[Chem. Pharm. Bull. 32(10)4043—4048(1984)]

The Effects of H_2-Receptor Antagonists and Imidazole on Testosterone Hydroxylations in Mouse Liver Microsomes

KUNIHIKO MORITA,[a] TAKESHI ONO,[a] HARUMI SHIMAKAWA*,[a] and FUMIO WADA[b]

Hospital Pharmacy, Shiga University of Medical Science,[a] Tsukinowa-cho, Seta, Otsu 520–21, Japan and Department of Endocrinology, Kagawa Medical School,[b] Miki-cho Kita-gun, Kagawa 761–07, Japan

(Received December 14, 1983)

A simple assay method for testosterone hydroxylase activity by thin-layer chromatography–ultraviolet spectrophotometry was developed. By using this method, the effects of various H_2-receptor antagonists (cimetidine, CIM; metiamide, MET; ranitidine, RAN; famotidine, FAM) or imidazole (IMZ) on the hydroxylations of testosterone by mouse liver microsomal enzymes were studied *in vitro*. CIM and MET inhibited the 6β-, 7α- and 16α-hydroxylations in a dose-dependent manner. RAN and FAM had little inhibitory effect on any hydroxylation. IMZ inhibited the 6β-hydroxylation to the same degree as CIM and MET, while the effects on the 7α- and 16α-hydroxylations were very weak. The kinetic data indicated that these drugs inhibited the three hydroxylation reactions competitively and the affinities for each hydroxylase varied from drug to drug. The results suggest that the inhibitory actions of CIM or MET on the hydroxylations of testosterone are due to the imidazole ring structure, and the side chain structures may play a role in the enhancement of affinity to the enzymes, particularly the 7α- and 16α-hydroxylases. On the other hand, RAN and FAM containing a ring structure different from imidazole had little effect on any testosterone hydroxylase.

Keywords——testosterone hydroxylation; H_2-receptor antagonist; cimetidine; metiamide; ranitidine; famotidine; imidazole; mouse liver microsome; TLC

It has been reported that cimetidine (CIM), an H_2-receptor antagonist widely used for the treatment of peptic ulcer, reduces the plasma clearance of a number of drugs in man[1–4] and has an inhibitory effect on the oxidative metabolism of xenobiotics by hepatic microsomal monooxygenases *in vitro*.[5–8] On the other hand, it is well known that this enzyme system mediates hydroxylations of various steroid hormones such as testosterone. Feely *et al.*[9] reported that CIM reduced the formation of 6β-hydroxycortisol from cortisol by inhibition of oxidative metabolism in man. On the basis of these findings, it seemed of value to investigate the inhibitory effects of H_2-receptor antagonists on the metabolism of steroid hormones by the liver microsomes.

In this study, we developed a simple assay method for testosterone hydroxylase activity by the use of thin-layer chromatography–ultraviolet (TLC–UV) spectrophotometry and investigated the effects of H_2-receptor antagonists such as imidazole, furan and thiazole derivatives on testosterone hydroxylations by mouse liver microsomes *in vitro*.

Materials and Methods

Materials——The drugs used were kindly supplied by the following companies: CIM and metiamide (MET) (Smith Klein & Fujisawa Co., Ltd., Tokyo, Japan); ranitidine (RAN) (Shin-Nippon Jitsugyo Co., Ltd., Tokyo, Japan); famotidine (FAM) (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan); 6β-hydroxytestosterone (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan). 16α-Hydroxytestosterone was purchased from Sigma Chemical

NII-Electronic Library Service

Co., Ltd., St. Louis, U.S.A. Imidazole (IMZ) and testosterone were purchased from Nakarai Chemical Co., Ltd., Kyoto, Japan. All other chemicals and solvents were of analytical grade.

Preparation of Microsomes——Liver from male ddY mice (4—6 weeks old) was homogenized in ice-cold 1.15% KCl (1 : 4, w/v), and the homogenate was centrifuged at 10000 *g* for 15 min. The supernatant fraction was centrifuged at 105000 *g* for 60 min to obtain a microsomal pellet. The microsomes were resuspended in 1.15% KCl to a concentration of about 5 mg protein/ml. Protein was determined by the method of Lowry *et al.*[10]

Enzyme Assay——The assay mixture contained (in a final volume of 10 ml) a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (0.5 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$ and 2 units of glucose-6-phosphate dehydrogenase), phosphate buffer (67.5 mM, pH 7.4), microsomal protein (0.5 mg/ml), testosterone (25—100 μM) and in some tubes an H_2-receptor antagonist or IMZ (0.25—1.0 mM). The reaction was started by adding 1 ml of microsomal suspension and carried out at 37 °C with shaking for 10 min. The reaction was stopped by adding 40 ml of dichloromethane. After addition of 0.5 ml of 0.2 mM digoxin as an internal standard and centrifugation at 900 *g* for 5 min, the organic layer was separated and the aqueous layer was extracted with a small amount of dichloromethane again. The combined organic layer was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of methanol and subjected to TLC.

Qualitative analysis of testosterone metabolites was carried out by two-dimensional TLC on TLC Silica gel 60 plate F_{254} (Merck No. 5715). The solvent systems were ethyl acetate–*n*-hexane–acetic acid (75 : 20 : 5 by vol.) (system A) in the first multiple developments and benzene–ethanol (90 : 10 by vol.) (system B) in the second development. The method of Lisboa *et al.*[11] was used. After two runs to 15 and 16 cm in system A, the plate was turned 90 degrees and development to 16 cm in the second direction was performed in system B. Each separated zone was detected by observation of the TLC plate under UV light (=254 nm). Testosterone, 6β- and 16α-hydroxytestosterone were identified by comparison with authentic standards (mobilities on TLC) and from the literature values.[11] An authentic sample of 7α-hydroxytestosterone was not available, so this compound was identified from the mobility given in the reference[11] (Table I).

Quantitative analysis of testosterone metabolites was carried out by one-dimensional ascending TLC and UV spectrophotometry. The metabolites were separated on high-performance thin-layer chromatography (HPTLC)

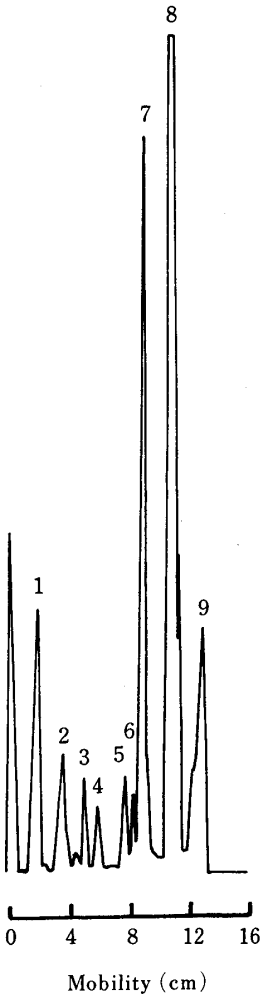

Fig. 1. Separation and Detection of 6β-, 7α- and 16α-Hydroxytestosterones by TLC–UV Spectrophotometry

1, internal standard (digoxin); 2, nicotinamide; 3, 16α-hydroxytestosterone; 4, 7α-hydroxytestosterone; 5, unknown; 6, unknown; 7, 6β-hydroxytestosterone; 8, testosterone; 9, microsomal membrane lipids.

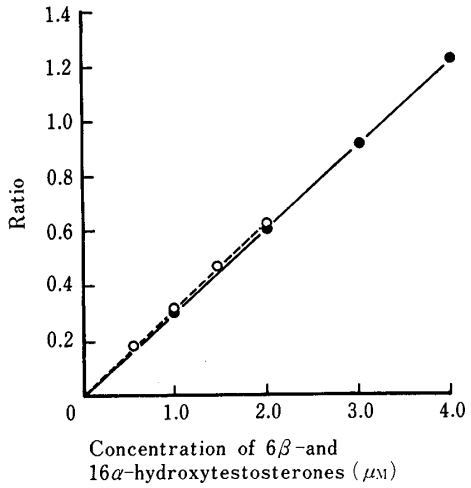

Fig. 2. Calibration Curves for 6β- and 16α-Hydroxytestosterones Added to the Incubation Mixture

The peak area ratio of metabolite to internal standard is plotted on the ordinate.
—●—, 6β-hydroxytestosterone; ---○---, 16α-hydroxytestosterone.

NII-Electronic Library Service

Silica gel 60 plate F_{254} (Merck No. 5642) with one-dimensional multiple developments in system A. This HPTLC plate was better for quantitative analysis than the plate used for identification. The chromatogram was obtained by using a dual-wavelength densitometer (Shimadzu TLC scanner, model CS-910) ($\lambda_1 = 238$ nm, $\lambda_2 = 350$ nm) (Fig. 1). The amounts of testosterone metabolites were calculated from the peak area ratio of each metabolite to the internal standard, obtained with an integrator (Shimadzu Chromatopac C-R2AX). The calibration curves for 6β- and 16α-hydroxytestosterones are shown in Fig. 2.

Results and Discussion

Assay Method for Testosterone Hydroxylase Activity by TLC–UV Spectrophotometry

In a number of studies,[12-17] assay for testosterone-metabolizing activity by liver microsomes has been carried out by determination of the testosterone metabolites labeled with radioactive isotopes. This method by paper chromatography-liquid scintillation spectrometry[12-17] is very sensitive, but requires a long time. In this study, a simple assay method not employing radioactive isotope-labeled compounds was developed. The principle of the new method is based on one-dimensional ascending TLC with multiple developments as reported by Lisboa *et al.*[11] and UV spectrophotometry.

Nine major peaks were detected by one-dimensional multiple developments in system A (Fig. 1). In order to check whether or not each of these peaks was a single component, development in the second direction was then performed in system B and the mobilities of the metabolites were compared with those of authentic standards and/or those given in the reference[11] (Table I). Each of the nine zones was confirmed to be a single component and peaks 3, 4, 7 and 8 coincided in mobility with 16α-, 7α- and 6β-hydroxytestosterone and testosterone, respectively. Peaks 1, 2 and 9 were identified as digoxin (internal standard), nicotinamide arising from NADP and a lipid component arising from microsomes, respectively. Peaks 5 and 6 are still unidentified, but the identification of the three hydroxylation products of testosterone in this study is supported by the existence of different cytochrome P-450s to hydroxylate testosterone at the 6β-, 7α- and 16α-positions in mouse liver microsomes.[18]

The calibration curves for 6β- and 16α-hydroxytestosterones showed good linearity when digoxin was used as an internal standard (Fig. 2). The calibration curve for 7α-hydroxytestosterone was expected to show similar linearity since the determination of these three hydroxylation products of testosterone by this method is based on measurement of UV

TABLE I. Mobilities of Standard Compounds and Metabolites Isolated by TLC

Peak number	Identification	Mobilities in solvent system (cm)			
		Standards		Metabolites	
		System A	System B	System A	System B
1	Digoxin (I.S.)	2.0	1.0	2.0	1.0
2	Nicotinamide	4.0	1.5	4.0	1.5
3	16α-Hydroxytestosterone	5.5 (5.4)	1.9 (1.9)	5.6	1.9
4	7α-Hydroxytestosterone	— (6.6)	— (2.0)	6.6	2.0
5				8.6	2.8
6				9.0	4.0
7	6β-Hydroxytestosterone	9.6 (9.6)	3.3 (3.3)	9.6	3.3
8	Testosterone	11.6 (11.5)	5.6 (5.6)	11.7	5.6
9	Membrane lipids (cholesterol *etc.*)	15.0	10.0	15.2	9.8

The values in parentheses are the mobility values given in the reference.[11]
Details of the solvent systems employed are given in Materials and Methods.

NII-Electronic Library Service

absorption specific for 3-oxo-Δ^4steroids (238 nm).

The Effects of H_2-Receptor Antagonists and Imidazole on Testosterone Hydroxylations in Mouse Liver Microsomes

The chemical structures of the H_2-receptor antagonists tested are shown in Fig. 3.

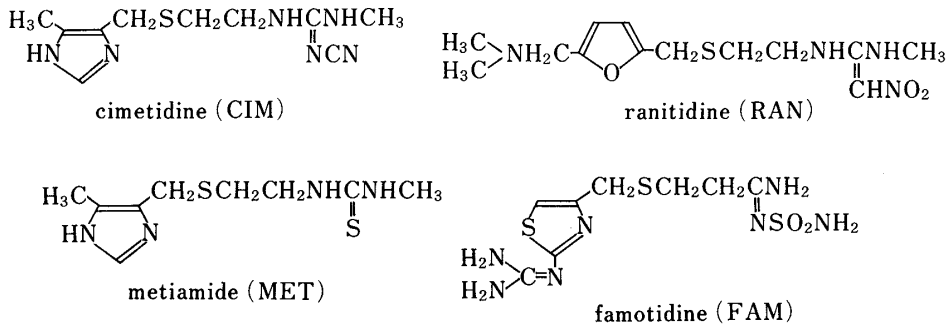

Fig. 3. Chemical Structures of H_2-Receptor Antagonists

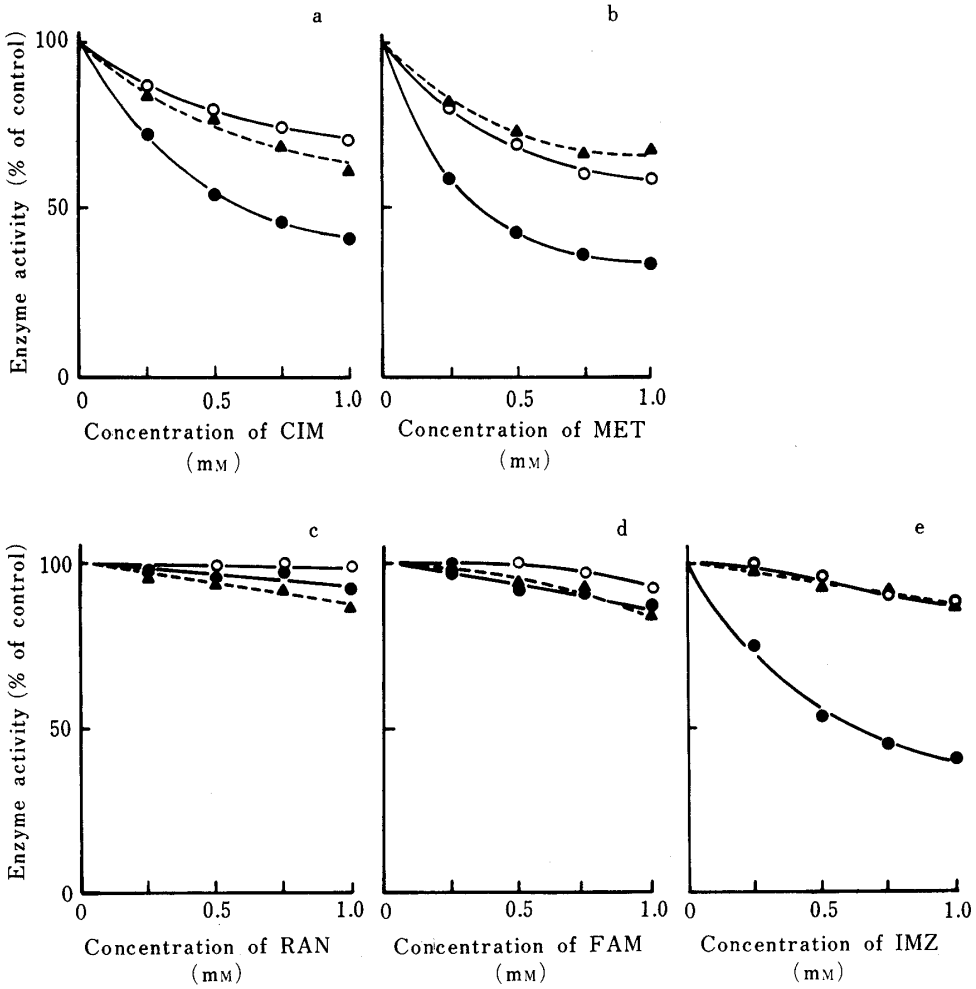

Fig. 4. Dose-Related Inhibition of Testosterone 6β-, 7α- and 16α-Hydroxylase Activities in Mouse Liver Microsomes by CIM (a), MET (b), RAN (c), FAM (d) and IMZ (e)

The concentration of testosterone in each experiment was 25 μM. Each curve is the mean of experiments with four different mouse liver microsomal preparations. Standard deviations for different data points were between 2 and 9 percent.
—●—, 6β-hydroxylation; ---▲---, 7α-hydroxylation; —○—, 16α-hydroxylation.

NII-Electronic Library Service

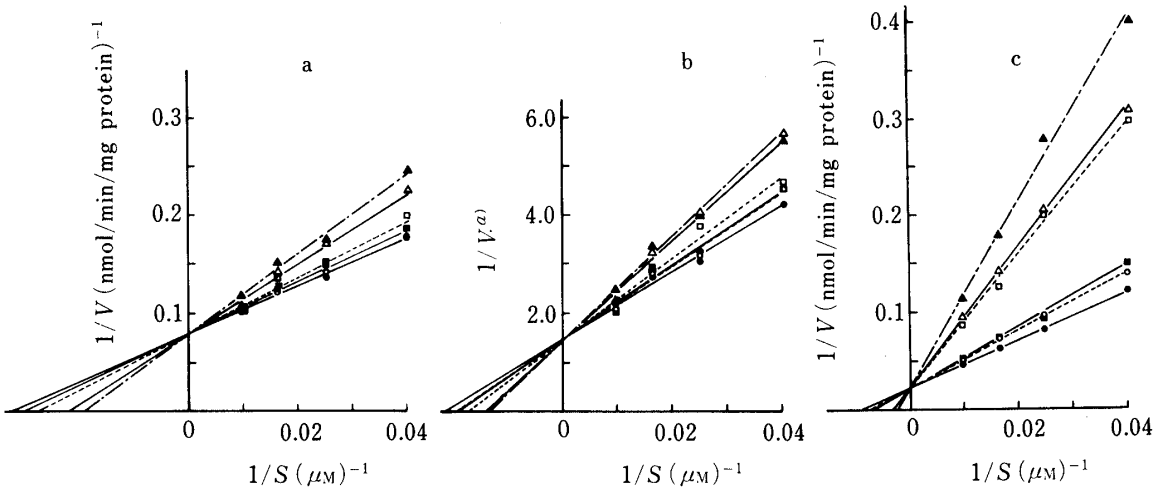

Fig. 5. Double-Reciprocal Plots Showing the Inhibitions of Testosterone 6β-, 7α- and 16α-Hydroxylations Obtained with Mouse Liver Microsomes in the Absence and Presence of 1.0 mM H_2-Receptor Antagonists and IMZ

a, 16α-hydroxylation; b, 7α-hydroxylation; c, 6β-hydroxylation.
—●—, control; —△—, CIM; ---▲---, MET; ---○---, RAN; —■—, FAM; ---■---, IMZ.
a) As authentic 7α-hydroxytestosterone was not available, the ordinate shows the value calculated from the peak area ratio of metabolite to internal standard.

TABLE II. Inhibition Constants (K_i) of H_2-Receptor Antagonists and IMZ for Testosterone Hydroxylases in Mouse Liver Microsomes

	K_i (mM)[a]		
	6β-OH	7α-OH	16α-OH
IMZ	0.68	6.50	6.34
CIM	0.67	3.49	2.33
MET	0.45	3.23	1.67
RAN	6.67	8.09	—[b]
FAM	6.09	8.09	9.00

a) Calculated from the double-reciprocal plots for testosterone hydroxylases (Fig. 5).
b) No significant inhibition could be demonstrated.

CIM and MET inhibited the 6β-, 7α- and 16α-hydroxylations of testosterone in a dose-dependent manner (Fig. 4a, b). In particular, the 6β-hydroxylation was inhibited more potently than the 7α- and 16α-hydroxylations. MET inhibited the 6β- and 16α-hydroxylations more strongly than CIM at the same molar concentrations. In contrast with CIM and MET (containing the imidazole ring structure), RAN and FAM (containing furan and thiazole ring structures, respectively) showed little inhibition of each hydroxylation (Fig. 4c, d). Knodell *et al.*[7] reported that CIM, but not RAN, showed competitive inhibition of meperidine demethylation and pentobarbital hydroxylation by hepatic microsomal drug-metabolizing enzymes of rat or man, and they suggested that these inhibitory actions by CIM are due to the interaction of its imidazole ring structure with cytochrome P-450. Thus, we investigated the effect of IMZ on each hydroxylation. IMZ inhibited the 6β-hydroxylation to the same degree as CIM and MET, but it had less inhibitory effect on the 7α- and 16α-hydroxylations than CIM and MET (Fig. 4e).

The modes of inhibitory activity on testosterone hydroxylases by H_2-receptor antagonists

NII-Electronic Library Service

and IMZ were investigated in detail by the use of double-reciprocal plots (Fig. 5). The kinetic data indicated that the inhibition of each testosterone hydroxylase activity by these compounds was of competitive type. From these data, the inhibition constants were calculated (Table II). MET, CIM and IMZ had potent inhibitory effects on 6β-hydroxylation, but RAN and FAM were weak inhibitors, since the values of the inhibition constants of RAN and FAM were larger by one order of magnitude than those of IMZ and CIM. On the other hand, CIM and MET showed 2—4 times stronger inhibitory effects on 7α- and 16α-hydroxylations than IMZ, RAN and FAM. From these results, it is suggested that CIM and MET have higher affinities than RAN and FAM for liver microsomal testosterone hydroxylases. It was also reported that CIM and MET had lower dissociation constants than RAN on the basis of spectral changes in binding studies with rat liver microsomes.[8] However, the factor involved in the affinities of CIM and MET for cytochrome P-450s cannot be only the imidazole ring structure, because differences in the inhibitory effects and in the inhibition constants of CIM, MET and IMZ against testosterone hydroxylases, particularly 7α- and 16α-hydroxylases, were found. Wilkinson *et al.*[19] examined the effects of a series of imidazole derivatives on rat liver microsomal enzyme activity, and reported that the greater lipophilicity of the 4(5)-substituted derivatives facilitates penetration of the lipid membrane and hydrophobic bonding to cytochrome P-450, and accounts for the markedly enhanced potency of these compounds compared with IMZ. Accordingly, it is considered that CIM and MET, imidazole derivatives, have higher lipophilicity than IMZ, and thus exhibit greater affinity for each testosterone hydroxylase.

Ultimately, our results suggest that the potent inhibitory activities of CIM and MET on 6β-, 7α- and 16α-hydroxylases of testosterone are essentially due to their imidazole ring structure, and that increased hydrophobicity based on the side chain structures may play a role in increasing the affinity for these enzymes, particularly 7α- and 16α-hydroxylases. On the other hand, RAN and FAM (containing another ring structure different from imidazole) had little effect on the testosterone hydroxylases. It is, of course, difficult to extrapolate these findings directly to man, but the possibility that a similar interaction could occur in patients seems to require investigation.

References

1) A. C. Flind, *Br. Med. J.*, **2**, 1367 (1978).
2) M. J. Serlin, S. Mossman, R. G. Sibeon, A. M. Breckenridge, J. R. B. Williams, J. L. Atwood and J. M. T. Willoughby, *Lancet*, **2**, 317 (1979).
3) U. Klotz and I. Reiman, *N. Engl. J. Med.*, **302**, 1012 (1980).
4) P. V. Desmond, R. V. Patwardhan, S. Schenker and K. V. Speeg, Jr., *Ann. Intern. Med.*, **93**, 266 (1980).
5) S. Rendic, V. Sunjic, R. Toso, F. Kajfez and H. H. Ruf, *Xenobiotica*, **9**, 555 (1979).
6) O. Pelkonen and J. Puurunen, *Biochem. Pharmacol.*, **29**, 3075 (1980).
7) R. G. Knodell, J. L. Holtzman, D. L. Crankshaw, N. M. Steele and L. N. Stanley, *Gastroenterology*, **82**, 84 (1982).
8) K. V. Speeg, Jr., R. V. Patwardhan, G. R. Avant, M. C. Michell and S. Schenker, *Gastroenterology*, **82**, 89 (1982).
9) J. Feely, D. Robertson, D. P. Island and A. J. J. Wood, *N. Engl. J. Med.*, **306**, 1054 (1982).
10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) B. P. Lisboa, J. Å. Gustafsson and J. Sjövall, *Eur. J. Biochem.*, **4**, 496 (1968).
12) A. H. Conney and A. Klutch, *J. Biol. Chem.*, **238**, 1611 (1963).
13) R. M. Welch, W. Levin and A. H. Conney, *J. Pharmacol. Exp. Ther.*, **155**, 167 (1967).
14) A. H. Conney, W. Levin, M. Ikeda and R. Kuntzman, *J. Biol. Chem.*, **243**, 3912 (1968).
15) W. Levin, A. Alvares, M. Jacobson and R. Kuntzman, *Biochem. Pharmacol.*, **18**, 883 (1969).
16) M. Y. Chan and A. Tse, *Biochem. Pharmacol.*, **27**, 1725 (1978).
17) R. Rahman and r. B. Clayton, *J. Biochem.* (Tokyo), **90**, 1567 (1981).
18) M. T. Huang, S. B. West and A. Y. H. Lu, *J. Biol. Chem.*, **251**, 4659 (1976).
19) C. F. Wilkinson, K. Hetnarski and T. O. Yellin, *Biochem. Pharmacol.*, **20**, 3187 (1971).

NII-Electronic Library Service