-one; 20α-dihydrocorticosterone: 11β,20α,21-trihydroxy-4-pregnen-3-one; 3α/β,5α-THDOC: 3α/β,21-dihydroxy-5α-pregnan-20-one; 3α/β,5α-THB: 3α/β,11β,21-trihydroxy-5α-pregnan-20-one; 3α/β,5α,20α-HHDOC: 5α-pregnane-3α/β,20α,21-triol.

Co. (Rockford, IL, U.S.A.), *bis*(trimethylsilyl)-trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) from Supelco (Bellefonte, PA, U.S.A.).

Reference steroids

Commercially available reference steroids were purchased from Makor Chemicals (Jerusalem, Israel), Ikapharm (Ramat-Gan, Israel) and Steraloids (Wilton, NH, U.S.A.).

Commercially unavailable 20α reduced steroids, 20α-dihydroDOC and 20α-dihydrocorticosterone were prepared by chemical reduction of the corresponding 20-oxo compounds. To 100 μg of substrate was added a suspension of 110 μg sodium borohydrate in 0.2 ml pyridine. The reaction mixture was left at room temperature for 4 h. After addition of water, the pyridine was evaporated and the steroids extracted from the aqueous phase three times with ethyl acetate. The ethyl acetate was dried with magnesium sulfate and evaporated. After derivative formation the mixture of 20α- and 20β-isomers was analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The isomers were identified by their retention times and mass spectra.

Labelled [4-^{14}C]deoxycorticosterone (sp. act., 55 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Cell cultures

The method used for the preparation of cell cultures was that previously described [5], adapted from the original method of Maume *et al.*[1, 3]. At 5 days the cells have attained confluency; the monolayer of cells attached to the dish bottom contained between 1 and 2×10^6 cells. The culture and incubation medium was of the following composition: Ham's F10 medium (Gibco, Paisley, U.K.), penicillin (100 U/ml), streptomycin (100 μg/ml), 10% of fetal calf serum (Gibco), and 10% newborn calf serum (Gibco). The dishes were kept in a culture oven at 37°C under an air–CO_2 (95:5, v/v) atmosphere saturated with water.

Incubations

Incubations were performed for 24 h periods starting on the 5th day of culture. The culture medium was replaced by 4.4 ml of new medium at 37°C at the beginning of the experimental period. Deoxycorticosterone (DOC) was added to each dish, 100 μg in 0.01 ml of ethanol. Adrenocorticotropin hormone (ACTH) from Choay (Paris, France) was added, 100 mU in 0.1 ml of medium without serum. The medium was changed every 24 h. The dishes involved in the ACTH experiments received a fresh dose of ACTH each time they were changed.

Steroid extractions and derivative formation

Steroid extraction was performed on Sep-Pak^R C_{18} columns from Waters Associates Inc. (Wilford, MA, U.S.A.) according to the method previously described [5]. Extraction yield was determined by addition of [4-^{14}C]DOC before extraction and measurement of the radioactivity extracted.

Preparation and purification of the methoxime–trimethylsilyl (MO–TMS) derivatives of the extracted steroids were performed as previously described [5], after addition of cholesteryl butyrate as internal standard. The methoxime formation of the extracts suspected of containing aldosterone was performed at room temperature for 12 h, followed by the usual trimethysilyl ether formation [9].

Thin-layer chromatography (TLC)

When necessary in order to obtain pure spectra for identification of compounds in the biological samples, a thin-layer purification was performed. Silica gel type 60 thin-layer plates from Merck (0.25 mm thick) with fluorescent indicator were used. They were washed once with ethanol and developed first in diisopropyl ether and then in chloroform–methanol (95:5, v/v). The desired zones were sonicated first in water and the steroids extracted with dichloromethane and then with ethyl acetate. Following extraction, they were derivatized and analyzed by the GC–MS method described below.

Gas–chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

Gas chromatography was carried out on 25 m long SE-30 wall-coated open tubular glass columns (0.21 mm internal diameter) made by Spiral (Dijon, France). A Packard model 427 chromatograph equipped with an all glass solid injector and a flame ionization detector was used. Injector temperature was 260°C; column temperature was programmed from 240 to 295°C at 1°C/min.

Quantitative measurements by GC were done using the response coefficients calculated for each steroid in a reference mixture with cholesteryl butyrate as internal standard [4]. Reference steroids not available were assumed to have a response coefficient equal to their closest homologues.

A Ribermag 10–10 quadrupole mass spectrometer (Nermag, Rueil-Malmaison, France) in the electron impact mode and a Finnigan 3300–1600 (Finnigan Corp., Sunnyvale, CA, U.S.A.) in the chemical ionization mode were used. They were both coupled to a capillary gas chromatographic column using helium as carrier gas.

RESULTS

The metabolism of DOC was determined in adrenocortical cells established and maintained in culture for 5 days in the absence of ACTH. Following this 5 day period, the culture was divided into two groups; one was left without ACTH while the other was treated with 22 mU ACTH/ml of culture medium. The metabolism of DOC followed through-

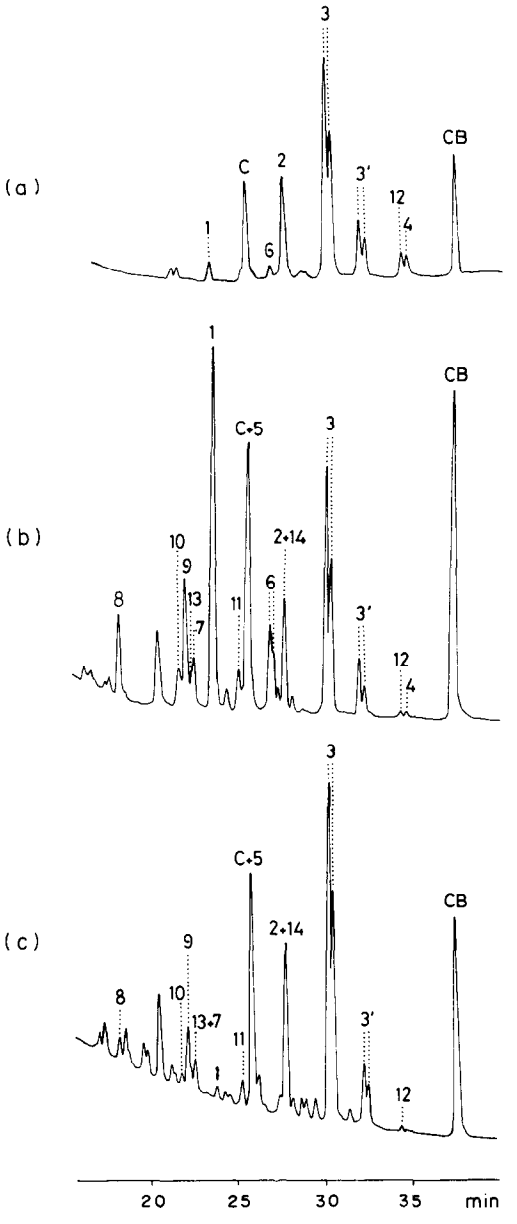

Fig. 1. Comparison of the GC recordings obtained during GC-MS analysis of the MO–TMS derivatives of the products of the incubation of DOC in: (a) a 5-day old culture, (b) a 13-day old culture and (c) a 13-day old culture treated with ACTH. Steroids indicated by numbers as listed in Table 1. Other compound codes as follow: C, cholesterol; 3', secondary peaks of corticosterone [4]; CB, cholesteryl butyrate.

out a 3 week period is presented below at 5 days and 13 days of culture.

Metabolism of DOC in 5-day old cultures

The GC–MS analysis of the products of the incubation of DOC in a 5-day old cell culture is shown in Fig. 1a. Five metabolites were identified by comparison of their retention times (Table 1) and mass spectra characteristics with those of reference steroids. The five metabolites are as follows: 20α-dihydroDOC (compound 6), 18-hydroxyDOC (compound 2), corticosterone (compound 3), 20α-dihydrocorticosterone (compound12), and 18-hydroxycorticosterone (compound 4), Figure 2a shows the mass spectrum of 20α-dihydroDOC (compound 6) as the methoxime–trimethylsilyl derivative. The base peak is at *m/z* 312 (M-103-90) and there is a prominent peak at *m/z* 402 (M-103). The abundant loss of the fragment of mass 103 is characteristic of a 20,21-di-trimethylsilyloxy structure [10]. The mass spectrum of 20α-dihydrocorticosterone (compound 12) is given in Fig. 2b. As in the spectra of 20α-dihydroDOC (Fig. 2a), the most prominent peaks are those comprising the loss of a fragment of mass 103. Figure 3 shows the mass spectrum of 18-hydroxycorticosterone (compound 4) as the methoxime–trimethylsilyl derivative. The most prominent peaks are at *m/z* 605 (M-31), 515 (M-31-90) and 484(M-152) as in the reference mass spectrum [11]. The fragment at *m/z* 484 (M-152) is specific of methoxime–trimethylsilylderivatives of 21-hydroxysteroids having a hydroxyl group in position 18 and a keto group in position 20 [12].

Metabolism of DOC in cell cultures kept without ACTH

The untreated cell cultures gradually lost most of their 11β/18-hydroxylating ability while the reduction metabolism of DOC increased. Figure 1b shows the GC recordings obtained during GC–MS analysis of the products of the incubation of DOC in a 13-day old untreated culture. In addition to the metabolites found after 5 days in culture, several other compounds were identified by comparison with reference steroids (Table 1). They are the products of the reductive metabolism of DOC and of corticosterone: 5α-dihydroDOC (compound 7), 3α, 5α-THDOC (compound 8), 3β,5α-THDOC (compound 9), 3α,5α,20α-HHDOC (compound 10), 3β,5α,20α-HHDOC (compound 11), 3α,5α-THB (compound 13) and 3β,5α-THB (compound 14). A metabolite eluted with cholesterol was identified as 6β-hydroxyDOC (compound 5) after TLC purification. Its mass spectrum is given in Fig. 4; the base peak at *m/z* 533 (M-15) and the fragment at *m/z* 501 (M-47) are characteristic of the 6-trimethylsilyloxy-3-methoxy-4-ene structure.

The quantities of steroids produced during 24 h at 5 days and 13 days of culture are given in Table 1. At 5 days the main products were corticosterone and 18-hydroxyDOC which amounted to 87.4% of the total metabolites. The only reduced metabolites were those reduced at position 20,20α-dihydroDOC and 20α-dihydrocorticosterone which represent 8.5%. At 13 days, the reduced metabolites accounted for 32.5% compared to 38.2% corticosterone and 18-hydroxyDOC. The reduced metabolites were composed of both 3α/β,5α-reduced (25.2%) and 20α-reduced (12.7%) compounds (Table 1). There is a large amount of unmetabolized DOC, 29.3%. After

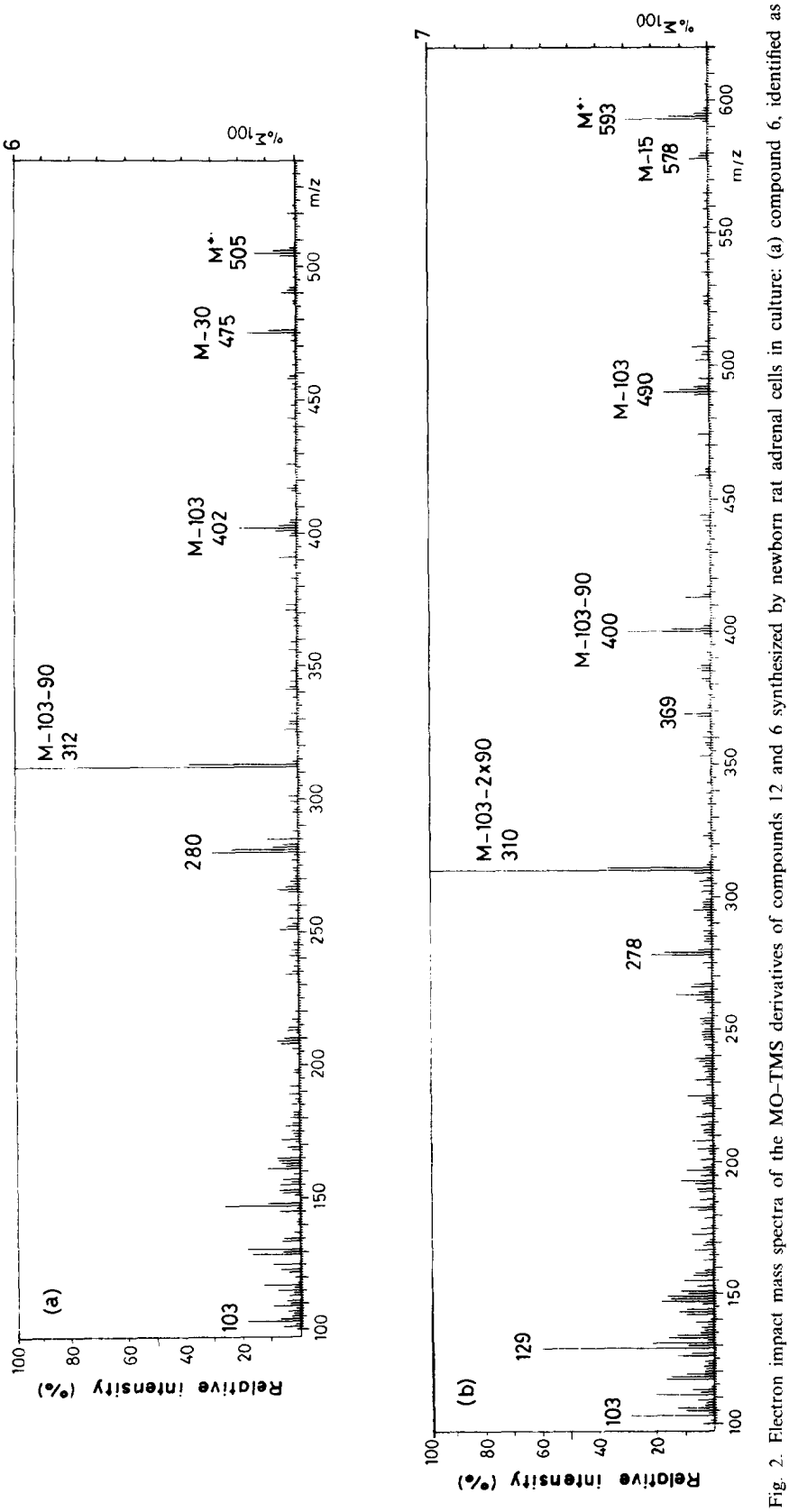

Fig. 2. Electron impact mass spectra of the MO–TMS derivatives of compounds 12 and 6 synthesized by newborn rat adrenal cells in culture: (a) compound 6, identified as 20α-dihydroDOC, (b) compound 12, identified as 20α-dihydrocorticosterone.

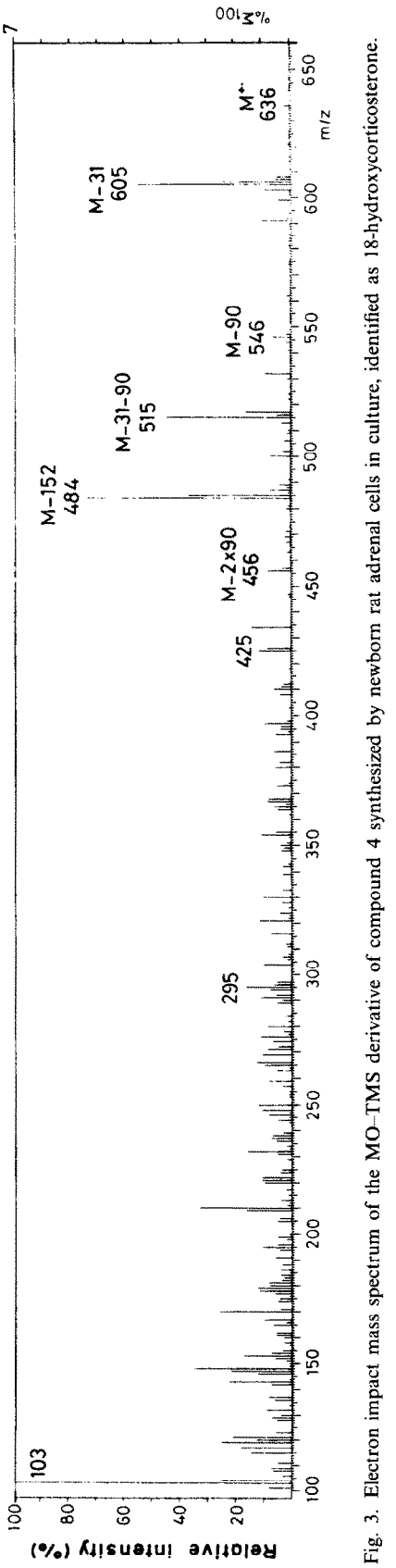

Fig. 3. Electron impact mass spectrum of the MO–TMS derivative of compound 4 synthesized by newborn rat adrenal cells in culture, identified as 18-hydroxycorticosterone.

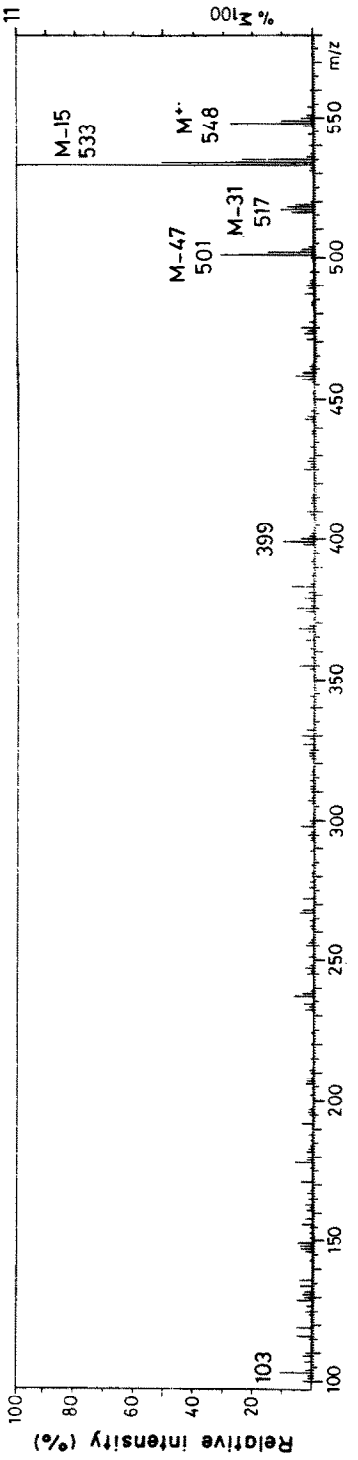

Fig. 4. Electron impact mass spectrum of the MO–TMS derivative of compound 5 synthesized by newborn rat adrenal cells in culture, identified as 6β-hydroxyDOC.

Table 1. Production of DOC metabolites (% of total steroids) at 5 days and 13 days after culture with and without ACTH

No.	Steroid	MU	Steroid production (% of total steroids) 5 days without ACTH	13 days without ACTH	13 days with ACTH
1	DOC	30.66	4.1	29.3	0.6
2	18-HydroxyDOC	31.66	28.2	11.8	26.9
3	Corticosterone	32.25* 32.31	55.8	22.6	50.8
4	18-Hydroxycorticosterone	33.43* 33.48	3.4	0.3	ND
5	6β-HydroxyDOC	31.10* 31.27	ND	3.5	4.0
6	20α-DihydroDOC	31.52* 31.55	3.7	7.0	0.2
7	5αDihydroDOC	30.38* 30.44	ND	3.9	1.9
8	3α,5α-THDOC	29.09	ND	4.1	2.3
9	3β,5α-THDOC	30.26	ND	5.2	3.6
10	3α,5α,20α-HHDOC	30.10	ND	2.3	1.8
11	3β,5α,20α-HHDOC	31.19	ND	3.1	2.6
12	20α-Dihydrocorticosterone	33.35* 33.42	4.8	0.3	0.3
13	3α,5α-THB	30.33	ND	2.9	2.1
14	3β,5α-THB	31.67	ND	3.7	2.9
	11β/18-Hydroxylated metabolites		87.4	38.2	81.6
	Reduced metabolites		8.5	32.5	17.6
	20α-Reduced metabolites		8.5	12.7	4.9
	5α-Reduced metabolites		0	25.2	17.1

Methylene units (MU) are given for MO–TMS derivatives chromatographed on capillary column as described in Experimental.
100 μg of DOC were incubated per dish for 24 h.
*syn and anti-isomers.
ND: not detected.

3 weeks in culture the reduced metabolites predominate accounting for over 50% of the products. The 11β/18-hydroxylated compounds account for no more than 8% of the products.

Metabolism of DOC in ACTH treated cell cultures

Cultures treated with ACTH retained their ability to metabolize DOC almost completely to corticosterone and 18-hydroxyDOC for over 3 weeks. The GC recordings obtained during analysis by GC–MS of the metabolites of DOC in a culture treated 1 week with ACTH is shown in Fig. 1c. The metabolites were the same as those produced by cells maintained over the same period of time without ACTH. However, there was almost no unmetabolized DOC and the reduced metabolites account for only 17.6% of the total metabolites while corticosterone and 18-hydroxyDOC represent 81.6% (Table 1). Comparison of the reduced metabolites at 5 days of culture and after 1 week of ACTH treatment shows that the 3α/β,5α-reduced compounds increased considerably (0–17.1%) while the 20α-reduced compounds decreased (8.5–4.9%). The proportion of the different metabolites is the same after 1 week and 3 weeks in culture with ACTH.

DISCUSSION

Newborn rat adrenal cells in primary culture conserve the metabolic functions of 11β- and 18-hydroxylation as well as those of reduction of the 20-oxo and 3-oxo-4-ene groups. They also express a 6β-hydroxylating activity never reported before in other adrenal cell culture systems. Two patterns of metabolism were evident as the culture aged with and without ACTH treatment. In the cell cultures that were not treated with ACTH, the metabolism evolved from that of almost total 11β/18-hydroxylation 5 days after culture to a predominantly reductive metabolism of DOC after several weeks. At 13 days after culture, there are approximately equal amounts of 11β/18-hydroxylated and reduced metabolites of DOC and corticosterone. In the cell cultures treated with ACTH, the predominant metabolism remains the 11β/18-hydroxylation of DOC throughout the life span of the cells. There is little 20α-reduction at 13 days of culture (7 days of ACTH) while 3α/β,5α-reduction is more important. In both cases there is increased 6β-hydroxylation. The evolution of the metabolism of DOC in untreated cultures from that of production of corticosterone and 18-hydroxy DOC to that of reduction could be explained by a decrease in 11β/18-steroid hydroxylase activity and/or an increase in the activity of the reductases.

It is evident from our results that ACTH is needed to maintain the high activity of the 11β/18-hydroxylating system, which otherwise diminishes rapidly as the culture ages. The stimulation of the 11β/18-hydroxylating system by ACTH has already been suggested from the study of endogenous steroid production in our cell culture system [6] and in foetal rat adrenal tissue cultures by Salmenpera and Kahri[8]. O'Hare and Neville[7] using radioactive DOC also found an increase in 11β/18-hydroxylation

due to ACTH in adult rat adrenal cells in culture; they reported no reduced products of DOC.

It is of interest to note that the conversion of DOC to corticosterone and 18-hydroxyDOC occurred in a ratio between 1.7 and 2.0 whatever the age of the culture or the treatment with ACTH. This reinforces the hypothesis first suggested by Rapp and Dahl[13] and strongly supported by Ulick[14] that the 11β- and 18-hydroxylating activities in the mitochondria of the adrenocortical cell are expressed by a single enzyme.

The increase in the reductive metabolism of DOC observed in relation to time of culture includes both the reduction of the 20-oxo group and that of the 3-oxo-4-ene group of ring A. These reductive activities are expressed by the adrenocortical cells in culture since fibroblast-like cell cultures originating from the outer part of the adrenal gland and cultured separately exhibit only minimal 5α- and 20α-reduction of exogenous progesterone (R. Raoux, personal communication). The corresponding metabolite from DOC (20α,21-dihydroxy-5α-pregnan-3-one) has not been detected in our cultures.

Both the 20-oxo and the 3-oxo-4-ene reductive activities are most likely alternate pathways in the metabolism of DOC which is available in larger amounts when there is less 11β/18-hydroxylation. Reduction of the 20-oxo group occurs in both culture groups but while it increases in the untreated cell cultures, it decreases in the ACTH treated cultures. A decrease of 20α-reduced progesterone metabolites during ACTH treatment has already been reported in newborn rat adrenal cells in culture[6] even though the biosynthesis of endogenous corticosteroids was increased. The present results reinforce the hypothesis that ACTH may control the importance of corticosteroid biosynthesis relative to the pathway of reductive catabolism. From a study of pregnenolone metabolism, O'Hare and Neville[7, 15] also suggested 20α-reduction as an alternate metabolic sequence when other biosynthetic enzymes were less active. However, in their adult rat cell cultures this 20α-reductive metabolism disappears completely under ACTH treatment whereas in our newborn rat cell cultures the metabolism of 20α-reduction of endogenous progesterone and of exogenous DOC persists even though it is minor. This could be linked to a basic 20α-oxido-reductase activity which is higher in newborn rat cell cultures than in those of adult cells. Ungar and Stabler[6] reported increased 20α-reductase activity in the female mouse during the first 2 weeks of life. Optimal activity was also found in newborn and prepubertal bovine adrenal glands [17].

The reduction of the 3-oxo-4-ene group is also seen in both groups of cultures and is again more important in the untreated group. However, in the ACTH treated cultures the increase relative to the 5-day old culture is quite large. Kitay *et al.*[18, 19] through studies of the hypophysectomized rat *in vivo* suggested that 5α-reduction could provide a mechanism for controlling the output of corticosterone. In further studies they performed, ACTH was found to increase the ratio of corticosterone to total steroids suggesting a decrease in 5α-reduction [20]. We observed in our study that though the quantity of 3α/β,5α-reduced compounds produced is lower in the ACTH treated cultures than in the untreated cultures, it nevertheless remains important. Due to the other metabolic pathways which compete for the substrate, these results do not permit us to reach a conclusion on the possible inhibition by ACTH of the reductive metabolism. However, if it does occur, it is only a partial inhibition. Further studies on the effect of ACTH on this reductive metabolism are under way.

The biosynthesis of 18-hydroxycorticosterone from DOC seen in our cultures has not been reported by other authors using comparable cell culture systems. Its formation in our cultures led us to question its biosynthetic pathway. The formation of 18-hydroxycorticosterone can occur in the glomerulosa zone of the adrenal gland catalyzed by the 18-methyloxidase (or corticosterone methyloxidase) cytochrome P450 system with concurrent formation of aldosterone [21]. It can also be synthesized by the 11β/18-hydroxylase cytochrome P450 system which is predominant in the fasciculata zone. In the 5-day old cultures where 18-hydroxycorticosterone represents 3.4% of the total metabolites aldosterone was searched through mass fragmentography of the purified extract. It was not detected at a threshold of 0.08%. Therefore, it is most likely that 18-hydroxycorticosterone is biosynthesized by the 11β/18-hydroxylase cytochrome P450 system through the 18-hydroxylation of corticosterone principally and the 11β-hydroxylation of 18-hydroxyDOC secondly [22]. This would indicate that our newborn rat adrenal cells in primary culture express specifically the functions of fasciculata-reticularis cells.

Acknowledgements—This study was supported by the Centre National de la Recherche Scientifique, ERA 267 and the Direction de la Recherche of the Ministère de l'Education Nationale. We wish to thank C. Millot, N. Pitoizet, O. Bonnard and M. Templer for their excellent technical assistance.

REFERENCES

1. Maume B. F. and Prost M.: Maintien des fonctions de stéroïdogénèse différenciée chez des cellules surrénaliennes isolées du rat nouveau-né en culture monocouche. *C.r. Soc. Biol.* **167** (1973) 1427–1430.
2. Ramirez L. C. and Maume B. F.: Métabolisme de stéroïdes exogènes par les cellules corticosurrénaliennes de rat en culture: place de l'hydroxylation en position 11β ou 18. *C.r. Soc. Biol.* **178** (1984) 77–83.
3. Maume B. F., Prost M. and Padieu P.: Steroid hormone biosynthesis in adrenal cell cultures from newborn rats. In *Advances in Mass Spectrometry in Biochemistry and Medicine* (Edited by A. Frigerio and N. Castagnoli). Spectrum Publications, New York, Vol. 1 (1976) pp. 525–540.
4. Maume B. F., Millot C., Mesnier D., Patouraux D., Doumas J. and Tomori E.: Quantitative analysis of

corticosteroids in adrenal cell cultures by capillary column gas chromatography combined with mass spectrometry. *J. Chromat.* **186** (1979) 581–594.
5. Ramirez L. C., Millot C. and Maume B. F.: Sample purification using a C_{18}-bonded reversed-phase cartridge for the quantitative analysis of corticosteroids in adrenal cell cultures by high-performance liquid chromatography or gas chromatography-mass spectrometry. *J. Chromat.* **229** (1982) 267–281.
6. Ramirez L. C., Millot C. and Maume B. F.: Effect of ACTH on endogenous steroid biosynthesis in long term primary cultures from newborn rat adrenal cells. *Acta endocr., Copenh.* **107** (1984) 401–412.
7. O'Hare M. J. and Neville A. M.: Steroid metabolism by adult rat adrenocortical cells in monolayer culture. *J. Endocr.* **58** (1973) 447–462.
8. Salmenpera M. and Kahri A. I.: Corticosterone, 18-OH-deoxycorticosterone, deoxycorticosterone and aldosterone secretion in tissue culture of foetal rat adrenals in the presence or absence of ACTH. *Acta endocr., Copenh.* **83** (1976) 781–793.
9. Horning E. C. and Maume B. F.: Derivatives of aldosterone for gas phase analysis. *J. Chromat Sci.* **7** (1969) 411–418.
10. Gustafsson J. A. and Sjovall J. : Steroids in germfree and conventional rats. 6. Identification of 15α- and 21-hydroxylated C_{21} steroids in faeces from germfree rats. *Eur. J. Biochem.* **6** (1968) 236–247.
11. Prost M. and Maume B. F.: Hormones stéroïdes de la surrénale de rat: analyse des 18-hydroxycorticostéroïdes par chromatographie gaz-liquide couplée à la spectrométrie de masse et par fragmentographie de masse. *J. steroid Biochem.* **5** (1974) 133–144.
12. Shackleton C. H. L. and Honour J. W.: Identification and measurement of 18-hydroxycorticosterone by gas chromatography-mass spectrometry. *J. steroid Biochem.* **8** (1977) 199–203.
13. Rapp J. P. and Dahl L. K.: Mendelian inheritance of 18- and 11β-steroid hydroxylase activities in the adrenals of rats genetically susceptible or resistant to hypertension. *Endocrinology* **90** (1972) 1435–1446.
14. Ulick S.: Adrenocortical factors in hypertension. I. Significance of 18-hydroxy-11-deoxycorticosterone. *Am. J. Cardiol.* **38** (1976) 814–824.
15. O'Hare M. J. and Neville A. M.: The steroidogenic response of adult rat adrenocortical cells in monolayer culture. *J. Endocr.* **56** (1973) 537–549.
16. Ungar F. and Stabler T. A.: 20α-Hydroxysteroid dehydrogenase activity and the X-zone of the female mouse adrenal. *J. steroid Biochem.* **13** (1978) 23–28.
17. Bongiovanni A. M., Marino J., Parks J. and Tenore A.: Bovine adrenal 20α-hydroxysteroid oxido-reductase and variations of activity with age. *J. steroid Biochem.* **7** (1976) 683–685.
18. Kitay J. I., Coyne M. D. and Swygert N. H.: Influence of gonadectomy and replacement with estradiol or testosterone on formation of 5α-reduced metabolites of corticosterone by the adrenal gland of the rat. *Endocrinology* **87** (1970) 1257–1265.
19. Kitay J. I., Coyne M. D. and Swygert N. H.: Effects of hypophysectomy and of cortisone or ACTH on adrenal 5α-reductase activity and steroid production. *Endocrinology* **89** (1971) 432–438.
20. Colby H. D. and Kitay J. I.: Interaction of testosterone and ACTH in the regulation of adrenal corticosterone secretion in the male rat. *Endocrinology* **91** (1972) 1247–1252.
21. Ulick S: Diagnosis and nomenclature of the disorders of the terminal portion of the aldosterone biosynthetic pathway. *J. clin. Endocr. Metab.* **43** (1976) 92–96.
22. Kim C. Y., Sugiyama T., Okamoto M. and Yamano T.: Regulation of 18-hydroxycorticosterone formation in bovine adrenocortical mitochondria. *J. steroid Biochem.* **18** (1983) 593–599.