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STUDIES ON ENZYME REACTIONS RELATED TO STEROID BIOSYNTHESIS

II. SUBMICROSOMAL DISTRIBUTION OF THE ENZYMES RELATED TO ANDROGEN PRODUCTION FROM PREGNENOLONE AND OF THE CYTOCHROME P-450 IN TESTICULAR GLAND OF RAT

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SUMMARY

A testicular microsomal fraction containing the enzyme systems necessary for the production of testosterone from pregnenolone was divided in two subfractions by sucrose density gradient centrifugation in the presence of CsCl. When the subfractions were examined by electron microscope, one consisted mainly of smooth-surfaced particles, the other of rough-surfaced particles with ribosomes on their outer surface. Enzymic and spectrometric investigation of these subfractions revealed that most of the enzyme activities related to the androgen synthesis, which remained after the gradient centrifugation, and also of the cytochrome P-450, which would be a direct site of activation of molecular oxygen for the 17α-hydroxylation and the side chain cleavage, were localized in the smooth-surfaced submicrosomal fraction. The enzyme activities of the submicrosomal fraction which were diminished by the gradient centrifugation were largely restored by addition of the heated 105,000 $\times$ *g* supernatant fluid.

Enzyme inhibitors such as Amphenone B (3,3-bis (*p*-aminophenyl)-2-butanone dihydrochloride) and SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) inhibited the testicular microsomal 17α-hydroxylase and the C_{17}–C_{20} lyase competitively. The apparent inhibition constants of Amphenone B were $9{\cdot}17 \times 10^{-5}$ M against 17α-hydroxylase and $2{\cdot}45 \times 10^{-4}$ M against C_{17}–C_{20} lyase, and those of SKF-525 A were $1{\cdot}61 \times 10^{-4}$ M against 17 α-hydroxylase and $1{\cdot}28 \times 10^{-5}$ M against the C_{17}–C_{20} lyase.

INTRODUCTION

IT WAS REPORTED that the cytochrome P-450[1] and the enzyme systems for testosterone* biosynthesis from pregnenolone[2] are located intracellularly in the testicular microsomal fraction (10,000–105,000 $\times$ *g* precipitate). Furthermore, as the 17α-hydroxylase (EC 1.14.1.7) and C_{17}–C_{20} lyase activities in this fraction were inhibited by previous bubbling with carbon monoxide, and then by introducing the mixture of carbon monoxide and oxygen as the gas phase during the incubation, the microsomal cytochrome P-450 appeared to be closely related to the functions of the enzymes referred to as the direct site of molecular oxygen activation[1]. The procedure of sucrose density gradient centrifugation[3] has been applied to the subfraction of adrenal microsomal fraction of rat and pig. It was confirmed that the adrenal microsomal enzymes such as the Δ^5-3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) together with the Δ^5–Δ^4 isomerase (EC 5.3.3.1), 17α- (EC 1.14.1.7) and 21-hydroxylases (EC 1.14.1.8) are localized

**Nomenclature of steroids:* The following trivial names are used in this text; pregnenolone: 3β-hydroxy-5-pregnen-20-one; progesterone: 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; androstenedione: 4-androstene-3,17-dione and testosterone: 17β-hydroxy-4-androsten-3-one.

in the smooth surfaced membrane structure in accordance with the known distribution of the microsomal cytochrome P-450 between the two subfractions [4, 5].

In the present work, testicular microsomal fraction of normal rat was further fractionated by means of a sucrose gradient centrifugation and then the distribution of the cytochrome P-450 and enzyme activities related to the steroidogenesis between the two submicrosomal fractions was examined. In addition, the mode of the inhibition of 17α-hydroxylation and of side chain cleavage by Amphenone B and by SKF-525A was examined in relation to the function of cytochrome P-450.

EXPERIMENTAL

Radioactive steroids

[4-^{14}C]-Pregnenolone (spec. activity 176μC/mg), [4-^{14}C]-progesterone (spec. activity 186μC/mg), [4-^{14}C]-17α-hydroxyprogesterone (spec. activity 114μC/mg) and [4-^{14}C]-androstenedione (spec. activity 122μC/mg) were purchased from the Radiochemical Centre, Amersham, England. The purities of the radioactive steroids were confirmed by thin layer chromatography immediately before use. These steroids were diluted with the corresponding non-radioactive substances before incubation.

Enzyme inhibitors

Amphenone B and SKF-525A were supplied by Dr. W. W. Tullner, Endocrinology Branch, National Cancer Institute, N.I.H., Bethesda, Md., and Dr. G. E. Ullyot, Smith, Kline and French Laboratories, Philadelphia, Pa., respectively.

Subcellular fractionation of rat testes

Normal 3 month old rats of the Wistar strain, bred in this Institute were used. Conventional differential centrifugation of the testicular homogenates gave the mitochondrial fraction (800–10,000 × *g* precipitate), the microsomal fraction (10,000–105,000 × *g* precipitate) and the supernatant fluid at 105,000 × *g* as reported previously [2]. The microsomal fraction was examined by electron microscope, and found to consist of rough and smooth surfaced membrane structures without any contamination by the mitochondria. Following the method used by Dallner *et al.*[3] for subfractionating hepatic microsomes, 4 ml of testicular microsomal fraction suspended in 0·25 M sucrose solution containing 15 mM CsCl was layered on top of 6·5 ml of 1·30 M sucrose solution containing 15 mM CsCl. After centrifugation for 60 min at 250,000 × *g* (Ultracentrifuge Model B-60 with A-321 rotor, International Equipment Company, Needham Heights, Massachusetts) a compact pellet was obtained at the bottom of the tube, while a fluffy layer remained near the position of the original boundary between the two sucrose solutions. Each subfraction was suspended in 0·25 M sucrose solution, and reprecipitated at 105,000 × *g* for 60 min. Cytochrome P-450 and the steroid metabolism enzymes were determined as outlined below.

The 105,000 × *g* supernatant fluid was heated in a boiling bath for 10 min, and then centrifuged at 10,000 × *g* for 20 min. to remove the denatured protein. This supernatant fluid was used as the "heated supernatant fluid".

Spectrometric determination of the cytochrome P-450

The cytochrome P-450 was detected as the peak at 450 mμ in the difference spectrum, where the preparation reduced by sodium dithionite and bubbled with carbon monoxide was compared with an identically reduced preparation as the reference [6] in a spectrophotometer with a diffuse reflectance attachment (Hitachi-Perkin-Elmer, Model EPS-3T).

Determination of RNA and protein in the submicrosomal fractions

After the submicrosomal fractions were washed exhaustively with isotonic KCl solution to remove the sucrose, RNA and protein were measured respectively by the methods of Ceriotti [7] and Lowry *et al.*[8].

Incubation and quantitation of the metabolites

Enzyme preparations were incubated with pregnenolone in the presence of NAD and with progesterone, 17α-hydroxyprogesterone and androstenedione in the presence of NADPH in an atmosphere of O_2:CO_2 (95:5) at 37·5°. Details of the incubation are given as a note in Table 2. At the end of the incubation, methylenedichloride (15 ml) was added and the mixture was shaken vigorously. The extract was subjected to thin layer chromatography on silica gel in the benzene-acetone (8:2) system.

The isolated metabolites were identified by the following methods and their amounts measured as previously reported[9]. 1, Identical chromatographic mobilities with those of the respective authentic preparation. 2, Identical chromatographic behaviour as those of the authentic preparation after oxidation and acetylation. 3, Recrystallization of radioactive metabolites with authentic steroids until constant specific activities of crystals and solid in the mother liquor were obtained with different solvent mixtures.

Measurement of the testicular enzyme activities related to androgen synthesis

As the four enzyme systems investigated were not separable, the enzyme activities are tentatively expressed as follows: The combined activities of the Δ^5-3β-hydroxysteroid dehydogenase with Δ^5–Δ^4 isomerase were denoted as the sum of Δ^4-3-oxosteroid derived from added pregnenolone. 17α-Hydroxylase activity was expressed as the sum of 17α-hydroxyprogesterone, androstenedione, testosterone and their metabolites formed from added progesterone. The C_{17}–C_{20} lyase activity was expressed as the sum of androstenedione, testosterone and other C-19-steroids derived from 17α-hydroxyprogesterone. Finally 17β-hydroxysteroid dehydrogenase (EC 1.1.1.51) activity was expressed as the yield of testosterone from androstenedione or the substrate[9].

RESULTS AND DISCUSSION

Electron microscopic examination of the submicrosomal fractions of rat testes

After the sucrose gradient centrifugation of the testicular microsomal fraction, the two subfractions were subjected to electron–microscopic examination. As shown in Fig. 1, the fraction obtained as the compact precipitate at the bottom of the tube in the presence of Cs^+ consists mostly of a rough-surfaced membrane structure with ribosomal particles on the outer surface. Hereafter, this fraction is named as the rough surfaced microsomal fraction. The other subfraction which was obtained as a fluffy layer in the middle of the tube consists of smooth-surfaced membrane structures without ribosomal particles on outer surfaces (Fig. 1A). This fraction is called as the smooth-surfaced microsomal fraction. The electron-microscopic characteristics of the two testicular subfractions were similar to those of hepatic subfractions[3]. In the case of adrenal microsomes, the subfraction which was obtained as the fluffy layer consists also of smooth-surfaced membrane structures, but the compact precipitate fraction, in the presence[4] and absence[5] of CsCl, consists largely of free ribosomes with limited amount of agranular membrane structures.

As the microsomal fraction was prepared from the testes of normal rats, the microsomal subfractions originate from both cells related to spermatogenesis and interstitial cells.

Protein and RNA content of testicular microsomal and submicrosomal fractions

Protein and RNA contents of the initial microsomal fraction and its two subfractions are given in Table 1. The ratio of nucleic acid to protein was found higher in the rough-surfaced fraction than in the smooth-surfaced one. This is in agreement with the electron-microscopic observation of ribosomal particles in the rough-surfaced submicrosomal fraction, and also with the result obtained in the case of hepatic submicrosomes[3]. On the other hand, the RNA content in the smooth-surfaced fraction suggests the involvement of RNA in the membrane structure[10] as well as the contamination of the smooth-surfaced fraction by the rough-surfaced one.

Distribution of the cytochrome P-450 among the subcellular components

As shown in Table 1, exclusive concentration of P-450 into the microsomal fraction was confirmed spectrometrically among the subcellular components[1].

Table 1. Contents of protein, RNA and P-450 in the subcellular fractions of rat testes

Subcellular fraction	RNA*/Protein*	ΔO.D.† (450–500 mμ)	Protein‡ (mg/ml)	ΔO.D./mg Protein/ml
Mitochondria	—§	0·000	11·5	0·000
Microsomes	0·19	0·075	20·0	0·004
Smooth-surfaced microsomes	0·14	0·085	7·5	0·011
Rough-surfaced microsomes	0·42	0·000	3·6	0·000
Supernatant fluid at 105,000 × *g*	—§	0·000	40·5	0·000

*After washing exhaustively with the isotonic KCl solution, protein and RNA concentration were determined by the copper-Folin [8] and orcinol reactions [7].

†Difference of the optical densities at 450 and 500 mμ in the carbon monoxide difference spectra.

‡Protein concentration measured without washing with KCl solution.

§Not measurement.

No significant peak at 450 mμ was observed in the difference spectrum of the mitochondrial fraction, while the supernatant fluid at 105,000 × *g* contained appreciable amount of P-420 instead. Furthermore, the P-450 in the microsomal fraction was found to reside mostly in the smooth-surfaced subfraction, while no significant peak at 450 mμ was observed in the rough-surfaced one.

The biased distribution of cytochrome P-450 suggests that at least, the enzyme systems in which the functions of P-450 are involved would show similar distribution between the two submicrosomal fractions to the submicrosomal distribution of the cytochrome itself, because the activities of 17α-hydroxylase and C_{17}–C_{20} lyase in testicular microsomes are inhibited by previous introduction of carbon monoxide [1].

As the microsomal concentration of the cytochrome P-450 is higher in X-irradiated than in normal testis and is enhanced by gonadotrophin, the P-450 was presumed to be concentrated in the radioresistant and gonadotrophin-stimulated cells, most likely the interstitial cells [1]. Therefore, the P-450 in the smooth-surfaced subfraction was considered to be derived mainly from the microsomal fraction of the testicular interstitial cells.

Submicrosomal distribution of testicular enzyme activities related to testosterone production from pregnenolone

The smooth- and rough-surfaced subfractions obtained by gradient centrifugation were separately incubated with the substrates. In addition, parallel incubations were carried out with the microsomal fraction and with the two subfractions following the addition of heated supernatant fluid.

After the gradient centrifugation, the combined activities of the two subfractions were found to be significantly lower than the activity of the initial microsomal fraction. Nevertheless, about 80 per cent or more of the enzyme activities which remained after the subfractionation were found in the smooth-surfaced microsomal fraction. The 17α-hydroxylase and C_{17}-C_{20} lyase which require molecular oxygen [11, 12] were found predominantly in the smooth-surfaced membrane structure, in accordance with the submicrosomal distribution of the

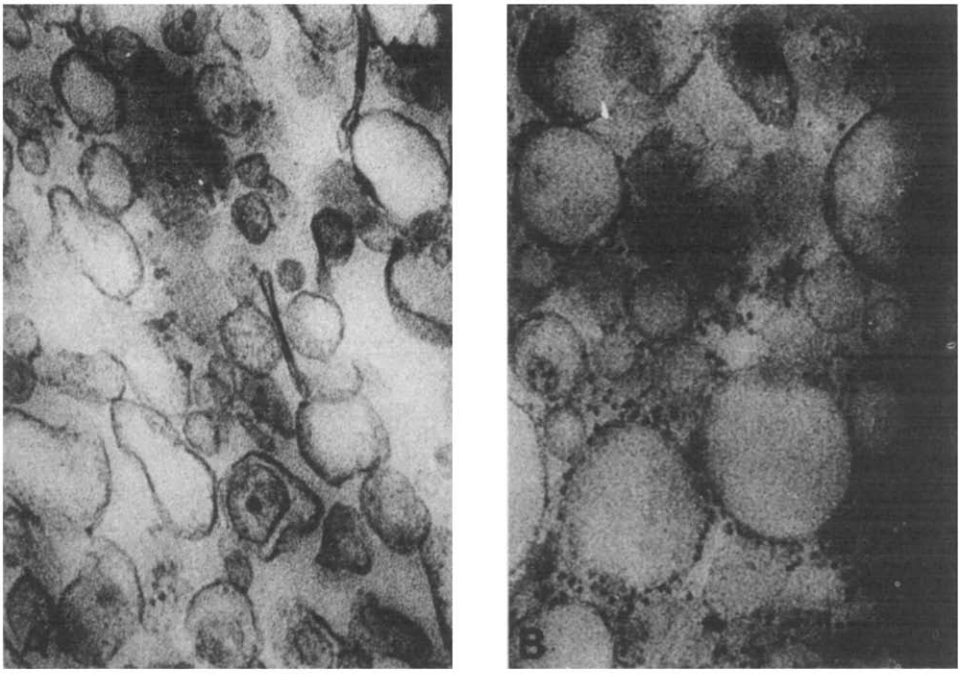

Fig. 1. Electron micrographs of the two different submicrosomal fractions of rat testes (× 75,000). (A) Submicrosomal fraction which was obtained as a fluffy layer in the middle of the tube after a sucrose density gradient centrifugation. The smooth-surfaced subfraction which bore no ribosome on their outer surface was mainly observed. (B) The other submicrosomal fraction which was obtained as a compact precipitate after the centrifugation. Rough-surfaced membrane structure which contained ribonucleoprotein particles attached to the outer surface was abundant.

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cytochrome P-450. On the other hand, the protein content of the smooth-surfaced subfraction was about twice that of the rough-surfaced one. Accordingly, the specific enzyme activity in the smooth-surfaced fraction was three times as much as that of the rough-surfaced subfraction. By adding the heated supernatant fluid, which, did not itself have enzymic activities, the apparent enzyme activities of the submicrosomal fractions were enhanced. In addition the sum of the two submicrosomal activities which were stimulated by the boiled supernatant fluid agreed reasonably well with activities of the intact microsomal fraction. This was particularly so in the case of the Δ^5-3β-hydroxysteroid dehydrogenase with Δ^5-Δ^4 isomerase and the 17α-hydroxylase. However, the activities of the C_{17}–C_{20} lyase and the 17β-hydroxysteroid dehydrogenase were remarkably enhanced by the heat stable components of the soluble fraction. They did not however reach the level of the enzyme activities of the intact microsomal fraction. It was reported that addition of the 105,000 × g supernatant fluid to the testicular microsomal fraction of rat[2] and guinea pig[13] tissue significantly increased the activities of the 17α-hydroxylase and the lyase. The supernatant fluid contained the 20α-hydroxysteroid dehydrogenase which converts 17α-hydroxyprogesterone to 17α,20α-dihydroxy-4-pregnen-3-one[14].

The 20α-hydroxysteroid dehydrogenase is believed to have a significant role in regulating the androgen production by competing with the microsomal lyase for utilization of the common substrate, and by competitively inhibiting the lyase activity by 17α,20α-dihydroxy-4-pregnen-3-one from 17α-hydroxyprogesterone [12]. Therefore, in order to avoid the possible inhibition of the C_{17}–C_{20} lyase activity by the 20α-hydroxysteroid dehydrogenase (or 20α-hydroxysteroid: NADP oxido-reductase) and its product, the soluble fraction of the heated supernatant fluid at 105,000 × g was employed.

Through the distribution of the enzyme activities which remain after the gradient centrifugation favors the smooth-surfaced submicrosomal fraction, the specific activities of the enzymes in the agranular submicrosomes were reduced, probably due to dilution of the submicrosomal fraction derived from the interstitial cell with the inactive smooth-surfaced submicrosomes which originate from other cells of testes than the interstitial cells.

One of the reasons why the enzyme activities related to testosterone synthesis of the two subfractions are significantly reduced after the gradient centrifugation is that one or more components which were essential for display of enzyme reactions but not P-450 itself, is partially solubilized by treatment of the microsomes with hypertonic sucrose solution, and the solubilized principle(s) was partly complemented with the heated supernatant fluid at 105,000 × g.

It was reported by Hagerman[15] that one of the smooth-surfaced submicrosomal fraction which was precipitated in the presence of Mg^{2+} but not of Cs^+ retained somewhat increased C_{17}–C_{20} lyase activity in comparison with the activity of the rough-surfaced fraction which still contained an appreciable enzyme activity.

On the other hand, in the adrenal submicrosomal fraction which did not bear ribosome on their outer surface the activities of the Δ^5-3β-hydroxysteroid dehydrogenase with the isomerase and the 17α- and 21-hydroxylases were enriched, in accordance with the submicrosomal distribution of the adrenal microsomal P-450[4, 5].

The results on the testicular and adrenal submicrosomal fractions indicate that the well-developed agranular membrane systems which are observed in the interstitial cell of testes and in adrenal cortex are engaged in the steroidogenesis.

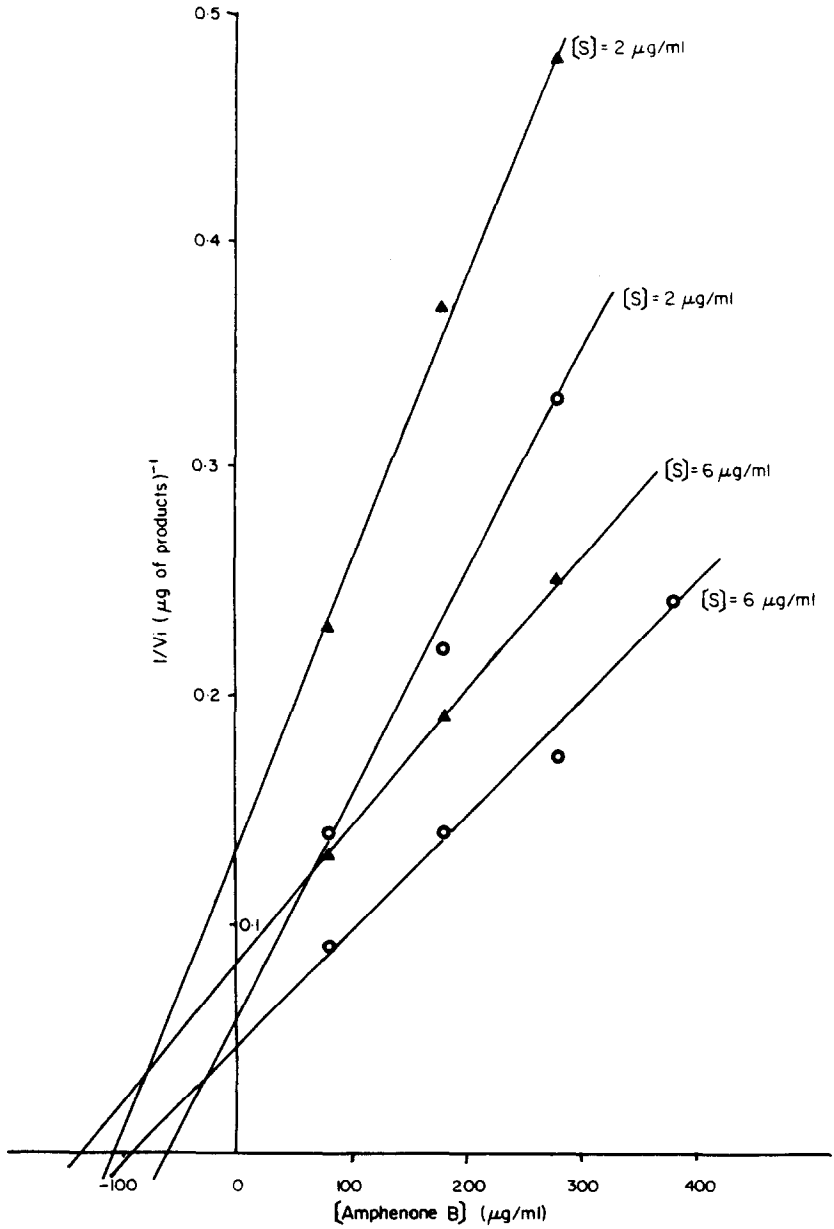

Fig. 2. Competitive inhibition of 17α-hydroxylase and C_{17}–C_{20} lyase by Amphenone B. Rat testicular microsomal fraction (18·3 mg of protein) was incubated with [4-^{14}C] progesterone (10 and 30 μg, 9·0 × 10^4 cpm) and [4-^{14}C] 17α-hydroxyprogesterone (10 and 30 μg, 7·4 × 10^4 cpm) in the presence of Amphenone B at various concentrations for 20 min at 37°. As cofactor, NADPH dissolved in 0·25 M sucrose solution containing 0·005 M $MgCl_2$ and 0·05 M Tris buffer (pH 7·4) was added in each incubation flask. Total volume of the incubation medium was adjusted to 5 ml. and final concentration of NADPH was 240 μM. The reciprocals of activities of the 17α-hydroxylase (○——○) and C_{17}–C_{20} lyase (▲——▲) were plotted against the concentration of the inhibitors by the method of Dixon [16].

Influence of Amphenone B and SKF-525A on the activities of 17α-hydroxylase and the lyase

The 17α-hydroxylation of progesterone and side-chain cleavage of 17α-hydroxyprogesterone by the testicular microsomal fraction were measured with a series of inhibitor concentrations and with the 2 or 3 different substrate concentrations. The results are analyzed by the method of Dixon[16], and suggest that both Amphenone B and SKF-525A are competitive inhibitors of the 17α-hydroxylase and C_{17}–C_{20} lyase, as shown in Figs. 2 and 3.

In the case of the 17α-hydroxylase, however, the inhibitor constant (Ki) for SKF-525A is 1·65 times as large as that for Amphenone B. On the other hand, the Ki of lyase with Amphenone B or SKF-525A were estimated as $2{\cdot}45 \times 10^{-4}$ and $1{\cdot}28 \times 10^{-5}$, respectively (Table 2). From these results, testicular 17α-hydroxyl-

Table 2. Distribution of the enzyme activities related to androgen production between the microsomal subfractions of rat testes, and influence of the heated supernatant fluid at 105,000 × *g* upon the activities

Fraction	Enzyme activities			
	Δ⁵-3β-hydroxysteroid dehydrogenase*	17α-hydroxylase	C_{17}–C_{20} lyase	17β-hydroxysteroid dehydrogenase
	μg products/mg progein			
MS	1·10	0·65	0·46	0·34
s-MS	4·18	1·67	1·14	0·94
r-MS	2·00	0·56	0·59	0·37
s-MS + h-SUP	6·36	4·09	2·14	1·14
r-MS + h-SUP	4·33	1·72	1·33	0·63
	μg products/testes			
MS	24·2	14·4	10·1	7·6
s-MS	11·7	4·7	3·2	2·7
r-MS	3·0	0·8	0·9	0·6
s-MS + h-SUP	17·8	57·3	30·0	15·9
r-MS + h-SUP	6·5	2·6	2·0	0·9

*With the Δ⁵-Δ⁴ isomerase.

[4-¹⁴C] Pregnenolone (50 μg, $3{\cdot}0 \times 10^4$ cpm) in the presence of 1 mg NAD, [4-¹⁴C] progesterone (20 μg, $4{\cdot}0 \times 10^4$ cpm), [4-¹⁴C] 17α-hydroxyprogesterone (20 μg, $3{\cdot}5 \times 10^4$ cpm) and [4-¹⁴C] androstenedione (20 μg, $3{\cdot}0 \times 10^4$ cpm) in the presence of 1 mg NADPH were incubated with the total microsomal (22 mg of protein), smooth-surfaced microsomal (2·8 mg of protein) and rough-surfaced microsomal (1·45 mg of protein) fractions of rat testes as shown in the Table. The abbreviations of MS, s-MS, r-MS and h-SUP represent respectively total microsomal fraction, smooth-surfaced microsomes, rough-surfaced microsomes and the heated 105,000 × *g* supernatant fluid. Incubation was carried out at 37° for 30 min in the atmosphere of O_2:CO_2 (95:5).

ase activity is concluded to have more affinity to Amphenone B than to SKF-525A, whereas the lyase is more strongly inhibited by SKF-525A than Amphenone B. The 17α-hydroxylase and lyase of testicular microsomal fraction[17] and the 11β-, 17α- and 21-hydroxylases in the adrenal subcellular fractions[18], all of which were concluded to involve the cytochrome P-450 as the site of molecular oxygen activation are inhibited by Amphenone B.

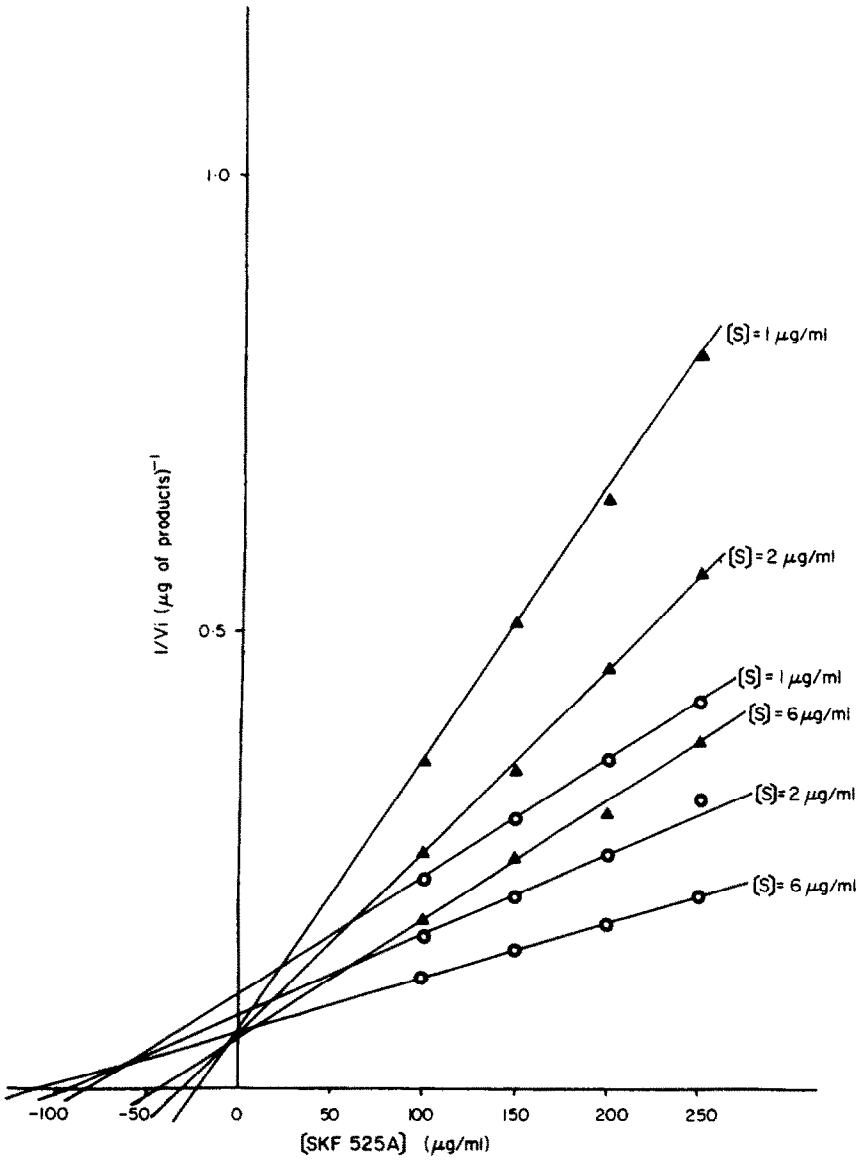

Fig. 3. Competitive inhibition of 17α-hydroxylase and C_{17}–C_{20} lyase by SKF-525A. Rat testicular microsomal fraction (17·2 mg) was incubated with [4-^{14}C] progesterone (5, 10 and 30 μg, $9{\cdot}0 \times 10^4$ cpm) and [4-^{14}C] 17α-hydroxyprogesterone (5, 10 and 30 μg, $7{\cdot}4 \times 10^4$ cpm) in the presence of SKF-525A at various concentrations for 20 min at 37°. As the cofactor, NADPH dissolved in 0·25 M sucrose solution containing 0·005 M $MgCl_2$ and 0·05 M Tris buffer (pH 7·4) was added to each incubation flask. Total volume of the incubation medium was adjusted to 5 ml, and final concentration of NADPH was 240 μM. The reciprocals of activities of the 17α-hydroxylase (○——○) and C_{17}–C_{20} lyase (▲——▲) were plotted against the concentration of the inhibitors by the method of Dixon[16].

Table 3. Contents of inhibition by Amphenone B and SKF-525A upon testicular microsomal 17α-hydroxylase and C_{17}–C_{20} lyase

Enzyme	Amphenone		SKF-525A	
	Inhibitor constant (Ki)*	Range of substrate constant (Ks)*	Inhibitor constant (Ki)*	Range of substrate constant (Ks)*
17α-Hydroxylase	$9{\cdot}17 \times 10^{-5}$	$6{\cdot}36$–$9{\cdot}54 \times 10^{-6}$	$1{\cdot}61 \times 10^{-4}$	$1{\cdot}05$–$3{\cdot}06 \times 10^{-5}$
C_{17}–C_{20} Lyase	$2{\cdot}45 \times 10^{-4}$	$1{\cdot}79$–$2{\cdot}59 \times 10^{-5}$	$1{\cdot}28 \times 10^{-5}$	$0{\cdot}86$–$2{\cdot}38 \times 10^{-6}$

*M/L.

It was reported that SU-8000 and SU-10603 competitively inhibited 17α-hydroxylase in rat testicular microsomal fraction, but also strongly non-competitively inhibited the lyase [19]. With the hepatic microsomal oxidase, it was reported that SKF-525A was a competitive inhibitor of the enzymic demethylation of N,N′-dimethyl-*p*-toluidine N-oxide, and the Ki value was 2×10^{-6} M [20]. On the other hand, the conversion of mevalonate to cholesterol and other non-saponifiable lipids was inhibited by SKF-525A [21, 22], and it was indicated that mode of the inhibition is of the so-called uncompetitive type [21].

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