Biosynthesis of Pregnenolone from Cholesterol by Mitochondrial Enzymes of Bovine Adrenal Cortex

The Question of the Participation of the 20(22)-Olefins and 20,22-Epoxides of Cholesterol

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The two isomeric 20(22)-olefins and the four isomeric 20,22-epoxides of cholesterol have been examined as possible intermediates in the conversion of cholesterol to pregnenolone both by crude extracts of acetone-dried bovine adrenal cortex mitochondria, and by a partially purified cytochrome P-450 specific for the side chain cleavage (P-450_{SCC}). The quantities of pregnenolone (measured by radioimmunoassay) formed from these putative intermediates and from cholesterol, (2057-20-hydroxycholesterol, (22R)-22-hydroxycholesterol, and (20R,22R)-20,22-dihydroxycholesterol have been compared. In both crude and purified systems, the conversion of the monohydroxycholesterols is somewhat more rapid than that of cholesterol. However, the dihydroxycholesterol is a very much more efficient precursor of pregnenolone. Whereas small amounts of pregnenolone were formed from the four 20,22-epoxycholesterols, and slightly larger amounts from the two 20(22)-didehydrocholesterols in the crude enzyme systems, almost no conversion of these six compounds was observed with the purified system containing cytochrome P-450_{SCC}, adrenodoxin, adrenodoxin reductase, and an NADPH-generating system. It is concluded that the olefins and epoxides are not obligatory intermediates in the conversion of cholesterol to pregnenolone, and the conversions of cholesterol olefins and epoxides observed by us and by others may reflect the presence of extraneous enzymes such as double bond reductases and epoxide hydratases which are unrelated to pregnenolone biosynthesis. The relative effectiveness of the cholesterol olefins and epoxides, as well as 20-isocholesterol in inhibiting the formation of pregnenolone from [4-14C]cholesterol has been determined. The compounds which have the same configuration as cholesterol at C-20 are more potent inhibitors of this conversion than the olefins in which the C-21, C-20, C-22, C-23 carbon system is planar, or than those compounds which have the opposite configuration to cholesterol at C-20 (i.e. 20-isocholesterol, (20S,22S)-20,22-epoxycholesterol and (20S,22R)-20,22-epoxycholesterol). The enzyme appears to be more fastidious in its steric requirement at C-20 than at C-22. Since the olefins and epoxides are stereocchemically rigidified around the C-20 to C-22 bond, these inhibitor studies suggest that the natural conformation of cholesterol on the side chain cleavage enzyme system is such that the side chain is fully extended and thus closely resembles the conformation of cholesterol in the crystal state.


Abbreviations. The following abbreviations are used: cytochrome P-450_{SCC}, a highly purified bovine adrenal cortex mitochondrial cytochrome P-450 that cleaves the side chain of cholesterol to pregnenolone and is inactive in the 11β-hydroxylation reaction; pregnenolone, 3β-hydroxypregn-5-en-20-one; cyanoketone, 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one.

Enzymes. Adrenodoxin reductase (EC 1.6.7.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).
Cleavage of the C-20 to C-22 bond of cholesterol (compound I) to pregnenolone (compound II) is an essential step in the biosynthesis of steroid hormones. This reaction requires three proteins obtainable from the adrenal cortex: an iron-sulfur protein known as adrenodoxin reductase, and the cytochrome P-450. One of oxygen and of NADPH are consumed in the conversion of one molecule of cholesterol to pregnenolone and isocaproaldehyde (4-methylhexanal) either by highly purified enzyme preparations or crude adrenal mitochondria. Despite substantial progress in our understanding of the enzymology of this reaction, its fundamental details remain controversial.

Several mechanisms of the side chain cleavage are currently in vogue. The now classical sequential hydroxylation mechanism holds that cholesterol first undergoes hydroxylation to (20S)-20-hydroxycholesterol (compound IX) or (22R)-22-hydroxycholesterol (compound X), which are then converted to (20R,22R)-20,22-dihydroxycholesterol (compound XI), and the latter is in turn cleaved to pregnenolone and isocaproaldehyde. Extensive efforts to support or refute this mechanism by kinetic measurements have been inconclusive [5-8]. The enzymatic formation of both (22R)-22-hydroxycholesterol (compound X) and (20R,22R)-20,22-dihydroxycholesterol (compound XI) is firmly established [9], and the turnover of these compounds as sequential substrates and products has been demonstrated by means of 18O isotope techniques [8].

Lieberman and co-workers have shown that analogues of cholesterol in which the C-22 to C-27 moiety of the side chain has been replaced by a tert-butyl or a p-tolyl group (and consequently incapable of being oxygenated at the carbon atom corresponding to C-22 of cholesterol) are hydroxylated at C-20 and converted to pregnenolone by adrenal mitochondria. Thus, these workers have suggested that the true intermediates are transient reactive species (ionic, free radical, or of a hybrid type) and that the hydroxylated cholesterol derivatives are side products. With highly purified side-chain cleavage enzyme preparations, however, Morisaki et al. [12] were unable to detect any conversion of (20R)-20-β-tolylpregn-5-ene-3β,20-diol to pregnenolone. This compound was erroneously assigned the (20S)-chirality by Morisaki et al. [12].

Recently, Kraaiipoel and co-workers observed that (20R)-22-dihydrocholesterol (compound III or IV, unspecified stereochemistry) markedly stimulates the oxygen consumption of respiring mitochondria, and increased the formation of pregnenolone. They proposed an olefin-epoxide mechanism which is analogous in some ways to the olefin-epoxide intermediates in the dealkylation of fucosterol in insects [15]. The mechanism of Kraaiipoel et al. requires the existence of enzymes capable of converting cholesterol to a 20(22)-didehydrocholesterol, epoxidizing the 20(22) double bond, and an epoxide hydratase forming the (20R,22R)-20,22-dihydroxycholesterol. Circumstantial evidence for the existence of an epoxide intermediate has been provided [3,13,14]. In the pathway proposed by Kraaiipoel et al. the (20S)-20-hydroxycholesterol and (22R)-22-hydroxycholesterol are regarded as side products resulting from the hydration (presumably, by additional enzymes) of the 20(22)-olefin(s).

In preliminary studies employing gas chromatography-mass spectrometry, Morisaki et al. [16] were unable to detect significant conversion of any of the four isomeric 20,22-epoxycholesterols (compounds V-VIII) or of E-20(22)-didehydrocholesterol (compound III) to pregnenolone by a highly purified side-chain cleavage enzyme system. Moreover, these putative intermediates did not trap any radioactivity from [3H]cholesterol in the enzyme systems that produced pregnenolone. Using gas chromatography-mass spectrometric techniques, Burstein and co-workers were likewise unable to detect significant conversion of E-20(22)-didehydrocholesterol (compound III), Z-20(22)-didehydrocholesterol (compound IV), or (20R,22R)-20,22-epoxycholesterol (compound V) to pregnenolone in crude adrenal mitochondrial acetone powders. These authors did, however, find that (20R,22S)-20,22-epoxycholesterol (compound VII) was converted to pregnenolone with 20% of the efficiency of cholesterol. The findings of Kraaiipoel et al. cannot easily be reconciled with those of Morisaki et al. [16] and of Burstein et al. [17], although the purity and manner of preparation of the side-chain cleavage enzyme systems used by these three groups were quite different.

In the light of these conflicting views on the mechanism of the enzymatic conversion of cholesterol to pregnenolone, we have undertaken a systematic and detailed examination of the possible conversion of the E and the Z 20(22)-didehydrocholesterols (compounds III and IV) and of the four isomeric 20,22-epoxycholesterols (compounds V-VIII) to pregnenolone. These experiments have been conducted in parallel with crude extracts of acetone-dried bovine adrenal mitochondria and with the refined enzymes obtained from this source by Shikita and Hall. Moreover, we have used highly sensitive, specific and quantitative radioimmunoassay procedures for measuring pregnenolone, thus obviating the necessity for uncertain corrections for yields entailed in chroma-
tohraphic and gas chromatographic-mass spectrometric procedures employed by other workers. The proposed intermediates have all been synthesized in our laboratories by independent stereoselective procedures and have been unambiguously characterized [18,19]. Our syntheses differ from those of other investigators [20,21]. The present paper constitutes the full description of our findings which have already been communicated in preliminary form [22].

As will be shown, the purified adrenal enzyme system fails to promote significant conversion of any of the cholesterol olefins or epoxides to pregnenolone, and consequently, we were able to obtain measurements of the inhibitory activities of these compounds on the conversion of cholesterol to pregnenolone. Since the olefin and epoxide functions impart a significant measure of conformational rigidity to the side chain, these inhibitor studies have led to inferences regarding the preferred conformation of the side chain at the active site of the cholesterol degrading enzyme.

MATERIALS AND METHODS

Materials

All solutions were prepared from reagent grade chemicals in deionized, glass-distilled water. The antisera to pregnenolone 3-hemisuccinate coupled to human serum albumin were obtained from Radioisoay Systems Laboratories (Carson, Calif.). [7,3H]-Pregnenolone (specific activity 20 Ci/mmol) and [4,14C]cholesterol (specific activity 55.5 Ci/mol) were purchased from New England Nuclear (Albany, MA.). The liquid scintillation fluor, known as PCS, was obtained from Amersham (Arlington Heights, Ill.). Crystalline yeast glucose-6-phosphate dehydrogenase was supplied by Sigma Chemical Company (St Louis, Mo.). Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Company (Chicago, Ill.). The E and Z isomers of 20(22)-didehydrocholesterol, the four isomers of 20,22-epoxycholesterol, and (20R,22R)-20,22-dihydroxycholesterol were synthesized in our laboratories [18,19]. Samples of (20S)-20-hydroxycholesterol and (22R)-22-hydroxycholesterol were prepared by established procedures [23,24]. 20-Isodehydrocholesterol was prepared by a novel stereospecific method [25]. The cyanoketone inhibitor used to retard the oxidation of pregnenolone to progesterone [26,26a] was synthesized according to Manson et al. [27].

Protein Determinations

The method of Lowry et al. [28] was used with crystalline bovine plasma albumin as standard.

Preparation of Crude Side-Chain Cleavage Enzyme from Bovine Adrenal Cortex Mitochondria

Acetone-dried powders of bovine adrenal mitochondria prepared according to standard procedures [5,28], were stable for many months when stored in a vacuum desiccator over P2O5 at −20 °C. For each experiment, the acetone powder (10 mg) was homogenized by hand in a tight-fitting all-glass tissue grinder in 2.0 ml of 20 mM potassium phosphate buffer, pH 7.8, containing 1 mM magnesium sulfate. The homogenate was centrifuged at 20000 × g (average) for 30 min. Aliquots of the resulting straw-colored supernatant were used as the side-chain cleavage enzyme. These preparations (protein concentration 0.35–0.55 mg/ml) retained enzymatic activity for several weeks when stored at −80 °C.

Adrenal Activator Preparation

Adrenal activator was obtained by homogenizing the acetone powder (10 mg/ml) in 20 mM potassium phosphate, pH 7.8, 1 mM magnesium sulfate, heating the preparations for 2 min at 100 °C, and centrifuging at 20000 × g (average) for 30 min. The supernatant fractions (protein concentration 0.76–1.25 mg/ml) served as activator [29–31].

Purified Side-Chain Cleavage Enzyme

Side-chain cleavage cytochrome P-450 was prepared as described [1,32]. The cytochrome P-450 concentrations in the preparation used for these experiments was 36 nmol/ml, as determined by CO difference spectra [33], and the protein concentration was 9.0 mg/ml. The preparation was stored at −20 °C in 50 % glycerol. Adrenodoxin and adrenodoxin reductase were prepared by described methods [34,35].

Incubation Conditions

The compositions of the incubation mixtures for the measurement of the formation of pregnenolone from cholesterol and other precursors are given with the individual protocols (see Fig. 2, Tables 1 and 2).

Analysis of Reaction Products

The enzymatic reactions were stopped by the addition of 2 ml of cyclohexane, mixed on a Vortex mixer for 1 min, and agitated for 30 min on a mechanical shaker. The organic solvent layer (centrifuged if necessary) was transferred to acid-washed 12 × 75-mm glass tubes, and the cyclohexane was evaporated on a vacuum centrifuge [36]. The aqueous residue was extracted with a second portion (2 ml) of cyclohexane, the extracts were combined and evaporated on the vacuum centrifuge. The dry residues were stored at...
RESULTS AND DISCUSSION

Formation of Pregnenolone from Cholesterol and Various Didehydro, Epoxy, and Hydroxy Derivatives of Cholesterol

The initial evaluation of these compounds as precursors of pregnenolone was carried out with crude centrifuged extracts of acetone-dried bovine adrenal mitochondria supplemented with a soluble heat-stable activator (obtained from similar extracts) and an NADPH-generating system. Table 1 presents the mean quantities of pregnenolone (measured by radioimmunoassay) formed from the various steroids in four separate experiments. The values have been normalized for each experiment in relation to the amount of pregnenolone formed from cholesterol (100). Although the results obtained at only a single low concentration of steroid (2.5 µM, or about one-tenth of the Kₘ value) are shown in Table 1, the relative rates of conversion were comparable at 5.0 and 12.5 µM concentrations of each steroid. Low concentrations of test compounds were selected to minimize the problems of steroid solubility, and this proved feasible because of the high sensitivity of the radioimmunoassay. Under these conditions, the two monohydroxylated derivatives of cholesterol are between 1.5 and 2 times more efficient precursors of pregnenolone than cholesterol itself, whereas (20R, 22R)-20,22-dihydroxycholesterol is converted to pregnenolone much more efficiently (6.7 times) than cholesterol, in agreement with the findings of other workers. There are also several reports on the conversion of the monohydroxycholesterols to pregnenolone, although the reported rates of these conversions vary considerably [6, 8].

In contrast, the two isomeric 20(22)-didehydrocholesterols and the four isomeric 20,22-epoxycholesterols gave rise to only between 15.8 and 66.2% of the amount of pregnenolone formed from cholesterol itself (Table 1). Although we conclude that the olefins and epoxides are not likely to be true intermediates in the biosynthesis of pregnenolone from cholesterol, the quantities of pregnenolone formed from these oxygenated species is not negligible, and these amounts increased with time, the amount of enzyme, and steroid concentrations (over a limited range). Moreover, the didehydrocholesterols and epoxycholesterols neither react nor interfere with the pregnenolone radioimmunoassay. The slow conversion of the dehydrocholesterols and epoxycholesterols to pregnenolone in these relatively crude enzyme systems could be attributed to the presence of ancillary enzymes.
Table 1. Conversion of cholesterol and various 20(22)-dihydro, 20,22-epoxy, and 20-hydroxy or 22-hydroxy cholesterol analogues to pregnenolone by crude extracts of bovine adrenal cortex mitochondria

The reaction systems contained in a final volume of 200 µl: 20 mM potassium phosphate, pH 7.8; 0.1% (w/v) bovine serum albumin; 5 mM reduced glutathione; 0.5 mM magnesium sulfate; 0.003% (v/v) Triton N-101; 6 µM cyanoketone in 0.1 µl of dimethylformamide; 2.5 µM (500 pmol) cholesterol or analogue in 5 µl of methanol; 25 µl of crude side-chain cleavage enzyme, and 100 µl of activator solution. After a preliminary incubation of 20 min at 37°C with gentle agitation, the reaction was initiated by addition of an NADPH-generating system composed of the following ingredients and giving the final concentrations: 5 mM glucose 6-phosphate; 0.2 mM NADP⁺; crystalline yeast glucose-6-phosphate dehydrogenase (0.26 µg); and 5 mM magnesium chloride. Reaction mixtures were incubated for 60 min at 37°C with gentle agitation. The reaction mixture was extracted with cyclohexane and the pregnenolone was analyzed on aliquots of the extracts by radioimmunoassay, as described under Methods. The results are expressed as the means (± S.E.M.) of four separate experiments, conducted with different enzyme preparations. The mean quantity of pregnenolone formed in the four experiments was 28.0 ± 6.9 pmol (range 11.5 – 69.5 pmol). The amount of pregnenolone formed from cholesterol in the four experiments has been normalized to 100.

The results were much more clear-cut when the same compounds were subjected to the action of the purified side-chain cleavage enzyme system, consisting of bovine adrenal cytochrome P-450scC, adrenodoxin, adrenodoxin reductase, and an NADPH-generating system (Fig. 2). At three concentrations of steroid (2.5, 7.5, and 15 µM), there was very little conversion of any of the four epoxides or of either of the two isomeric olefins to pregnenolone, and the small amount of pregnenolone formed from these compounds did not rise with increasing steroid concentration. The actual quantities of endogenous pregnenolone detected in the reaction system were negligible. The enzymes that might cleave the epoxides (compounds V – VIII) to diols (some of which might then enter the sequential hydroxylation pathway), or might reduce the 20,22 double bond of the olefins (protected in the reaction system were negligible. The enzymes that might cleave the epoxides (compounds adrenodoxin reductase, and an NADPH-generating system, consisting of the sequential hydroxylation pathway), or might be obligatory intermediates in the formation of pregnenolone from cholesterol. Our findings are in agreement with those of Morisaki et al. [16] and Burstein and Gut [8]. Wang and Kimura [43] have independently concluded that the participation of an epoxide hydratase in the cholesterol side-chain cleavage reac-

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<thead>
<tr>
<th>Steroid added</th>
<th>Relative amounts of pregnenolone formed</th>
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<tr>
<td>Cholesterol (I)</td>
<td>100</td>
</tr>
<tr>
<td>E-20(22)-Didehydrocholesterol (III)</td>
<td>65.2 ± 7.9</td>
</tr>
<tr>
<td>Z-20(22)-Didehydrocholesterol (IV)</td>
<td>66.2 ± 12.3</td>
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<tr>
<td>(20S,22S)-20,22-Epoxysterol (VI)</td>
<td>26.4 ± 6.5</td>
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<tr>
<td>(20S,22R)-20,22-Epoxysterol (VII)</td>
<td>15.8 ± 6.1</td>
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<td>(20R,22S)-20,22-Epoxysterol (VIII)</td>
<td>25.8 ± 8.6</td>
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<td>(20R,22R)-20,22-Epoxysterol (V)</td>
<td>34.1 ± 6.4</td>
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<tr>
<td>(20S)-20-Hydroxysterol (IX)</td>
<td>154.8 ± 12.6</td>
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<tr>
<td>(22R)-22-Hydroxysterol (X)</td>
<td>182.4 ± 32.4</td>
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<tr>
<td>(20R,22R)-20,22-Dihydroxysterol (XI)</td>
<td>671.8 ± 68.3</td>
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Fig. 1. Structures of steroids and their designations by Roman numerals. Systematic names are given in Tables 1 and 2.

rates of formation of pregnenolone from the two monohydroxycholesterols and the dihydroxycholesterol are in the same relative order as with the crude system (cf. Fig. 2 and Table 1). At low substrate concentrations, the amount of pregnenolone formed from the epoxycholesterols or dihydrocholesterols is a larger fraction of that formed from cholesterol, whereas at the higher substrate concentrations, the yield is negligible, amounting to no more than 15% of the product formed from cholesterol or 4% of that formed from equimolar concentrations of (20R,22R)-20,22-dihydroxycholesterol. Although the significance of the apparent formation of very small quantities of pregnenolone from the olefins and epoxides in the crude enzyme system is not clear, the conversion of these compounds becomes negligible as the enzyme system is purified and consequently, these compounds cannot be obligatory intermediates in the formation of pregnenolone from cholesterol. Our findings are in agreement with those of Morisaki et al. [16] and Burstein and Gut [8]. Wang and Kimura [43] have independently concluded that the participation of an epoxide hydratase in the cholesterol side-chain cleavage reac-
tion is unlikely. Moreover, the purified enzyme preparations used in these studies [1] do not appear to contain epoxide hydratase activity. Our results do not support the mechanism of Kraaipoel et al. [3, 13, 14] who do not give quantitative information on the relative efficiency of the conversion of their 20(22)-dihydrocholesterol (of unspecified stereochemistry) to pregnenolone, and consequently their experiments cannot be assessed fully. The transient putative epoxide intermediate detected on thin-layer chromatography [13] was claimed by these workers to be converted to (20R,22R)-20,22-dihydroxycholesterol upon treatment with perchloric acid. However, as already pointed out by Morisaki et al. [16] the chromatographic mobility of this compound is inappropriate for any of the 20,22-epoxides of cholesterol. The mechanism of Kraaipoel must therefore be rejected.

If (20R,22R)-20,22-dihydroxycholesterol is considered an obligatory intermediate, the manner of its formation by an epoxide hydratase from the 20,22-epoxycholesterols is governed by certain steric limitations. The (20S,22S)-20,22-epoxycholesterol is not capable of yielding (20R,22R)-20,22-dihydroxycholesterol by any chemically plausible mechanisms. The formation of the diol from the (20R,22R)-epoxide requires enzymatic cis hydration which appears to be rare [44] and has not been observed in animal tissues (which normally carry out specific trans hydrations). The two remaining epoxide isomers could yield the (20R,22R)-20,22-dihydroxycholesterol by trans hydration. The preferred enzymatic ring opening would involve attack at the least substituted carbon atom [45], which in the case of the 20,22-epoxycholesterols is C-22, and leads to the prediction that the preferred epoxide substrate should have been (20R,22S)-20,22-epoxycholesterol. Nevertheless, the efficiency of all of the four epoxides as precursors of pregnenolone in crude enzymatic systems (Table 1) is low and does not vary with stereochemistry.

**Effects of Various 20(22)-Didehydro, 20,22-Epoxy, and 20-Hydroxy or 22-Hydroxy Cholesterol Analogues on the Conversion of Cholesterol to Pregnenolone**

The effects of two concentrations (2.5 and 12.5 μM) of the various 20(22)-didehydrocholesterols and 20,22-epoxycholesterols on the conversion of [4-14C]cholesterol (1.75 μM) to pregnenolone by purified side-chain cleavage enzyme are shown in Table 2. These experiments were feasible since none of the compounds appear to be substrates for this enzyme. Because conformational restrictions rigidly define the relative spatial disposition of C-17, C-20, C-21, C-22, and C-23 in these compounds, their inhibitory potency may indicate the conformation of cholesterol and its hydroxylated derivatives at the active site of the side-chain cleavage enzyme. Although complete studies of inhibitory constants have not been undertaken, Table 2 indicates the relative potency of the various inhibitors, and the approximate concentrations (IC50 values) required to obtain 50% inhibition. The stereochemical configurations at both C-20 and C-22 are important for binding to the side-chain cleavage enzyme, although the former is somewhat more critical than the latter. This finding is entirely consistent with the conclusions of Morisaki et al. [12] on the stereochemical specificity at C-20 and C-22 of hydroxylated
cholesterols in this enzymatic system. Both the E and Z olefins in which the C-21 methyl group lies rigidly in the same plane as the side chain extending from C-23 compete only poorly with cholesterol for the active site(s) of the cleavage enzyme, as seen in projection structures.

The chirality of the configurations at C-20 of 20-isocholesterol, (20S,22S)-20,22-epoxycholesterol, and (20S,22R)-20,22-epoxycholesterol is opposite to that of cholesterol.

It is not surprising that these compounds are much poorer inhibitors of the side-chain cleavage enzyme than (20R,22S)-20,22-epoxycholesterol and (20R,22R)-20,22-epoxycholesterol, both of which have the same configuration at C-20 as cholesterol.

The (20R,22R)-20,22-epoxycholesterol is a more potent inhibitor than (20R,22S)-20,22-epoxycholesterol suggesting again that the enzyme is more fastidious in its stereochemical requirements at C-20 than at C-22, and establishing that the preferred conformation of C-17, C-20, C-22, and C-23 of the cholesterol side chain on the cleavage enzyme is maximally extended. This conformation of the side chain is very similar to that of crystalline cholesterol (anhydrous and monohydrate) in which the C-22 to C-23 bond points away from the ring system [46–49]. For (20R,22R)-20,22-epoxycholesterol, the three-membered epoxide ring also rigidly constrains the side chain segment comprising C-22 and C-23 to assume a conformation trans to the ring system. The side chain is similarly disposed in (20R,22R)-20,22-dihydroxycholesterol, if the assumption is made that the two hydroxyl groups both occupy positions analogous to that of the oxygen bridge of the epoxide.

It is not surprising that the most effectively bound epoxide is not a substrate, since (20R,22R)-20,22-epoxycholesterol cannot give rise to (20R,22R)-20,22-dihydroxycholesterol by trans hydration, which appears to be by far the most common mechanism for epoxide opening in biological systems.

The effects of adding (20S)-20-hydroxycholesterol, (22R)-22-hydroxycholesterol, and (20R,22R)-20,22-dihydroxycholesterol to the highly purified enzyme system converting [4-14C]cholesterol to pregnenolone are also shown in Table 2. Since these compounds are all substrates of the reaction, the apparent inhibitions undoubtedly reflect complex events including isotope dilution of intermediates. The results indicate that these compounds have access to the active site of the enzyme, and offer some measure of their ability to suppress formation of pregnenolone from added [4-14C]cholesterol.
Table 2. Effect of various dihydro, epoxy, and hydroxy cholesterol derivatives on the conversion of [4-14C]cholesterol to pregnenolone

The incubation system contained in a final volume of 200 μl: 2.25 μg (9 pmol) of purified cytochrome P-450pmec, 21 μg of adrenodoxin, and 7.5 μg of adrenodoxin reductase; 50 mM potassium phosphate, pH 7.0; 5 mM L-cysteine; 1 mM EDTA; 1.75 pM [4-14C]cholesterol, and 2.5 or 12.5 μM of the other steroid in a total of 5 μl of methanol. The reaction was started by addition of the NADPH-generating system which gave a final concentration of: 5 mM glucose 6-phosphate, 0.25 mM NADP+, 2.5 mM magnesium chloride, and 0.26 μg (1.5 units) of crystalline yeast glucose-6-phosphate dehydrogenase. The reaction mixtures were incubated for 15 min at 37 °C, the mixtures extracted once with 2 ml of cyclohexane and once with 2 ml of ethyl acetate, and the products were separated on a silica gel chromatogram sheet (see Methods). The regions corresponding to pregnenolone were counted. In the control vessels, in the absence of inhibitors, 52 ± 9 pmol of cholesterol were converted to pregnenolone. This value has been set at 100.

<table>
<thead>
<tr>
<th>Steroid added</th>
<th>Relative quantities of [14C]pregnenolone formed in the presence of inhibitors or intermediates at</th>
<th>Apparent quantities of [14C]pregnenolone formed in the presence of inhibitors or intermediates at</th>
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<tr>
<td></td>
<td>2.5 μM</td>
<td>12.5 μM</td>
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<th>Non-substrates</th>
<th>50 pM</th>
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<tr>
<td>E-20(22) Didehydrocholesterol (III)</td>
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<td>100</td>
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<td>Z-20(22) Didehydrocholesterol (IV)</td>
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<td>(20R,22S)-20,22-Epoxycholesterol (VII)</td>
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<tr>
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<td>50</td>
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<td>20-Isocolesterol</td>
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<table>
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<th>Substrates</th>
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<td>(20S)-20-Hydroxycholesterol (IX)</td>
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<td>50</td>
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<td>(22R)-22-Hydroxycholesterol (X)</td>
<td>61</td>
<td>50</td>
</tr>
<tr>
<td>(20R,22R)-20,22-Dihydroxycholesterol (XI)</td>
<td>100</td>
<td>50</td>
</tr>
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</table>

The relatively weak inhibitory power of the 20(22)-olefins of cholesterol (Table 2) is in agreement with the findings of Morisaki et al. [16]. However, in contrast, we observed that the (20R,22S)-20,22-epoxycholesterols and the (20R,22R)-20,22-epoxycholesterols are more potent inhibitors than the corresponding diastereomeric epoxides. Although these conflicting results were both obtained with highly purified enzymes, the experimental conditions differed in several respects. Burstein et al. [50] have studied the effects of many cholesterol analogues on the formation of [14C]pregnenolone from [14C]cholesterol in crude adrenal mitochondrial acetone powder extracts. Since an indeterminate number of the compounds tested by Burstein et al. [50] were also substrates, it is somewhat difficult to interpret their findings conclusively. Nevertheless, these authors found 20-isocholesterol to be a considerably more potent inhibitor of the enzymatic process than is indicated by our experiments.

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