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EFFECTS OF INDUCERS AND INHIBITORS OF RAT LIVER MONO-OXYGENASES ON DIGITOXIN METABOLISM*

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Abstract—In rats, microsomal mono-oxygenases are involved in the cleavage and hydroxylation of digitoxin (dt-3).† Previous to hydrolytic cleavage the terminal digitoxosyl has to be oxidized to the corresponding dehydro-digitoxosyl. The microsomal formation rate of 15′-dehydro-dt-3 could be enhanced 3 to 7 fold by pretreatment with pregnenolone-16α-carbonitril, spironolactone, cancrenoate and cyproterone. Phenobarbital and polychlorinated biphenyls had no effect, whereas polyaromatic hydrocarbons caused a significant decrease of both C12-β-hydroxylation and sugar oxidation. The inducing effects of the steroids were less pronounced in the formation of 9′-dehydro-dt-2 and 3′-dehydro-dt-1. For the latter, phenobarbital evoked an inducing effect by factor 1.7. The sugar chain oxidation could be inhibited by metyrapone, spironolactone, cancrenoate and digitoxigenin when added *in vitro*. a-Naphthoflavone was a weak inhibitor only. The results indicate that the terminal digitoxosyl oxidation is due to a specific isocytochrome P450 (or pattern of isocytochromes) inducible by some synthetic steroids.

In rats digitoxin is metablised by C12-β-hydroxylation, cleavage of the sugar chain and conjugation of predominantly the cleavage product dt-1 [1–5]. Excreted $CHCl_3$-soluble metabolites consist mainly of dt-2, dt-1 and dg-1. In addition, 15′-dehydro-dt-3 and 9′-dehydro-dt-2 are present in the $CHCl_3$ extract of the bile [4, 6]. Only traces of dg-3, dg-1 and unchanged dt-3 can be detected. The water-soluble fraction of dt-3 metabolites contains mainly glucuronosides and sulfates of dt-1. The excretion of water-soluble metabolites can be enhanced by pretreatment with PB or SP, whereas MC causes a decrease of the elimination rate [4, 7–9].

Previous studies suggested that not only the C12-β-hydroxylation but also the stepwise cleavage of the sugar chain is catalysed by microsomal mono-oxygenases [10]. Appartently, the essential step of this oxidative cleavage is the dehydrogenation of the axial OH-group of the terminal digitoxosyl [11]. The resulting dehydro-digitoxyosyl can be split off, presumably by β-elimination, in the presence of microsomal protein. The purpose of the present investigation was: (1) to evaluate which mono-oxygenase species is involved in the sugar chain cleavage, and (2) to find out whether the elimination rates after pretreatment with various inducers can be explained by changes of the mono-oxygenase activity.

MATERIALS AND METHODS

Materials. The following compounds were generous gifts: PCN from Dr. Ronald E. Talcott, University of California (Riverside, CA), Benz[a]anthracene from Prof. Dr. G. Grimmer, Biochem. Inst. für Umweltcarcinogene (Ahrensburg, Germany); PCB (Aroclor® 1254) from Monsanto, Germany (Düsseldorf, Germany), SP from Boehringer (Mannheim, Germany), cyproterone from Schering (Berlin, Germany), metyrapone from Ciba (Switzerland, Basel), 20[22]-^{3}H-digitoxin (660Ci/mole) from Dr. G. Haberland, Beiersdorf (Hamburg, Germany). 20[22]-^{3}H-dt-2 and 20[22]-^{3}H-dt-1 were prepared from 20[22]-^{3}H-dt-3 by the method of Satoh and Aoyama [12]. 15′-dehydro-dt-3, 9′-dehyro-dt-2 and 3′-dehydro-dt-1 were synthesized as reported previously [13].

Digitioxosides of dt-0 and dg-0, isocitrate dehydrogenase (E.C. 1.1.1.42), pyridine nucleotides and CANR postassium (Aldactone® *pro injectione*) were purchased from Beohringer (Mannheim, Germany). BNF and ANF (i.e. 7,8-benzoflavone and 5,6-benzoflavone, resp.) were from Roth (Karlsruhe, Germany); Instagel from Packard (Frankfurt, Germany); all other chemicals were of analytical grade from Merck (Darmstadt, Germany).

Male Wistar rats were obtained from E. Jautz (Kiesslegg, Germany).

Treatment of animals. Solutions for i.p. injections were 40 mg polyaromatic hydrocarbon or BNF/ml arachis oil, 200 mg PCB/ml arachis oil, 50 mg cyproterone or PCN/2 ml 5% Tween® 80 suspension, 100 mg SP/2 ml 0.5% carboxymethylcellulose, 100 mg CANR/2 ml water, 80 mg PB/ml water. BNF

* Some results of this work have been presented at the annual meeting of the Deutsche Pharmazeutische Gesellschaft, Tübingen, Germany, September, 1978, and at the spring meeting of the Deutsche Pharmakologische Gesellschaft, Mainz, Germany, 1979 [A. Schmoldt, *Naunyn-Schmiedeberg's Archs. Pharmac.* **305**, 261 (1978) and *ibid.* **306**, R24 (1979)].

† Abbreviations used in the text are as follows: ANF, α-naphtoflavone; BNF, β-naphthoflavone; CANR, canrenoate potassium; dg-0, digoxigenin; dg-1, dg-2, dg-3, digoxigenin mono-, bis-, tridigitoxoside; dt-0, digitoxigenin; dt-1, dt-2, dt-3, digitoxigenin mono-, bis-, tridigitoxoside; MC, 3-methylcholanthrene; PB, phenobarbital; PCN, 16-α-pregnenolone carbonitril; SP, spironolactone.

Table 1. Effect of inhibitors on microsomal dt-3 metabolism

Variation of complete system	Metabolites in per cent of controls ± S.D. dg-3	dg-2	15'-dehydro-dt-3
— (controls)*	100 ± 10	100 ± 7	100 ± 6
1. −ICDH, −IC, −NADPH	8.3 ± 4.2†	5.3 ± 2.9†	8.8 ± 0.4†
2. −ICDH, −IC, −NADPH −NADH, + NADP, + NAD (0.3. mM)	7.4 ± 3.6†	4.9 ± 2.4†	7.8 ± 5.1†
3. −air + N_2	9.2 ± 3.2†	6.3 ± 0.5†	6.8 ± 3.2†
4. + air/CO (3 + 2, v/v)	54 ± 6†	29 ± 2†	29 ± 1†
5. +NaN_3 (0.2 mM)	102 ± 15	117 ± 14	106 ± 4
6. +KCN (0.1 mM)	101 ± 17	103 ± 3	99 ± 2
7. +ethanol (22 mM)	89 ± 9	105 ± 9	100 ± 2
8. +EDTA (2.5 mM)	88 ± 5	96 ± 3	91 ± 5
9. +metyrapone (10 μM)	74 ± 3†	9.2 ± 3.1†	3.8 ± 2.0†
10. +ANF (75 μM)	57 ± 4†	63 ± 3†	59 ± 1†
11. +SKF-525A (0.1 mM)	62 ± 7†	11 ± 3†	22 ± 4†
12. +PCB (150 μM)	32 ± 8†	38 ± 6†	26 ± 6†
13. +dt-0 (28 μM)‡	49	51	31
(56 μM)	11	9	21
14. +SP (50 μM)	32 ± 4†	16 ± 1†	15 ± 1†
15. +CANR (50 μM)	20 ± 2†	14 ± 3†	13 ± 1†
	100% of controls correspond to metabolites/mg microsomal protein/15 min		
Variation no.			
1, 2, 3	360 ± 30	468 ± 56	530 ± 71
4, 11	606 ± 74	904 ± 55	874 ± 164
5, 9, 10	583 ± 52	798 ± 63	910 ± 83
6, 7, 8	424 ± 60	573 ± 17	819 ± 32
12, 14, 15	674 ± 61	834 ± 97	865 ± 85
13	275	284	452

* The complete system taken as controls contained per ml: 30 μM ^{3}H-dt-3, 1.5 mg microsomal protein, 50 mM Tris buffer (pH 7.45), 0.15 M KCl, 5 mM $MgCl_2$, 0.3 mM NADH, 0.5 mM NADPH, 8 mM isocitrate (IC), 400 mU isocitrate dehydrogenase (ICDH). Values are means ± S.D. of 3 separate experiments.

† Significant difference to controls ($P < 0.01$).

‡ Means of 2 experiments; dt-3 concentration: 14 μM.

or polyaromatic hydrocarbon (40 mg/kg/day), SP or CANR (100 mg/kg/day) and PB (80 mg/kg/day) were given for 3 days; PCB (200 mg/kg) was adminstered once 4 days before killing. Control animals received the vehicle doses at the same times. The various vehicles had no different effects on the dt-3 metabolism. Animals were fed *ad lib.* on a normal laboratory diet.

Preparation of microsomes and assays. Liver microsomes were prepared by the method of Kutt and Fouts [14]. Protein concentrations were determined by the Lowry method and cytochrome P450 contents by the method of Omura and Sato [15], except that Tris–HCl (pH 7.5) was used instead of phosphate buffer. Binding spectra with dt-3 (dissolved in dimethylsulfoxide) were recorded using an Aminco-DW2 spectrophotometer (split-beam mode) as described by Schenkman *et al.* [16]. ^{3}H-digitoxosides (10–200 μM, [1–1.5 μCi]) were incubated in the total volume of 0.25 ml containing 150 μM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.45), 8 mM isocitrate, 0.3 mM NADH, 0.5 mM NADPH, 400 mU isocitrate dehydrogenase and 0.38 mg microsomal protein. Incubation was at 37° with shaking in air. After 15 min, aliquots of the incubation mixture were stopped by adding equal volumes of freshly distilled acetone. Thin layer chromatography (on silica gel plates, development in ethylacetate, $CHCl_3$–methanol (92:8, v/v), or $CHCl_3$–acetone (65:35, v/v)) and counting of radioactivity were the same as previously described [10, 11]. Blank values were obtained by stopping the incubation at zero time, incubation without microsomes or by omission of NADPH and NADPH regenerating system. No significant differences could be found between these three procedures.

Statistical significances were determined by Student's *t*-test for independent values of two groups, and analysis of variance and Sheffé-test [17] for comparing more than 2 means. In any case absolute values were compared statistically even when presented in percent of controls (Tables 1 and 2).

RESULTS

15'-dehydro-dt-3, dt-2 and dg-3 could only be formed in the presence of oxygen and NADPH (Fig. 1, Table 1). Oxidized pyridine nucleotides and anaerobic conditions prevented 12-β-hydroxylation as well as oxidation and splitting off of the terminal digitoxose. There was no effect on the metabolism when catalase (by azide) or the cyanide sensitive factor (CSF) were inhibited. Since carbon monoxide inhibited the reaction, it can be concluded that both the 12-β-hydroxylation and the oxidation of the ter-

Table 2. Digitoxin metabolism by microsomes by variously pretreated rats

Pretreatment	cyt. P450		Per cent of controls ± S.D. metabolites dg-3	dt-2	15′-dehydro-dt-2
— (controls)*		(a)†	100 ± 12	100 ± 11	100 ± 10
	100 ± 9	(b)	100 ± 13	100 ± 9	100 ± 8
1. PB		(a)	85 ± 19	100 ± 6	106 ± 6
	205 ± 30‡	(b)	46 ± 10‡	51 ± 9‡	51 ± 13‡
2. MC		(a)	76 ± 9‡	46 ± 5‡	41 ± 5‡
	167 ±27‡	(b)	46 ± 6‡	30 ± 3‡	26 ± 4‡
3. benzo[*a*]anthracene		(a)	72 ± 11‡	61 ± 6‡	59 ± 10‡
	128 ± 13‡	(b)	57 ± 13‡	46 ± 8‡	44 ± 11‡
4. benzo[*a*]pyrene		(a)	74 ± 10‡	52 ± 7‡	49 ± 7‡
	132 ± 7‡	(b)	57 ± 12‡	40 ± 11‡	37 ± 6‡
5. PCB		(a)	136 ± 38	123 ± 14	122 ± 8
	315 ± 61‡	(b)	43 ± 12‡	39 ± 7‡	39 ± 4‡
6. SP		(a)	223 ± 22‡	530 ± 14‡	598 ± 66‡
	109 ± 9	(b)	201 ± 21‡	518 ± 15‡	551 ± 36‡
7. CANR		(a)	163 ± 22‡	426 ± 12‡	384 ± 53‡
	108 ± 6	(b)	164 ± 24‡	414 ± 31‡	363 ± 44‡
8. PCN		(a)	550 ± 92‡	592 ± 60‡	690 ± 75‡
	168 ± 23‡	(b)	347 ± 83‡	327 ± 29‡	445 ± 69‡
9. cyproterone		(a)	209 ± 22‡	298 ± 20‡	289 ± 32‡
	112 ± 5	(b)	186 ± 19‡	266 ± 15‡	258 ± 28‡
	100% of controls correspond to nmoles cyt. P450 or pmoles metabolites per mg microsomal protein				
No.					
1, 8	0.83 ± 0.07		738 ± 70	1019 ± 226	1539 ± 290
2, 5	0.68 ± 0.09		638 ± 61	604 ± 104	865 ± 84
3, 4	0.74 ± 0.06		771 ± 54	824 ± 95	1221 ± 153
6	0.64 ± 0.07		594 ± 148	548 ± 63	878 ± 69
7	0.88 ± 0.1		602 ± 82	792 ± 80	1320 ± 161
9	0.69 ± 0.05		517 ± 57	682 ± 47	771 ± 76

30 μM ^{3}H-dt-3 was incubated with liver microsomes and a NADPH regenerating system for 15 min, as given in the legend to Table 1. Values are means ± S.D. of 3–4 rats.

† (a) per mg microsomal protein; (b) per nmole cyt. P450.

‡ Significant difference to controls.

minal digitoxyosyl are cytochrome P450-linked reactions.

In order to determine which of the P450 isocytochromes is involved in microsomal dt-3 metabolism, some inhibitors were tested. The cyt. P450-specific inhibitor, metyrapone, caused an almost complete inhibition of the sugar oxidation at a concentration of 10 μM. The 12-β-hydroxylation was less affected. The cyt. P 448 specific inhibitor ANF, however, was a weak inhibitor. About 40 percent inhibition was obtained for both 12-β-hydroxylation and sugar oxidation (and subsequent cleavage) at a concentration of 75 μM. High inhibition values could be observed also in the presence of 100 μM SKF-525A, 50 μM SP and CANR. The SP inhibition proved to be noncompetitive (K_i = 7 μM, estimated graphically from Lineweaver–Burk plots).

The findings, inhibition by metyrapone, dt-0 and PCB, suggested that mainly cyt. P450 catalyses the dt-3 oxidation. In order to confirm this conclusion, microsomes of PB pretreated rats were tested. However, no significant enhanced oxidation rates could be obtained (Table 2), in spite of a two-fold cyt. P450 increase. With regard to oxidation per nmole cytochrome, this means a significant decrease. A more pronounced decrease was seen with microsomes of rats pretreated with MC and other polycyclic hydrocarbons (benz[*a*]anthracene, benz[*a*]pyrene), even when expressed as oxidation per mg microsomal protein. BNF caused no significant changes. Even PCB, known as inducer of both cyt. P450 and P448, caused only a slight increase due to the very high induction of total cytochromes.

In contrast to that, pretreatment with SP, PCN, the SP metabolite CANR and cyproterone evoked a 3–7 fold formation rate of 15′-dehydro dt-3. Furthermore, after PCN, a fourfold increase of 12-β hydroxylation was found, whereas the other steroids were less active in this respect. The total cyt. P450 concentration increased only slightly, this means a remarkable increase of the 'specific activity'. Spectral binding studies with these microsomes revealed a type I spectrum for dt-3 with a peak at 338 nm and a trough at 420 nm (Fig. 2).

In order to yield dt-1 or dt-0 as substrate for conjugating enzymes *in vivo*, the dt-3 cleavage product dt-2 and its cleavage products dt-1 have also to be oxidized at their terminal digitoxyosyl. Therefore,

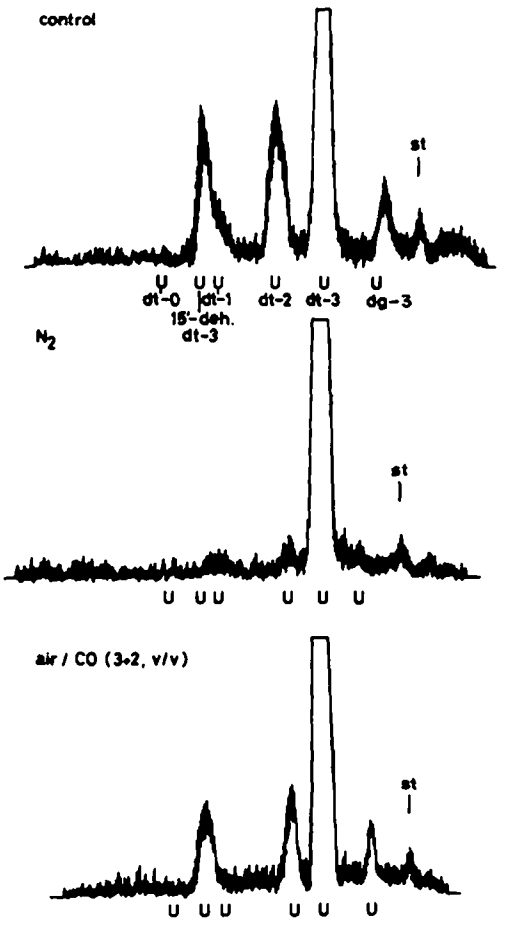

Fig. 1. Thin layer chromatography and ^{3}H-scanning after incubation of ^{3}H-dt-3 (30 μM) with rat liver microsomes (1.5 mg/ml) in the presence of NADPH (control, upper panel), without O_2 (middle panel) and in the presence of CO (lower panel). Development in ethyl acetate together with reference compounds (U). St = start.

these glycosides also were incubated under the same conditions (Table 3). In all cases, dt-2 was metabolized at a slower rate than dt-3 or dt-1. The inducing effects of CANR were considerably lower for dt-1 than for dt-3. Similar effects were obtained after pretreatment with SP [18] and PCN (10.5, 6.4 and 8.3 times higher than control values for sugar oxidation (+ cleavage) of dt-3, dt-2 and dt-1, respectively). In contrast to that, PB caused an increase for dt-1, but was without effect in dt-3 or dt-2 metabolism. Furthermore, Table 3 shows that dt-2 and dt-1 were better substrates for 12-β-hydroxylation than dt-3.

DISCUSSION

15'-dehydro-dt-3, 9-dehydro-dt-2 and 3'dehydro-dt-1 are metabolites of dt-3. They were identified by chromatographic and chemical comparison with the corresponding synthesized compounds. They are important for the metabolism because the terminal digitoxosyl can only be split off after formation of these dehydrodigitoxosides [11].

With reservation of experiments with purified components of the rat liver mono-oxygenase complex, the results of this study strongly suggest that microsomal P450 cytochromes are involved in the formation of these dehydro-digitoxosides. The dt-3 oxidation rates were not influenced by inhibitors of catalase or the cyanide sensitive factor, but were markedly inhibited by typical mono-oxygenase inhibitors. The nature of this cyt. P450 linked oxidation remains unclear because the formation of an oxo-group at C15' corresponds formally to a dehydrogenation. However, that corresponds to the microsomal ethanol oxidation for which it is also shown to depend partially on cyt. P450 [19–21]. The ethanol oxidizing system itself seem not to be involved in dt-3 oxidation since ethanol could not inhibit the reaction (Table 2).

With regard to the mono-oxygenase species, it is evident that one (or more) of those P450 isocyto-

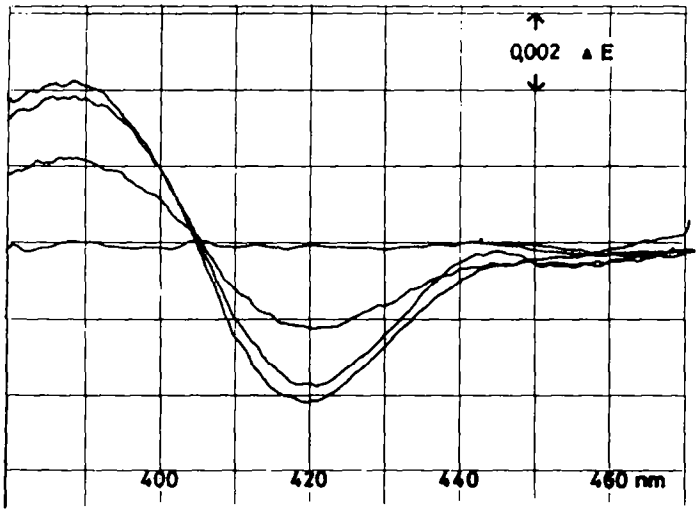

Fig. 2. Dt-3 induced difference spectra in liver microsomes of SP-pretreated rats (0.01 nmole cyt. P450/ml Tris buffer). Dt-3 concentrations were 36, 72 and 108 μM. The reference cuvette contained the same concentrations of the solvent (dimethyl sulfoxide, final concentration: 5.5 μl/ml).

Table 3. Comparison of the metabolism of dt-3, dt-2 and dt-1 by microsomes of normal and pretreated rats

Pretreatment	Substrate (30 μM)	12-β-hydroxylation	Digitoxosyl oxidation and cleavage
		(pmoles/mg microsomal protein/15 min)*	
	dt-3	602 ± 85	1738 ± 180
PB	dt-3	486 ± 68‡	1929 ± 114
CANR	dt-3	872 ± 74‡	6432 ± 282‡
	dt-2	911 ± 90†	1182 ± 83†
PB	dt-2	888 ± 84†	1360 ± 142†
CANR	dt-2	1372 ± 175†‡	3425 ± 83†‡
	dt-1	981 ± 87†	1713 ± 135
PB	dt-1	1421 ± 121†‡	2870 ± 193†‡
CANR	dt-1	1216 ± 113†‡	3695 ± 205†‡

† Values are means ± S.D. of 3 experiments with pooled microsomes of 3–4 rats. Incubations were made as described in the legend to Table 1. Cyt. P450 concentrations (nmole/mg microsomal protein) were: 0.82 (controls) 1.75 (PB) and 0.93 (SP).

† Significantly different to corresponding dt-3 metabolites ($P < 0.01$).

‡ Significantly different to controls ($P < 0.01$).

chromes are involved in digitoxosyl oxidation which can be incuded by PCN, SP, CANR and cyproterone. The formation rate per nmole P450 rose up to 5 fold values, whereas phenobarbital and polycyclic hydrocarbons decreased it to 60 and 40 per cent, respectively, when compared to controls. The lower inducing effect of CANR on dt-1 oxidation can be explained possibly by a shift of the affinity to PB inducible cyt. P450. This view is supported by the effect of PB, which significantly stimulated dt-1 oxidation and is known to enhance dt-0 metabolism as well as cyproterone and SP [22–24]. *In vivo*, however, the induction of dt-1 metabolism seems to be irrelevant, since dt-1 is conjugated as soon as it is formed [3, 4, 6].

Obviously, dt-2 cannot be metabolized at the same rate as dt-3. Therefore, one may speculate that not dt-3 oxidation, but dt-2 oxidation is the rate limiting step in dt-3 degradation *in vivo*. This hypothesis is supported by the observation that the $CHCl_3$-soluble fraction of the bile contains preferably dt-2, although it is more lipophilic than dt-3.

With regard to *in vivo* conditions, the results of this study suggest that PCN, SP and cyproterone enchance dt-3 elimination by a rather specific induction of P450 cytochromes involved in dt-3- and dt-2 sugar oxidation. The comparable low effect of PB, however, could be explained by a proliferation of endoplasmic reticulum with a nearly constant specific activity.

Finally, the impaired biliary dt-3 elimination rate of dt-3 metabolites after induction of cytochrome P448 is probably due to the low affinity of this cytochrome to dt-3.

REFERENCES

1. K. Repke, *Naunyn-Schmiedeberg's Archs. exp. Path. Pharmac.* **237**, 155 (1959).
2. K. Repke and F. Lauterbach, *Verh. dt. Ges. Kreisl-Forsch.* **25**, 290 (1959).
3. M. C. Castle and G. L. Lage, *Res. Commun. Chem. Path. Pharmac.* **5**, 99 (1973).
4. A. Schmoldt, H. F. Benthe and W. Grote, *Naunyn-Schmiedeberg's Archs. Pharmac.* **282**, Suppl. R85 (1974).
5. N. Rietbrock, H. F. Vöhringer and L. Weller, *Naunyn-Schmiedeberg's Archs. Pharmac.* **227**, Suppl. R62 (1973).
6. A. Schmoldt, H. F. Benthe and G. Haberland, *Toxic. appl. Pharmac.* **47**, 483 (1979).
7. C. D. Klassen, *J. Pharmac. exp. Ther.* **191**, 201 (1974).
8. M. C. Castle and G. L. Lage, *Toxic. appl. Pharmac.* **27**, 99 (1973).
9. H. F. Vöhringer, L. Weller and N. Rietbrock, *Naunyn-Schmiedeberg's Archs. Pharmac.* **287**, 129 (1975).
10. A. Schmoldt, H. F. Benthe and G. Haberland, *Biochem. Pharmac.* **24**, 1639 (1975).
11. A. Schmoldt and C. Rohloff, *Naunyn-Schmiedeberg's Archs. Pharmac.* **305**, 167 (1978).
12. D. Satoh and K. Aoyama, *Chem. pharm. Bull. Tokyo* **18**, 94 (1970).
13. A. Schmoldt and C. Rohloff, *Arch. Pharm.* **311**, 833 (1978).
14. H. Kutt and J. R. Fouts, *J. Pharmac. exp. Ther.* **176**, 11 (1971).
15. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
16. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
17. H. Sheffe, *Biometrica* **40**, 87 (1953).
18. A. Schmoldt, *Naunyn-Schmiedeberg's Archs. Pharmac.* **306**, 261 (1979).
19. R. Teschke, K. Ohnishi, Y. Hasumura and Ch. S. Lieber, in *Microsomes and Drug Oxidations* (Eds. V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney), p. 103. Pergamon Press, Oxford (1977).
20. W. H. Orme-Johnson and D. M. Ziegler, *Biochem. Biophys. Res. Commun.* **21**, 78 (1965).
21. A. Hildebrandt, M. Speck and I. Roots, *Naunyn-Schmiedeberg's Archs. Pharmac.* **281**, 371 (1974).
22. J. L. Spratt, *Biochem. Pharmac.* **22**, 1669 (1973).
23. S. J. Stohs, L. A. Reinke and M. M. El Olemy, *Biochem. Pharmac.* **20**, 437 (1971).
24. R. E. Talcott and S. J. Stohs, *J. Pharmac. exp. Ther.* **184**, 419 (1973).