The Stoichiometry of the Conversion of Cholesterol and Hydroxycholesterols to Pregnenolone (3 β -Hydroxypregn-5-en-20-one) Catalysed by Adrenal Cytochrome P-450

(mitochondria/adrenal cortex/steroidogenesis)

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ABSTRACT The stoichiometry of the side-chain cleavage of cholesterol (cholest-5-en-3 β -ol), 20 α -hydroxycholesterol (cholest-5-ene-3 β ,20 α -diol), and 20 α ,22-dihydroxycholesterol (cholest-5-ene-3 β ,20 α ,22-triol) has been examined with respect to TPNH, oxygen and H⁺. Side-chain cleavage of cholesterol can be described by the following equation:

Stoichiometry of 20 α -hydroxycholesterol is 2TPNH and 2O₂ per mole of cleavage and with 20 α , 22-dihydroxycholesterol the values are 1:1:1. In addition 1 mole of H⁺ is consumed per mole of TPNH oxidized in each of these reactions. These observations are in keeping with a mechanism previously proposed in the literature, namely:

Discordant observations are reviewed and it is concluded that insufficient data are available to sustain objections to this pathway.

Cytochrome P-450 catalyzes mixed function or monooxygenase reactions which lead to hydroxylation of the substrate (1, 2). Cytochromes P-450 are present in the mitochondria of steroid-forming organs where they catalyze cleavage of the C₂₀-C₂₂ bond of cholesterol to form pregnenolone (3 β -hydroxypregn-5-en-20-one, refs. 3-5). We have recently purified a P-450 from bovine adrenocortical mitochondria to near homogeneity (6, 7).

It has been proposed that the mechanism of side-chain cleavage of cholesterol involves hydroxylation of C₂₀ and C₂₂ with cleavage of the vicinal glycol to yield pregnenolone and isocaproaldehyde (8-10):

Abbreviations and Nomenclature: cholesterol, cholest-5-en-3 β -ol; 20 α -hydroxycholesterol, cholest-5-ene-3 β ,20 α -diol; 20 α , 22-dihydroxycholesterol, cholest-5-ene-3 β ,20 α ,22-triol; pregnenolone, 3 β -hydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione.

The requirement for steroid hydroxylation which the scheme proposes is consistent with the involvement of P-450 in side-chain cleavage.

To catalyze side-chain cleavage, P-450 requires TPNH, atmospheric oxygen and two electron carrier proteins, namely TPNH flavoprotein dehydrogenase (reduced TPN: ferredoxin oxidoreductase, EC 1.6.99.4) and adrenodoxin (non-heme iron; adrenal ferredoxin) (11, 12). Some doubt has been cast upon this as the only pathway for side-chain cleavage (13-17). In any case the mechanism of the cleavage step (III) remains uncertain, although this step appears to require TPNH and oxygen (18). The proposed mechanism suggests a stoichiometry of at least two molecules of TPNH and two molecules of oxygen per molecule of side-chain cleavage. Knowledge of the stoichiometry of the overall reaction could suggest possible mechanisms for the cleavage step or might indicate the need for reevaluation of the proposed mechanism shown above. Using purified P-450, prepared by modification of a method recently reported from this laboratory (6), we have determined the stoichiometry of side-chain cleavage of cholesterol and of the two hydroxylated derivatives shown in the preceding scheme.

EXPERIMENTAL PROCEDURE

Preparation of Enzymes: Cytochrome P-450. Acetone powder was prepared from adrenocortical mitochondria (4). A ratio of acetone to mitochondrial suspension of 5:1 was used. The precipitate was washed repeatedly with acetone (-20°) and then with ether (0°). The desiccated powder was extracted twice by sonication in KCl (154 mM, pH 7.4) and a third time with potassium phosphate buffer (100 mM, pH 7.4). The three extracts were pooled and centrifuged at 100,000 $\times g$ for 60 min. The pellet was treated with sodium cholate and the purification of P-450 was continued according to a method already published (6). The purified preparation contained 3.0 nmoles of P-450 per mg protein (6).

Adrenodoxin and TPNH Flavoprotein Reductase. Adrenodoxin was prepared as described by Suhara *et al.*; this preparation reduced 7.2 nmoles of cytochrome *c* per min/mg of protein (19) and TPNH flavoprotein reductase was purified by a method reported from the same laboratory (20); this preparation reduced 3.9 μ moles of cytochrome *c* per min/mg of pro-

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FIG. 1. Utilization of TPNH (left) and oxygen (right) with and without substrate as a function of concentration of *P*-450. The figures also show the ratios $\Delta\text{TPNH}/\text{pregnenolone}$ formed and $\Delta\text{oxygen}/\text{pregnenolone}$ formed. The conditions used were those described in the text and incubation was continued for 15 min.

tein (20). The two proteins, as prepared by these methods, are purified to homogeneity according to criteria stated in these publications.

Oxidation of TPNH. Reactions were performed at 37° in a reaction cuvette placed in a Gilford recording spectrophotometer with an attachment to regulate temperature. The reaction mixture consisted of potassium phosphate (15 mM, pH 7.6) in a final volume of 2 ml, TPNH (60 nmoles), adrenodoxin (1.7 nmoles), flavoprotein reductase [0.9 DCPIP (2,6-dichloroindophenol) units—see ref. 4], *P*-450 (0.26 nmoles: 0.17 mg of protein) and substrate (50 nmoles in 50 μ l of *N,N*-dimethylformamide) unless otherwise stated. The reaction was started by addition of the flavoprotein reductase and absorbance at 340 nm was recorded as a function of time. The reaction was stopped by addition of dichloromethane and the mixture was extracted with this solvent to measure the amount of pregnenolone formed.

Consumption of Oxygen. Concentration of oxygen was measured at 37° with a Clark electrode. The reaction mixture was that described above for measuring the rate of oxidation of TPNH except that more adrenodoxin (3.4 nmoles) was used. Concentration of oxygen was recorded as a function of time and the system was calibrated by a published method based on the extent of oxidation of DPNH (21). The reaction was started by addition of TPNH; the reaction was stopped as described above and pregnenolone was isolated and measured.

Hydrogen Ion Concentration. To measure the rate of change of hydrogen ions by side-chain cleavage reaction, the incubation mixture was the same as that used in experiments with the oxygen electrode except that in order to facilitate measurement of small changes in pH, the concentration of phosphate buffer was reduced to 1 mM. Changes in pH were recorded by means of a pH-meter (Radiometer: Digital pH meter pHM52).

The pH meter was connected to a recorder and calibrated so that addition of 100 nmoles of HCl added to the incubation medium gave a full scale recorder deflection. The mV setting on the pH meter was used and voltage was recorded as a function of time. Replicate determinations agreed within the range of $\pm 5\%$.

Pregnenolone and [^{14}C]Pregnenolone. The products of side-chain cleavage (pregnenolone or [^{14}C]pregnenolone) were converted to progesterone or [^{14}C]progesterone by means of a microbial enzyme system containing 3β -hydroxysteroid dehydrogenase (3β -hydroxy- Δ^5 -steroid:NAD $^{+}$ 3-oxidoreductase, EC 1.1.1.145) plus 3 oxosteroid Δ^4 - Δ^5 isomerase, EC 5.3.3.1). The [^{14}C]progesterone so formed was purified by thin-layer chromatography and measured by liquid scintillation spectrometry; the progesterone formed from pregnenolone was measured by a fluorescence assay for DPNH (6, 22). In our hands, duplicate determinations by this method agreed within the range of $\pm 5\%$.

Chemicals. In addition to those reported previously (6), the following chemicals were used. 20α , 22 -dihydroxycholesterol was generously provided by Dr. K. Shimizu of Totori University, Yonago, Japan. The purity of cholesterol and both hydroxycholesterols was demonstrated by values for melting point and by behavior on thin layer, partition (15) and gas chromatography (6). Superoxide dismutase was purchased from Truett Laboratories, Dallas, Texas.

RESULTS

Stoichiometry of side-chain cleavage

Fig. 1 shows a number of important characteristics of the side-chain cleavage of cholesterol when the oxidation of TPNH and the consumption of oxygen are measured as a function of the concentration of *P*-450. Firstly, it can be seen that the oxida-

FIG. 2. Stoichiometry of side-chain cleavage of cholesterol. *A* shows Δ TPNH and pregnenolone formed as function of duration of incubation together with the ratio Δ TPNH/pregnenolone formed. *B* shows separate experiments with analogous findings for consumption of oxygen. Each flask contained 0.26 nmoles of *P*-450.

tion of TPNH and the consumption of oxygen take place in the absence of added substrate (cholesterol). This background reaction is not accompanied by side-chain cleavage because the enzyme is essentially devoid of endogenous cholesterol (<0.002 nmoles/nmole of *P*-450) (6) and no pregnenolone (<0.05 nmoles per flask) is formed during this reaction. The nature of this background reaction is not clear but a number of interesting features of the reaction will be discussed below. Secondly, if the stoichiometry of side-chain cleavage is calculated on the basis of *total* rate of consumption of TPNH and oxygen (i.e., background not subtracted) stoichiometry varies with concentration of *P*-450 from 4.5 to 7.0. On the other hand, the stoichiometry, calculated as increase of oxidation of TPNH and utilization of oxygen above background (referred to here as Δ TPNH and Δ oxygen), is 3:3:1 (TPNH, oxygen, side-chain cleavage) and is independent of the concentration of *P*-450. Nineteen determinations using three separate preparations of *P*-450 revealed ratios of TPNH/side-chain cleavage of 3.0 ± 0.2 (range) and for O_2 /side-chain cleavage 3.2 ± 0.4 (range).

Fig. 2 shows the production of pregnenolone and oxidation of TPNH (*A*) and consumption of oxygen (*B*) as a function of duration of incubation. Again the stoichiometry is 3.0 for

both parameters at all time intervals tested. The ratios shown in Fig. 2 are calculated after subtracting the background reaction; if total consumption of TPNH and oxygen are used, the ratio varies with time since the time courses of the two reactions (background and side-chain cleavage) are somewhat different. Table 1 shows that the stoichiometry with 20α -hydroxycholesterol is 2:2:1. In 12 determinations with 20α -hydroxycholesterol the following values were obtained (means and ranges): TPNH oxidized/cholesterol cleaved, 1.9 ± 0.3 ; oxygen used per cholesterol cleaved, 2.1 ± 0.3 ; with $20\alpha,22$ -dihydroxycholesterol the corresponding values were 1.0 ± 0.3 and 0.9 ± 0.4 . As expected, the previous comments apply to the background reaction observed in these studies except that in the case of 20α -hydroxycholesterol this reaction constitutes a smaller proportion of the total consumption of TPNH and oxygen (approximately 15%) because the rate of side-chain cleavage with this substrate is considerably greater than that observed with the other two substrates (cholesterol and $20\alpha,22$ -dihydroxycholesterol). Table 1 also shows the stoichiometry with cholesterol (3:3:1). The value for the stoichiometry with oxygen (3.6) is the highest value observed in these studies. Stoichiometry did not change when higher substrate concentrations were used ($20.0 \mu M$).

TABLE 1. Stoichiometry of side-chain cleavage of cholesterol and hydroxycholesterols*

Substrate	Rate of formation of pregnenolone (nmoles/20 min)	Rate of consumption of TPNH	Δ TPNH <hr/> pregnenolone	Rate of formation of pregnenolone (nmoles/20 min)	Rate of consumption of oxygen	Δ O ₂ <hr/> pregnenolone
Cholesterol	2.4	7.9	3.3	3.5	12.7	3.6
20 α -Hydroxycholesterol	7.5	14.7	2.0	9.1	17.1	1.9
20 α ,22-Dihydroxycholesterol	7.2	7.1	1.0	7.4	11.0	1.5

* See Experimental Procedure for conditions used.

TABLE 2. Consumption of hydrogen ions during side-chain cleavage of cholesterol*

Rate of formation of pregnenolone (nmoles/20 min)	Rate of consumption of hydrogen ions	ΔH^+ Pregnenolone
3.2	9.7	3.03
2.1	6.5	3.0
3.7	11.4	3.10

* See *Experimental Procedure* for conditions used.

The consumption of hydrogen ions in relation to side-chain cleavage is shown in Table 2. It is clear that, as expected, one mole of H^+ is consumed for each mole of TPNH oxidized and hence 3 moles of H^+ are consumed per mole of cleavage with cholesterol as substrate.

Side-chain cleavage of cholesterol and 20α -hydroxycholesterol was not accompanied by accumulation of reaction intermediates, so that formation of pregnenolone was accounted for by disappearance of cholesterol or 20α -hydroxycholesterol within the limits of experimental error (6). The various reactions (cleavage, oxygen consumption, TPNH oxidation) are all linear for at least 5 min.

Background reaction

The background reaction shows the following characteristics: (a) oxidation of TPNH and consumption of oxygen occur at a ratio of approximately 1.0 (TPNH/ O_2 :1.1 $\pm$ 0.2 in eight determinations). (b) The rate of the reaction per nmole of *P*-450 is independent of the state of purity of *P*-450 and that of the two electron carriers once these proteins are partly purified. (c) The background reaction is slightly increased by certain hemoproteins (cytochrome *c*, catalase, and peroxidase) and is inhibited by superoxide dismutase (data not shown).

Effect of hemoproteins and dismutase

Peroxidase, cytochrome *c*, hemoglobin at high concentrations (1–2 μ M), and catalase inhibited side-chain cleavage as much as 30%. As stated above, these hemoproteins stimulated the rate of oxidation of TPNH in the absence of added cholesterol. At lower concentrations (<0.2 μ M), the hemoproteins had no effect on the rate of side-chain cleavage and in no case did they inhibit the rate of oxidation of TPNH in the absence of cholesterol.

It was repeatedly observed that side-chain cleavage was not inhibited by dismutase (Table 3) and that any decrease in the rate of oxidation of TPNH observed was consistent with inhibition of the background reaction with the result that the stoichiometry of Δ TPNH/side-chain cleavage was not altered by addition of dismutase (Table 3). The effect of dismutase was the same when studied as a function of the duration of incubation at times between 5 and 20 min.

DISCUSSION

The observations presented here show that when the side-chain cleavage of cholesterol is catalyzed by purified cytochrome *P*-450, the stoichiometry of the reaction is as follows:

Such stoichiometry is consistent with the scheme presented

TABLE 3. Effect of superoxide dismutase on oxidation of TPNH by side-chain cleavage system*

Dismutase (μ g)	Basal rate of oxidation of TPNH	Δ TPNH in presence of cholesterol (nmoles/20 min)	Rate of pregnenolone formation	Δ TPNH pregnenolone
0	9.1	14.6	5.2	2.8
25	6.8	15.1	5.8	2.6

* See *Experimental Procedure* for conditions used.

in the introduction. If this pathway does describe the true course of the reaction, the cleavage step itself must require one mole of TPNH and one mole of oxygen per mole of pregnenolone formed. The stoichiometry with 20α -hydroxycholesterol as substrate is $2O_2$ and 2TPNH per molecule of pregnenolone formed—one molecule of each substance for the hydroxylation of C_{22} and one of each for cleavage of the C_{20} , C_{22} bond. The stoichiometry of 1:1:1 for 20α ,22-dihydroxycholesterol is consistent with these findings and with an earlier report showing that oxygen and TPNH are required for the conversion of this substance to pregnenolone (18).

One technical problem, associated with these experiments, must be discussed. Fig. 1 shows that significant consumption of TPNH and oxygen occurs without added substrate and hence in the absence of side-chain cleavage since the enzyme used is virtually devoid of endogenous substrate (see *Results*). It is necessary to consider whether it is justified to subtract the background consumption of TPNH and oxygen from that seen in the presence of substrate. The range observed with replicate determinations indicates that the methods used are sufficiently accurate to give reproducible results in spite of the background reaction. The question is whether the reaction with substrate alters the background reaction, for example, by competing for electrons. This seems unlikely for the following reasons: In the first place when background (i.e., reaction without substrate) is subtracted, the calculated stoichiometry is always the same and close to a whole number with each substrate. When total consumption of TPNH and oxygen are used to calculate stoichiometry, the values found are seen to vary with the concentration of *P*-450 (Fig. 1 and *Results*) and with duration of incubation (*Results*). It is difficult to imagine a mechanism that would permit such variations in stoichiometry. Partial competition between the two reactions which varies with concentration of *P*-450 and with time, in such a manner as to yield a false but consistent value for stoichiometry with each substrate in every experiment, is equally improbable. In the second place, if the background reaction is decreased in the presence of side-chain cleavage, the calculated stoichiometry is too low. It is not easy to suggest a mechanism for the side-chain cleavage of cholesterol requiring more than three molecules of oxygen per molecule of pregnenolone formed. In the third place the three substrates would not be expected to affect the background reaction to a different extent in such a way as to reveal false stoichiometries of 3, 2, and 1. Moreover, in the case of 20α ,22-dihydroxycholesterol the substrate could not be increasing the background reaction because this would mean that the cleavage step shows a stoichiometry of less than 1. Fourthly, the fact that dismutase inhibits the background reaction but not the increased rate of oxidation of

TPNH seen when cholesterol is present (Table 3), suggests that these two reactions can proceed independently.

Clearly our observations are most readily explained by assuming that side-chain cleavage is without significant effect on the background reaction and that the two reactions differ with respect to such variables as time and concentration of *P*-450.

The nature of the background reaction is uncertain and may involve more than one reaction, especially since dismutase causes significant but only partial inhibition. The reaction requires the presence of *P*-450 and the two electron carriers. It apparently does not involve formation of peroxide since it is inhibited by neither catalase nor peroxidase. Moreover the rate of the background reaction is not demonstrably altered by the state of purity of *P*-450 or the electron carriers (data not shown). The preparation of *P*-450 used in these studies has a molecular weight of 210,000. This form of the enzyme consists of 4 subunits (6, 7) and was chosen for these studies because the background reaction is considerably less than that observed with the larger (16 subunits) form of the enzyme originally isolated (6, 7). The reason for this difference between the two forms of the enzyme is not clear at this time.

The stoichiometries for the reactions studied here provide indirect support for the proposed pathway for side-chain cleavage. It has been shown that the oxygen at C₂₀ of 20 α -hydroxycholesterol remains as the ketone group of pregnenolone and that the oxygen at C₂₂ in 20 α ,22-dihydroxycholesterol appears in isocaproaldehyde (23). Moreover, it is highly improbable that the oxygenated substrates are first converted to cholesterol which is then cleaved by a mechanism not involving hydroxylated derivatives because no external source of energy was available to reverse the hydroxylation reactions. Furthermore, such reversal would release one atom of oxygen from 20 α -hydroxycholesterol, so that the stoichiometry with this substrate (oxygen *versus* side-chain cleavage) as measured by the methods used here, would not be a whole number.

At least two observations in the literature appears to be inconsistent with the present findings. Firstly, Hall and Koritz (13) reported that when [³H]cholesterol was incubated with an acetone powder of adrenocortical mitochondria, exogenous 20 α -hydroxycholesterol added to the incubation medium could be isolated, after incubation, without detectable tritium. Under a variety of experimental conditions the production of [³H]pregnenolone could be accounted for by disappearance of [³H]cholesterol within the limits of experimental error. If 20 α -hydroxycholesterol is an intermediate in this reaction, it must be present in extremely small amounts in a form which does not permit exchange with exogenous 20 α -hydroxycholesterol.

A second discordant observation was reported by Luttrell *et al.* (17) who showed that an analogue of 20 α -hydroxycholesterol in which C₂₂ is completely substituted by methyl

groups, is converted to pregnenolone by a crude enzyme system from adrenocortical mitochondria (17). Since one product of side-chain cleavage of the analogue as well as the stoichiometry and the rate of the reaction are not known, it is not possible to choose between various theoretically acceptable mechanisms for cleavage of the analogue. At this point the relevance of the cleavage of the analogue to that of cholesterol is uncertain.

It is, therefore, concluded that our findings appear to uphold the mechanism for side-chain cleavage postulated in earlier studies (8–10) and are difficult to reconcile with ionic and free radical mechanisms (17).

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