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# STEROID METABOLISM BY CONSTITUTIVE CYTOCHROMES *P*450

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**Summary**—In rat liver endoplasmic reticulum some 16 different cytochromes *P*450 have been identified as constitutive, sequenced from recombinant DNA, and shown to be distinct gene products. These forms are "multipurpose", i.e. functional in xenobiotic metabolism as well as endogenous substrate metabolism. In the latter case, these forms metabolize steroids, fatty acids, prostaglandins and even ketone bodies, indicating an involvement in homeostasis. In steroid metabolism, in contrast to "biosynthetic" forms of *P*450 which generally yield one product, the multipurpose forms exhibit broad, overlapping metabolite profiles, with isomeric and epimeric specificity and different mechanisms of product formation. The nature of the substrate docking region is of much interest and attempts have been made to rationalize the manner in which multiple metabolites are produced from a single substrate. Brain, with a very low level of *P*450 relative to liver also catalyzes steroid metabolism. The nature of the forms involved are not yet known.

Cytochromes *P*450 exist in every phylum in which the hemoprotein presence has been sought. In the mammalian system *P*450 is found in just about every tissue, mainly in the endoplasmic reticulum and in the mitochondria [1]. All of the mammalian forms of *P*450 can be grouped into two categories (Table 1), the biosynthetic forms and the multipurpose forms. The majority of papers in this Symposium proceedings are concerned with the biosynthetic forms of cytochrome *P*450 (see general review [2]), forms involved in steroid biosynthesis. This paper and the several following papers [3–6], however, are concerned with multipurpose forms of cytochrome *P*450, forms which may or may not metabolize steroids, but which also metabolize a wide variety of chemicals and drugs, and often a number of other endogenous substrates, like free fatty acids, ketone bodies and prostaglandins. The forms of cytochrome *P*450 in this latter group may be constitutively expressed in one or another tissue, or may require challenge with some chemical (inducer) *in vivo* for expression, e.g. 1A1, 2B1 and 3A1.

In the liver endoplasmic reticulum, or its fragments the microsomes, exist a large number of constitutively expressed forms of cytochrome *P*450. Some 16 forms are present constitutively (C) in the rat (Table 2) and these have already been isolated, cloned, sequenced, and been given names relating them to individual gene families [7]. Other forms appear only upon induction (I) and these have also been given gene subfamily assignments [7]. A number of other *P*450 forms are only known from cDNA clone sequencing, and some have been purified but as yet have not been fully sequenced [8]. As shown in Table 2, most of the multipurpose forms of *P*450 are able to metabolize steroids (testosterone shown). Steroid metabolism by these forms is stereo-specific; they exhibit broad overlapping metabolite patterns with isomeric and epimeric specificities.

Studies employing antibodies to specific forms of hepatic *P*450 have revealed that in a number of instances one or another of the steroid metabolite produced by liver microsomes is mainly the product of a single form of cytochrome *P*450. Such metabolites are designated with an asterisk in Table 2. For example, although *P*450s 1A2, 2A2, 2C13, 2D1, 2D2, and 3A2 all produce 6$\beta$-hydroxytestosterone from testosterone in the untreated animal, antibodies monospecific for 3A2 inhibit microsomal 6$\beta$-hydroxytestosterone production by almost 85% [9], even though this latter hemoprotein only represents about 10% of the microsomal

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Table 1. Categories of mammalian cytochromes *P*450

| Biosynthetic forms | Multipurpose forms |
|---|---|
| Lanosterol 14-demethylase | Induced forms |
| Cholesterol 7α-hydroxylase | Constitutive forms |
| Cholesterol 12α-hydroxylase | |
| Cholesterol side-chain cleavage | |
| Steroid 17α-hydroxylase | |
| Aromatase | |
| 11β-Hydroxylase | |
| 21-Hydroxylase | |
| 26-Hydroxylase | |
| 25(OH)D3-1α-hydroxylase | |

content of *P*450. Similarly, about 90% of the 2α-hydroxy- and about 90% of the 16α-hydroxytestosterone are produced by *P*450 2C11 [10]. The picture changes when homeostatis is perturbed, as for example by inducer treatments; thus, when levels of 3A1 are elevated by treatment of rats with pregnenolone 16α-carbonitrile (PCN), this form becomes the source of 90% of the 6β-hydroxytestosterone produced by the liver microsomes [10]. Figure 1 shows thin layer plate separation of metabolites of testosterone produced by liver microsomes (MS), in the presence of NADPH. Earlier, Conney's group [11] noted that a number of factors influenced the ratios of the different products produced from testosterone, suggesting more than one enzyme is responsible for the metabolites produced. Of interest, it could be shown [12, Fig. 1] that purified, constitutive forms of cytochrome *P*450 in a reconstituted system produced multiple metabolites, often with overlapping patterns, from testosterone [13] and other steroids, such as estradiol-17β and progesterone [14]. The multiplicity of metabolites produced by the individual multipurpose forms of *P*450 is in distinct contrast with the biosynthetic forms of *P*450, which generally produce a single steroid metabolite (see section 6, of Ref. [1]).

Lineweaver–Burk plots of substrate dependence for production of the individual metabolites of testosterone by *P*450 2C11 intersect at a common point on the abscissa (Fig. 2), suggesting the multiple metabolites are produced in a common binding site. Of concern was the fact that one of the monohydroxy metabolites was the result of an A-ring oxidation (2α-) and another was a D-ring oxidation (16α-). It was difficult to visualize how a common substrate-binding site could accommodate such different substrate orientations as would be necessary for production of these products.

Using 3-dimensional imaging of the testosterone molecule relative to an active oxygen provided an indication of how one docking site could yield several isomeric- and epimeric-specific metabolites. The imaging showed that the testosterone molecule could be rotated 180° in a vertical plane and oriented such that similar surfaces are presented to the docking site. Figure 3 shows two superimposed ball and stick models of testosterone, one rotated 180° in the vertical plane of the picture. On the right, the 17-hydroxy oxygen of one molecule is adjacent to the 3-keto group of the other molecule. In this arrangement, the 16α-hydrogen of one molecule superimposes the 2α-hydrogen of the

Table 2. Rat hepatic *P*450 forms

| Gene | C/I | Inducers | Testosterone stereoselectivity | Marker substrates |
|---|---|---|---|---|
| 1A1 | I | MC, Ar | 6β | B(*a*)P, 7-EC |
| 1A2 | C | MC | 6β | E2 (2) |
| 2A1 | C | MC, Ar | *7α,tΔ6 | — |
| 2A2 | C | — | *15α,7β(18),6β,7α,15β | — |
| 2B1 | I | PB | *17,*16α,*16β | BZP |
| 2B2 | C | PB | t-17,16α,16β | — |
| 2C6 | C | PB | t-16α | — |
| 2C7 | C | — | — | — |
| 2C11 | C | — | *16α,*2α,17 | E2 (2,16α,17) |
| 2C12 | C | — | *f15α | 15β-And., PGE(W-3) |
| 2C13 | C | — | 6β,15α,16α | — |
| 2D1 | C | MC, D, PB | 6β | debrisoquine |
| 2D2 | C | — | 6β | — |
| 2E1 | C | Ac, i | — | acetone, acetol, NDMA |
| 3A1 | I | D, PCN | *6β | — |
| 3A2 | C | PB | *6β,2β | — |
| 4A1 | C | Clofibrate | — | C12:0 W |
| 4A2 | C | Clofibrate | — | — |
| 4A3 | C | Clofibrate | — | — |

Induction of constitutive (C) and inducible (I) forms of rat microsomal cytochrome *P*450 by 3-methylcholanthrene (MC), Arochlor 1254 (Ar), phenobarbital (PB), dexamethasone (D), acetone (Ac), pregnenolone 16α-carbonitrile (PCN) and isoniazid (i). Asterisks indicate metabolites for which the individual forms contribute over 85% to microsomal metabolism. Marker substrates include benz(*a*)pyrene [B(*a*)P], 7-ethoxy coumarin (7-EC), β-estradiol (E2), benzphetamine (BZP), prostaglandin E (PGE), *N*-dimethylaniline (NDMA) and Lauric acid (C12:0).

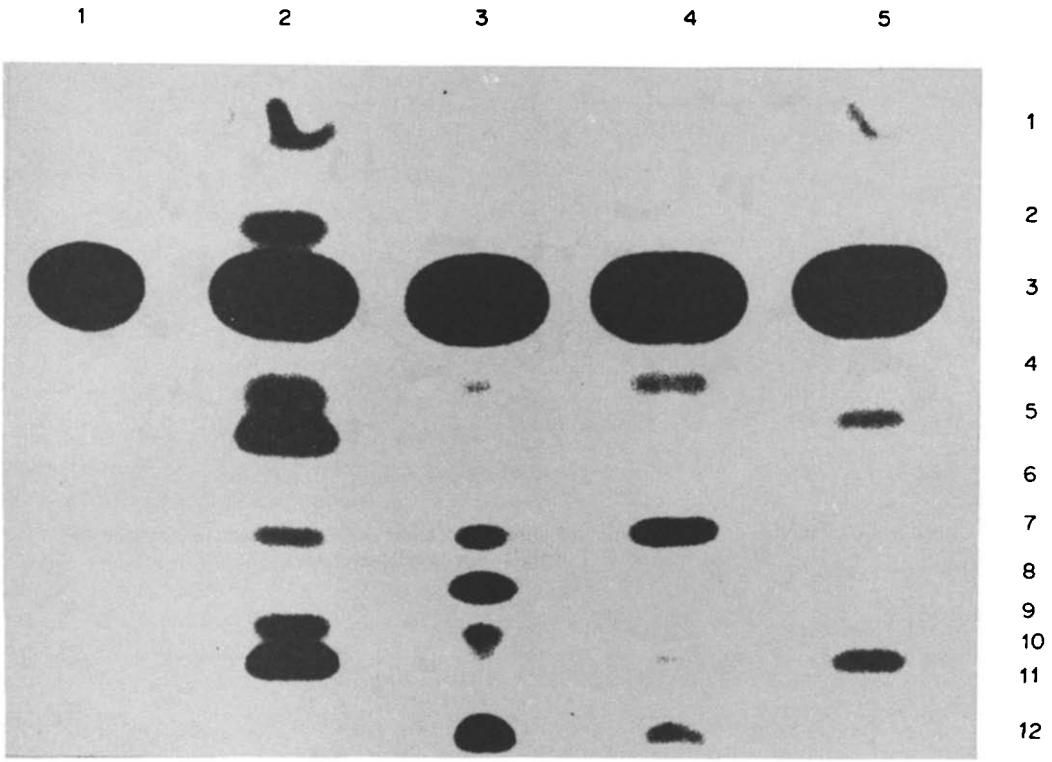


Fig. 1. Thin layer chromatographic separation of metabolites of testosterone (120 $\mu$M) by rat liver microsomes (0.2 nmol *P*450), or 0.15 nmol of purified forms of *P*450, 2A2, 2C13 and 2C11, respectively in tracks 2–5. The thin layer plate was developed with chloroform–ethyl acetate–ethanol (4:1:0.7) and autoradiographed. Data from [16] with permission.

other molecule. Note the similar arrangement of the oxygens on the left side. Even more important, the angular methyl groups are positioned upward and are $<0.5$ A apart on the two molecules. Thus, it would appear that the A- and D-ring metabolites owe their existence to structural similarities in two different orientations relative to the substrate docking site. The angular methyl groups appear to have importance in the docking, as binding of 19-hydroxytestosterone to *P*450 2C11 was very poor (I. Jansson, personal communication).

*P*450 2C11 also metabolizes testosterone to androstenedione [12], a reaction that results in conversion of a 17$\beta$-hydroxyl group to a ketone. Two possible mechanisms for the reaction were considered [13]: the first mechanism involves hydroxylation of carbon 17 (monooxygenation). This would cause formation of a gemdiol (Fig. 4). The gemdiol would spontaneously dehydrate to the ketone. The second mechanism involves a double abstraction of hydrogen atoms from position 17, a peroxidase-like reaction (Fig. 4). The two mechanisms were tested by carrying out testosterone metabolism by 2C11 in a reconstituted system under $^{18}O_2$ [13]. If *P*450 functioned as a monooxygenase in the producton of androstenedione, then one would expect about half of the 17-position oxygens should be $^{18}O$. On the other hand, if 2C11 used a peroxidase-like mechanism to produce androstenedione, i.e. dual hydrogen

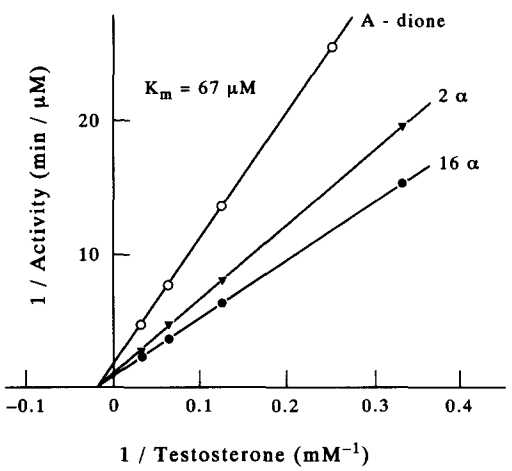


Fig. 2. Lineweaver–Burk plot of testosterone metabolism by *P*450 2C11. Data was obtained as in Fig. 1 and spots were scraped from the plates for quantitation by scintillation counting.

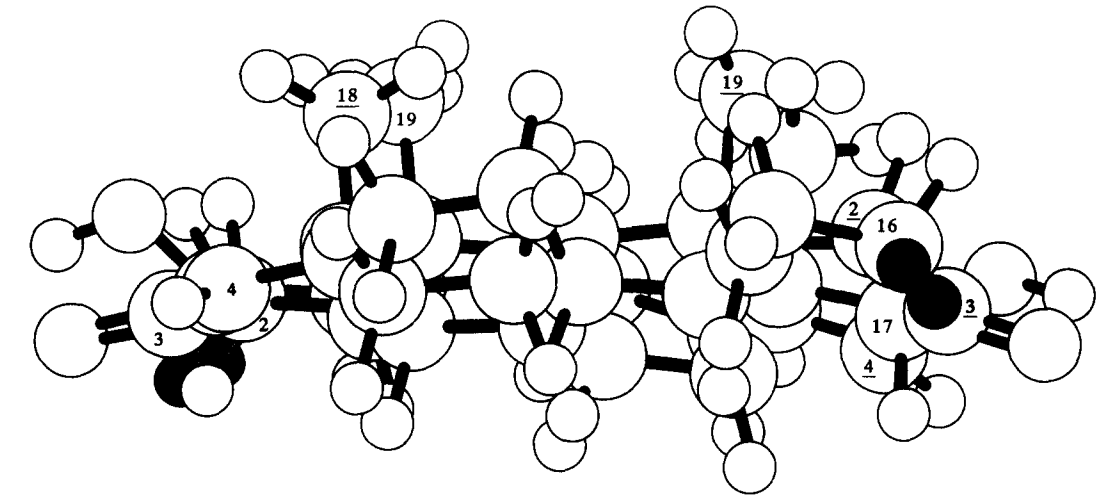


Fig. 3. Ball and stick models of two superimposed testosterone molecules. One molecule was rotated 180° so that C2 and C17 of the two molecules overlap.

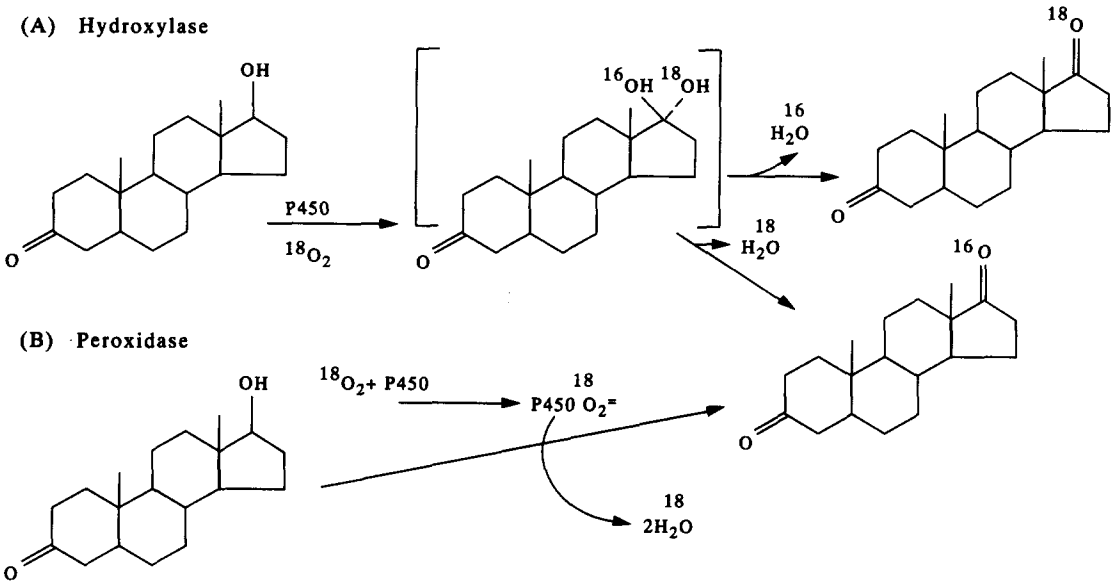


Fig. 4. Sketch of possible mechanisms of androstenedione formation from testosterone. Modified from [13].

abstraction, then there would be no $^{18}O$ enrichment of the androstenedione formed. Table 3 shows that while 2α-hydroxytestosterone produced in this reaction medium had major $^{18}O$ enrichment, essentially no enrichment was seen in androstenedione [13]. Recently, evidence was provided for another metabolite produced by dual hydrogen abstraction, 17β-hydroxy-4,6-androstradiene-3-one, of testosterone [15]. The activity was elevated in liver microsomes of PCN or dexamethasone-treated rats. These inducers cause major elevations of *P*450 3A1 levels, and this form of *P*450 is the form primarily responsible for 6β-hydroxylation of testos-

Table 3. Isotope ratios of metabolites formed by reconstituted cytochrome *P*450 2C11 in air or in $^{18}O_2$ atmosphere

| Metabolite spot | Ion masses | Mass ratio | |
|---|---|---|---|
| | | In air | In $^{18}O_2$ |
| 2αOH-Testosterone | *m/e* 306/(*m/e* 304 + *m/e* 306) | 0.027 | 0.825 |
| | *m/e* 262/(*m/e* 260 + *m/e* 262) | 0.028 | 0.116 |
| Androstenedione | *m/e* 288/(*m/e* 286 + *m/e* 288) | 0.019 | 0.040 |
| | *m/e* 246/(*m/e* 244 + *m/e* 246) | 0.025 | 0.018 |

Data from [13].

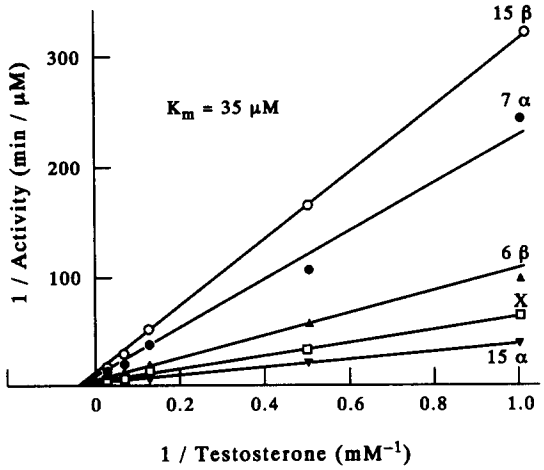


Fig. 5. Lineweaver–Burk plot of testosterone metabolism by *P*450 2A2. Conditions were as in Fig. 2. From [16] with permission.

terone (Table 2) in such induced animal liver microsomes [9, 10]; it was found that 6,7-desaturation of testosterone paralleled 6β-hydroxylation activity. Further, 16-methylprogesterone, an inhibitor of 6β-hydroxylase activity also inhibited 6,7-desaturation of testosterone, indicating *P*450 3A1 is probably the form of *P*450 in microsomes responsible for this reaction [15].

While *P*450 2C11 produces three main metabolites (Table 2, Fig. 2), *P*450 2A2 produces a greater number. Six main metabolites plus a trace of minor metabolites of testosterone were reported [16]. A Lineweaver–Burk plot of production of these metabolites as a function of testosterone concentration is shown in Fig. 5. The plots meet in a common point on the abscissa ($K_m = 35\,\mu M$) suggesting a common substrate binding site on the enzyme. The metabolites plotted are 15β-, 7α-, 6β-, an as-yet unidentified major metabolite and 15α-hydroxytestosterone. In addition, trace amounts of androstenedione were found [16]. The unidentified metabolite was suggested, on the basis of fragmentation pattern, to be 7β-hydroxytestosterone [16], but this was later shown not to be the case [17], when an authentic sample of 7β-hydroxytestosterone was generously made available to us by Searle (courtesy of Mr Tony B. Martinez, Skokie, IL). In a subsequent study on this enzyme, other trace metabolites, including 16α-hydroxytestosterone and 14-hydroxytestosterone were reported [18]. In that study,

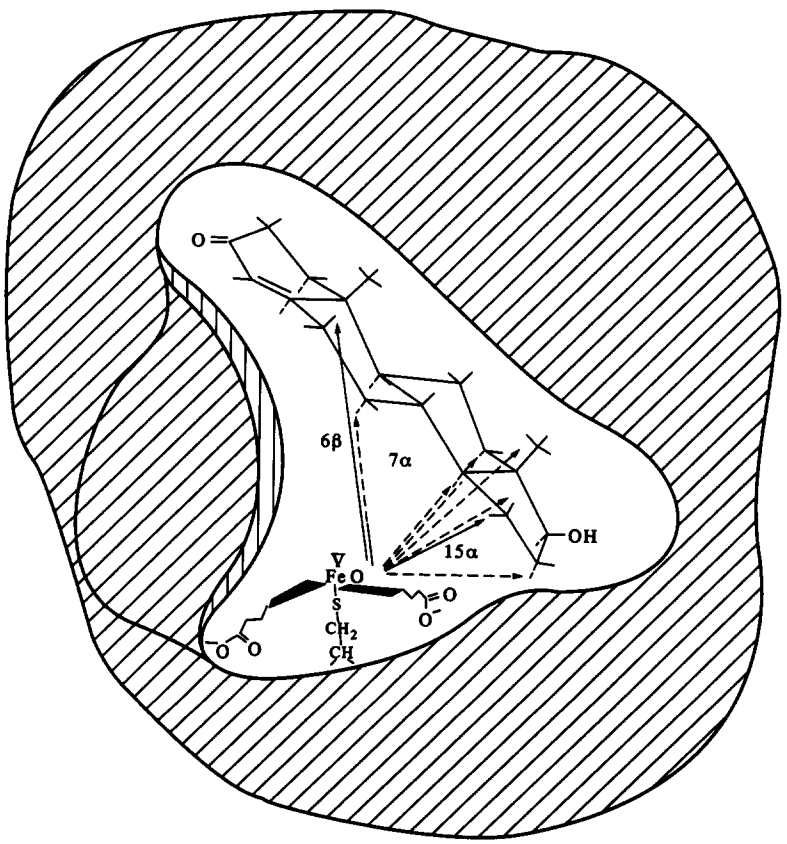


Fig. 6. Cartoon of possible alignment of testosterone in the active site of *P*450 2A2 to account for the multiple metabolites produced.

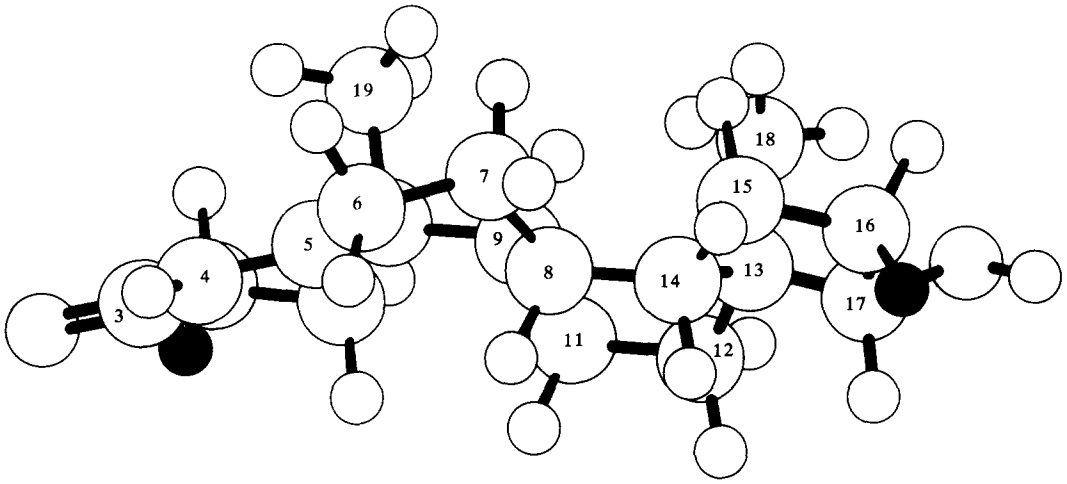


Fig. 7. Ball and stick model of testosterone at an orientation showing relationships between hydroxylatable sites by *P*450 2A2. This form of cytochrome *P*450 hydroxylates testosterone mainly at positions 15α, possibly 12α, 6β, 7α and 15β, with very minor amounts of androstenedione and of 14-hydroxytestosterone and 16α-hydroxytestosterone being produced.

the unknown major metabolite was suggested to be 18-hydroxytestosterone on the basis of its HPLC retention time.

Using 3-dimensional imaging, we attempted to rationalize the manner in which *P*450 2A2 could produce so many isomeric and epimeric metabolites from testosterone. Figure 6 is a cartoon depicting how a number of these metabolites could be produced rationalizing a single substrate docking site. The known metabolites were triangulated from a common heme iron-bound active oxygen to the respective isomeric and epimeric positions, with distance expressed as the reciprocal of the rate of production of the metabolite. Although the unknown major metabolite triangulated well to the 7β-position (Fig. 6), its retention time and fragmentation pattern in the GC/MS indicated it was not this metabolite. If we look at a ball and stick model of testosterone (Fig. 7) we see that access to the 18 carbon, the angular methyl group, from other accessible identified sites of oxidation would require a rotation of the molecule around the horizontal axis.

With respect to the unknown metabolite, based upon its fragmentation pattern, we can eliminate the possibility of it being an A-, B- or D-ring metabolite. The possibility of an 11-oxymetabolite is slim, as liver does not appear to make 11-oxysteroids, plus the pattern of 11-oxysteroid fragmentation is distinctly different. By the process of elimination we suspect the unknown metabolite may be a 12-hydroxy compound. According to Dr Ken Korzekwa (personal communication, this symposium), his laboratory has been able to conclusively show the unknown major metabolite produced by *P*450 2A2 is 12α-hydroxytestosterone. If this is indeed the case, then it will be necessary to re-evaluate the nature of the substrate binding site(s) of the cytochrome *P*450 enzymes. In this respect, it is important to note that substrate binding specificity may be determined by as few as a single amino acid residue. Dr Negishi [3] has recently been able to show, using site-directed mutagenesis, that the alteration of a single amino acid residue of mouse Cyp 2a-4 (coumarin hydroxylase) or Cyp 2a-5 (testosterone 15α-hydroxylase) can change the substrate specificity and metabolite formed [19]. These two forms of *P*450 are extremely similar, differing by only 11 out of 494 residues. The conversion of residue 209 of Cyp 2a-4 to that of Cyp 2a-5 changed the coumarin hydroxylase to a testosterone 15α-hydroxylase; the reverse effect was obtained by changing this Cyp 2a-5 amino acid residue to that of Cyp 2a-4 [19].

In addition to the liver, brain microsomes are known to metabolize steroids [20–27]. Brain has also been shown spectrophotometrically to contain cytochrome *P*450 [23, 24]. In rat whole brain microsomes the content of cytochrome *P*450 was determined as 1% of rat liver microsomal *P*450 content [25]. From the very low level of *P*450 it is still too early to determine the presence and identity of individual constitutive endoplasmic reticulum multipurpose *P*450 forms. However, using immunofluorescence one constitutive form, Cyp 2E1 [25] and several inducible forms [26–29] have been found. In

Table 4. Whole brain steroid metabolism

| Activity | Products (pmol/min/mg ms) |
|---|---|
| Estradiol 2-hydroxylase | 0.5-4[a] |
| Estradiol 4-hydroxylase | 0.8-5[a] |
| 3β-Androstanediol hydroxylase (total) | 10[b] |
| Estradiol oxidase (total) | 1.8 |
| Testosterone oxidase (total) | 5.8 |

[a]Calculated from [30].
[b]Calculated from [31].

brain microsomes, the level of steroid metabolizing activity is very low (Table 4). Consequently, it is possible that the role of the endoplasmic reticulum forms of brain P450 is to generate neuroactive steroids, rather than to inactivate those steroids synthesized in brain or which enter the brain. This very important possibility will undoubtedly attract many more studies on the nature of brain P450.

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