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SHORT COMMUNICATIONS

Increase in the hepatic glucuronidation and clearance of bilirubin in clofibrate-treated rats*

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Clofibrate (ethyl-4-chlorophenoxyisobutyrate) is widely used as an hypolipidemic drug. It effectively lowers the levels of serum cholesterol, triglycerides and free fatty acids in man and experimental animals [1]. Nevertheless the mechanisms of this action have not yet been clearly elucidated since the effects of the drug vary and seem to act upon many sites. In other respects, lipid metabolism is not the only one to be concerned and it has been shown that other metabolisms are modified after administration of clofibrate, such as those of steroid hormones [2, 3]. Its chronic administration to rats produces an elevation in liver weight [4–8] and a proliferation of the smooth endoplasmic reticulum of liver [5]. This very-like inducer property of clofibrate was confirmed by Lewis *et al.* [9] who reported an acceleration of ethylmorphine and aminopyrine metabolisms, accompanied by an elevation of Cytochrome *P*-450 in rat liver microsomes. The present paper reports the action of clofibrate on bilirubin-UDP-glucuronyltransferase (BGT) activity paralleling the increase in *Z* hepatic content.

Male Sprague–Dawley rats (Charles River France), aged 6 weeks, fed with a controlled diet were given clofibrate (I.C.I., France) (6.25–25 mg/100 g B.W. in propyleneglycol) i.p. once daily. Controls received propyleneglycol by the same route for the same time. Animals were killed under light ether anesthesia 24 hr after the last drug dose. The liver was removed, washed immediately and weighed. A small lobe was taken to measure total protein content and BGT activity. The remaining liver was perfused through hepatic veins with 50 ml of ice-cold isotonic saline. A 25% homogenate was prepared in 0.25 M sucrose-0.01 M phosphate buffer (pH 7.4), and supernatant fractions (cytosol) harvested after centrifugation at 105,000 *g* for 120 min in a Beckman L2-65B ultracentrifuge (Beckman Instruments, France). Specimens were stored at −18° if not used immediately. Protein concentration in total liver, supernatant fractions and fractions *Y* and *Z* was determined by the method of Lowry *et al.* [10]. *Y* (ligandin) and *Z* protein concentrations were estimated by bromsulphthalein (BSP) binding after standardized chromatography of the 105,000 *g* supernatant on Sephadex G-75 according to the method described by Levi *et al.* [11] as previously reported [12]. Liver homogenate BGT activity was measured according to the method of Black *et al.* [13]. To evaluate the effect of clofibrate *in vivo* on the hepatic transport of anions, we studied the following parameters: (1) the initial disappearance rate from plasma of a single intravenous dose of BSP (5 mg/100 g B.W.), expressed as the first order rate constant, K_1, was determined from four carefully timed plasma samples obtained from 2 to 8 min after dye administration. (2) the plasma bilirubin disappearance was determined after a single intravenous injection of unconjugated bilirubin (1 mg/100 g B.W. solubilized in Na_2CO_3 0.1 M). Blood samples were collected at 2.5, 5, 7.5 and 10 min after bilirubin administration. Bilirubin content in the plasma samples was determined by the method of Jendrassik and Grof [14]. Rats pretreated with clofibrate (18.8 mg/100 g B.W.) once daily for 10 days, along with the corresponding controls were tested in this manner. Student's *t* test was used for statistical analysis of results.

Administration of clofibrate for 10 days significantly increased total lever weight and total liver protein content. There was no difference in body weight between the control and treated animals. Table 1 shows the effect of clofibrate treatment on the cytosolic proteins *Y* and *Z* and on BGT activity of rat liver. *In vitro* BSP binding to protein *Y* was not modified with clofibrate treatment whilst it was dramatically increased for protein *Z* (180 per cent of the controls). Protein content in fractions *Z* was higher in treated rats than in controls. *In vitro* glucuronidation of bilirubin in rat liver homogenates was also stimulated, it increased 200 per cent above control values. This effect of clofibrate on both parameters was shown to increase with dose (Fig. 1); it appeared in a few days after the beginning of the treatment. Clofibrate administration to rats for 10 days failed to increase BSP K_1 whatsoever the dose used. Figure 2 illustrates the results of plasma bilirubin disappearance studies: the clofibrate-treated animals exhibited significantly lower bilirubin levels at all four

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Table 1. Effect of clofibrate treatment on bilirubin-glucuronyltransferase activity and bromosulfophthalein binding to *Y* and *Z* proteins in rat liver

	Liver weight (% body wt)	Total liver protein (mg/g)	BGT (mgCB/g liver/hr)	BSP binding (nm/g liver)	
				Y	*Z*
Control	3.66 ± 0.13	167.6 ± 4.4	1.33 ± 0.10	101 ± 4	68 ± 4
Clofibrate treated	6.53 ± 0.13	195.2 ± 3.7	3.96 ± 0.22	95 ± 5	188 ± 10
P	<0.001	<0.001	<0.001	N.S.	<0.001

Values represent the mean ± S.E.M. for 6 animals. Clofibrate was given i.p. (25 mg/100 g B.W.) once daily for 10 days. N.S.: not significant.

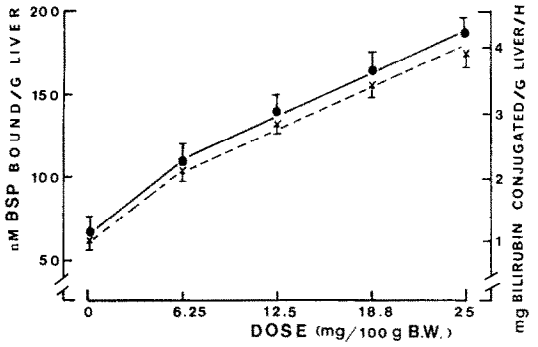

Fig. 1. Dose–response study of effect of clofibrate on *in vitro* BSP binding to protein Z (●——●) and BGT activity (×——×) of rat liver. The rats were given injections i.p. of clofibrate or an equivalent volume of propylene-glycol for 10 days. The data are expressed as the mean ± S.E.M. of 6 animals in each group.

times periods studied. Clofibrate, therefore, significantly enhanced the disappearance of exogenously administered bilirubin from the plasma.

Our results confirm the increase in liver weight and protein content produced by clofibrate administration as previously reported by several authors [4–7]. Since no rise in hepatic DNA content was shown [7, 15] this increase in liver weight is attributed to an hypertrophy rather than an hyperplasy. The import of this hypertrophy is not clear. Whether it is an increase in hepatic function or a sign of toxicity remains an open question as the same effect has been noted with numerous compounds so different as phenobarbitone, DDT and CCl_4 [16].

Our experiments confirm that clofibrate administration increases Z protein [17–19] as determined by BSP binding. This enhanced capacity of Z to bind the dye is accompanied by an increase in protein amount. Y protein is unaffected by the treatment. In the same way, the hepatic level of protein Y or ligandin is increased by numerous drugs [20, 21] without change in the concentration of Z protein. BSP along with many other organic anions have been shown to be bound to cytosolic proteins Y and Z in the liver [11, 20]. These proteins are thought to faciliatate both the uptake and the storage of organic agnions by the liver. This increase of Z protein by clofibrate could result in an enhanced uptake of BSP as affected by BSP binding to Z. Yet the BSP uptake as indirectly measured by BSP K_1 determination is not modified. In contrast, the data presented in Fig. 2 indicate that clofibrate stimulation of BGT activity is associated with enhanced disappearance of exogenously administered bilirubin from plasma.

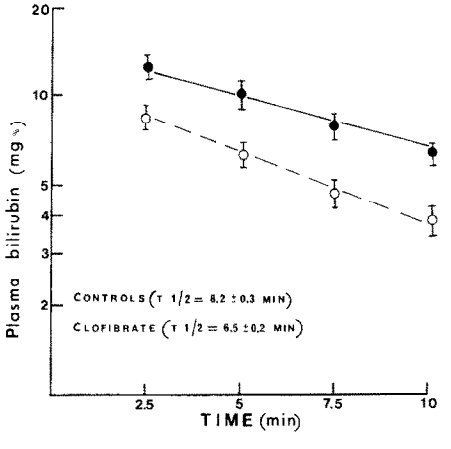

Fig. 2. Disappearance of an exogenous bilirubin load (1 mg/100 g B.W., i.v.) from plasma of clofibrate-pretreated rats (18.8 mg/100 g B.W., i.p. once daily for 20 days prior to bilirubin administration). Values represent the mean ± S.E.M. for 10 animals. The 2.5, 5, 7.5 and 10 min plasma bilirubin levels were significantly lower in the clofibrate-pretreated animals (○) than in the controls (●) ($P < 0.001$).

These differences observed in bilirubin and BSP hepatic uptake under the influence of clofibrate treatment seem to privilege the role of ligandin in this process. Phenobarbitone which increases liver ligandin and BGT activity enhances both the disappearance of bilirubin [22–25] and BSP [26] from plasma whilst clofibrate seems to affect only bilirubin. Recently, similarities have been noted in molecular weight and binding properties between one of the glutathione-S-transferases, S-aryltransferase B which is responsible of the conjugation of BSP, and ligandin [27, 28]. So the difference of effect observed with clofibrate on bilirubin and BSP uptake by the liver could be explained by a stimulated or not conjugating enzyme activity.

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REFERENCES

1. A. M. Barrett and J. M. Thorp, *Br. J. Pharmac. Chemother.* **32,** 381, (1968).
2. K. Einarsson, J. Gustafsson and K. Hellström, *Biochem. J.* **136,** 623 (1973).
3. R. A. Salvador, S. Haber, C. Atkins, B. W. Gommi and R. M. Welch, *Life Sci.* **9,** 397 (1970).
4. M. M. Best and C. H. Duncan, *J. Lab. clin. Med.* **64,** 634 (1964).
5. R. Hess, W. Staubli and W. Reiss, *Nature, Lond.* **208,** 856 (1965).
6. D. S. Platt and J. M. Thorp, *Biochem. Pharmac.* **15,** 915 (1966).
7. R. B. Beckett, R. Weiss, R. E. Stitzel and R. J. Genedella, *Toxic. Appl. Pharmac.* **23,** 42 (1972).
8. A. Hruban, M. Gotoh, A. Slesers and S. F. Chou, *Lab. Invest.* **30,** 64 (1974).
9. N. J. Lewis, D. T. Witiak and D. R. Feller, *Proc. Soc. exp. Biol. Med.* **145,** 281 (1974).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
11. A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **48,** 2156 (1969).
12. A. Foliot, E. Housset, J. P. Ploussard, J. P. Petite and R. Infante, *Biomedical,* **19,** 488 (1973).
13. M. Black, B. H. Billing and K. P. M. Heirwegh, *Clinica. chim. Acta* **29,** 27 (1970).
14. L. Jendrassik and P. Grof, *Harn. Biochem. J.* **296,** 71 (1938).
15. C. Dalton, W. C. Hope, H. R. Hope, and H. Sheppard, *Biochem. Pharmac.* **23,** 685 (1974).
16. D. S. Platt and B. L. Cockrill, *Biochem. Pharmac.* **16,** 2257 (1967).
17. A. Foliot, B. Christoforov, E. Housset, J. P. Petite, J. P. Ploussard and J. P. Etienne, *Biol. Gastroenterol.* **7,** 332 (1974).
18. A. Foliot, E. Housset, J. N. Fiessinger, B. Christoforov and J. P. Petite, *Digestion* **12,** 256 (1975).

19. G. Fleischner, D. K. F. Meijer, W. G. Levine, Z. Gatmaitan, R. Gluck and I. M. Arias, *Biochem. biophys. Res. Commun.* **67**, 1401 (1975).
20. H. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *J. clin. Invest.* **50**, 2242 (1971).
21. A. Foliot, Ph. D. Thesis, Paris (1975).
22. R. J. Roberts and G. L. Plaa, *Biochem. Pharmac.* **16**, 827 (1967).
23. D. R. Davis and R. A. Yeary, *Proc. Soc. exp. Biol. Med.* **148**, 9 (1975).
24. B. Christoforov, R. Deraedt, A. Foliot and J. P. Petite, *Path. Biol.* **18**, 663 (1970).
25. S. H. Robinson, C. Yannoni and S. Nagasawa, *J. clin. Invest.* **50**, 2606 (1971).
26. H. Reyes, A. J. Levi, Z. Gatmaitan and I. M. Arias, *Proc. natn. Acad. Sci. U.S.A.* **64**, 168 (1969).
27. N. Kaplowitz, I. W. Perey-Robb and N. B. Javitt, *J. exp. Med.* **138**, 483 (1973).
28. M. J. Pabst, W. H. Habig and W. B. Jacoby, *Biochem. biophys. Res. Commun.* **52**, 1123 (1973).

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A kinetic method for the determination of the multiple forms of microsomal cytochrome P-450

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The identification and characterization of the multiple forms of cytochrome P-450 associated with liver microsomes are currently being extensively studied [1–8]. Such studies may lead to the categorization of the various enzymes involved into activity profiles which in turn would provide information as to their relative roles as detoxifying catalysts or as catalysts which generate toxic electrophilic intermediates.

The study of the multiple forms of cytochrome P-450 has been approached by two main lines of investigation. The first and most direct has been biochemical investigations which have focused on the physical isolation, purification and characterization of both normal and inducible forms [9–13]. The second method has approached the problem indirectly by measuring changes produced in the enzymatic profiles of the system by some perturbation, typically the use of inducing agents such as phenobarbital (PB) and 3-methylcholanthrene (3-MC) and inhibitors such as carbon monoxide or SKF-525A [14–20]. In interpreting such changes in terms of multiplicity, most investigators have operated under the same set of assumptions, but to our knowledge neither the assumptions nor the rules for their applications have been explicitly stated.

The purpose of this communication is to state the assumptions initially for a system involving a single substrate, a single enzymatic site and multiple products. Given these assumptions, a systematic framework for the interpretation of changes in microsomal enzymatic profiles of such a system brought about by various perturbations to the system will be proposed. In developing the framework, we assume that product formation is irreversible and that the steady state kinetics of Briggs and Haldane [21] are applicable.

For a single substrate, single enzymatic site, multiple product system, the following two cases are possible.

Case 1. The substrate combines with an enzyme to form a single enzyme–substrate complex which dissociates to multiple products, that is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \begin{cases} \xrightarrow{k_2} P_1 + E \\ \xrightarrow{k_3} P_2 + E \\ \xrightarrow{k_4} P_3 + E \end{cases}$$

Deriving the expression for $E\cdot S$ by the method of King and Altman [22] as described by Segel [23] and rearranging to the form of the Michaelis–Menten equation yields

$$E\cdot S = \frac{(E_0)(S)}{\dfrac{k_{-1} + k_2 + k_3 + k_4 + S}{k_1}}$$

Therefore, a single K_m

$$K_m = \frac{k_{-1} + k_2 + k_3 + k_4}{k_1}$$

characterizes the entire system and is independent of either enzyme or substrate concentration. Since the individual velocities for each of the products are $dP_1/dt = k_2(E\cdot S)$, $dP_2/dt = k_3(E\cdot S)$ and $dP_3/dt = k_4(E\cdot S)$, the ratio of any two will be constant and independent of either the concentration of E or S.

Case 2. The substrate combines with an enzyme to form energetically distinct $E\cdot S$ complexes each of which dissociates to a different product, that is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_4} P_1$$

$$\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot S' \xrightarrow{k_5} P_2$$

$$\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot S'' \xrightarrow{k_6} P_3$$

Once again, deriving the expressions for $E\cdot S$, $E\cdot S'$ and $E\cdot S''$ by the method of King and Altman [22] and rearranging in the form of the Michaelis–Menten equation yield three equations:

$$E\cdot S = \frac{\dfrac{k_1(k_{-2} + k_5)(k_{-3} + k_6)[E_0][S]}{R}}{K_m + S}$$

$$E\cdot S' = \frac{\dfrac{k_2(k_{-1} + k_4)(k_{-3} + k_6)[E_0][S]}{R}}{K_m + S}$$

$$E\cdot S'' = \frac{\dfrac{k_3(k_{-1} + k_4)(k_{-2} + k_5)[E_0][S]}{R}}{K_m + S}$$

Where

$$R = k_1(k_{-2} + k_5)(k_{-3} + k_6) + k_2(k_{-1} + k_4)(k_{-3} + k_6) + k_3(k_{-1} + k_4)(k_{-2} + k_5)$$

and

$$K_m = \frac{(k_{-1} + k_4)(k_{-2} + k_5)(k_{-3} + k_6)}{R}$$