Steroid Hydroxylation in Bovine Adrenocortical Mitochondria. Competition between Side-Chain Cleavage of Cholesterol and 11β Hydroxylation*

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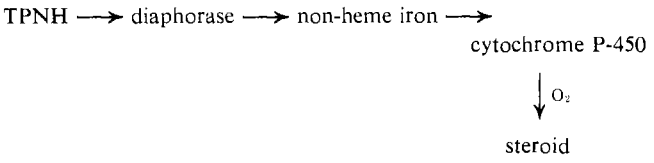
ABSTRACT: The influence of a substrate for 11β hydroxylation (11-deoxycorticosterone or DOC) upon the conversion of cholesterol into pregnenolone (side-chain cleavage of cholesterol) was examined using bovine adrenocortical mitochondria—both intact organelles and submitochondrial systems. With intact mitochondria DOC inhibits side-chain cleavage but not *vice versa*. This inhibition occurs when reducing equivalents are generated by a variety of oxidizable substrates, although at very high concentrations of substrate (>10 mM) inhibition is not observed. Inhibition of side-chain cleavage by DOC was also seen when exogenous TPNH and Ca^{2+} were used to provide reducing equivalents for the two reactions. It is suggested that competition for TPNH may play a part in regulating these reactions *in vivo via* negative feedback inhibition of side-chain cleavage. With submitochondrial systems DOC also inhibits side-chain cleavage although not by competing for TPNH. Metopirone inhibits 11β hydroxylation and greatly decreases the capacity of DOC to inhibit side-chain cleavage. It is concluded that these observations can be explained by competition between cholesterol and DOC for cytochrome P-450 or for some other factor associated with the cytochrome.

The conversion of cholesterol into corticosteroids by the cells of the adrenal cortex includes two important contributions by mitochondria, namely the initial steps in which cholesterol is converted to pregnenolone (side-chain cleavage of cholesterol) (Halkerston *et al.*, 1961) and 11β hydroxylation (Grant, 1956) which results in the conversion of 11-deoxycorticosterone (DOC)[1] into corticosterone. Side-chain cleavage appears to involve hydroxylation reactions (Shimizu *et al.*, 1961; Constantopoulos and Tchen, 1961) which in common with 11β hydroxylation require reduced TPN, an electron-transport system consisting of a diaphorase and non-heme iron, molecular oxygen, and cytochrome P-450 (Simpson and Boyd, 1966; Hall, 1967; Omura *et al.*, 1966):



Special importance attaches to side-chain cleavage of cholesterol because this step appears to regulate the rate of synthesis of corticosteroids and is specifically stimulated by ACTH (Karaboyas and Koritz, 1965; Hall and Young, 1968). The following experiments were designed to determine whether side-chain cleavage and 11β hydroxylation compete for mitochondrial factors shared by the two reactions or whether the two reactions are regulated independently. It will be shown that 11β hydroxylation inhibits side-chain cleavage.

Experimental Section

Mitochondria. The preparation of bovine adrenocortical mitochondria has been reported previously (Hall, 1967). The method was modified only in that sucrose (0.25 M) was prepared in Tris buffer (pH 7.4, 0.25 M). Mitochondria were incubated in 25-ml erlenmeyer flasks at 37° for various times in a total volume of 2 ml. The amount of mitochondrial protein (approximately 2.5 mg/flask) is given with the various experiments to be described. Additions were made to flasks in the following order: potassium chloride (0.154 M), sodium chloride (11.5 mM), nicotinamide (50 mM), Tris-sucrose (pH 7.4, 20 mM), Ca^{2+} (1.1 mM), and TPNH or oxidizable substrate, bovine serum albumin 1% (w/v), steroids in 0.2 ml, *N,N*-dimethylformamide, and finally mitochondria; in each case the concentration shown was the final concentration in the incubation flasks.

Submitochondrial Systems. Two submitochondrial systems were used: (i) A system composed of TPNH–cytochrome P-450 reductase (called S_2) and a fraction containing cytochrome P-450 (called P_3); these fractions were prepared as described by Omura *et al.* (1966). This system will be called the submitochondrial system ($S_2 + P_3$). (ii) A purified enzyme system consisting of three fractions: TPNH diaphorase prepared according to Omura *et al.* (1966); non-heme iron prepared by the method of Kimura and Suzuki (1967); and cytochrome P-450 prepared by the method of Mitani and Horie (1969). This enzyme system will be referred to as the reconstituted enzyme system. In several experiments the diaphorase and non-heme iron were incubated with P_3; these experiments are identified in the accompanying legends.

The details of procedures for incubating the submitochondrial systems are given elsewhere (Young and Hall, 1969). Following incubation of mitochondria or submitochondrial systems the reaction was stopped by adding methyl-

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[1] The following trivial names are used: corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; 11-deoxycorticosterone (DOC), 21-hydroxypregn-4-ene-3,20-dione; metopirone (SU-4885), 2-methyl-1,2-bis(3-pyridyl)-L-propanone; pregnenolone, 3β-hydroxypregn-5-en-20-one.

ene chloride to the incubation flasks which were then stood on ice until extraction was performed.

Side-Chain Cleavage of Cholesterol. The conversion of cholesterol into pregnenolone was measured by extracting pregnenolone into methylene chloride following incubation of mitochondria or submitochondrial systems with cholesterol. Pregnenolone was purified by paper chromatography followed by thin-layer chromatography (Hall, 1967) and was measured by gas–liquid partition chromatography. Recovery of pregnenolone was measured by addition of pregnenolone-7α-*t* to the incubation medium after incubation. Details of these procedures have been reported elsewhere (Young and Hall, 1969).

11β Hydroxylation. The conversion of 11-deoxycorticosterone (DOC) into corticosterone was measured by isolation and fluorimetric determination of corticosterone following incubation of mitochondria and submitochondrial systems with DOC (Silber *et al.*, 1958). Recovery of corticosterone was measured by addition of corticosterone-*4*-^{14}C to the incubation medium before extraction with methylene chloride. Corticosterone was purified by two systems of thin-layer chromatography on silica gel G: (I) chloroform–ethanol–water (92:8:0.5, v/v) and (ii) ethyl acetate–chloroform (90:10, v/v). Corticosterone was eluted from the first system, taken to dryness under nitrogen and applied to the second system. Corticosterone was eluted from the second system and aliquots were taken for measurement of mass by fluorimetry and of radioactivity by liquid scintillation spectrometry.

When corticosterone and pregnenolone were to be measured in the same samples, the methylene chloride extract was applied to paper in the system ligroin–propylene glycol for 14 hr. Corticosterone remained at the starting line and pregnenolone moved approximately 20 cm from the origin. Corticosterone and pregnenolone were eluted and further purified by thin-layer chromatography using the two systems described above for corticosterone and a system described elsewhere (Hall, 1967) for pregnenolone.

Liquid Scintillation Spectrometry. Details of the procedure used to measure radioactivity have been published (Means and Hall, 1967).

Determination of Protein. Protein was measured by the method of Lowry *et al.* (1951).

Chemicals. Corticosterone-*4*-^{14}C was purchased from New England Nuclear Corp. (lot no. 66-134-81), corticosterone from Sigma Chemical Corp., and Metopirone was generously donated by Ciba Limited, Basel, Switzerland. The sources of other chemicals have been given previously (Young and Hall, 1969).

Results

Side-Chain Cleavage and 11β Hydroxylation in Whole Mitochondria. EXOGENOUS TPNH AND Ca^{2+}. Figure 1 shows the results of an experiment in which bovine adrenocortical mitochondria were incubated with Ca^{2+} and excess TPNH together with cholesterol or DOC or both. Side-chain cleavage and 11β hydroxylation were measured. It is clear that DOC inhibits side-chain cleavage (Figure 1A), whereas cholesterol does not inhibit 11β hydroxylation or does so to no more than a trivial extent (Figure 1B).

In a second preparation of mitochondria the influence of DOC and cholesterol upon the same reactions was studied as a function of the concentrations of these substances in the incubation medium (Figure 1C). Whereas 600 mμmoles of DOC produced almost 80% inhibition of side-chain cleavage

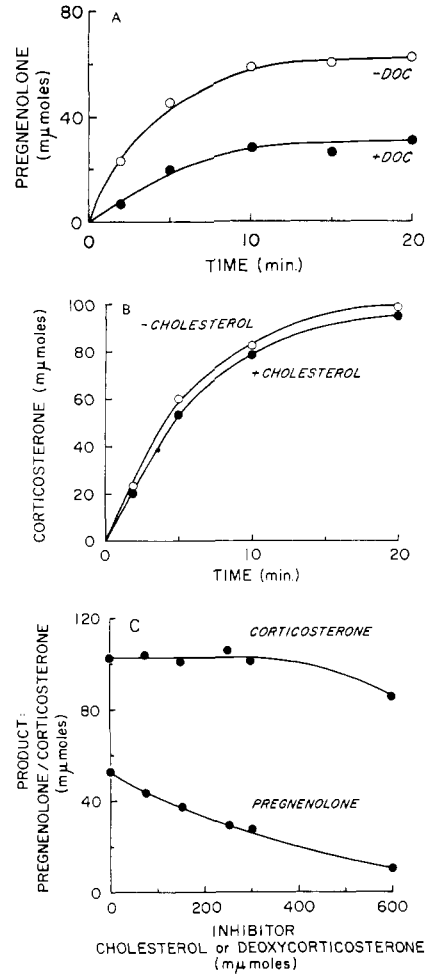

FIGURE 1: Bovine adrenocortical mitochondria (2.5 mg of protein/flask) were incubated with cholesterol or DOC dissolved in *N,N*-dimethylformamide (final concentration of each steroid 0.2 mM). Calcium chloride and TPNH were added to each flask (final concentrations 11 mM and 0.5 M, respectively). Other additions are described under Experimental Section. Incubation was performed for the times shown. Following incubation pregnenolone and corticosterone were isolated and measured as described under Experimental Section. In A and B the curves with and without inhibitor are labeled on the graph. In C the curves are labeled according to the product of the reaction, *i.e.*, the curve for 11β hydroxylation is labeled corticosterone and that for side-chain cleavage is labeled pregnenolone. In C incubation was for 15 min.

(curve labeled pregnenolone) the same concentration of cholesterol produced less than 20% inhibition of 11β hydroxylation (curve labeled corticosterone). Whereas 300 mμmoles of cholesterol did not inhibit 11β hydroxylation, this concentration of DOC caused approximately 50% inhibition of side-chain cleavage. Under the conditions used in these experiments and in the absence of inhibitors, 11β hydroxylation is approximately twice as active as side-chain cleavage and in some preparations this difference was greater.

Extensive experience with bovine adrenocortical mitochondria demonstrated that the concentration of TPNH used in these experiments (0.5 M) was saturating, that is greater concentrations of TPNH influenced neither the enzyme activities nor the extent of inhibition. Table I shows that exogenous TPNH accelerates side-chain cleavage and that DOC inhibits this reaction at various concentrations of TPNH. It is also clear that TPNH accelerates 11β hydroxylation as expected (Grant, 1956).

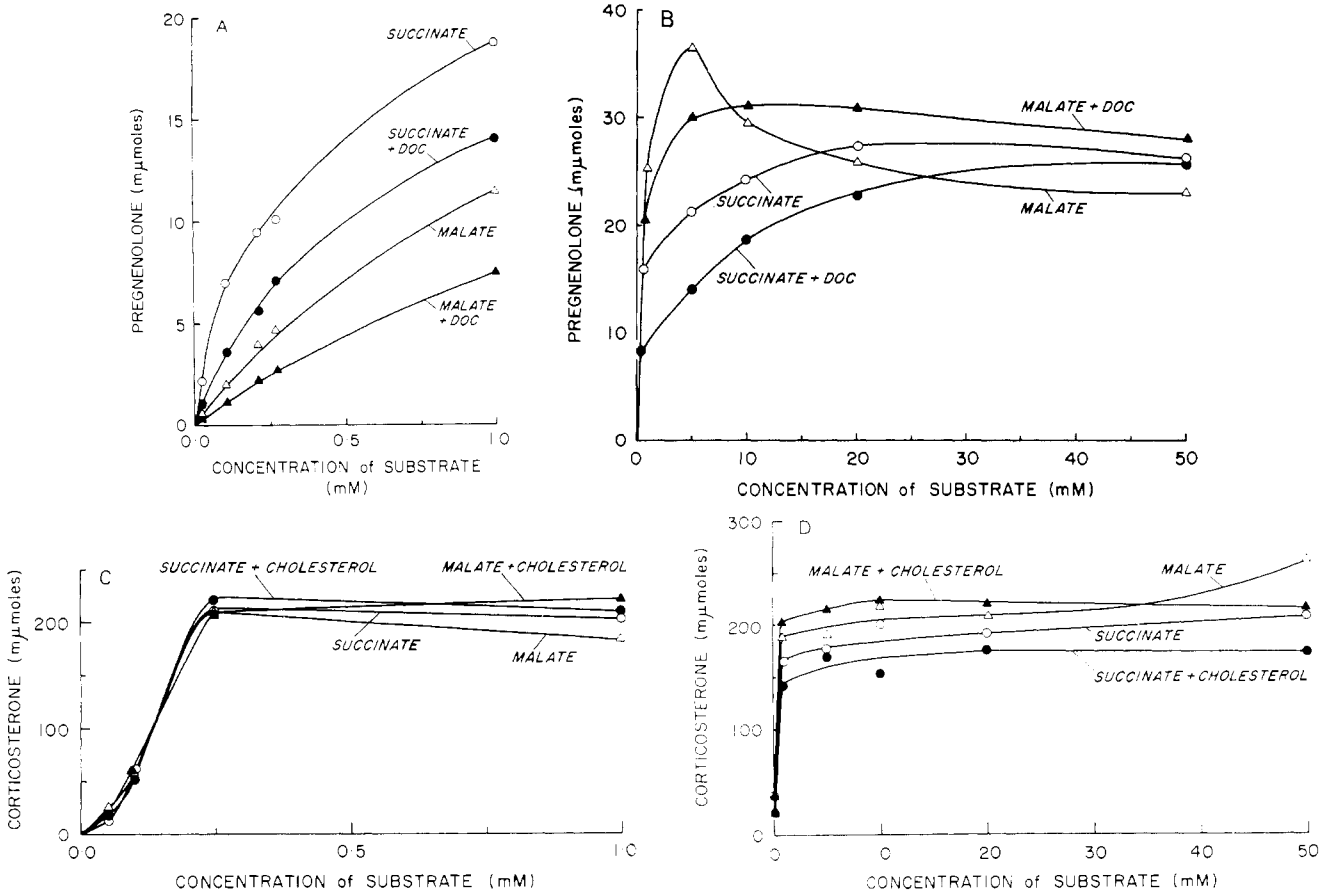

FIGURE 2: Bovine adrenocortical mitochondria (A and C, 3.5 mg of protein/flask; B and D, 4.7 mg of protein/flask) were prepared and incubated for 15 min as described for Figure 1. The concentrations of substrate used are shown. Neither TPNH nor Ca^{2+} was added. Cholesterol and DOC were added as indicated at a final concentration of 0.2 mM for each steroid. Following incubation pregnenolone and corticosterone were isolated and measured (Experimental Section). A and C were performed with one preparation of mitochondria, B and D with a second preparation.

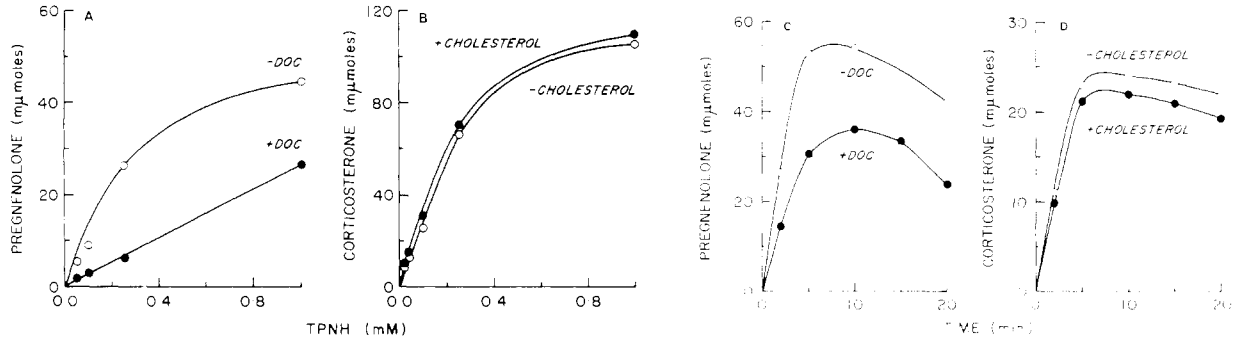

FIGURE 3: Cytochrome P-450 reductase (S_2) and cytochrome P-450 (P_3) were prepared from bovine adrenocortical mitochondria as described by Omura *et al.* (1966). S_2 (4.4 mg of protein/flask) and P_3 (2.4 mg of protein/flask) were incubated with cholesterol and DOC as shown; final concentration for each steroid was 0.12 mM. The following additions were made to each flask: sodium chloride (144 μmoles), potassium chloride (100 μmoles), magnesium chloride (10 μmoles), glycylglycine buffer (pH 7.4, 23 μmoles), and bovine serum albumin (20 mg). The reaction was started by addition of a TPNH-generating system (Young and Hall, 1969); the concentration of TPN^+ used was 2 mM. Pregnenolone and corticosterone were isolated and measured following incubation (Experimental Section).

OXIDIZABLE SUBSTRATES. Figure 2A,B shows the influence of DOC upon the side-chain cleavage of cholesterol in the presence of low (A) and high (B) concentrations of succinate and malate. Figure 2C,D shows similar studies for the influence of cholesterol upon 11β hydroxylation with the same Krebs' cycle intermediates. The same preparation of mitochondria was used for A and C; a second preparation was used for B and D.

Comparison of A and B shows that whereas with relatively low concentrations of succinate and malate (<1 mM), DOC inhibits side-chain cleavage, at high concentrations inhibition is no longer evident or is considerably reduced. It should be noted that the scale of the ordinates differ for the curves in A and B. By contrast, C and D show that cholesterol causes little inhibition of 11β hydroxylation at any concentration of succinate or malate. With isocitrate and fumarate as sub-

TABLE I: Inhibition of Side-Chain Cleavage by DOC in Adrenocortical Mitochondria with Exogenous TPNH.[a]

TPNH (μM)	Pregnenolone (mμmoles)	
	−DOC	+DOC
	0.08	0.04
	0.2	0.06
1	17.7	6.1
1	20.4	8.3
10	31.0	15.9
10	33.2	13.1
1000	51.0	21.8
1000	47.3	25.3

[a] This experiment was performed with one preparation of bovine adrenocortical mitochondria. The final concentrations of cholesterol and of DOC (when added) were 0.2 mM. The experimental procedure was exactly as described under Figure 1 except for the concentration of TPN^+ added with the reducing system (glucose-6-PO_4, 2.72 μmoles, and glucose-6-PO_4 dehydrogenase, 0.5 Kornberg unit).

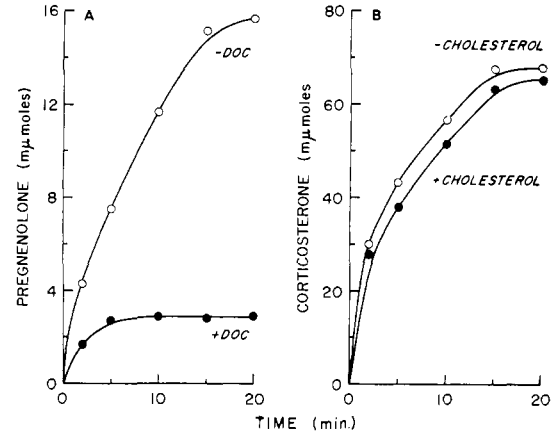

FIGURE 4: This experiment was performed as described under Figure 3 except that malate (10 mM) was added to each flask. S_2 (4.4 mg of protein) and P_3 (2.4 mg of protein) were also added to each flask). Neither TPN^+ nor a reducing system was added.

strates the same responses were observed, *i.e.*, DOC inhibits side-chain cleavage at relatively low concentrations of Krebs' cycle intermediates but cholesterol does not inhibit 11β hydroxylation at any concentration (data not shown).

Submitochondrial Systems. MITOCHONDRIAL FRACTIONS (S_2 + P_3). The influence of DOC on side-chain cleavage and that of cholesterol on 11β hydroxylation of DOC were studied with mitochondrial fractions consisting of cytochrome P-450 reductase (S_2) and cytochrome P-450 (P_3) (see Experimental Section).

Exogenous TPNH as Substrate. With exogenous TPNH the mitochondrial fractions responded to cholesterol and DOC in the same way as whole mitochondria (Figure 3A,B). DOC inhibited side-chain cleavage while cholesterol caused only slight inhibition of 11β hydroxylation over the range of concentrations of TPNH examined. Figure 3C,D shows the effects of DOC upon side-chain cleavage and of cholesterol upon 11β hydroxylation of DOC as a function of time with a second enzyme preparation. Again DOC inhibited side-chain cleavage (Figure 3C) especially at short incubation times and cholesterol produced little inhibition of 11β hydroxylation (Figure 3D) using the same enzyme preparation. The concentration of TPNH used for the studies shown in C and D was above the level required for maximal activity of both enzyme activities (2 mM).

Malate as Substrate. Evidently S_2 and P_3 contained malic dehydrogenase or some system which permits malate to generate TPNH from endogenous TPN^+, because malate supported side-chain cleavage and 11β hydroxylation (Figure 4A,B). Again DOC caused considerable inhibition of side-chain cleavage in contrast to minimal inhibition of 11β hydroxylation by cholesterol. The concentration of malate used was shown (with the same enzyme system) to produce half-maximal stimulation of enzyme activity. Similar results were obtained when exogenous TPN^+ (0.5 mM) was added to the system (data not shown).

RECONSTITUTED ENZYME SYSTEM. DOC also inhibited side-chain cleavage of cholesterol when the enzyme system consisted of a purified diaphorase, non-heme iron, and cytochrome P-450 (Figure 5A); the concentrations of DOC used were in the range (0.5–5) K_m for 11β hydroxylation on the basis of K_m of 60 μM (see below). Cholesterol produced little inhibition of 11β hydroxylation in this system (Figure 5B).

Measurement of Side-Chain Cleavage Plus 11β Hydroxylation Using Reconstituted Enzyme System. Table II shows values for side-chain cleavage and 11β hydroxylation with one substrate and values for both activities when both substrates were present. The enzyme system consisted of purified enzymes (see Experimental Section). The concentrations of substrates TPNH, diaphorase, and non-heme iron used exceeded those required for maximal enzyme activity. Three experiments are shown with three different preparations of enzyme. In each case DOC has inhibited side-chain cleavage but addition of cholesterol has not inhibited 11β hydroxylation. Moreover in the presence of excess cofactors (including diaphorase and non-heme iron), the sum of the two enzymatic activities when the two substrates were added separately is greater than the total activity observed when the two substrates are incubated together. The concentration of substrates used (2 mM) was more than 30 times greater than values for K_m of each substrate for the relevant enzyme system, using K_m cholesterol (50 μM) (Young and Hall, 1969) and K_m DOC (60 μM) (see below). Levels of endogenous cholesterol in expt 1 and 3 were <0.2 μM in each case. Two other similar experiments were performed with a fourth enzyme preparation. Mean decrease in total enzyme activity when both substrates were present together was 35% for the five experiments.

Studies with Metopirone in Reconstituted Enzyme System. The experiment reported in Table III shows that metopirone inhibits 11β hydroxylation using the reconstituted enzyme system in confirmation of numerous studies of other workers (*e.g.*, Dominguez and Samuels, 1963). Metopirone (75 μM) produces some inhibition of side-chain cleavage. When side-chain cleavage and 11β hydroxylation were measured by adding the two substrates cholesterol and DOC together, inhibition of 11β hydroxylation by metopirone persisted. However the usual inhibition of side-chain cleavage produced by DOC was decreased in the presence of metopirone (Table III).

Kinetics of 11β Hydroxylation in Reconstituted Enzyme System. Four separate experiments were performed using four different enzyme systems in the range for substrate concentration (5–200 μM). K_m was determined by means of a least-squares fit of the experimental data to the initial velocity

TABLE II: Side-Chain Cleavage and 11β Hydroxylation by a Reconstituted Adrenocortical Enzyme System Measured Separately and Together.[a]

Expt	Reaction	Product Formed (mμmoles/10 min) Pregnenolone	Corticosterone	Sum
1	Side-chain cleavage	21.2 ± 1.5		
	11β hydroxylation		42.2 ± 0.6	63.4
	Side-chain cleavage + 11β hydroxylation	9.0 ± 0.2	40.1 ± 1.3	49.1
2	Side-chain cleavage	59.3 ± 2.6		
	11α hydroxylation		35.8 ± 1.9	95.1
	Side-chain cleavage + 11β hydroxylation	21.9 ± 3.1	37.5 ± 2.1	59.4
3	Side-chain cleavage	14.2 ± 2.7		
	11β hydroxylation		21.1 ± 3.2	35.3
	Side-chain cleavage + 11β hydroxylation	5.6 ± 1.9	19.4 ± 3.0	25.0

[a] Side-chain cleavage and 11β hydroxylation were measured by incubating the reconstituted enzyme system with cholesterol or DOC or both. Following incubation pregnenolone and corticosterone were isolated and measured. Details of the relevant methods are given under Experimental Section. The enzyme was made up as follows: expt 1: diaphorase 2.5, non-heme iron 0.5 and P_3 (Omura *et al.*, 1966) 1.6; expt 2: diaphorase 2.4, non-heme iron 0.5, and cytochrome P-450 (Mitani and Horie, 1969) 1.0; expt 3: diaphorase 2.4, non-heme iron 0.5, and cytochrome P-450 (Mitani and Horie, 1969) 1.0; units in each case; milligrams of protein per flask. The final concentration of substrate was 2 mM in each case.

TABLE III: Influence of Metopirone on the Inhibition of Side-Chain Cleavage by DOC.[a]

Reaction	Metopirone (μM)	Product Formed (mμmoles/10 min) Pregnenolone	Corticosterone	Sum
Side-chain cleavage		36.6 ± 1.9		
11β hydroxylation			40.7 ± 1.5	
				77.3
Side-chain cleavage	25	31.8 ± 0.8		
11β hydroxylation	25		8.8 ± 0.8	
				40.6
Side-chain cleavage	75	27.0		
11β hydroxylation	75		4.1	
				31.1
Side-chain cleavage + 11β hydroxylation		24.6 ± 0.3	36.1 ± 2.1	60.7
Side-chain cleavage + 11β hydroxylation	25	34.1 ± 0.6	5.0 ± 1.1	39.1
Side-chain cleavage + 11β hydroxylation	75	32.0	3.0	35.0

[a] Side-chain cleavage and 11β hydroxylation were measured with and without addition of metopirone by incubating the reconstituted enzyme system with cholesterol and DOC. The formation of pregnenolone and of corticosterone were measured following incubation (see Experimental Section). The composition of the reconstituted enzyme system was that shown for expt 2 under Table II. Simultaneous incubation of the two substrates is indicated by +.

equation $v = VS/K_m + S$. Values for K_m ranged between 50 and 70 μM. These values are all considerably higher than those reported for an enzyme preparation from bovine adrenocortical mitochondria by Sharma *et al.* (1963). The enzyme system prepared by these authors involved an entirely different method of preparation from that used by the present authors. No explanation can be offered at present for the different values observed for K_m by the two groups.

Side-Chain Cleavage with and without Corticosterone. Corticosterone does not inhibit side-chain cleavage of cholesterol (Table IV). This has been demonstrated with four different preparations of the reconstituted enzyme system (two other studies not shown in Table IV) which together with five experiments with $S_2 + P_3$ all showed that corticosterone is without effect on side-chain cleavage.

Discussion

When side-chain cleavage and 11β hydroxylation are measured *in vitro* using bovine adrenocortical mitochondria, side-chain cleavage is inhibited by addition of DOC, while on the other hand, addition of cholesterol does not inhibit 11β hydroxylation. Inhibition of side-chain cleavage by addition of DOC is seen when the reducing equivalents for the two reactions (side-chain cleavage and 11β hydroxylation) are generated by various oxidizable substrates, although very high concentrations of such substrates permit maximal activity of the side-chain cleavage system even in the presence of DOC (Figure 2); such concentrations of substrate (>10 mM) are quite unphysiological. When exogenous TPNH is added as the source of reducing equivalents with Ca^{2+} to render mitochondria permeable to the pyridine nucleotide, inhibition of side-chain cleavage by addition of DOC is evident even at very high levels of TPNH (Figure 1 and Table I).

The interest in these observations lies in the possible significance of such inhibition in the regulation of the rate of steroid biosynthesis. If mitochondria permit 11β hydroxylation to proceed at the expense of side-chain cleavage, the products of 11β hydroxylation will leave the mitochondrion and so permit accelerated side-chain cleavage; side-chain cleavage in turn will provide substrate for 11β hydroxylation which will inhibit side-chain cleavage and so on. The system would operate by a form of negative feedback inhibition with the complications provided by movement of substrates between cytoplasm and mitochondria. The concentration of oxidizable substrates available *in vivo* is un-

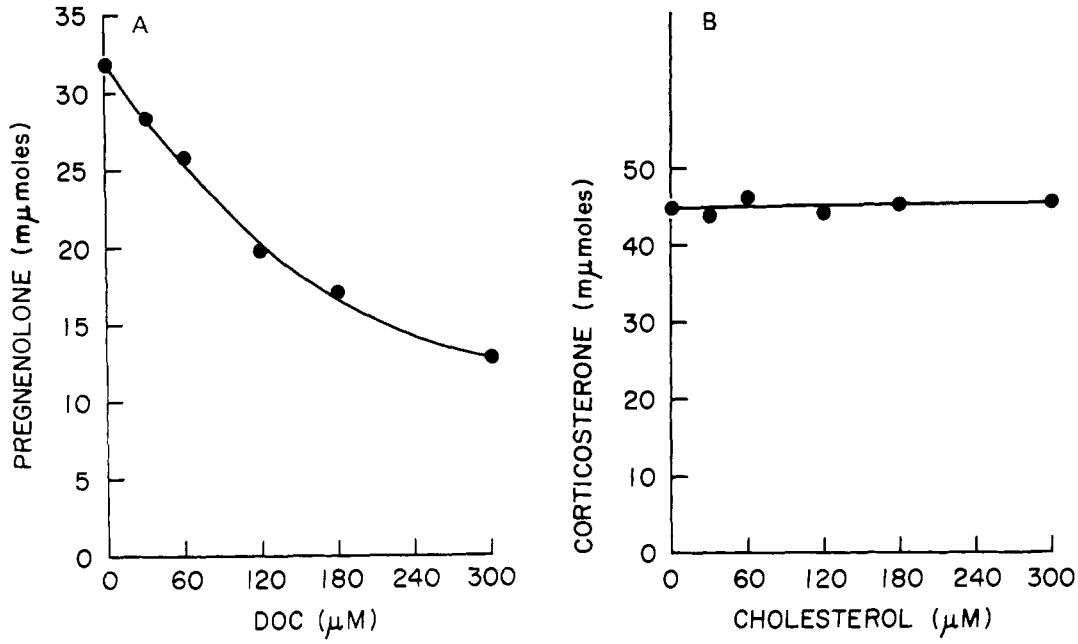

FIGURE 5: Inhibition of side-chain cleavage was examined as a function of the concentration of DOC (A), using a reconstituted enzyme (diaphorase 2.5 mg of protein/flask, non-heme iron 0.5 mg of protein/flask, and cytochrome P-450 0.7 mg of protein/flask). Similar measurements of 11β hydroxylation were made as a function of the concentration of cholesterol with the same enzyme preparation (B).

likely to reach the levels required to overcome inhibition of side-chain cleavage, although we have not tested possible combinations of such substrates. Presumably excess TPNH fails to overcome inhibition because the concentration of this substance entering the mitochondrion *in vitro* with the aid of Ca^{2+} (and in particular that part of the mitochondrion at which steroid hydroxylation occurs), is less than the TPNH available for these reactions when the pyridine nucleotide is reduced *in situ* by oxidizable substrates. In any case competition for TPNH seems a likely explanation for the inhibitory effect of DOC on side-chain cleavage in whole mitochondria. Firstly the various oxidizable substrates gave the same results with respect to inhibition of side-chain cleavage. Since the conversion of cholesterol into pregnenolone is known to require TPNH, oxidizable substrates must act by reducing TPN^+. High substrate concentrations provide sufficient reduced TPN to permit both reactions to occur at maximal rates. Secondly exogenous TPNH accelerates both reactions showing that availability of TPNH is rate determining for both reactions in isolated mitochondria (Table I and Hall, 1967). Inhibition is seen whether high or low concentrations of TPNH are added (Table I). The suggestion that the reactions compete for TPNH is reinforced by the fact that side-chain cleavage involves more than one hydroxylation reaction and therefore should require more TPNH than 11β hydroxylation (Shimizu *et al.*, 1961; Constantopoulos and Tchen, 1961).

The possibility that side-chain cleavage influences 11β hydroxylation needs further consideration since mitochondria contain cholesterol some of which is available for conversion into pregnenolone; therefore 11β hydroxylation has not been measured in the absence of side-chain cleavage using intact mitochondria. If side-chain cleavage does inhibit 11β hydroxylation, such inhibition is not likely to be physiologically important because 11β hydroxylation in mitochondria proceeds much more rapidly than side-chain cleavage even in the presence of exogenous cholesterol (Figure 1). Moreover any possible inhibition by endogenous cholesterol must represent maximal inhibition since addition of exogenous cholestero does not inhibit 11β hydroxylation (Figure 1B).

It seems reasonable therefore to suggest that 11β hydroxylation utilizes available TPNH at the expense of side-chain cleavage, so that at steady state a balance would be achieved between the relative rates of the two reactions competing for available TPNH.

With submitochondrial systems certain complications encountered with intact mitochondria are no longer found. For example, possible compartmentation of enzymes is presumably abolished. With the system $S_2 + P_3$, DOC inhibits

TABLE IV: Side-Chain Cleavage of Cholesterol with and without Corticosterone.[a]

Corticosterone (mμmoles/Flask)	Pregnenolone (mμmoles)
0	27
0	24
100	26
200	23
0	14
0	17
100	15
200	20

[a] The conversion of cholesterol to pregnenolone by the reconstituted enzyme system was measured. Corticosterone was added where shown. The table shows two experiments each performed with a different enzyme preparation. The composition of the enzyme system is shown for expt 3 under Table II.

side-chain cleavage but exogenous cholesterol does not inhibit 11β hydroxylation when excess TPNH or malate are added. With the reconstituted enzyme system, DOC was again found to inhibit side-chain cleavage and cholesterol did not inhibit 11β hydroxylation. In one such preparation, endogenous cholesterol was present in very small amounts (<0.3 mμmole/flask, *i.e.*, <0.2 μM). It seems unlikely that endogenous cholesterol significantly inhibits 11β hydroxylation in this system. These findings cannot be explained on the basis of competition for TPNH. Moreover measurement of both reactions together and separately (Table II) suggests competitive inhibition of side chain cleavage by addition of DOC. In these studies excess TPNH, diaphorase, non-heme iron, and substrates (>30 K_m) were present so that competition presumably occurs for cytochrome P-450 or for something associated with this cytochrome.

When 11β hydroxylation was inhibited by addition of metopirone, the inhibition of side-chain cleavage was greatly decreased (Table III). It has been shown that metopirone acts by binding to cytochrome P-450 with consequent inhibition of binding of DOC (Williamson and O'Donnell, 1969). Metopirone produces some inhibition of side-chain cleavage (Table III) but much greater inhibition of 11β hydroxylation. Presumably the inhibitor is a less effective competitor for the binding of cholesterol (as opposed to DOC) to cytochrome P-450. It should be added that the product of 11β hydroxylation of DOC (*i.e.*, corticosterone) is not responsible for the inhibition observed in these studies (Table IV). These data do not permit a single explanation for the competitive inhibition produced by DOC. At least two possibilities will need to be considered: competition for a single cytochrome P-450 and competition for some protein factor associated with the cytochrome (*i.e.*, a hydroxylase enzyme). Moreover the relative importance *in vivo*, of the two types of inhibition revealed by these studies, *i.e.*, competition for TPNH in whole mitochondria and competition for the cytochrome P-450 fraction, remains to be determined.

Acknowledgment

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