MODIFICATION OF STEROIDOGENESIS IN A MOUSE ADRENAL CELL LINE (Y-1) TRANSFORMED BY SIMIAN ADENOVIRUS SA-7

Anick LEFEVRE[*], Nicole FAUCON-BIGUET[**], Danièle MATHIEU[***], Paul TOURNIER[***] and José M. SAEZ[*]

[*] Unité de Recherches INSERM U 162. Hôpital Debrousse. 69322 Lyon (France)
[**] Unité de Recherches INSERM U 51. 1 Place Pr. J. Renaut. 69008 Lyon (France)
[***] IRSC. B.P. 8. 94008 Villejuif (France).

Received 10-24-80

ABSTRACT

Transformation of a steroidogenic mouse adrenal cell line (Y-1) by simian adenovirus SA7 produced a cell line with low apparent steroidogenic activity. The effect of ACTH and cholera toxin on cyclic AMP production was similar in both not transformed and virus-transformed cells and activity of cyclic AMP-dependent protein kinase was also similar in both cells. In transformed cells, cholesterol was metabolized to Δ^5-3β-hydroxysteroids, mainly 20α-dihydropregnenolone while in not transformed cells, the major metabolites were Δ^4-3 ketosteroids (20α-dihydro- and 11β-hydroxy-20α-dihydroprogesterone). In both cell lines ACTH increased the metabolism of cholesterol. Further studies with labelled pregnenolone and progesterone revealed a loss of Δ^5-3β-hydroxysteroid dehydrogenase/isomerase and 11β-hydroxylase activity in the transformed cells.

Transformation and malignancy are associated with cell changes which include modification of protein composition and enzymatic activities of cell membranes (1, 2). In some cases transformation is associated with modifications of adenylate cyclase activity (3). In the past, attention has been focused mainly on the modification of activity of this enzyme and its hormonal regulation in transformed cells (3, 4), but less attention has been paid to the eventual alterations of specific cell function and its hormonal regulation. Recently Auesperg et al (5, 6) have reported a modification of pregnenolone metabolism in rat adrenocortical cells transformed by Kirsten murine sarcoma virus. However, they did not investigate whether or not the transformed cells remain sensitive to the steroidogenic effects of ACTH, which is one of the main characteristics of adrenal cell differentiation. The present study describes the steroidogenic response to ACTH and the alterations in the steroidogenic pathway of a mouse adrenal cell line (Y-1) transformed by simian adenovirus (SA 7) (7, 8).

MATERIALS

[^{14}C] cholesterol (SA 47 mCi/mmole), [^{14}C] pregnenolone (SA 54 mCi/mmole) and [^{14}C] progesterone (SA 58 mCi/mmole) were obtained from Saclay, France ; [^{3}H] pregnenolone (SA 48 Ci/mmole), [^{3}H] progesterone (SA 52 Ci/mmole) and [^{3}H] 20α-dihydroprogesterone (SA 47 Ci/mmole) from New England Nuclear, Germany, nutrient medium and horse and fetal calf serum from Gibco and all other chemicals from Sigma. $ACTH_{1-24}$ was provided by CIBA, Basel, and 11β-hydroxy-20α-dihydroprogesterone was a gift from Dr. Kline, MCR, London.

Cells culture. Y-1 mouse adrenal cells (9) were routinely grown in Ham's F–10 medium supplemented with 10 % horse serum and 2.5 % foetal calf serum. SA–Y1 cells were obtained by transformation of the Y-1 cells by oncogenic simian adenovirus SA–7 as described elsewhere (7, 8). The cells were grown in MEM medium containing 5 % calf serum.

Incubation of cells with [^{14}C] cholesterol. Confluent cells were incubated with Ham's F-10 medium containing 8 % human serum without lipoproteins, 1 % calf serum and [^{14}C] cholesterol (0.5 μCi/ml) for 36 h. The medium was removed and discarded and replaced by fresh medium containing the same amount of [^{14}C] cholesterol. After 24 h the medium was removed and saved for analysis. Next the cells were incubated for 24 additional hours at 37° in fresh medium containing [^{14}C] cholesterol and ACTH (10^{-7} M). At the end of incubation the medium was removed and saved.

Incubation of cells with [^{14}C] pregnenolone or [^{14}C] progesterone. Confluent cells were incubated with either [^{14}C] pregnenolone (1.6×10^{-6} M) or [^{14}C] progesterone ($2 \cdot 10^{-6}$ M) at 37°C. Aliquot of the medium was taken at 1, 2, 4, 6, 8 and 24 h after addition of the labeled steroid and saved for analysis.

Chromatography systems The following solvent systems were used with paper chromatography (Whatman 3 MM) : A = hexane:methanol:water (10:9:1) ; B = hexane:benzene:methanol:water (66:33:80:20) ; C = benzene:methanol:water (100:55:45). With silica gel thin layer chromatography (Merck GF 254) the following solvent systems were used : 1, chloroform:acetone (97:3) ; 2, chloroform:ethanol (2:1) ; 3, benzene:acetone (11:4) ; 4, hexane:acetone (100:12).

Preparation of [^{3}H] 11β-hydroxy-20α-dihydroprogesterone. Since this compound is not commercially available, we prepared it as follows : Y-1 cells were plated in a 75 cm^2 flask (4×10^6 cells). The next day the medium was removed and replaced by Ham's F-10 medium containing 0.1 % fetal calf serum. After 48 h the medium was replaced by fresh medium containing 200 μCi of [^{3}H] 20α-dihydroprogesterone ($4 \cdot 10^{-7}$ M) and the incubation continued for 6 h. The medium was then removed and extracted 3 times with two volumes of ethyl acetate and the extract chromatographed in solvent system C for 3 h. The area corresponding to 11β-hydroxy-20α-dihydroprogesterone was eluted, evaporated and rechromatographed in solvent system 3. After acetylation overnight (200 μl of pyridine and 50 μl of acetic anhydride) the sample was evaporated and rechromatographed in solvent system 2. The area corresponding to 11β-hydroxy-20α-dihydroprogesterone 20-acetate was eluted and saponified overnight with a 0.07 N NaOH in 80 % methanol. After saponification the sample was submitted to chromatography using solvent system 2 and the area corresponding to 11β-hydroxy-20α-dihydroprogesterone eluted. The yield of conversion of [^{3}H] 20α-dihydroprogesterone in

[^{3}H] 11β-hydroxy-20α-dihydroprogesterone was about 60 % and the specific activity of the isolated steroid about 1 Ci/mmole.

Extraction and isolation of steroid metabolites. Before extraction [^{3}H] labelled cholesterol, pregnenolone, progesterone, 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone and 100 μg of unlabelled 20α-dihydropregnenolone were added to the medium from cholesterol incubation for recovery purpose. The same labelled and unlabelled steroids except cholesterol were added to the medium from [^{14}C] pregnenolone and [^{14}C] progesterone incubations. The medium was extracted 3 times with 3 volumes of ethyl acetate, the recovery yield with this extraction being greater than 95 % in all cases.

The extract from [^{14}C] cholesterol incubation was chromatographed in solvent system A for 3 hours. Scanning of the paper revealed four areas of radioactivity with mobilities corresponding to : 1) cholesterol in the front of the solvent ; 2) pregnenolone plus progesterone ; 3) 20α-dihydroprogesterone plus 20α-dihydropregnenolone and 4) 11β-hydroxy-20α-dihydroprogesterone.

Cholesterol was submitted to two-dimensional thin-layer chromatography using solvent systems 1 and 4, eluted and counted. Pregnenolone and progesterone were separated by chromatography with solvent system 1. The eluted samples were acetylated and rechromatographed in solvent system 4. The areas corresponding to pregnenolone acetate and progesterone were eluted and counted.

The eluted area corresponding to the mobility of 20α-dihydroprogesterone and 20α-dihydropregnenolone was rechromatographed in solvent system B for 3 h to separate both compounds. After elution and prior to acetylation each compound was chromatographed in solvent system 2. After acetylation each sample was submitted to thin layer chromatography using solvent system 3. Then areas corresponding to 20α-dihydroprogesterone monoacetate and 20α-dihydropregnenolone diacetate were eluted and counted.

The dried extract corresponding to 11β-hydroxy-20α-dihydroprogesterone was purified as indicated above, except that saponification of the acetate and last thin layer chromatography were omitted.

At the end of the isolation procedure, the recovery for each compound was determined either by counting for tritium or by the colorimetric procedure (10) in the case of 20α-dihydropregnenolone.

Other methods. Cytosolic cyclic AMP protein kinase (11) and adenylate cyclase (12) activities, were determined as previously described. Cyclic AMP and 20α-dihydroprogesterone were measured by radioimmunoassay (12). Total Δ^4-3-ketosteroids were measured fluorometrically in ethanolic sulfuric acid and expressed in equivalents of 20α-dihydroprogesterone.

RESULTS

Steroids and cyclic AMP production by Y-1 and SA7-Y1 cells. Steroidogenesis in Y-1 cells could be stimulated by ACTH, cholera toxin and dibutyryl cyclic AMP (Table 1). These results were similar to those reported by many other laboratories (12-17). By contrast, SA7-Y1 cells were incapable, both under basal conditions and after stimulation, of secreting significant amounts of steroids measured by

TABLE 1

Effects of ACTH, dibutyryl cyclic AMP and cholera toxin on the stimulation of steroidogenesis in Y-1 and SA7-Y-1 cells ($\mu g/10^6$ cells/day)

	Y-1	SA7-Y-1
Control	5.6 ± 0.8	< 0.03
ACTH 10^{-7} M	19.4 ± 2.1	< 0.03
Cholera toxin (1 μg/ml)	24.1 ± 3.4	< 0.03
Dibutyryl cAMP 10^{-3}M	18.9 ± 2.9	< 0.03

Triplicate cells monolayers were incubated for 24 h as indicated. At the end of incubation, steroids were extracted from the medium with methylene chloride, quantitated by fluorescence in ethanol sulfuric acid and expressed as equivalents of 20α-dihydroprogesterone.

TABLE 2

Steroid and cAMP production by Y-1 and SA7-Y1 cells under several conditions

	Y-1		SA7-Y1	
	20α-dihydro-progesterone ng/2 h	cAMP pmoles/2 h	20α-dihydro-progesterone ng/2 h	cAMP pmoles/2 h
Basal	7.1 ± 1.2	3.8 ± 0.8	0.06 ± 0.01	6.9 ± 1.2
ACTH 10^{-8} M	32 ± 3	340 ± 38	0.19 ± 0.02	414 ± 48
Cholera toxin (1 μg/ml)	28 ± 2	250 ± 38	0.19 ± 0.02	320 ± 41
Dibutyryl cAMP (10^{-3}M)	36 ± 4	-	0.18 ± 0.02	-

Y1 and SA7-Y-1 cells were plated at the same density into 25 cm^2 flasks either in Ham's F-10 medium containing 10 % horse serum and 2.5 % of foetal calf serum (Y-1) or MEM containing 5 % calf serum. 48 h later the medium was removed and replaced by the same medium with or without ACTH, cholera toxin or dibutyryl cyclic AMP. 2 hours later the medium was removed for measuring 20α -dihydroprogesterone and cyclic AMP by radioimmunoassay. The results are the mean ± SEM of triplicate determinations of three different flasks. At the end of incubation the number of Y-1 and SA7-Y-1 cells per flask was $1.8 \pm 0.1 \cdot 10^6$ and $2.1 \pm 0.2 \cdot 10^6$ respectively.

fluorescence. Given the relative low sensitivity of this method, we measure 20α-dihydroprogesterone by radioimmunoassay. The results (Table 2) confirm the very low steroid secretion by SA7-Y1 cells, although there was a three fold increase after stimulation by ACTH, cholera toxin and dibutyryl cyclic AMP. On the other hand, the stimulatory effect of both ACTH and cholera toxin on cyclic AMP production was even greater on SA7-Y1 cells than on Y-1 cells, indicating that low production of steroids by SA7-Y1 cells was not related to any alteration of the receptor-adenylate complex nor to an increase of phosphodiesterase activity in transformed cells. Indeed, the results depicted on Table 3 clearly demonstrate that adenylate cyclase activity of SA7-Y1 cells was similar to that of Y-1 cells.

TABLE 3

Adenylate cyclase activity associated with plasma membranes of Y-1 and SA-7

	cyclic AMP - pmoles/min/mg protein				
	Basal	ACTH 10^{-6}M	Gpp(NH)p 10^{-5}M	ACTH + Gpp(NH)p	NaF 10 mM
Y-1	18 ± 4	80 ± 10	130 ± 12	376 ± 24	240 ± 18
SA7-Y-1	16 ± 3	84 ± 7	145 ± 15	350 ± 28	210 ± 20

The first step which might have been modified after cyclic AMP formation is the activity of cyclic AMP-dependent protein kinase. Figure 1 shows that basal protein kinase activity as well as the Km of the enzyme for cyclic AMP were similar in SA7-Y1 and in Y-1 cells.

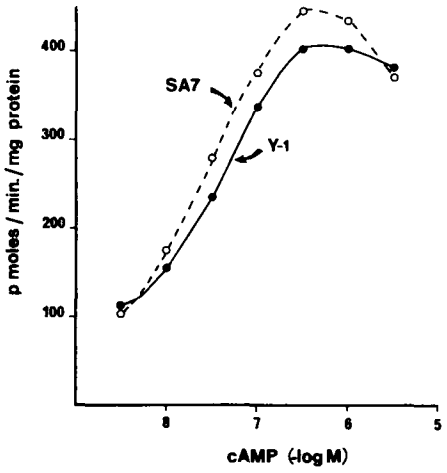

Figure 1. Cyclic AMP-dependent protein kinase in cytosol of Y-1 ( ) and SA7-Y1 ( ) cells. Protein kinase activity was measured in the presence of increasing concentrations of cyclic AMP. The results are the mean of triplicate determinations.

Metabolism of [^{14}C]-cholesterol. In Y-1 cells the major metabolites of cholesterol were 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone (Table 4), confirming the data of Pierson (18) and Kowal (13, 17). Moreover ACTH increases the conversion of cholesterol to both metabolites. On the other hand, no significant radioactivity could be found corresponding to either 20α-dihydro- or 11β-hydroxy-20α-dihydroprogesterone after incubation of SA-7 cells. The major steroid isolated was 20α-dihydropregnenolone whose production was increased by ACTH, and whose presence could explain the small amount of steroid measured using 20α-dihydroprogesterone antiserum (Table 1). The cross-reactivity of 20α-dihydropregnenolone for this antibody is about 500 times lower than that of 20α-dihydroprogesterone (data not shown).

TABLE 4

Metabolism of [^{14}C] cholesterol by Y-1 and SA-7 cells. % of radioactivity

	Y-1		SA7-Y-Il	
	Control	ACTH	Control	ACTH
Cholesterol	51 ; 50	40 ; 44	62 ; 57	54 ; 52
Pregnenolone	0.05 ; 0.04	0.05 ; 0.04	0.03 ; 0.02	0.03 ; 0.02
Progesterone	0.24 ; 0.19	0.16 ; 0.19	< 0.001	< 0.001
20α-dihydro-progesterone	5.4 ; 5.5	6.7 ; 7.8	< 0.001	< 0.001
20α-dihydro-pregnenolone	0.12 ; 0.14	0.10 ; 0.11	18 ; 21	31 ; 34
11β-hydroxy-20α-dihydroprogesterone	16.3 ; 15.5	37 ; 35	< 0.001	< 0.001

Cells were preincubated with medium containing [^{14}C] cholesterol (0.5 μCi/ml) for 36 h. Then the medium was removed and discarded. Fresh medium containing [^{14}C] cholesterol was added and incubation continued for 24 h. At the end of the incubation the medium was removed and saved for analysis (control). Fresh medium with the same concentration of [^{14}C]cholesterol plus ACTH (10^{-7} M) was added. After 24 h the medium was removed and analysed (ACTH). The data represent the values obtained from two different flasks. At the end of incubation the number of Y-1 and SA-7 cells per flask were $4.9 \cdot 10^6$ and $6 \cdot 10^6$ respectively.

Metabolism of [^{14}C] pregnenolone and [^{14}C] progesterone. Pregnenolone was metabolised by Y-1 cells very rapidly (Fig. 2), so that at 2 h only 1 % remained unmetabolized. At this time the major metabolite was 20α-dihydroprogesterone which represented about 40 %, while progesterone and 11β-hydroxy-20α-dihydroprogesterone represented about 10 %. Thereafter, both progesterone and 20α-dihydroprogesterone decreased, while 11β-hydroxy-20α-dihydroprogesterone

increased progressively. At 24 h this steroid represents about 70 % of the pregnenolone added. At any time 20α-dihydropregnenolone represents no more than 0.2 % of the total radioactivity.

Pregnenolone was metabolized slowly in SA-7 cells as compared to Y-1 cells. The major metabolite isolated was 20α-dihydropregnenolone, which represented about 70 % of the total radioactivity at the end of the incubation. At no time could $[^{14}C]$ labeled progesterone, 20α-dihydroprogesterone or 11β-hydroxy-20α-dihydroprogesterone be detected.

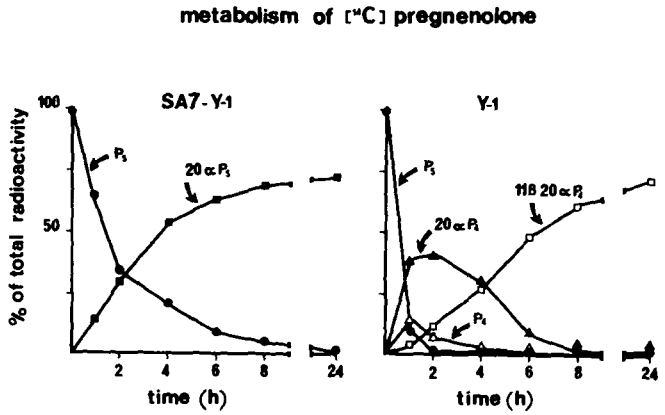

Figure 2. Time course metabolism of $[^{14}C]$ pregnenolone by Y-1 (right) and SA7-Y-1 (left) cells. Cells were incubated with $[^{14}C]$ pregnenolone (1.6×10^{-6} M) and at indicated times an aliquot was taken. The steroids were purified and isolated as described under Methods. Pregnenolone ( ), progesterone ( ), 20α-dihydroprogesterone ( ), 11β-hydroxy-20α-dihydroprogesterone ( ) and 20α-dihydropregnenolone ( ). The results are expressed as % of the total radioactivity in the extracts and represent the mean of duplicate determinations from two different flasks. At the end of incubation the number of Y-1 and SA7-Y-1 cells per flask was 3.9×10^6 and 4.2×10^6 respectively.

The metabolism of $[^{14}C]$ progesterone by Y-1 cells was also very rapid (Fig. 3). Initially the major metabolite was 20α-dihydroprogesterone but thereafter this steroid decreased and at the end of incubation the main steroid was 11β-hydroxy-20α-dihydroprogesterone. Likewise, SA-7 cells metabolised progesterone very rapidly, but only 20α-dihydroprogesterone could be identified during the first few hours. Between 2 and 8 hours this compound represented about 40 % of the total radioactivity. However at the end of the 24 hr incubation it represented only 3 % of the total radioactivity.

In no instance, either with Y-1 or SA-7 cells, did the sum of radioactivity of all metabolites identified reach the 100 % of radioactivity that was in the initial extract, indicating that ^{14}C-progesterone was also metabolised into other unknown steroids. At 24 hours one compound (compound c in fig. 3) less polar than 20α-dihydroprogesterone on solvent system B was present in the extracts of both Y-1 and SA-7 mediums. At this step of purification compound c represented 5 and 20 % of the total radioactivity for Y-1 and SA-7 respectively.

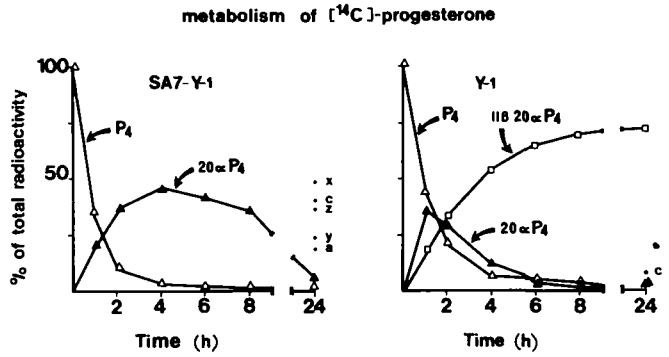

Figure 3. Time course metabolism of [^{14}C] progesterone by Y-1 (right) and SA-7-Y-1 (left) cells. Cells were incubated with [^{14}C] progesterone (2 . 10^{-6}M) and at indicated times an aliquot was taken. The steroids were purified and isolated as described under Methods. Progesterone ( ), 20α-dihydroprogesterone ( ), 11β-hydroxy-20α-dihydroprogesterone ( ). The results are expressed as % of the total radioactivity in the initial extract and represent the mean of duplicate determination of two different flasks. At the end of incubation the number of Y-1 and SA7-Y-1 cells per flask was 3.1 x 10^6 and 3.2 x 10^6 respectively. a, c, x, y and z represented unidentified metabolites (see text).

In the same paper chromatography, a compound (compound a in Fig. 3) more polar than 20α-dihydroprogesterone was detected in the extracts from SA-7 cells only and represented about 10 % of the total radioactivity.

Likewise, following paper chromatography in solvent system C, three compounds (called x, y and z in Fig. 3) all more polar than 11β-hydroxy-20α-dihydroprogesterone were detected in the 24 h extract from SA-7 cells medium, but not in that from Y-1 cells. They represented 28, 12 and 17 % of the total radioactivity respectively.

None of the above steroids has been identified but it can be said that they are neither 11β-hydroxyprogesterone nor 11-keto-20α-dihydroprogesterone.

DISCUSSION

The main steroidogenic pathway in Y-1 cells has been identified as follows : cholesterol → pregnenolone → progesterone → 20α-dihydroprogesterone → 11β-hydroxy-20α-dihydroprogesterone (13, 17, 18), the last two compounds being the major steroids secreted by these cells. Our results confirm this sequence and also show that in Y-1 cells like in normal adrenal cells the rate limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone and that this step is accelerated by acute stimulation with ACTH.

The simian virus transformed Y-1 cells did not produce significant amounts of fluorimetrically measurable steroids either under basal conditions or after ACTH stimulation. This apparent insensitivity to the hormone was not due to an alteration of the hormone receptor similar to that described in some mutants of Y-1 cells (19) and human adrenocortical tumors (20), since ACTH stimulated cAMP production in both broken and intact cells and since dibutyryl cyclic AMP was unable to stimulate steroidogenesis. An alteration of cyclic AMP dependent protein kinase (21, 22) is also unlikely to be responsible for the steroidogenic refractoriness to ACTH, cholera toxin and dibutyryl cyclic AMP as the in vitro activity of this enzyme was similar in Y-1 and in SA7–Y1 cells. Thus an alteration of the steroidogenic pathway seemed the most likely explanation for the apparent steroidogenic refractoriness and this hypothesis was confirmed by studying the metabolism of labelled steroids.

In preliminary experiment we have found that after preincubation for 36 h of Y-1 cells with ^{14}C-cholesterol in the medium, as indicated under Methods, the specific activity of total cellular cholesterol (ester plus unconjugated forms) was similar to that of secreted 20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone, indicating uniform labelling of all endogenous cholesterol pools. Moreover, the specific activity of cellular cholesterol, after 36 h preincubation with ^{14}C-cholesterol, was almost identical in Y-1 (32100 ± 2000 cpm/μg) than in SA7-Y-1 (34200 ± 2900 cpm/μg) suggesting that the uptake of cholesterol was similar in both cell lines. Therefore, the experiments described in table 3 were performed at steady state equilibrium and express the ability of the cells to cleave the side chain of cholesterol. Taking in consideration that the number of SA7–Y–1 cells was higher than that of Y-1 cells, the cytochrome P450 cholesterol side chain cleavage activity of the former was lower (about 20 %) than that of Y–1 cells. However, the stimulation of the metabolism of cholesterol by ACTH was similar in both cell lines. In the medium from SA7–Y–1 cells no Δ^4-3-

ketosteroids could be identified, suggesting a loss of 3β-hydroxysteroid dehydrogenase activity in these cells. This was confirmed by the lack of conversion of ^{14}C-pregnenolone to Δ^4-3-ketosteroids isomerase. Indeed, the main metabolite of pregnenolone was 20α-dihydropregnenolone. The metabolism rate of pregnenolone in SA7-Y-1 cells was slower than in Y-1, while that of progesterone was similar. This might suggest that the affinity and/or the Vmax of 20-ketosteroid 20α-reductase is higher for progesterone than for pregnenolone.

The second main alteration in the steroidogenic pathway of SA7-Y1 is an apparent loss of 11β-hydroxylase activity, since no 11β-hydroxyl derivatives could be isolated after incubation with either cholesterol, pregnenolone or progesterone.

In normal adrenal cells 3β-hydroxysteroid dehydrogenase/isomerase is located in both mitochondria and microsomes (23) while 11β-hydroxylase is exclusively located in mitochondria. The mechanism by which the viral transformation produced this loss of both enzymatic activities is unknown but it should be noted that in some human adrenocortical carcinomas secreting high levels of steroids there is a decrease in the activity of both enzymes (24, 25). It is also interesting to note that an increase of mitochondrial DNA synthesis has been found in chick embryo fibroblasts transformed by Rous sarcoma virus (26). Whether or not this increase is present in SA7-Y-1 cells and what the relations are, if any, with the alteration described in the present paper require further studies.

ACKNOWLEDGEMENTS

This work was supported by la Ligue Nationale Française contre le Cancer. We thank Dr. M. Donaldson for his editorial help nand Ms. J. Bois for her expert secretarial assistance.

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The following trivial names have been used : Pregnenolone : 3β-hydroxy-5-pregnen-20-one ; 20α-dihydropregnenolone : 5-pregnen-3β,20α-diol ; 20α-dihydroprogesterone : 20α-hydroxy-4-pregnen-3-one ; 11β-hydroxy-20α-dihydroprogesterone : 11β,20α-dihydroxy-4-pregnen-3-one ; 11-keto-20α-dihydroprogesterone : 20α-hydroxy-4-pregnen-3,11-dione.

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