The Influence of Age on Steroidogenic Enzyme Activities of the Rat Adrenal Gland: Enhanced Expression of Cholesterol Side-Chain Cleavage Activity*

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ABSTRACT. The ability of isolated adrenocortical cells to secrete corticosterone in response to ACTH challenge declines as rats age, but the site or mechanism(s) of this impairment is still unknown. To test the functionality of steroidogenic capacity *per se*, we measured the key enzyme activities involved in corticosterone biosynthesis. We also measured the mitochondrial cytochrome P-450 content and nonsteroidogenic enzymes specific for subcellular fractions. Mitochondria and microsomal fractions were isolated from the adrenals of 2-, 12-, and 18-month-old animals and used for various enzyme measurements.

Mitochondrial side-chain cleavage enzyme activity (nanomoles per min mg protein⁻¹) increased from a mean of 0.43 ± 0.06 in 2-month-old rats to 1.26 ± 0.11 and 1.51 ± 0.06 in 12- and 18-month old rats, respectively. After incubation with 5-cholesten-3 β ,25-diol (25-hydroxycholesterol; 25 μ g/ml) side-chain cleavage activity rose to 5.0 ± 0.6 , 12.4 ± 1.2 , and 16 ± 1.4 nmol min⁻¹ mg protein⁻¹ in adrenal mitochondrial fractions from

2-, 12-, and 18-month-old rats, respectively. In contrast, mitochondrial cytochrome P-450 content did not vary with advancing age. Microsomal Δ^5 -3 β -hydroxysteroid dehydrogenase- Δ^5 - Δ^4 -isomerase activities were similar in 2- and 12-month-old rats, but 21-hydroxylase (nanomoles per min mg protein⁻¹) activity was significantly increased in 12-month-old rats (2-month-old, 5.2 ± 0.2 ; 12-month-old, 7.7 ± 0.5). Finally, mitochondrial 11 β -hydroxylase was comparable in both age groups. In addition, activities of mitochondrial nonsteroidogenic enzymes, such as monoamine oxidase, amytal insensitive NADH cytochrome c reductase, cytochrome c oxidase, succinate dehydrogenase, and malate dehydrogenase, did not change with age. It appears from the evidence presented that the activities of the steroidogenic enzymes are not responsible for the diminished capacity in corticosterone production seen with aging in the rat. (*Endocrinology* 120: 2521-2528, 1987)

IT HAS been reported previously from this laboratory that the steroidogenic capacity of adrenocortical cells declines as rats age (1). Furthermore, this defect seems to be due to biochemical events distal to binding of ACTH to its receptor and stimulation of cAMP production (1). Although the exact mechanism of this defect is yet to be defined, the possibility that this phenomenon may represent a direct effect on the steroidogenic process *per se* is quite appealing.

In rat adrenals, steroid (corticosterone) biosynthesis involves the active participation of cholesterol side-chain cleavage, Δ^5 -3 β -hydroxysteroid dehydrogenase- Δ^5 - Δ^4 -isomerase (3 β -HSD), 21-hydroxylase, and 11 β -hydroxylase activities (2-8). Among these, cholesterol side-chain cleavage (cytochrome P-450_{sc}) catalyzes the first step in corticosterone biosynthesis (*i.e.* the conversion of cholesterol to pregnenolone) and is generally considered to be

the rate-limiting step in steroidogenesis (2-8). Furthermore, the levels of various steroidogenic enzyme activities have been shown to be influenced and/or regulated by ACTH (2-15). Therefore, the age-related decline in corticosterone secretion would be expected to have a profound effect on the activity of some or all steroidogenic enzymes. Moreover, this age-related defect may, in fact, result from alterations in the key enzyme(s) involved in corticosterone biosynthesis.

The present studies were conducted, therefore, to examine the effects of aging on steroidogenic enzymes responsible for the production of corticosterone. Additionally, changes in mitochondrial cytochrome P-450 were compared with the activity of cholesterol side-chain cleavage. Finally, to assess nonspecific age-related effects, the activities of several unrelated enzymes associated with specific compartments of mitochondria were also measured.

Materials and Methods

Materials

[1,2-N-³H]Cholesterol (SA, 40-60 Ci/mmol), [7-N-³H]pregnenolone (SA, 20-25 Ci/mmol), [4-¹⁴C]progesterone (SA, 50-

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60 mCi/mmol), [1,2-N-³H]corticosterone (SA, 40–60 Ci/mmol), and [1,2-N-³H]progesterone (SA, 40–60 Ci/mmol) were purchased from E.I. DuPont de Nemours and Co. (Inc.) NEN Research Products (Boston MA). [7-¹⁴C]Benzylamine hydrochloride (SA, 50–60 mCi/mmol) and [1,2-N-³H]deoxycorticosterone (SA, 35–60 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Fatty acid-free BSA, glucose-6-phosphate, adenine nucleotides, sodium succinate, and cytochrome *c* were the products of Sigma Chemical Co. (St. Louis, MO). All unlabeled steroids were supplied by Research Plus Laboratories (Denville, NJ). 5-Cholesten-3 β ,25-diol (25-hydroxycholesterol) was purchased from Steraloids, Inc. (Wilton, NY). All other reagents used were of analytical grade.

Animals

Male retired breeder Sprague-Dawley rats [Cr1:CoBs CD (SD)BR], 9–10 months of age, were obtained from Charles Rivers Breeding Laboratories (Wilmington, MA) and allowed to age to 12 and 18 months in our facility for aging animals, which is isolated from the general animal quarters in this V.A. Center. Two-month-old rats of the same strain were obtained from the same source. The rats were housed under conditions of controlled light (constant light from 0600–1800 h each day) and temperature (22–24 C) and given free access to Purina laboratory chow (no. 5012, Ralston-Purina, St. Louis, MO) and water. The animals in this colony are routinely monitored for the following viruses and infectious agents: pneumonia virus of mice (PVM), Rev-3 virus, encephalomyelitis virus (GD-V11), Sendai virus, Kilham rat virus, rat corona virus/sialodacryoadenitis (RCV/SDA), and *Mycoplasma pulmonis*. Occasional animals tested positively for PVM, h-1 virus and REV/SDA, but all animals were negative for the remaining agents. Routine diagnostic necropsies were not performed. In addition, animals were checked for gross pathology before use. Those with visible tumors or apparent defects were discarded.

Methods

Preparation of mitochondria. Rats were killed by cervical dislocation, and the adrenals were rapidly removed and trimmed of excess fat and external connective tissue. Adrenal mitochondria were isolated by a slightly modified procedure of Toaff *et al.* (16), as described previously (17, 18). Briefly, adrenals from four to six rats were homogenized in 5 times their wet weight of ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, 25 mM HEPES (pH 7.0) and 10 mg/ml fatty acid-free BSA (buffer A). The homogenate was then centrifuged at 600 $\times g$ (JA 20 rotor, Beckman J21, Palo Alto, CA) for 10 min to remove intact cells, nuclei, and debris. The supernatant was then subjected to centrifugation at 8700 $\times g$ for 10 min to sediment mitochondria. The pellet (sedimented mitochondria) was resuspended in buffer A and centrifuged at 600 $\times g$ for 10 min, and mitochondria were resedimented at 8700 $\times g$ for a further 10 min. The resulting pellet was resuspended in buffer B (0.2 M sucrose, 20 mM KCl, 5 mM MgCl₂, and 25 mM Tris-HCl pH 7.4) to a desired protein concentration and immediately used for enzyme measurements.

Preparation of microsomal fraction. The 8,700 $\times g$ supernatant

was centrifuged at 15,000 $\times g$ for 15 min, and the pellet was discarded. The supernatant fraction was next centrifuged at 105,000 $\times g$ for 60 min (60-Ti rotor, Beckman LM 8 Ultracentrifuge) to sediment microsomal fraction. The pellet was washed in buffer A (without BSA) and again collected at 105,000 $\times g$ for 60 min. The sediment was resuspended in buffer C (0.25 M sucrose, 1 mM EDTA, and 10 mM potassium phosphate, pH 7.0) and immediately used for enzyme assays.

Assay for cholesterol side-chain cleavage activity. The enzyme activity was measured by a slight modification of the procedure of Toaff *et al.* (16), as described by Mori and Marsh (17). The incubation medium in a final volume of 1 ml contained 0.2 M sucrose, 10 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 10 mM potassium phosphate, 25 mM Tris-HCl (pH 7.4), 1 mg/ml BSA (essentially fatty acid free), 3 mM sodium succinate, 6 μ M cyanoketone, and a suitable aliquot of mitochondrial preparation (50–100 μ g protein/ml) in the presence or absence of 25-hydroxycholesterol (25 μ g/ml). After incubation at 37 C for 5–10 min, the reaction was terminated by quick freezing at –60 C. The reaction product, pregnenolone, was extracted from the incubation mixture with hexane (three times, 2 ml each) and assayed by specific RIA (19). Cyanoketone was included to inhibit further metabolism of pregnenolone to progesterone. All assays were run in triplicate.

The cholesterol side-chain cleavage activity is expressed as nanomoles of pregnenolone formed per min mg protein^{–1}.

Assay for 3 β -HSD. 3 β -HSD activity was determined by measuring the conversion of [³H]pregnenolone to [³H]progesterone as described by Shaw *et al.* (20). Briefly, incubation medium in a final volume of 1.0 ml contained 50 mM potassium phosphate buffer (pH 7.4), 50 μ M [³H]pregnenolone (400 dpm/pmol), 0.5 mM NAD⁺, and a suitable aliquot of microsomal fraction. The reaction, maintained at 37 C for 15–30 min, was initiated by the addition of [³H]pregnenolone (dissolved in 30 μ l dimethylsulfoxide). The reaction was stopped by the addition of 0.1 ml 1 N NaOH, and unlabeled pregnenolone (50 μ g) and progesterone (50 μ g) were added in addition to [¹⁴C]progesterone (5000 dpm) to monitor recovery. The steroids were extracted two times with 5 ml diethyl ether, and pooled diethyl ether extracts were dried under N₂. Pregnenolone and progesterone were separated by ascending TLC on silica gel H using chloroform-diethyl ether (5:1, vol/vol) as a solvent system. The radioactivity associated with progesterone was measured by liquid scintillation spectrometry (Beckman LS3801 scintillation counter). The purity of the [³H]progesterone was determined by repeated recrystallization. All enzyme assays were carried out in triplicate. 3 β -HSD activity is expressed as nanomoles of progesterone produced per min mg protein^{–1}.

Assay for 21-hydroxylase activity. Enzyme activity was determined by measuring the formation of [¹⁴C]deoxycorticosterone from [¹⁴C]progesterone according to the procedure of Menard *et al.* (21). All incubations were performed at 37 C for 15–20 min. The incubation medium in a final volume of 0.4 ml contained 50 mM Tris-HCl (pH 7.4), 0.6 mM NADPH, 10 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 5 mM MgCl₂, 1 mM [¹⁴C]progesterone (10,000 dpm/nmol in 2.5% propylene glycol), and a suitable aliquot of the micro-

somal fraction. The reaction was initiated by the addition of microsomal fraction and stopped by the addition of 0.04 ml 1 N NaOH. Fifty micrograms each of progesterone and deoxycorticosterone (containing 5000 dpm [^3H]deoxycorticosterone to monitor recovery) were added to each tube, and steroids were extracted with dichloromethane. Steroids were separated by TLC (Silica gel F₂₅₄ plates) using dichloromethane-butyl alcohol (1:1, vol/vol) as a mobile phase. The radioactivity associated with deoxycorticosterone was measured by liquid scintillation spectrometry, as described above. All assays were carried out in triplicate.

The enzyme activity is expressed as nanomoles of deoxycorticosterone formed per min mg protein⁻¹.

Assay for 11 β -hydroxylase. The enzyme activity was measured by following the conversion of [^3H]deoxycorticosterone to [^3H]corticosterone, as described by Churchill *et al.* (22). Briefly, incubation medium in a final volume of 0.5 ml contained 50 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 1 mM [^3H]deoxycorticosterone (20,000 dpm/nmol in 2.5% propylene glycol), 0.25 M sucrose, 4 mM sodium succinate, and a suitable aliquot of mitochondrial preparation. All incubations were carried out at 37 C for 15–20 min. The reaction was initiated by the addition of mitochondrial suspension and terminated by the addition of 0.05 ml 1 N NaOH. Fifty micrograms each of deoxycorticosterone and corticosterone were added to each tube, and steroids were extracted with dichloromethane. Steroid were separated by TLC using silica gel F₂₅₄ plates and dichloromethane-butyl alcohol (1:1, vol/vol) as a solvent system. The plates were run three times in same solvent system, and the corticosterone-containing areas were isolated, eluted, and counted for radioactivity by liquid scintillation spectrometry, as described above. All enzyme assays were carried out in triplicate.

The enzyme activity is expressed as nanomoles of corticosterone formed per min mg protein⁻¹.

Measurement of cytochrome P-450. Mitochondrial cytochrome P-450 was measured according to the method of Omura and Sato (23). The extinction coefficient of 91 cm⁻¹ mM⁻¹ was used to calculate the cytochrome P-450 concentration from the absorbancy difference between 450 and 490 nm.

Miscellaneous enzyme assays. Succinate dehydrogenase activity was measured according to the method of Pennington (24). The activity is expressed as nanomoles of 2-(*p*-iodophenyl)3-(*p*-nitrophenyl)5-phenyltetrazolium reduced per min mg protein⁻¹. Malate dehydrogenase was assayed by a modification of the procedure of Ochoa (25), as described by Vardanis (26), with activity expressed as nanomoles of NADH oxidized per min mg protein⁻¹. The radiochemical procedure of McCaman *et al.* (27) was used to quantitate monoamine oxidase, with activity expressed as nanomoles of benzaldehyde produced per min mg protein⁻¹. Amytal-insensitive NADH cytochrome *c* reductase was measured by a combination of the procedures of Sottocasa *et al.* (28) and Privalle *et al.* (29). The enzyme activity is expressed as nanomoles of cytochrome *c* reduced per min mg protein⁻¹. Cytochrome oxidase activity was measured according to the methods of Cooperstein and Lazarow (30), with activity expressed as nanomoles of cytochrome *c* oxidized per min mg protein⁻¹.

The protein content of all fractions was measured by the modified procedure of Lowry *et al.* (31), as described by Markwell *et al.* (32).

Statistical analyses. The results are expressed as the mean $\pm$ SE. The results were analyzed by analysis of variance. The analysis used the general linear models procedures of SAS (SAS Institute, Cary, NC) when giving a significant F value; Scheffe's posttests were used to determine differences between any two age groups. $P < 0.05$ was considered statistically significant.

Results

Cholesterol side-chain cleavage enzyme activity, the rate-limiting enzyme in corticosterone biosynthesis, was measured in adrenal mitochondria isolated from 2-, 12-, and 18-month-old male Sprague-Dawley rats. All assays were carried out in the presence and absence of 25-hydroxycholesterol (25 $\mu\text{g}/\text{ml}$). The enzyme-catalyzed reaction product, pregnenolone, was quantitated by a specific RIA.

Results presented in Figs. 1 and 2 show incubation time- and enzyme concentration-dependent pregnenolone formation by the adrenal mitochondria isolated from 2- and 12-month-old rats. Under basal conditions, the rate of pregnenolone formation was low and exhibited a biphasic response (Fig. 1, *inset*). In contrast, addition of oxygenated sterol (25-hydroxycholesterol) greatly enhanced pregnenolone production, and again this effect was biphasic. Furthermore, at each time point studied, mitochondria from senescent rats synthesized 2–3 times more pregnenolone compared to corresponding values from young control groups. Similarly, the rate of pregnenolone formation was linear with protein concentration up to 100 $\mu\text{g}/\text{ml}$ in both age groups examined (Fig. 2). Like time-course studies, enzyme concentration studies also revealed enhanced steroidogenic capacity of mitochondria from 12-month-old rats, and this effect was potentiated in the presence of 25-hydroxycholesterol.

We next examined the side-chain cleavage enzyme activity in mitochondria isolated from rats 2, 12, and 18 months of age, and the results are presented in Fig. 3. Under basal conditions (without 25-hydroxycholesterol), cholesterol side-chain cleavage activity (expressed as nanomoles of pregnenolone produced per min mg protein⁻¹) increased ($P < 0.001$) from a mean ($\pm$ SE) of 0.43 ± 0.06 in 2-month-old rats to 1.26 ± 0.11 and 1.51 ± 0.06 in the 12- and 18-month-old rats, respectively. Similarly, mitochondria isolated from 12- and 18-month-old rats showed significantly higher activity in the presence of more polar exogenous substrate (25-hydroxycholesterol) compared to the 2-month-old groups (18-month-old, 16 ± 1.4 ; 12-month-old, 12.4 ± 1.2 ; 2-month-old, 5.0 ± 0.6). All of these enzyme assays were carried out under a near-linear range of time and enzyme protein concentration.

Thus mitochondria isolated from the 12- or 18-month-

FIG. 1. Time course of pregnenolone formation by mitochondria isolated from adrenals of 2- and 12-month-old rats. Incubation conditions are described in *Materials and Methods*. All incubations were carried out with or without 25-hydroxycholesterol (25 $\mu\text{g/ml}$). Pregnenolone production was measured by RIA. Results are the mean $\pm$ SE of three separate experiments. $\bigcirc$ — $\bigcirc$ and $\bullet$ — $\bullet$, 2-month-old animals; $\triangle$ — $\triangle$ and $\blacktriangle$ — $\blacktriangle$, 12-month-old animals; $\bigcirc$ — $\bigcirc$ and $\triangle$ — $\triangle$, basal pregnenolone production; $\bullet$ — $\bullet$ and $\blacktriangle$ — $\blacktriangle$, pregnenolone production in the presence of 25-hydroxycholesterol (25 $\mu\text{g/ml}$). Inset, Basal pregnenolone production shown on expanded ordinate.

old rats exhibited an enhanced capacity to synthesize pregnenolone compared to mitochondria from control groups. Moreover, addition of 25-hydroxycholesterol produced a further substantial increase in pregnenolone formation in mitochondria isolated from older animals, and levels were significantly higher than those in mitochondria from control groups. In contrast to progressive changes in side-chain cleavage activity, mitochondrial cytochrome-P450 contents did not change with age (Table 1).

A comparison of the activities of other steroidogenic enzymes, 3β -HSD 21-hydroxylase, and 11β -hydroxylase,

FIG. 2. Effect of protein concentration on pregnenolone production by mitochondria. Incubation conditions were similar to those described in *Materials and Methods*, except reactions were carried out for 10 min at 37 C in the presence and absence of 25-hydroxycholesterol (25 $\mu\text{g/ml}$). Pregnenolone production was quantitated by RIA. Results are the mean $\pm$ SE of three separate experiments. $\bigcirc$ — $\bigcirc$ and $\bullet$ — $\bullet$, 2-month-old animals; $\triangle$ — $\triangle$ and $\blacktriangle$ — $\blacktriangle$, 12-month-old animals; $\bigcirc$ — $\bigcirc$ and $\triangle$ — $\triangle$, basal pregnenolone production; $\bullet$ — $\bullet$ and $\blacktriangle$ — $\blacktriangle$, pregnenolone production in the presence of 25-hydroxycholesterol (25 $\mu\text{g/ml}$).

in microsomal and mitochondrial fractions of adrenals from 2- and 12-month-old animals is shown in Table 2. No significant age-related changes were noted for 3β -HSD (29.8 ± 1.2 nmol min^{-1} mg protein $^{-1}$ in 2-month-old rats *vs.* 29.5 ± 2.2 nmol min^{-1} mg protein $^{-1}$ in 12-month-old rats; $P = \text{NS}$). Similarly, mitochondrial 11β -hydroxylase activity was comparable in both age groups examined (1.21 ± 0.15 nmol min^{-1} mg protein $^{-1}$ in 2-month-olds *vs.* 1.19 ± 0.09 nmol min^{-1} mg protein $^{-1}$ in 12-month-olds; $P = \text{NS}$). In contrast microsomal 21β -hydroxylase activity was slightly but significantly ($P <$

FIG. 3. Effect of age on cholesterol side-chain cleavage activity in intact mitochondria isolated from adrenals of 2-, 12-, and 18-month-old rats. The enzyme activity is expressed as nanomoles of pregnenolone produced per min mg protein⁻¹. The results are the mean $\pm$ SE of six separate experiments.

TABLE 1. Effect of age on cytochrome P-450 contents of adrenal mitochondria from 2-, 12-, and 18-month-old rats

Age (months)	Cytochrome P-450 (nmol mg protein ⁻¹)
2	1.28 $\pm$ 0.06
12	1.17 $\pm$ 0.03
18	1.32 $\pm$ 0.01

Results are the mean $\pm$ SE of six separate experiments.

0.001) increased in 12-month-old rats (7.7 ± 0.5 nmol min⁻¹ mg protein⁻¹) compared to that in 2-month-old animals (5.2 ± 0.2 nmol min⁻¹ mg protein⁻¹).

Mean activities ($\pm$ SE) of monoamine oxidase, amytal-insensitive NADH cytochrome *c* reductase, succinate dehydrogenase, cytochrome oxidase, and malate dehydrogenase are presented in Table 3.

Monoamine oxidase (33, 34) and amytal-insensitive

TABLE 2. The effect of age on mean ($\pm$ SE) activities of microsomal 3 β -HSD, microsomal 21-hydroxylase, and mitochondrial 11 β -hydroxylase activities in subcellular fractions of rat adrenals

Enzyme	Age (months)	
	2	12
3 β -HSD ^a	29.8 $\pm$ 1.2 (6)	29.5 $\pm$ 2.2 (6) P = NS
21-Hydroxylase ^b	5.2 $\pm$ 0.2 (8)	7.7 $\pm$ 0.5 (8) p < .001
11 β -Hydroxylase ^c	1.2 $\pm$ 0.06 (7)	1.19 $\pm$ 0.09 (7) P = NS

Results are the mean $\pm$ SE. The number of experiments is given in parentheses.

^a Expressed as nanomoles of progesterone produced per min mg protein⁻¹.

^b Expressed as nanomoles of deoxycorticosterone produced per min mg protein⁻¹.

^c Expressed as nanomoles of corticosterone produced per min mg protein⁻¹.

TABLE 3. Mean ($\pm$ SE) activities of monoamine oxidase, amytal-insensitive NADH cytochrome *c* reductase, succinate dehydrogenase, cytochrome *c* oxidase, and malate dehydrogenase in mitochondrial fractions of adrenals isolated from 2- and 12-month-old rats

Enzyme	Age (months)	
	2	12
Monoamine oxidase ^a	2.14 $\pm$ 0.10	2.73 $\pm$ 12
Amytal-insensitive NADH cytochrome <i>c</i> reductase ^b	368 $\pm$ 21	349 $\pm$ 39
Succinate dehydrogenase ^c	55.6 $\pm$ 4.2	57.6 $\pm$ 2.5
Cytochrome <i>c</i> oxidase ^d	709 $\pm$ 54	638 $\pm$ 25
Malate dehydrogenase ^e	763 $\pm$ 99	797 $\pm$ 10

Results are the mean $\pm$ SE of eight separate experiments.

^a Expressed as nanomoles of benzaldehyde produced per min mg protein⁻¹.

^b Expressed as nanomoles of cytochrome *c* reduced per min mg protein⁻¹.

^c Expressed as nanomoles of iodonitrotetrazolium reduced per min mg protein⁻¹.

^d Expressed as nanomoles of cytochrome *c* oxidized per min mg protein⁻¹.

^e Expressed as nanomoles of NADH oxidized per min mg protein⁻¹.

NADH cytochrome *c* reductase (18, 28, 29, 35) are located on the outer mitochondrial membrane and were chosen for study to serve as a control for the increase in side-chain cleavage activity associated with preparation of mitochondrial fractions. Succinate dehydrogenase and cytochrome oxidase are conventional markers of the inner mitochondrial membrane (26) and were chosen as a control for the possibility that age leads to a generalized change in the activity of adrenal enzymes. Malate dehydrogenase is a classical marker for mitochondrial matrix (26, 29) and was employed to serve as a control for mitochondrial integrity. The fact that the activity of none of these enzymes changed as rats grew older provides support for the relative specificity of the age-related increase in cholesterol side-chain cleavage activity.

Discussion

The ability of isolated adrenocortical cells to respond to ACTH declines with advancing age (1, 36). We have shown previously that this aging defect lies distal to both binding of ACTH to its receptor and the consequent production of its secondary messenger cAMP (1). In the present studies the possible relationship between steroidogenic enzyme activities and steroidogenesis in rat adrenals during aging was explored.

Accordingly, we measured cholesterol side-chain cleavage, 3β -HSD, 21-hydroxylase, 11β -hydroxylase activities, and cytochrome P450 contents in appropriate mitochondrial and microsomal fractions of adrenals from 2-, 12-, and 18-month-old rats. An unexpected finding was that cholesterol side-chain cleavage activity showed a progressive increase with advancing age. A slight but significant increase in the activity of 21-hydroxylase was also noted in adrenal microsomal fractions from senescent rats. In contrast, activities of 3β -HSD and 11β -hydroxylase were insensitive to the aging process.

Interestingly, the current results have clearly indicated that adrenal side-chain cleavage enzyme activity increased at a time when ACTH-induced cellular steroidogenesis has been more than 60% depressed (1, 36). Several mechanisms might account for the stimulatory effect of aging on cholesterol side-chain cleavage activity. One possibility is an augmentation of enzyme synthesis. In addition, if degradation of cholesterol side-chain cleavage enzyme protein were to proceed at a lower rate than in young rats, the levels of this enzyme would be expected to be higher, since the steady state level of any given protein is achieved by its relative rates of synthesis and degradation. A second possibility may be that an increased activity represents cellular adaptive changes to deal with the diminished capacity of the adrenal gland to mobilize and use cholesterol esters for steroidogenesis. Indeed, we have recently shown that cholesterol ester content increases with age and that this defect was related to specific changes in neutral cholesterol esterase (Poppellwell, P. Y., and S. Azhar, submitted). Thus, an increase in cholesterol side-chain cleavage activity can be viewed as a compensation on the part of the enzyme to the elevated cholesterol esters levels. That is, mitochondrial side-chain cleavage enzymes increase in activity in order to effectively use that cholesterol which becomes available from the stored cholesterol ester. However, the fact that the adrenal steroidogenic response is still blunted in senescent rats demonstrates this to be a partially successful compensation. Another possibility of equal importance is that the observed increase in cholesterol side-chain activity may be in response to vastly elevated levels of plasma ACTH, previously reported in senescent rats (37). This hyperstimulation

hypothesis is supported by the observations that the cholesterol side-chain cleavage step is the rate-limiting step in steroidogenesis and, as such, activated/regulated by ACTH (2, 4, 5). Finally, evidence has recently been presented to support the idea that adrenal mitochondria contain more than one cholesterol side-chain cleavage system (38–40). Thus, it is conceivable that elevated levels represent stimulation of only one isoenzymic form which may or may not actively participate in steroidogenesis under *in vivo* situations. Obviously more rigorous experimental approaches are needed to sort out these various possibilities. Such investigations are currently in progress in our laboratory.

We were unable to document any age-related changes in mitochondrial cytochrome P450 levels. These results point to a dissociation between cytochrome P450 levels and cholesterol side-chain cleavage enzyme activity. This discrepancy could be attributed to a variation in the relative compositions of various forms of cytochrome P450 in total mitochondrial preparations from young and senescent rat adrenals (41, 42). In addition, a pool of cytochrome P450 not associated with steroidogenic enzymes (*i.e.* cytochrome P450_{sc} or cytochrome P450_{11 β}) may exist (12), which, in turn, could mask a real age effect on specific cytochrome P450_{sc}. The above hypothesis is of interest in view of the previous finding demonstrating a dissociation in half-lives of 21-hydroxylase and cytochrome P450 (4.5 *vs.* 2.9 days) in rat adrenals after hypophysectomy (12). Additional evidence for this hypothesis is the recent finding of Naumoff and Stevenson (43), who observed a delayed developmental maturation pattern for cholesterol side-chain activity compared to cytochrome P450 in rat ovaries after gonadotropin treatment. In view of these observations, it is not unreasonable to speculate that levels of cytochrome P450 and side-chain cleavage are modulated differentially as rats grow old. Finally, demonstration of any specific age effect on cytochrome P450_{sc} will require the purification of a side-chain cleavage enzyme system from adrenals of young and old rats.

Results of the present study also suggest that the decline in steroidogenesis observed in adrenocortical cells isolated from senescent rats is not related to a reduction in cholesterol side-chain cleavage activity or other steroidogenic responses, as measured *in vitro* in isolated subcellular components. These *in vitro* results do not exclude the possibility, however, that side-chain cleavage activity is reduced (or blunted) in intact cells or *in vivo*. Furthermore, the possibility that the aging process directly affects the performance (*in vivo*) of recently identified regulator (29, 44–55) of the side-chain cleavage enzyme activity should also be considered. Previous reports indicate that the aging process in mouse and rat adrenals is associated with changes in 3β -HSD (55, 56).

We realize that our inability to demonstrate an age effect on this enzyme are in conflict with earlier studies (55, 56). Several technical/physiological differences in the experimental approach to the problem may account for the observed incongruent findings. For instance, the earlier studies were carried out with female rats or mice, as opposed to male rats employed in the present studies. Additionally, variations in the assay mixture and other experimental conditions may account for the observed discrepancy. Likewise, alterations in nutritional status, animal strain, or other physiological factors could also contribute significantly. Finally, since this enzyme is not rate limiting, even a modest decline in its activity should have very little adverse effect on the overall steroidogenic capacity of the adrenal gland.

Significant changes in some steroidogenic enzymes in other steroid-producing tissues have been demonstrated in senescent rats. For example, testicular 3β -HSD activities have been shown to decline with advancing age (57–59). Similarly, other researchers have reported significant age-related loss of this enzyme activity in rat ovary (60–62). Likewise, other researchers have presented evidence that activities of 17α -hydroxylase (57, 59, 63), C17–20 lyase (63), as well as microsomal cytochrome P-450 (63) are depressed in testes from aged rats. However, comparison of data from different steroidogenic tissues suggests that changes in enzyme activities that occur with aging in one steroid-producing tissue may not necessarily be applicable to other steroid-producing tissues.

In conclusion, the results of the present study suggest that the impairment of corticosterone secretion in adrenocortical cells isolated from senescent rats is probably not related to a reduction in the activity of steroidogenic enzymes, as measured *in vitro*. However, since the transport of cholesterol to cytochrome P450_{sc} rather than the activity of cytochrome P450_{sc} *per se* limits the rate of steroidogenesis, it is possible at this time to at least speculate that the age-related decline in steroidogenesis is related to a decrease in the transport of cholesterol to the mitochondria. The roles of microtubules (44), microfilaments (44), phospholipids (40, 45–47, 52), sterol carrier proteins (48–51), and other factors (28, 53, 54) have all been described as playing a part in cholesterol transport. Failure of or changes in any of these mechanisms with age would reduce the amount of cholesterol substrate available for steroidogenesis within the mitochondria, and we are presently studying the possibility that cholesterol transport is also altered with age.

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