Metabolic Regulation of Steroidogenesis in Isolated Adrenal Cell. Investigation of the Adrenocorticotropic Hormone, Guanosine 3′,5′-Monophosphate, and Adenosine 3′,5′-Monophosphate Control Step†

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ABSTRACT: Using a technique which allows incorporation of radioactive cholesterol into the endogenous precursor pool utilized for steroidogenesis, the precise step in the transformation of cholesterol to corticosterone has been investigated in isolated adrenal cells. The data demonstrate that adrenocorticotropic hormone (ACTH), guanosine 3′,5′-monophosphate (cGMP), and adenosine 3′,5′-monophosphate (cAMP) stimulate the transformation of cholesterol to corticosterone. This transformation is both cycloheximide and aminoglutethemide sensitive. While cycloheximide does not have any effect on the transformation of (20*S*)-20-hydroxycholesterol to corticosterone [Sharma, R. K. (1973), *J. Biol. Chem. 248*, 5473], aminoglutethemide inhibits this transformation. This indicates that the aminoglutethemide-sensitive step is the side-chain cleavage of cholesterol. These data also indicate that, while cycloheximide does not have any effect on the mitochondrial cholesterol side-chain cleavage, it blocks the synthetic step which converts cholesterol to hydroxylated cholesterol. That the cycloheximide-sensitive step is at the level of the premitochondrial cholesterol precursor pool is further supported by the finding that no stimulatory effect of ACTH, cGMP, and cAMP on the transformation of mitochondrial cholesterol to corticosterone is observed. These studies, therefore, provide indirect evidence that the cycloheximide-sensitive step of ACTH-induced steroidogenesis is the entry of the cytoplasmic cholesterol pool into the mitochondria. The same step is regulated by cGMP and cAMP. By inference this would suggest that this step is under the translational control of the hormone.

In adrenal steroidogenesis, the first step, and the rate-limiting step, is the cleavage of the cholesterol side chain to synthesize pregnenolone[1] and isocaproaldehyde which is rapidly converted to isocaproic acid (Stone and Hechter, 1954; Karaboyas and Koritz, 1965; Constantopoulos and Tchen, 1961). The overall rate of cleavage of the cholesterol side chain is dictated by the rate of hydroxylation at C-20 (Shimizu et al., 1961; Koritz, 1962; Tchen, 1968). Studies with bovine adrenal slices (Hall and Young, 1968) indicated that ACTH, instead of stimulating the transformation of (20*S*)-20-hydroxycholesterol to cortisol, slightly inhibited this step.

Isolated adrenal cells are comprised mainly of fasciculata cells (Kitabchi and Sharma, 1971; Sharma et al., 1972). These cells have undetectable levels of cAMP and cGMP phosphodiesterase activity (Kitabchi et al., 1971a), respond to microunit amounts of ACTH in the synthesis of corticosterone (Kitabchi et al., 1971b), and can convert exogenous (20*S*)-20-hydroxycholesterol (Sharma and Brush, 1973; Sharma, 1973a), pregnenolone, progesterone, and deoxycorticosterone to corticosterone (Sharma, 1973b, 1974; Sawhney and Sharma, 1976). The studies carried out with this model system have demonstrated that, although cycloheximide inhibits the ACTH-triggered process of steroidogenesis (Kitabchi and Sharma, 1971; Sharma, 1973b) presumably from endogenous cholesterol, it does not interfere with the subsequent steroidogenic steps from (20*S*)-20-hydroxycholesterol (Sharma, 1973a). It was, therefore, evident that the cycloheximide-sensitive step involved in ACTH-induced steroidogenesis was not cleavage of the cholesterol side chain but was on events preceding this transformation (Sharma, 1973a). Subsequently, various investigators have attempted to further narrow down the step that is activated by ACTH (Kahnt et al., 1974; Mahaffee et al., 1974; Bell and Harding, 1974; Paul et al., 1976). These studies have suggested that ACTH control of steroidogenesis is governed by the regulation of the size of the mitochondrial precursor pool of cholesterol rather than through a direct effect on the mitochondrial enzyme system that converts cholesterol to pregnenolone. These investigations, however, have been indirect since they were carried out with the mitochondrial preparations isolated from ACTH- or cAMP-treated animals. Direct studies to demonstrate the ACTH-control step in the scission of cholesterol side chain have been hampered since tracer exogenous cholesterol could not be incorporated into the endogenous cholesterol pool of the isolated adrenal cell (Sharma, 1973a; Neher and Milani, 1974). This paper describes unique techniques utilized to directly study the isolated events which lead to the cleavage of cholesterol side chain. With the use of two inhibitors, aminoglutethemide and cycloheximide, double labeled precursors, cholesterol and (20*S*)-20-hydroxycholesterol, it has been possible to assign the probable steroid biosynthetic step which is under the translational control of ACTH. Furthermore, for the first time, the present paper investigates the regulatory role of cGMP, the nucleotide proposed to play an important role in the mediation of the ACTH-induced steroidogenesis in an isolated adrenal

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[1] The trival names and abbreviations used are: ACTH, adrenocorticotropic hormone; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; pregnenolone, 5-pregnen-3β-ol-20-one; progesterone, 4-pregnen-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

0006-2960/78/0417-0316$01.00/0

cell (Sharma et al., 1974, 1976; Sharma, 1974; Perchellet et al., 1978). From these studies a hypothesis is developed which links cGMP- and cAMP-mediated events with ACTH induction of steroidogenesis.

Experimental Procedure

Tissue. The isolated adrenal cells were prepared by trypsin digestion of adrenal glands obtained from male Holtzman rats weighing 300 to 400 g (Kitabchi and Sharma, 1971; Sharma et al., 1972; Sayers et al., 1971).

Chemicals. ACTH, a United States Pharmacopeia standard, was purchased from United States Pharmacopeia. Each vial contained 1.5 IU of ACTH and was diluted with 0.2 mL of vehicle to give the desired concentration. The vehicle consisted of a solution of 0.5% albumin in 0.9% NaCl adjusted to pH 3.5. [4-^{14}C]Cholesterol (specific activity 50–60 mCi/mmol), [1,2-^{3}H]cholesterol (specific activity 40–60 Ci/mmol), and (20*S*)-20-hydroxy-[7α-^{3}H]cholesterol (specific activity, 25 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. cGMP and cAMP were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Boehringer Mannheim (New York). Aminoglutethemide phosphate was a gift from Dr. J. M. Saez, INSERM, Lyon, France.

Synthesis of (20S)-20-Hydroxy[4-^{14}C]cholesterol. In our hands the experimental conditions published for the synthesis of this compound were found to be unsatisfactory (Petrow and Stuart-Webb, 1956). The reaction was, however, successful when an excess amount of isohexyl bromide was added and when the reaction was carried out under nitrogen.

A benzene solution containing 2 mg of [4-^{14}C]pregnenolone (20 μCi, specific activity 10 μCi/mg) was added to the stirring Grignard solution prepared from magnesium (9 mg), isohexylbromide (25 mg), and dry ether (2 mL). The mixture was stirred for 2 h under gentle heat and subsequently distilled to remove ether. The reaction mixture was then refluxed for 4 h, cooled to room temperature, decomposed with saturated ammonium chloride solution and the product was extracted with benzene (3 × 10 mL). Distillation of the benzene yielded an oily product which was purified by thin-layer chromatography (ethyl acetate–*n*-hexane, 3:7 v/v). A sample of authentic nonradioactive (20*S*)-20-hydroxycholesterol was cochromatographed, the (20*S*)-20-hydroxycholesterol was detected by iodine vapors and the area of radioactivity corresponding to the zone was extracted. The product was acetylated (Sharma, 1973b) and purified by thin-layer chromatography (ethyl acetate–*n*-hexane, 3:7). This yielded colorless crystals of 3β-acetoxy-(20*S*)-20-hydroxy[4-^{14}C]cholesterol, mp 156–157 °C. The acetate was converted to (20*S*)-20-hydroxy[4-^{14}C]cholesterol, mp 125–126 °C. The purity was ascertained by infrared spectroscopy, mixed melting point, and cochromatography on thin-layer plates with authentic sample. The purified product (8.6 μCi) had a specific activity of 7 μCi/mg.

All other chemicals were reagent grade and were obtained commercially.

Chromatography. Precoated silica gel plates (silica gel F_{254}, Brinkmann, New York) were used for thin-layer chromatography in the indicated solvent systems. Thin-layer plates were used for the final purification of deoxycorticosterone and corticosterone as previously described (Sharma, 1973a,b).

Counting. Counting was carried out in the Searle-Isocap 300 automatic liquid scintillation counter. The samples were dissolved in 15 mL of a scintillation solution of toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per 1000 mL. Counting efficiencies for ^{3}H and ^{14}C under these conditions were 40 ± 1% and 63 ± 1%, respectively.

Method of Incubation. The method of incubation for ACTH and NADPH was that already described (Kitabchi and Sharma, 1971). In general, for each isolated adrenal cell preparation, adrenal glands from 16 rats were used, and the cells from each adrenal gland (approximately 2 × 10^6 cells) were resuspended in 0.8 mL of Krebs–Ringer/bicarbonate buffer, pH 7.4, containing 4% albumin and 0.2% glucose. Corticosterone was measured fluorometrically (Glick et al., 1964).

The method of incubation with single- and double-labeled radioactive precursors was as previously described (Sharma, 1973a,b). In double-labeled experiments an appropriate amount of nonradioactive cholesterol was added to the flasks containing [^{3}H]cholesterol so that the specific activity of the ^{3}H- and ^{14}C-labeled precursors were the same. Incubation was carried out in Teflon flasks for 150 min. Each flask contained 20 mL of suspended isolated adrenal cells (Kitabchi and Sharma, 1971; Sharma et al., 1972) obtained from ten adrenal glands.

In single-label experiments each flask contained an appropriate amount of [1,2-^{3}H]cholesterol or (20*S*)-20-hydroxy[7α-^{3}H]cholesterol along with ACTH or other agent. In the experiments where cycloheximide (10 mM) or aminoglutethemide (100 μM) was used, suspended cells were preincubated with these inhibitors for 15 min, and then radioactive steroid precursors were added, and the incubation was continued. Control incubation flasks which did not contain cycloheximide or aminoglutethemide were processed similarly.

Double-label experiments were carried out as previously described (Sharma, 1973b). In the experiments where incubation was carried out in three Teflon flasks, the first flask contained, in addition to the appropriate cell suspension, a mixture of 5 μCi of ^{14}C-labeled steroid [cholesterol] and 50 μCi of corresponding ^{3}H-labeled steroid (^{3}H/^{14}C ratio 10.00) along with NADPH (1 mM); the second flask contained 5 μCi of ^{14}C-labeled steroid + NADPH (1 mM); and the third flask 50 μCi of ^{3}H-labeled steroid + cycloheximide (10 μM) and ACTH (100 μU per mL), cGMP (10 mM) or cAMP (10 mM). In the experiments where incubation was carried out in five Teflon flasks, the first flask contained, in addition to the appropriate cell suspension, a mixture of 5 μCi of [4-^{14}C]-cholesterol and 50 μCi of [1,2-^{3}H]cholesterol + NADPH (1 mM) (^{3}H/^{14}C ratio 10.00); the second flask contained 5 μCi of [4-^{14}C]cholesterol + NADPH (1 mM); the third flask 50 μCi of [1,2-^{3}H]cholesterol + NADPH (1 mM) + ACTH (100 μU per mL), cGMP (10 mM) or cAMP (10 mM); the fourth flask contained 5 μCi of [4-^{14}C]cholesterol + NADPH (1 mM); and the fifth flask 50 μCi of [1,2-$^{3}H_2$]cholesterol + NADPH (1 mM) + cycloheximide (10 μM) or aminoglutethemide (100 μM) and ACTH (100 μU per mL), cGMP (10 mM) or cAMP (10 mM). The cells in the flasks in which exogenous radioactive cholesterol was added along with NADPH were preincubated for 15 min with these agents to equilibrate the exogenously added cholesterol with the endogenous cholesterol. The incubation was stopped by the addition of 75 mL of methylene chloride to each flask. The contents of the second flask were mixed with the contents of the third flask and the contents of the fourth flask with the contents of the fifth flask. To the reaction mixture of each flask, 10 mg of corticosterone and to some flasks 15 mg of deoxycorticosterone along with corticosterone were added and the products processed identically (Sharma, 1973a,b).

The methylene chloride extract from each flask was washed

TABLE I: Effect of NADPH on ACTH-Induced Steroidogenesis as Measured Fluorometrically.[a]

Additions	Corticosterone (μg/2 h)
Control	0.050
ACTH (100 microunits/mL)	1.800
NADPH (1 mM)	0.045
ACTH + NADPH	1.750

[a] Incubation system: adrenal cell suspension, 0.8 mL; reagents dissolved in 0.2 mL of vehicle. Total volume of incubation 1 mL. Results are the average of two observations. Control value has been subtracted from the experimental results.

with 2 × 10 mL of 5% sodium hydroxide and then with 2 × 10 mL of distilled water. The methylene chloride extract was dried over sodium sulfate and filtered. The solvent was evaporated to dryness under nitrogen and deoxycorticosterone and corticosterone isolated.

Isolation of Deoxycorticosterone and Corticosterone. Deoxycorticosterone and corticosterone were purified by thin-layer chromatography (Sharma, 1973a,b). The isolated deoxycorticosterone was acetylated (Sharma, 1973b), further purified by thin-layer chromatography (Sharma, 1973b; Sharma, 1974), and crystallized from acetone–*n*-hexane to constant specific activity and $^3H/^{14}C$ ratio. The purified corticosterone was crystallized from acetone–ligroin until the specific activity and $^3H/^{14}C$ ratios were constant.

The initial $^3H/^{14}C$ ratios of the incubated cholesterol in each flask were always checked by purifying the cholesterol on thin-layer chromatograms (Sharma, 1973a,b).

All experiments, single and double labeled, were conducted at least five times. The results reported in this manuscript are typical of one such experiment and good reproducibility of the results was obtained in each study.

Results

Effect of NADPH on ACTH-Induced Steroidogenesis as Measured Fluorometrically. It has been shown previously that, in contrast to a cell suspension containing intact cell membranes, a cell preparation with damaged cell membranes elicits steroidogenesis in response to NADPH (Halkerston et al., 1968; Tsang and Stachenko, 1970). This is based on the assumption that NADPH is unable to penetrate through the intact plasma cell membrane. A subsequent study has shown that NADPH did not activate ACTH-induced steroidogenesis utilizing an isolated adrenal cell preparation but did stimulate the transformation of deoxycorticosterone to corticosterone (Kitabchi et al., 1974).

In order to further rule out the possibility that the effect of NADPH on ACTH-induced steroidogenesis may be due to the cells having damaged plasma membranes, ACTH was incubated with isolated adrenal cells together with NADPH. The results in Table I demonstrate that, in accord with the previously published report, exogenous NADPH did not stimulate formation of corticosterone in intact cells

Effect of NADPH on the Transformation of Exogenous Cholesterol to Deoxycorticosterone and Corticosterone. Since it is established that exogenous cholesterol by itself is not incorporated into the endogenous precursor pool (Sharma, 1973a; Neher and Milani, 1974), we followed the preliminary evidence that the exogenous radioactive cholesterol in the presence of NADPH might equilibrate with the endogenous cholesterol pool and subsequently be converted to corticosterone (Neher and Milani, 1974). In order to test this possibility,

TABLE II: Effect of ACTH, cGMP, and cAMP on the Transformation of Exogenous [1,2-^{3}H]Cholesterol into Corticosterone in the Presence of NADPH in Isolated Adrenal Cells.[a]

Additions	$^3H/^{14}C$ ratio of corticosterone	% age stimulation
Control	14.56	
+ACTH	33.80	132
Control	11.61	
+cGMP	29.98	158
Control	12.77	
+cAMP	34.98	174

[a] The $^3H/^{14}C$ ratios of corticosterone obtained after the incubation of [4-^{14}C, 1,2-^{3}H]cholesterol + NADPH with isolated adrenal cells. Incubation was carried out in appropriate flasks containing 20 mL of isolated adrenal cell preparation as mentioned in Experimental Procedure. Flask 1 (control) contained a mixture of [1,2-^{3}H]cholesterol (50 μCi) + [4-^{14}C]cholesterol (5 μCi) ($^3H/^{14}C$ ratio 10.00) + NADPH (1 mM); flask 2 contained [4-^{14}C]cholesterol (5 μCi) + NADPH (1 mM); and the flask 3 contained [1,2-^{3}H]cholesterol + NADPH and ACTH (100 μU per mL) or cGMP (10 mM) or cAMP (10 mM). The incubation was for 2.5 h and the reaction was stopped by the addition of 75 mL of methylene chloride to each flask. The contents of the second and third flask were mixed and corticosterone isolated as described under Experimental Procedure.

isolated adrenal cells were incubated with [1,2-^{3}H]cholesterol along with ACTH or NADPH. In accord with the previous studies, it was found that exogenous cholesterol by itself is not converted into corticosterone even in the presence of ACTH (Sharma, 1973a; Neher and Milani, 1974). Significant incorporation of cholesterol into corticosterone (0.18%) was observed in the presence of NADPH (data not shown). It is, therefore, obvious that the fluorometric technique (Glick et al., 1964) used previously to measure corticosterone is not sensitive enough to detect the incorporation of such a small increment into corticosterone. Considering that the cell suspension derived from one adrenal gland contains (Sharma et al., 1972) total endogenous cholesterol equivalent to approximately 20 μg, the amount of exogenous cholesterol converted, assuming no internal dilution, corresponded to approximately 38% of the total intracellular cholesterol pool.

Effect of Cycloheximide on the Transformation of Exogenous Cholesterol into Corticosterone in the Presence of NADPH. Studies carried out in the isolated adrenal cells where one group of cells was incubated with [1,2-^{3}H]cholesterol + NADPH + cycloheximide and the other group with [4-^{14}C]cholesterol + NADPH and the incubation solutions of the two groups mixed after terminating the reaction, showed (data not shown) no significant change in the $^3H/^{14}C$ ratios of the NADPH-treated cells from that noted in the nontreated cells. This indicates that cycloheximide has no inhibitory effect on the transformation of cholesterol to corticosterone observed in the presence of NADPH.

Effect of ACTH, cGMP and cAMP on the Transformation of Exogenous Cholesterol to Corticosterone. In order that the activation of steroidogenesis by ACTH, cGMP, and cAMP could be differentiated from that obtained with NADPH, one group of cells was incubated with [1,2-^{3}H]cholesterol + NADPH + ACTH, cGMP or cAMP and the other group with [4-^{14}C]cholesterol + NADPH and the incubation mixtures of the two groups were mixed after the reaction. The results in Table II show that ACTH, cGMP, and cAMP stimulate more than twofold the transformation of cholesterol to corticosterone over and above to that obtained in the presence of NADPH. These data also indicate that this stimulation by

TABLE III: Effect of Cycloheximide on the ACTH-, cGMP-, and cAMP-Stimulated Transformation of Exogenous [1,2-^{3}H]Cholesterol into Corticosterone in Isolated Adrenal Cells.[a]

Additions	$^3H/^{14}C$ ratio of corticosterone	
Control	10.00	10.00
+ACTH	29.24	26.19
+cGMP	32.01	28.37
+cAMP	32.34	29.10
+ACTH + cycloheximide or aminoglutethemide	9.17	17.16
+cGMP + cycloheximide or aminoglutethemide	20.79	7.30
+cAMP + cycloheximide or aminoglutethemide	18.13	15.00

[a] The $^3H/^{14}C$ ratios of the products obtained after the incubation of [4-^{14}C, 1,2-^{3}H]cholesterol + NADPH with isolated adrenal cells. Conditions of the experiment were identical with the experiment in Table II except the increased incorporation of [1,2-^{3}H]cholesterol due to the presence of NADPH was subtracted. This value was calculated from the control flask 1 which was then normalized to the value of $^3H/^{14}C$ ratio of 10.00 corresponding to the initial ratio of cholesterol used in the incubation medium. Flask 1 (control) contained a mixture of [1,2-^{3}H]cholesterol + [4-^{14}C]cholesterol; flask 2 contained [4-^{14}C]cholesterol; flask 3 contained [1,2-^{3}H]cholesterol + ACTH or cGMP or cAMP; flask 4 contained [4-^{14}C]cholesterol; and flask 5 contained [1,2-^{3}H]cholesterol + cycloheximide and ACTH or cGMP or cAMP. The contents of the second flask were mixed with the third flask and of the fourth flask with the fifth and the samples processed identically. The data provided in the first column relate to the values obtained with the experiment conducted with cycloheximide and the second to the experiment conducted with aminoglutethemide.

ACTH could not be due to its effect on the cells with the damaged plasma membranes since to date ACTH-induced corticosterone synthesis has not been demonstrated in cell free systems.

Effect of Cycloheximide on the ACTH-, cGMP-, and cAMP-Stimulated Transformation of Exogenous Cholesterol to Corticosterone. Puromycin and cycloheximide are known to inhibit the adrenal steroidogenic response to ACTH in vivo (Davis and Garren, 1968), in vitro (Ferguson, 1962), and in isolated adrenal cell (Kitabchi and Sharma, 1971). In an attempt to investigate the direct effect of cycloheximide on the conversion of cholesterol to corticosterone, isolated adrenal cells were incubated with cycloheximide (10 μM) + [1,2-^{3}H]cholesterol + NADPH + ACTH, cGMP, or cAMP.

The results in Table III show that cycloheximide inhibits the ACTH-, cGMP-, and cAMP-activated conversion of cholesterol to corticosterone. These data also indicate that the stimulation of steroidogenesis obtained in the presence of NADPH clearly proceeds via a mechanism which is cycloheximide independent.

Effect of Aminoglutethemide on the ACTH-, cGMP, and cAMP-Stimulated Transformation of Exogenous Cholesterol to Corticosterone. Aminoglutethemide is a known inhibitor of the cholesterol side-chain cleavage (Kahnt and Neher, 1966; Dexter et al., 1967), a process which apparently involves two or more steps (Burstein and Gut, 1971; Burstein et al., 1975; Kraipool et al., 1975). The initial event is the side-chain hydroxylation of cholesterol and the subsequent step(s) leads to the cleavage of the side chain between the C-20 and C-22 atoms yielding pregnenolone. In order to differentiate which of these events is aminoglutethemide sensitive, isolated adrenal cells were incubated with this inhibitor (100 μM) along with [1,2-^{3}H]cholesterol + NADPH + ACTH, cGMP or cAMP.

The results in Table III demonstrate that the ACTH-, cGMP-, and cAMP-activated transformation of cholesterol to corticosterone is inhibited by aminoglutethemide.

Effect of Aminoglutethemide on the Transformation of (20S)-20-Hydroxycholesterol to Corticosterone. After it was found that aminoglutethemide interferes with the conversion of cholesterol to corticosterone in an isolated adrenal cell preparation (vide supra), an examination of the effect of this inhibitor on the cholesterol side-chain cleavage activity subsequent to the hydroxylation of cholesterol was made.

Although the question whether the hydroxylation of cholesterol takes place at positions 20*S*, 20*S*,22*R*, or 22*R* in an adrenal cell is not yet settled, there is general agreement that side-chain cleavage of cholesterol follows the hydroxylation reactions (Burstein and Gut, 1971; Kraipool et al., 1975). On the other hand, the recent studies of Morisaki et al. (1976) have clearly indicated the intermediary role of (20*S*)-20-hydroxycholesterol in the conversion of cholesterol to pregnenolone. For the sake of convenience and simplicity, our studies have chosen the latter substrate as the precursor of pregnenolone.

One group of adrenal cells was incubated with (20*S*)-20-hydroxy[7α-^{3}H]cholesterol + aminoglutethemide and the other group with (2]*S*)-20-hydroxy[4-^{14}C]cholesterol and the incubation solutions mixed after terminating the reaction. The results showed that aminoglutethemide inhibited more than 80% the conversion of (20*S*)-20-hydroxycholesterol to corticosterone (data not shown) indicating that the cleavage of the side chain is aminoglutethemide sensitive. Taken together with the evidence that the biosynthetic transformation of mitochondrial cholesterol to corticosterone is also blocked by aminoglutethemide, these data indicate that the steroidogenic step inhibited by aminoglutethemide is the scission of the cholesterol side chain.

Effect of ACTH, cGMP, and cAMP on the Transformation of Mitochondrial Cholesterol to Corticosterone. In order to differentiate whether the ACTH-induced cycloheximide-sensitive step is on the pre- or mitochondrial precursor pool of cholesterol, isolated adrenal cells were preincubated with aminoglutethemide and [1,2-^{3}H]cholesterol was added along with NADPH + ACTH to transport the cytoplasmic cholesterol pool into mitochondria. After 1 h of incubation, the cells were washed to remove aminoglutethemide. The cells were divided into two groups. One group (control) contained only the cells and the other group contained cycloheximide + ACTH. Cycloheximide was added to further ensure that contaminant cytoplasmic cholesterol did not enter the mitochondria during the second incubation. The results obtained without the addition of cycloheximide during the second incubation were, however, essentially the same.

The results thus obtained showed that subsequent to the entry of cholesterol into the mitochondria, ACTH, cGMP, and cAMP do not affect the cleavage of the cholesterol side chain (data not shown). These data also indicate that the cycloheximide-sensitive step in the process of steroidogenesis is related to the migration of cholesterol from the cytoplasm into the mitochondria.

Discussion

There is considerable divergence of opinion regarding the mechanism of action of ACTH in adrenal steroidogenesis. According to one proposal, ACTH regulates the transport of pregnenolone, an inhibitor of cholesterol side-chain cleavage, out of mitochondria (Koritz and Kumar, 1970). This proposal does not appear to be valid since utilizing rat adrenal slices an increased synthesis of pregnenolone was observed in the presence of ACTH. This increased synthesis was observed even

when cyanoketone, an inhibitor of pregnenolone to progesterone transformation, was present (Farese, 1971). It has also been postulated (Roberts et al., 1967) that cAMP directly regulates the mitochondrial enzyme activity responsible for the transformation of cholesterol to pregnenolone. This proposal is not substantiated in various other studies (Koritz et al., 1968; Cohen and Morinvki, 1969). The hypothesis which at this time appears to be most rational and which is supported indirectly by various laboratories (Kahnt et al., 1974; Bell and Harding, 1974; Jefcoate et al., 1974) is that ACTH regulates steroidogenesis by transporting cholesterol, via a labile protein, from the adrenal cytoplasm into the mitochondria (Garren et al., 1971). Another group (Mahaffee et al., 1974), while in general agreement with this proposal, questions the site of inhibition by cycloheximide on adrenal steroidogenesis. These investigators were unable to observe the inhibitory effect of cycloheximide on the premitochondrial precursor pool of cholesterol.

On the other hand, studies with the isolated adrenal cell (Kitabchi and Sharma, 1971; Sharma et al., 1972) indicate that cGMP plays a crucial role in the regulation of steroidogenesis by ACTH (Sharma et al., 1974; Sharma, 1974, 1976; Perchellet et al., 1978). The techniques described in the present study provide means to directly study the effect of these hormonal agents on individual steroidogenic steps beginning from cholesterol in an intact isolated adrenal cell. These studies with intact cells show that ACTH-, cGMP-, and cAMP-triggered reactions stimulate the conversion of cholesterol to corticosterone. Cycloheximide, a protein synthesis inhibitor, blocks this transformation but does not inhibit the conversion of (20*S*)-20-hydroxycholesterol to corticosterone (Sharma, 1973a). Previously, it has been shown that neither cGMP (Sharma, 1974) nor cAMP (Sharma, 1973a) had any effect on the transformation of (20*S*)-20-hydroxycholesterol to corticosterone. This indicates that cycloheximide interferes with the hormonally mediated events which lead to the side-chain hydroxylation of cholesterol, rather than the subsequent scission of the cholesterol side chain. Aminoglutethemide inhibits both the ACTH-, cGMP-, and cAMP-activated conversion of cholesterol to corticosterone as well as the transformation of (20*S*)-20-hydroxycholesterol to corticosterone. This indicates that the inhibitory effect is on the cleavage of the cholesterol side chain and most probably not on the events resulting in the hydroxylation of cholesterol. These results are in accord with the conclusions of others (Kahnt and Neher, 1966; Dexter et al., 1967), and with the more exact study (Uzgiris et al., 1977) where the site of inhibition by this inhibitor has been narrowed down to the cytochrome P-450 associated with the cholesterol side-chain cleavage. Taken together with our present findings, this indicates that aminoglutethemide acts on the steroidogenic step after the cholesterol bound to the cytochrome P-450 is hydroxylated. The pyridine nucleotide-dependent hydroxylation step (Constantopoulos et al., 1962) involves the transfer of molecular oxygen which leads to either the formation of (20*R*,22*R*)-20,22-dihydroxycholesterol—a process which occurs by the simultaneous addition of two oxygen atoms (Burstein et al., 1975)—or to the synthesis of both (20*S*)-20-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol (Morisaki et al., 1976). Based on the latter contention, our present studies suggest that the side-chain hydroxyl reactions of cholesterol would not be interfered by aminoglutethemide but the final scission of the C-20(22) bond would be.

Previous studies with the isolated adrenal cells have shown that the stimulatory effect of ACTH, cGMP, and cAMP on steroidogenesis utilizing endogenous precursor, presumably cholesterol, is blocked by cycloheximide. This occurs after the activation of protein kinase and before the synthesis of pregnenolone (Sharma, 1973b; Sharma et al., 1976). This indicates that the cycloheximide-sensitive ACTH, cGMP, and cAMP control step in the process of steroidogenesis is before the biochemical events which lead to the hydroxylation of the side-chain of cholesterol. This particular control step is further narrowed down since no stimulation by ACTH, cGMP, and cAMP is observed on the conversion of mitochondrial cholesterol to corticosterone. This indicates that the ACTH-activated step, which is also cycloheximide sensitive, is at the entry of cytoplasmic cholesterol into the mitochondria. By inference these data suggest that this very step is under the translational control of the hormone and is mediated by cGMP and cAMP. Whether a labile protein is involved in such a process, although an attractive possibility, remains speculative at this time. To date no such protein has been isolated. The reported (Kan and Unger, 1973) sterol carrier protein appears to have a function other than the transport of cholesterol across the mitochondrial membrane. Considering these factors, the mechanism of the control of ACTH in adrenal steroidogenesis may be summarized as follows.

ACTH binds to the hormone receptor located on the adrenal cell plasma membrane (Lefkowitz et al., 1970). At physiological concentrations the hormone raises the cGMP level, presumably by activating guanylate cyclase enzyme, whereas at supraphysiological concentrations it stimulates the synthesis of cAMP by activating the adenylate cyclase system (Sharma et al., 1974, 1976; Sharma, 1976; Grahme-Smith et al., 1967). The two nucleotides in turn activate their respective protein kinase activities (Sharma et al., 1976) which in turn leads to the translation of a hypothetical preexistent mRNA (Davis and Garren, 1968; Garren et al., 1971). The new protein thus synthesized controls the entry of the cytoplasmic cholesterol into the mitochondrial precursor pool of cholesterol.

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