Biochemical Pharmacology. Vol. 25, pp. 2403–2406. Pergamon Press, 1976. Printed in Great Britain.

Differential effect of clofibrate on hepatic drug oxidation and cholesterol 7α-hydroxylation

(*Received* 18 *August* 1975; *accepted* 26 *March* 1976)

The rate-limiting step of cholesterol biosynthesis has been proposed to be the formation of mevalonic acid from 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), a reaction catalyzed by HMG-CoA reductase (EC 1.1.1.34) [1 3]. With regard to the regulation of hepatic cholesterol biosynthesis, a major hypocholesterolemic action of clofibrate (ethyl ester of α-*p*-chlorophenoxyisobutyrate) is related to an inhibition of the microsomal reduction of HMG-CoA to mevalonate [4]. In contrast to its inhibitory effects upon hepatic microsomal cholesterol biosynthesis, clofibrate stimulates many drug and steroid hydroxylation pathways mediated via the hepatic microsomal mixed function oxidase system [5–9].

Several investigators [5, 10–12] have demonstrated that clofibrate pretreatment results in elevations of liver weight along with a marked change in the liver cells of several species with the principal change being an increase in microbodies and proliferation of the smooth endoplasmic reticulum (SER). The changes in protein content and SER occur during the early stages of clofibrate treatment with the increase in protein synthesis induced by clofibrate preceding the elevation in liver weight [11, 12]. Few investigators have attempted to fully examine the influence of prior treatment with clofibrate on drug oxidations in liver microsomes. Cholesterol 7α-hydroxylase (EC 1.14) has also been shown to be a component of the cytochrome P-450-mediated hepatic microsomal mixed function oxidase system [13–17]. In this paper, we have investigated the effect of clofibrate pretreatment upon hepatic microsomal enzyme activities associated with drug oxidation and cholesterol 7α-hydroxylation.

Materials and Methods. Male, albino Wistar rats (Harlan Laboratories, Cumberland, Ind.), weighing 60–80 g, were fed Purina chow and received water *ad lib.* All animals were maintained in a vivarium at 25–26° on an alternating 12-hr light and dark cycle. Prior treatment of clofibrate (100 mg/kg) or an equal volume of corn oil was given, i.p., twice daily for 10 consecutive days. Injections were given in a volume of 0.1 ml/100 g body wt. Cholestyramine was given by mixing it with the diet (5%, w/w). [4-^{14}C]cholesterol (sp. act. 56 mCi/m-mole), [2-^{14}C]HMG-CoA (sp. act. 11.7 mCi/m-mole) and [5-^{3}H]mevalonic acid, DBED salt (sp. act. 6.74 Ci/m-mole) were obtained from New England Nuclear Corp., Boston, Mass. The reference compounds, 7α- and 7β-hydroxycholesterol, were prepared from the reduction of 7-ketocholesterol as described by Mosbach *et al.* [18]. Prior to use, the labeled cholesterol was purified by thin-layer chromatography using Silica gel G (Merck) developed in benzene–ethyl acetate (7:13). Radiochemical purity for HMG-CoA and cholesterol was >98 per cent. Mevalonic acid was used directly in these experiments.

Animals were sacrificed and their livers excised immediately, rinsed in ice-cold buffer, weighed and minced, and then the hepatic microsomal 9000 *g* supernatant fraction was prepared as described previously [8] except that the homogenizing buffer consisted of 0.1 M Tris–HCl buffer, pH 7.4, containing 1.15% (w/v) KCl and 1 mM EDTA. A portion of the 9000'*g* supernatant fraction was used for the cholesterol 7α-hydroxylase assay. The microsomal fraction was obtained by centrifuging the 9000 *g* supernatant at 105,000 *g* for 1 hr. The microsomal pellet was resuspended in 0.1 M Tris HCl buffer, pH 7.4, containing KCl 1.15% and spun at 105,000 *g* for 30 min. The final 105,000 *g* pellet was resuspended in the above buffer and kept on ice until use.

The method of Huber *et al.* [19] was utilized to measure the activity of HMG-CoA reductase in liver microsomes. Incubation mixtures contained 0.1 M Tris–HCl buffer, pH 7.2, with KCl 1.15%; 20 mM Na-EDTA; 0.1 M cysteine HCl, neutralized; an NADPH-generating system (10 mM glucose 6-phosphate, 1 mM NADP, 1 enzyme unit glucose 6-phosphate dehydrogenase, and 4 mM $MgCl_2$); 0.107 mM HMG-CoA[2-^{14}C], and 0.0625 mg of microsomal protein in a final volume of 0.2 ml. The incubation was carried out in air using a Dubnoff metabolic incubator at 37° with 100 oscillations/min for 1 hr. The reaction was terminated with methanol and H_2SO_4, while the addition of [5-^{3}H]mevalonic acid (50,000 dis./min) to each flask served as a measure of product recovery. Anion exchange resin (Bio-Rad 1 × 8, Cl form, 100–200 mesh) column eluates containing [^{3}H]- and [^{14}C]mevalonolactone were combined with a 3a40 scintillation mixture (RPI, Elk Grove, Ill.), and double isotope analysis using liquid scintillation spectrometry was applied to determine HMG-CoA reductase activity. Quenching was monitored by automatic external standardization. Counting efficiencies for ^{3}H and ^{14}C varied between 35 and 40 per cent and 84 and 88 per cent respectively.

Cholesterol 7α-hydroxylase activity was determined by adding [4-^{14}C]cholesterol (2.6 μM) in 25 μl of acetone to 2.0 ml of 9000 *g* supernatant, 30 mM nicotinamide, and fortified with 1 mM NADP, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 0.1 M Tris–HCl buffer, pH 7.4, with 1.15 per cent KCl in a final volume of 5.0 ml. The addition of nicotinamide was necessary to prevent NADP destruction during prolonged incubation. The incubation was carried out in a Dubnoff metabolic shaker in an atmosphere of air at 37°, 100 oscillations/min, for 1 hr. The reaction was terminated with and extracted with 7 vol. of chloroform–methanol (2:1, v/v). Further analysis of the chloroform–methanol extracts was performed according to the chromatographic method described previously [15].

Drug metabolism *in vitro* was determined in an incubation mixture consisting of 0.1 M Tris–HCl buffer, pH 7.4, an NADPH-generating system (5 mM $MgCl_2$, 0.044 mM NADP, 5.9 mM glucose 6-phosphate, and 2 enzyme units glucose 6-phosphate dehydrogenase), 5 mg microsomal protein and substrate, in a final volume of 3.0 ml for incubations with ethylmorphine and benzphetamine and 5.0 ml for aniline. After the addition of ethylmorphine (0.071 to 3.3 mM), benzphetamine (0.017 to 1.0 mM) or aniline (2.0 mM), the reaction mixtures were placed in a Dubnoff metabolic shaker and incubated in air at 37° with a shaking speed of 100 oscillations/min for times indicated in the tables. The method of Nash [20] was used to estimate the amount of formaldehyde formed from ethylmorphine

and benzphetamine. Conversion of aniline to *p*-aminophenol was assayed by the procedure described by Kato and Gillette [21].

For spectral binding studies, all samples were diluted to a final protein concentration of 2.0 mg/ml with 0.1 M Tris–HCl buffer, pH 7.4, containing 1.15% KCl. The magnitude of the spectral changes following consecutive additions of substrate was measured with an Aminco–Chance dual-wavelength splitbeam spectrophotometer (American Instrument Co., Silver Spring, Md.), as described by Schenkman *et al.* [22]. Two 3.0-ml aliquots of a microsomal suspension were placed in separate cuvettes, and a baseline was recorded at room temperature. The differences in the troughs relative to an isobestic point were measured after successive additions (1 μl) of ethylmorphine (0.033 to 0.2 mM, final concentration) or benzphetamine (8.3 to 70 μM, final concentration).

The assay for cytochrome P-450 was determined as described by Omura and Sato [23]. NADPH-cytochrome *c* reductase activity was measured by the method of Phillips and Langdon [24] and microsomal protein was estimated by the method of Lowry *et al.* [25] using crystalline bovine serum albumin as a standard. The kinetic and spectral binding constants were obtained by constructing regression lines from Lineweaver and Burk plots [26] which were analyzed statistically by the method of least squares to give the apparent K_m, the concentration of substrate which results in half the maximal activity; (V_{max}); and the apparent spectral dissociation constants (K_s) and the absorbance maxima (A_{max}). Where appropriate, statistical comparisons of independent sample means were made using the Student *t*-test.

Results. In agreement with preliminary studies carried out in our laboratory [8] in immature male rats, chronic clofibrate administration did not exhibit changes in liver weights and liver/body weight ratios, but revealed a 28 per cent elevation in microsomal protein (Table 1). Thus, clofibrate pretreatment in immature rats appears to produce increases in microsomal protein content, while not altering total liver weight. In addition to changes in microsomal protein, treatment with clofibrate resulted in significant alterations of cytochrome P-450 content and NADPH-cytochrome *c* reductase activity (Table 1). The hemoprotein P-450 levels were elevated 37 per cent over control values, while NADPH-cytochrome *c* reductase activity was increased by 39 per cent.

Since it has previously been shown by several investigators [5–8] that clofibrate administration results in an elevation of microsomal drug oxidations, it was of interest to determine whether this increase was due to quantitative and/or qualitative changes in the enzyme system. An examination of these changes in hepatic microsomal enzyme(s) activity is also presented in Table 1. The V_{max} values obtained for ethylmorphine and benzphetamine *N*-demethylase were increased significantly by 55 and 70 per cent, respectively, by clofibrate pretreatment, whereas drug treatment did not modify the apparent K_m for either enzyme. Furthermore, clofibrate pretreatment was unable to alter aniline hydroxylase activity in liver microsomes.

To further delineate the phenomenon of microsomal induction due to prior treatment with clofibrate, substrate binding to the microsomal hemoprotein P-450 was investigated. In accordance with the results obtained from the apparent K_m data, there was no alteration in apparent K_s due to clofibrate administration. However, clofibrate resulted in a 23 per cent significant increase in the A_{max} for the binding of ethylmorphine, whereas a non-significant increase (12 per cent) in the A_{max} of benzphetamine was observed. Since clofibrate is able to differentially affect hepatic microsomal enzymes, i.e. inhibit HMG-CoA reductase while inducing drug and steroid hydroxylation reactions, we tested whether clofibrate could alter cytochrome P-450-mediated cholesterol 7α-hydroxylase. Employing the 9000 *g* supernatant fraction resulted in measurable quantities of 7α-hydroxycholesterol, while peroxidation products (i.e. 7β-hydroxycholesterol and 7-ketocholesterol) of cho-

Table 1. Effect of clofibrate pretreatment on various parameters of hepatic drug metabolism in male rats*

Parameter	Treatment†		% of Control
	Control	Clofibrate	
Liver wt (g)	6.88 ± 0.84	6.74 ± 1.05	98
Liver/body wt (%)	4.77 ± 0.36	4.92 ± 0.51	103
Microsomal protein (mg/g liver)	31.50 ± 1.73	40.25 ± 0.56	128‡
Cytochrome P-450 (nmoles/mg protein)	0.574 ± 0.096	0.784 ± 0.050	137‡
NADPH-cytochrome *c* reductase (nmoles/mg protein/min)	42.7 ± 12.5	59.4 ± 7.4	139‡
Drug substrates			
Ethylmorphine *N*-demethylase			
V_{max} (nmoles/mg/20 min)	75.9 ± 17.6	117.6 ± 20.0	155‡
K_m (mM)	0.670 ± 0.231	0.740 ± 0.122	110
Benzphetamine *N*-demethylase			
V_{max} (nmoles/mg/15 min)	11.6 ± 1.7	19.7 ± 4.9	170‡
K_m (mM)	0.104 ± 0.024	0.098 ± 0.004	94
Aniline hydroxylase			
V_{max} (nmoles/mg/20 min)	20.73 ± 3.10	25.90 ± 5.13	125
Spectral binding studies			
Ethylmorphine			
A_{max} (ΔO.D.$_{403-420}$/mg protein)§	0.0021 ± 0.0003	0.0026 ± 0.0002	123‡
K_s (mM)	0.050 ± 0.029	0.048 ± 0.005	96
Benzphetamine			
A_{max} (ΔO.D.$_{407-421}$/mg protein)§	0.0032 ± 0.0005	0.0036 ± 0.0004	112
K_s (mM)	0.0106 ± 0.0028	0.0104 ± 0.0019	98

* Values are expressed as the mean ± S. D. of six rats for each treatment group.
† Animals received clofibrate (100 mg/kg), or corn oil, i.p., twice daily for 10 days.
‡ Significant difference ($P < 0.05$) from corresponding control group.
§ The ΔO.D. were obtained by the difference in the isobestic point relative to the trough (λ_{min}) for each substrate.

lesterol were virtually absent in these preparations. When the dried chloroform extracts of the 9000 *g* supernatant fraction were spotted on Silica gel G plates and chromatographed in benzene–ethyl acetate (7:13) using a saturated chamber, 7α-hydroxycholesterol and cholesterol migrated with R_f values of 0.34 and 0.77, respectively, while standards of 7β-hydroxycholesterol and 7-ketocholesterol resulted in R_f values of 0.42 and 0.64. Moreover, 7α-hydroxycholesterol can be resolved from 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol using ethyl acetate–cyclohexane (3:2) on Silica gel G plates (R_f = 0.22, 0.28, 0.32 and 0.58 respectively). As presented in Fig. 1A, microsomes obtained from clofibrate-pretreated animals showed a significant reduction (35 per cent) in the amount of 7α-hydroxycholesterol formed. By comparison, disrupting the enteroheptic circulation of bile salts by administration of cholestyramine resulted in a 254 per cent increase in hepatic microsomal cholesterol 7α-hydroxylase activity.

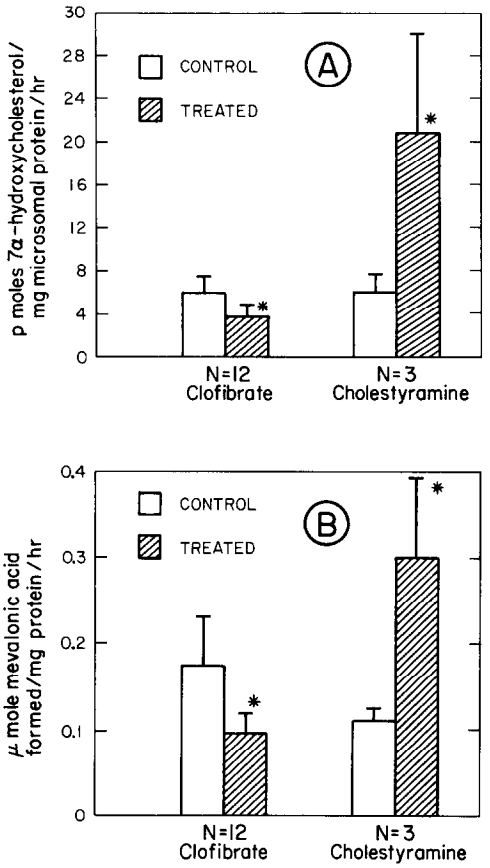

Fig. 1. Effect of clofibrate (100 mg/kg, twice daily, for 10 days) and cholestyramine (5% in diet for 5 days) pretreatment on hepatic microsomal HMG-CoA reductase and cholesterol 7α-hydroxylase activities in immature male rats. (A) Formation of 7α-hydroxycholesterol from [4-^{14}C]cholesterol by the 9000 *g* supernatant fraction of rat liver after various drug pretreatments. The conversions were calculated from the amounts of radioactivity present in each zone of the thin-layer chromatogram, based on the amount of microsomal protein in each incubation. (B) Effect of drug pretreatment on HMG-CoA-reductase activity. Mean values ± S. D. are presented for each treatment group. The asterisk indicates a significant difference ($P < 0.05$) between the treatment and control groups.

The ability of clofibrate to inhibit the formation of mevalonic acid from HMG-CoA reductase activity is well established [4]. In contrast, cholestyramine fed in the diet (5 per cent) for 5 days stimulated the formation of mevalonic acid by 169 per cent (Fig. 1B). Thus, in immature rats, clofibrate remains effective in reducing HMG-CoA reductase activity significantly, while the anion exchange resin cholestyramine increases the formation of mevalonic acid.

Discussion. Clofibrate has been reported to cause increases in liver weight and microsomal protein in rats [5, 10–12], albeit these changes have been observed to be dependent upon the age of the animals [8]. In this present study, liver weight and liver/body weight ratios did not change with clofibrate pretreatment, while microsomal protein content was increased significantly (Table 1). These findings are consistent with those of Lewis *et al.* [8]. It has been established that elevations in microsomal hepatic protein are associated with proliferation of the smooth endoplasmic reticulum and that these alterations occur during the early stages of clofibrate treatment with the increase in protein synthesis preceding the elevation in liver weight [12].

It has been well established that numerous compounds when administered to animals result in the induction of the hepatic microsomal mixed function oxidase system which mediates both drug and steroid oxidations [27]. Clofibrate administration has been reported to selectively elevate the activities of microsomal enzymes for the metabolism of several drug substrates [5–8], testosterone [6], and 4-androstene-3,17-dione [9]. Although clofibrate-pretreated mature and immature rats possess the ability to metabolize various substrates [5–9], the elevation of protein synthesis and hepatic drug oxidations without concomitant hepatomegaly occurs only in immature animals [8]. Thus, the present experiments utilize immature rats to enable correlation of various hepatic microsomal enzyme activities with the initial stage of protein synthesis and without the associated enlargement of liver cells due to clofibrate treatment as described by Kaneko *et al.* [5]. It is noteworthy that the changes in various microsomal enzymes associated with clofibrate pretreatment [5, 8] were also accompanied by an elevation in cytochrome P-450, a component of the electron transport system usually coexistent with changes in hepatic drug oxidations. The increase in cytochrome P-450 content that can be seen in Table 1 serves to substantiate these earlier findings. Moreover, an increase in NADPH-cytochrome *c* reductase activity, usually associated with a concomitant elevation in the reduction of cytochrome P-450, was observed which further reflects changes in the electron transport system induced by clofibrate administration. It is also of interest to note that several analogs of clofibrate cause stimulation of hepatic drug metabolism with concomitant alterations in cytochrome P-450 levels [28].

The results given in Table 1 also demonstrate that the increased hepatic metabolism of ethylmorphine and benzphetamine is dependent upon a quantitative rather than a qualitative change in the nature of the microsomal enzyme system. Prior treatment with clofibrate did not alter the apparent K_m for ethylmorphine or benzphetamine *N*-demethylase, whereas total metabolism was increased by 55 and 70 per cent respectively. However, the hydroxylation of the type II substrate aniline was not altered by clofibrate administration. This differential inductive effect displaced by clofibrate on a limited series of type I and II substrates has also been reported by other workers [7, 8]. In concurrence with the increases of maximal rates of type I substrate metabolism, clofibrate administration produced an increase in the ΔA_{max} values for the binding of ethylmorphine (23 per cent) and benzphetamine (12 per cent) to microsomal proteins. In comparison, the apparent K_s for both substrates was not changed by pretreatment

with clofibrate. It is of interest to note that the magnitude of the maximal binding spectral change for both ethylmorphine and benzphetamine was smaller than the maximal rate of metabolism. Thus, the increased hemoprotein concentration in liver microsomes induced by clofibrate is apparently not the only factor responsible for enhancement of substrate metabolism. Consequently, other elements such as the increase in NADPH-cytochrome P-450 reductase may play an integral role in the greater metabolism that occurred in clofibrate-treated rats.

Several of the enzymes necessary for cholesterol biosynthesis and degradation are found in the hepatic endoplasmic reticulum. The rate-limiting step in this biosynthetic sequence is HMG-CoA reductase [2, 3], while the 7α-hydroxylation of cholesterol is the major rate-limiting step in the overall conversion of cholesterol to bile acids [29]. A number of investigators [13, 17, 30–32] have indicated the participation of the hemoprotein P-450 in various steps of cholesterol biosynethesis. Moreover, the hypolipidemic agent clofibrate has been shown to stimulate drug oxidations in liver microsomes [5–8], while also inhibiting the formation of mevalonic acid via HMG-CoA reductase [4]. Our results using immature rats in this study corroborate earlier findings on the ability of clofibrate to significantly reduce HMG-CoA reductase activity (Fig. 1B). Furthermore, the administration of the bile acid sequestering agent cholestyramine resulted in a 1.69-fold increase in HMG-CoA reductase activity via the removal of the feedback inhibitory action of cholesterol on HMG-CoA reductase [1, 2], thus enhancing both cholesterol biosynthesis and 7α-hydroxycholesterol production (Fig. 1). The 7α-hydroxylation of cholesterol is catalyzed by the microsomal fraction of liver homogenates and involves the participation of cytochrome P-450 and of NADPH-cytochrome P-450 reductase as well as requiring NADPH and molecular oxygen [16, 33]. Furthermore, the formation of bile acids in the liver is regulated by homeostatic mechanisms whereby an interruption of the enterohepatic circulation results in a several-fold increase in bile acid synthesis. When the anionic exchange resin cholestyramine (5%) was fed to rats for 5 days, a 254 per cent increase in cholesterol 7α-hydroxylase activity ensued (Fig. 1A). In contrast to the results of Einarsson *et al.* [9], prior treatment with clofibrate resulted in a decrease in cholesterol 7α-hydroxylase activity. Therefore, our results have shown that clofibrate administration caused an induction of the hepatic microsomal drug-metabolizing enzyme(s) involved with the *N*-demethylation of ethylmorphine and benzphetamine and produced an apparent inhibition of cholesterol 7α-hydroxylase.

The evidence for the similarity of the mixed function oxidase system which participates in drug oxidations and that which catalyzes the hydroxylation of cholesterol is somewhat equivocal. Studies are currently being conducted in our laboratory to investigate the action of clofibrate on cholesterol 7α-hydroxylase by determination of the mass of 7α-hydroxycholesterol and to further elucidate the interrelationship of drug and steroid hydroxylations with that of cholesterol 7α-hydroxylase.

Acknowledgement—W. S. M. gratefully acknowledges support of Medicinal Chemistry Training Grant GM 1949 from the National Institutes of Health. The present work was supported by a grant from the National Institutes of Health (HL 12740) from the U.S. Public Health Service. Several drugs were generously supplied by the following companies: clofibrate (Atromid S), Ayerst; benzphetamine (Didrex), Upjohn; ethylmorphine (Dionin), Merck; and cholestyramine (Questran), Mead Johnson & Co.

Divisions of Pharmacology and Medicinal Chemistry,
College of Pharmacy,
The Ohio State University,
Columbus, Ohio 43210, *U.S.A.*

WILLIAM S. MELLON
ALAN P. GOLDBERG
DONALD T. WITIAK
DENNIS R. FELLER

REFERENCES

1. N. L. R. Bucher, P. Overath and F. Lynen, *Biochim. biophys. Acta* **40**, 491 (1960).
2. M. D. Siperstein and V. M. Fagan, *J. biol. Chem.* **241**, 602 (1966).
3. L. H. White and H. Rudney, *Biochemistry* **9**, 2725 (1970).
4. L. H. White, *J. Pharmac. exp. Ther.* **178**, 361 (1971).
5. A. Kaneko, S. Sakamoto, M. Morita and T. Onoe, *Tohuku J. exp. Med.* **99**, 81 (1969).
6. R. A. Salvador, S. Haber, C. Atkins, B. W. Gommi and R. M. Welch, *Life Sci.* **9**, 397 (1970).
7. D. L. Azarnoff, *Fedn Proc.* **30**, 827 (1971).
8. N. J. Lewis, D. T. Witiak and D. R. Feller, *Proc. Soc. exp. Biol. Med.* **145**, 281 (1974).
9. K. Einarsson, J. Gustafsson and K. Hellström, *Biochem. Pharmac.* **23**, 13 (1974).
10. M. M. Best and C. H. Duncan, *J. Lab. clin. Med.* **64**, 634 (1964).
11. R. Hess, W. Stäubli and W. Riess, *Nature, Lond.* **208**, 856 (1965).
12. D. S. Platt and J. M. Thorp, *Biochem. Pharmac.* **15**, 915 (1966).
13. F. Wada, K. Hirata, H. Shibata, K. Higashi and Y. Sakamoto, *J. Biochem., Tokyo* **62**, 134 (1967).
14. F. Wada, K. Hirata, K. Nakao and Y. Sakamoto, *J. Biochem., Tokyo* **66**, 699 (1969).
15. J. R. Mitton, N. A. Scholan and G. S. Boyd, *Eur. J. Biochem.* **20**, 569 (1971).
16. G. S. Boyd, A. M. Grimwade and M. E. Lawson, *Eur. J. Biochem.* **37**, 334 (1973).
17. S. D. Atkin, E. D. Palmer, P. D. English, B. Morgan, M. A. Cawthorne and J. Green, *Biochem. J.* **128**, 237 (1972).
18. E. H. Mosbach, M. A. Rothschild, I. Bekersky, M. Oratz and J. Mongelli, *J. clin. Invest.* **50**, 1720 (1971).
19. J. Huber, S. Latzin and B. Hamprecht, *Hoppe-Seyler's Z. physiol. Chem.* **354**, 1645 (1973).
20. T. Nash, *Biochem. J.* **55**, 415 (1953).
21. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150**, 279 (1965).
22. J. B. Schenkman, I. Frey, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 516 (1967).
23. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
24. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
25. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
26. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
27. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
28. N. J. Lewis, D. R. Feller, G. K. Poochikian and D. T. Witiak, *J. med. Chem.* **17**, 41 (1974).
29. H. Danielsson, K. Einarsson and G. Johansson, *Eur. J. Biochem.* **2**, 44 (1967).
30. R. Kato, K. Onoda and Y. Omori, *Jap. J. Pharmac.* **18**, 514 (1968).
31. F. Wada, K. Hirata and Y. Sakamoto, *J. Biochem., Tokyo* **65**, 171 (1969).
32. G. F. Gibbons and K. A. Mitropoulos, *Eur. J. Biochem.* **40**, 267 (1973).
33. I. Björkhem, H. Danielsson and K. Wikvall, *Biochem. biophys. Res. Commun.* **61**, 934 (1974).