The Role of Cytochrome *P*-450 in Cholesterol Biogenesis and Catabolism

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1. Adjuvant-induced arthritis in rats is accompanied by a loss of activity of the drug-metabolizing enzyme system and a decrease in hepatic cytochrome *P*-450. 2. Arthritic rats have normal serum and liver cholesterol concentrations. 3. The rate of biogenesis of cholesterol *in vivo* and *in vitro* from either [¹⁴C]acetate or [¹⁴C]mevalonate in arthritic rats was the same as or greater than that found in control rats. 4. Treatment of rats with carbon disulphide (1 ml/kg) resulted in a loss of drug-metabolizing-enzyme activity and increased cholesterol biogenesis. 5. The activity of cholesterol 7 α -hydroxylase in adjuvant-induced arthritic rats did not differ significantly from that in control rats. 6. Rats fed with cholestyramine had an elevated hepatic cholesterol 7 α -hydroxylase activity, but neither the concentration of cytochrome *P*-450 nor the activity of the drug-hydroxylating enzyme, aminopyrine demethylase, was affected. 7. The relationships between drug hydroxylation and cholesterol metabolism are discussed.

Cytochrome *P*-450, a microsomal haemoprotein, is reduced by NADPH-cytochrome *c* reductase (EC 1.6.2.3) in the presence of NADPH (Orrenius *et al.*, 1964). The reduced form of cytochrome *P*-450 reacts with molecular O₂ and is also capable of binding to CO (Klingenberg, 1958). Cytochrome *P*-450 functions in the activation of O₂ for the hydroxylation of drugs in the liver and steroids in the adrenals (Conney, 1967).

Tchen & Bloch (1957) have shown that one of the enzyme systems involved in cholesterol biosynthesis, namely that which converts squalene into lanosterol, requires O₂ and NADPH. Also, a number of authors have noted that inhibitors of cholesterol biosynthesis such as SKF 525-A (β -diethylaminoethyl-diphenyl-propylacetate hydrochloride), triparanol, benzmalacene and AY 9944 [*trans*-1,4-bis(2-chlorobenzyl-aminoethyl)cyclohexanediolhydrochloride] are also inhibitors of drug metabolism (Kato *et al.*, 1963; Marton & Chappel, 1964). F. Wada *et al.* (1969*a*) found that cholesterol biogenesis from [¹⁴C]acetate, [¹⁴C]mevalonate or [¹⁴C]squalene was inhibited by CO and they therefore suggested that cytochrome *P*-450 is functional in cholesterol synthesis. F. Wada *et al.* (1969*a*) also showed, however, that the inhibitory effect of CO on cholesterol synthesis was smaller than the inhibition of drug hydroxylation.

The formation of 7 α -hydroxycholesterol is thought to be the primary rate-limiting event in the conversion of cholesterol into bile acids. F. Wada *et al.* (1969*b*) showed that the 7 α -hydroxylation was inhibited by CO. Boyd *et al.* (1971) demonstrated that the inhibition by CO could be reversed by monochromatic light at 450 nm; however, they found that cholesterol 7 α -hydroxylase activity was not increased

by pretreatment of the rat with phenobarbitone, a known inducer of the drug-metabolizing-enzyme system.

The evidence for the involvement of cytochrome *P*-450 in cholesterol metabolism is clearly contradictory. The problem might be resolved by measuring the rate of cholesterol biogenesis and catabolism in rats experimentally depleted of cytochrome *P*-450. Morton & Chatfield (1970) found that the livers of rats with adjuvant-induced polyarthritis (Pearson, 1956) contained little cytochrome *P*-450 and had low drug-metabolizing-enzyme activity. The present paper concerns a study of cholesterol metabolism in arthritic rats and in normal rats in which some of the hepatic cytochrome *P*-450 had been destroyed by treatment of the rat with carbon disulphide (Bond & De Matteis, 1969).

Materials and Methods

Animals

Male CFHB rats were obtained from Carworth (Europe) Ltd., Alconbury, Hunts, U.K. The rats were fed a stock pelleted diet (Diet FFG; E. Dixon & Sons, Ware, Herts, U.K.) unless otherwise stated.

Adjuvant-induced arthritis was produced by a single subcutaneous injection of 0.50 mg of *Mycobacterium butyricum* (Difco Laboratories, Detroit, Mich., U.S.A.), suspended in 0.1 ml of liquid paraffin, into the foot pad of the right-hand paw (plantar). Secondary arthritic lesions occurred after a delay of approx. 10 days. Control rats were given an injection of liquid paraffin only.

A single oral dose of carbon disulphide (1 ml/kg body wt.) was administered as a 50% (v/v) solution in

arachis oil. Control rats received arachis oil only (2ml/kg body wt.).

Rats were killed by cervical dislocation and blood was obtained from the jugular vein.

Methods

Liver cholesterol synthesis in vitro. Liver slices, approx. 0.5mm thick, were cut with a Stadie-Riggs microtome. The slices (approx. 250mg) were incubated in Erlenmeyer flasks at 37°C for 3 h with 10 μ mol (2 μ Ci) of sodium [1-¹⁴C]acetate or 2.5 μ mol (0.5 μ Ci) of DL-[2-¹⁴C]mevalonic acid (sodium salt) in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.4, containing 30 mM-nicotinamide and 4 mM-MgCl₂. The flasks were shaken at 100 oscillations/min with air as the gas phase.

At the end of the incubation period, the contents of each flask were saponified at 70°C with 5 ml of 15% (w/v) KOH in 90% (v/v) ethanol for 30 min. The non-saponifiable fraction was extracted three times with 20 ml of light petroleum (b.p. 40–60°C) and the digitonin-precipitable sterols were isolated as described by De Matteis (1968). The radioactivity of this fraction, which is mainly cholesterol, was taken as a measure of cholesterol synthesis. The digitonides were dissolved in methanol and a portion was taken for liquid-scintillation counting of radioactivity.

Liver cholesterol synthesis in vivo. Rats were each given 10 μ Ci of sodium [1-¹⁴C]acetate (61 mCi/nmol) in 0.1 ml of NaCl (0.9%, w/v) by intraperitoneal injection. Exactly 1 h later, the rats were killed by cervical dislocation and their livers were quickly removed, rinsed in ice-cold saline, blotted dry and weighed.

After saponification, the digitonin-precipitable sterols were extracted into light petroleum (b.p. 40–60°C) and isolated (De Matteis, 1968).

Cholesterol 7 α -hydroxylase assay. Rat liver was passed through a stainless-steel mincer (Cornforth *et al.*, 1958). A 20% (w/v) homogenate of the mince was prepared in modified Bucher medium (Bergström & Gloor, 1955) by making three passes in a glass-Teflon homogenizer with a 0.5 mm gap. The homogenate was centrifuged at 20000g for 15 min at 4°C and the supernatant was used as the enzyme source.

[4-¹⁴C]Cholesterol (1 μ Ci; 61.7 mCi/mmol), dissolved in 30 μ l of acetone, was added to the 20000g supernatant (3 ml) and incubated for 1 h at 37°C. A portion (1 ml) was then removed and added to 19 ml of chloroform-methanol (2:1, v/v). A two-phase system was formed by the addition of water and methanol (Bligh & Dyer, 1959) and the chloroform phase was evaporated to dryness under a stream of N₂. The residue was subjected to t.l.c. as described by Danielsson *et al.* (1967) and cholesterol and 7 α -hydroxycholesterol were detected by spraying with

an ethanolic solution of ferric chloride (1%, w/v). The areas corresponding to cholesterol and 7 α -hydroxycholesterol were scraped off the plates directly into vials containing scintillation fluid.

Radioactivity-counting procedures. The scintillation fluid consisted of 10 ml of 0.6% (w/v) 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD; Intertechnique Ltd., Brighton, Sussex, U.K.) in toluene. All samples were analysed in an Intertechnique ABAC SL 40 spectrometer and corrected for quenching by the external standard channels-ratio method.

Content of cholesterol in serum. Total cholesterol in serum was measured by using the Technicon Autoanalyser method N24A (Technicon Instruments Corp., 1965), as modified by Seigel & Bowdoin (1971).

Liver aminopyrine demethylase assay. A 20% (w/v) liver homogenate was prepared in 0.25 M-sucrose buffered to pH 7.4 with 0.1 M-sodium phosphate and 0.001 M-EDTA. After filtering through gauze, the homogenate was centrifuged at 10000g for 15 min. The supernatant was used as the enzyme source. The incubation mixture had the composition: KH₂PO₄ buffer, pH 7.4, 83.3 mM; MgCl₂, 3.3 mM; NADP, 0.4 mM; sodium isocitrate, 8 mM; isocitrate dehydrogenase, 0.5 unit; aminopyrine, 25 mM; semicarbazide (as a formaldehyde trapping agent), 0.75 mM; and enzyme from 200 mg of liver. The total volume of the incubation mixture was 4 ml. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 2 ml of 1.0 M-NaOH and 2 ml of 20% (w/v) ZnSO₄. Formaldehyde was measured in the protein-free supernatant by the method of Nash (1953).

Content of cytochrome P-450 in liver. A 20% (w/v) liver homogenate was prepared in 0.15 M-KCl buffered to pH 7.4 with 0.05 M-sodium phosphate and filtered through gauze. The homogenate was centrifuged at 10000g for 15 min. The supernatant was then recentrifuged at 100000g for 60 min and the microsomal pellet was washed twice with the KCl buffer to remove any residual adherent haemoglobin. The microsomal preparation was then resuspended in 0.1 M-KCl buffered to pH 7.4 with 0.05 M-sodium phosphate and the cytochrome P-450 concentration was determined by the method of Omura & Sato (1964).

Protein concentration. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals

Sodium [1-¹⁴C]acetate, [2-¹⁴C]mevalonic acid lactone and [4-¹⁴C]cholesterol were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NADPH, isocitrate dehydrogenase and trisodium isocitrate were obtained from Sigma

(London) Chemical Co. Ltd., London S.W.6, U.K. Aminopyrine was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. The 7 α -hydroxy-cholesterol was the gift of Dr. P. P. Shah, of these laboratories. Cholestyramine was obtained from Mead Johnson, Evansville, Ind., U.S.A.

Experimental and Results

Effect of adjuvant treatment on cholesterol biogenesis and drug-metabolizing enzymes

Treatment of rats with the adjuvant preparation induced, after 10 days, the occurrence of a poly-arthritic condition, whereas treatment with liquid paraffin had no effect. The livers of arthritic rats had

a decreased capacity to metabolize aminopyrine *in vitro* (Table 1) and had a low concentration of hepatic cytochrome P-450. These findings agree with those of Morton & Chatfield (1970). Table 1 shows also that both the serum and liver cholesterol concentrations of adjuvant-treated arthritic rats are normal. In Expt. 1, the hepatic biogenesis of cholesterol from sodium [1-¹⁴C]acetate was measured *in vitro* and it was found that the arthritic rats synthesized more cholesterol than the controls did (means of 129 and 41 nmol/h per g of liver, respectively), but this difference was not significant, owing to the considerable variation between the individual values. However, in Expt. 2 (Table 1), when the rate of hepatic biogenesis of cholesterol from [2-¹⁴C]-mevalonic acid was determined, adjuvant-treated

Table 1. *Effect of adjuvant-induced loss of cytochrome P-450 on cholesterol biogenesis in vitro from [1-¹⁴C]acetate or [2-¹⁴C]mevalonate*

The rats (180–200g) were treated with 0.1 ml of liquid paraffin (control) or *M. butyricum* in liquid paraffin (adjuvant-treated). They were killed 14 days later. In Expt. 1, the formation of digitonin-precipitable [¹⁴C]sterols from [¹⁴C]acetate was measured; in Expt. 2, [¹⁴C]mevalonate was used as the substrate. The results are given as means $\pm$ s.e.m.; the number of determinations are in parentheses.

Group	Serum cholesterol (mg/100ml)	Wt. of liver as a percentage of body wt.	Liver cholesterol (mg/g of liver)	Cholesterol biosynthesis (nmol incorporated/h per g of liver)	Aminopyrine demethylase (μ mol of formaldehyde formed/h per g of liver)	Hepatic cytochrome P-450 (nmol/mg of protein)
Expt. 1						
Control	—	4.4 $\pm$ 0.1 (8)	—	41.4 $\pm$ 12.7 (6)	7.5 $\pm$ 0.6 (6)	0.41 $\pm$ 0.07 (7)
Adjuvant-treated	—	4.7 $\pm$ 0.3 (8)	—	128.9 $\pm$ 38.2 (6)	1.7 $\pm$ 0.6 (6)*	0.20 $\pm$ 0.09 (8)†
Expt. 2						
Control	55 $\pm$ 3 (7)	4.8 $\pm$ 0.2 (8)	3.00 $\pm$ 0.14 (8)	162.8 $\pm$ 17.7 (5)	8.0 $\pm$ 1.3 (3)	0.39 $\pm$ 0.04 (8)
Adjuvant-treated	53 $\pm$ 4 (8)	4.6 $\pm$ 0.1 (8)	3.48 $\pm$ 0.08 (8)	227.0 $\pm$ 15.6 (5)‡	2.3 $\pm$ 1.5 (3)‡	0.22 $\pm$ 0.06 (3)

* Significantly less than controls ($P < 0.001$)

† Significantly less than controls ($P < 0.01$)

‡ Significantly different from controls ($P < 0.05$)

Table 2. *Effect of adjuvant treatment on cholesterol biogenesis in vivo*

Each rat was given 10 μ Ci of sodium [1-¹⁴C]acetate by intraperitoneal injection 1 h before it was killed. Digitonin-precipitable [¹⁴C]sterols were isolated and measured as described in the text. Results are the means $\pm$ s.e.m. of eight analyses; each analysis was of tissue from a single rat.

Group	Liver			[¹⁴ C]Acetate incorporated (d.p.m./g of liver)		
	Wt. as a percentage of body wt.	Cholesterol (mg/g of liver)	Serum cholesterol (mg/100ml)	Total lipid	Non-saponifiable lipids	Digitonin-precipitable sterols
Control	4.4 $\pm$ 0.1	2.78 $\pm$ 0.14	59 $\pm$ 2	50616 $\pm$ 7568	10241 $\pm$ 4123	617 $\pm$ 163
Adjuvant-treated	4.6 $\pm$ 0.2	2.91 $\pm$ 0.06	53 $\pm$ 2	56535 $\pm$ 14030	12182 $\pm$ 3734	1439 $\pm$ 346

rats had a significantly greater enzymic activity than control rats ($P < 0.05$).

In a subsequent experiment (Table 2), it was found that the incorporation of sodium [$1\text{-}^{14}\text{C}$]acetate into digitonin-precipitable sterols *in vivo* was similar in arthritic and control rats.

Effect of cholestyramine and adjuvant treatment on cholesterol catabolism and drug hydroxylation

Arthritis was induced in a group of 16 rats by adjuvant treatment and another group of 16 rats, given liquid paraffin only, served as controls. Both groups of rats were given diet FFG in powdered form for 9 days. Eight rats were then selected at random from each group and were given diet FFG containing 3% of the bile-acid-sequestering agent, cholestyramine, for a further 5 days. The remaining eight rats in each group continued to receive the unsupplemented FFG diet for the same period. At 14 days after the injections, all the rats were killed and hepatic cholesterol 7α -hydroxylase, aminopyrine

demethylase and cytochrome *P*-450 were measured (Table 3).

Treatment of rats with cholestyramine alone, although increasing the activity of hepatic cholesterol 7α -hydroxylase, had no effect on aminopyrine demethylase or on the concentration of cytochrome *P*-450. Conversely, adjuvant treatment produced a loss of aminopyrine demethylase in rats fed on either the normal or the cholestyramine-containing diet but had no effect on cholesterol 7α -hydroxylase.

Effect of carbon disulphide on drug metabolism and cholesterol biogenesis

The rats were given either CS_2 or arachis oil orally and killed 24 h later. The livers of the CS_2 -treated rats contained significantly less cytochrome *P*-450 than control rats and consequently the activity of hepatic aminopyrine demethylase was decreased. These findings agree with those of Bond & De Matteis (1969). However, the mean incorporation of sodium

Table 3. *Effect of cholestyramine and adjuvant treatment on cholesterol 7α -hydroxylase and drug metabolism*

The rats (230–270 g) were killed by cervical dislocation. There were eight rats in each group. Liver wt. and cholesterol 7α -hydroxylase results are the means $\pm$ S.E.M. of eight values. Livers were combined in pairs for the determination of cytochrome *P*-450 and aminopyrine demethylase activity and therefore these results are the means of four values.

Dietary supplement	Treatment	Wt. of liver as a percentage of body wt.	Aminopyrine demethylase (μmol of formaldehyde formed/h per g of liver)	Cytochrome <i>P</i> -450 in liver (nmol/mg of protein)	Hepatic cholesterol 7α -hydroxylase (percentage incorporation of substrate/100 mg of protein)
None	Control	4.3 ± 0.1	$10.8 \pm 0.9^*$	0.35 ± 0.02	1.41 ± 0.32
	Adjuvant-treated	4.5 ± 0.2	5.4 ± 1.7	0.22 ± 0.07	0.98 ± 0.19
Cholestyramine	Control	4.4 ± 0.1	$11.8 \pm 1.0^*$	0.44 ± 0.07	3.27 ± 0.36
	Adjuvant-treated	4.2 ± 0.2	6.3 ± 0.5	0.29 ± 0.06	2.59 ± 0.27

* Significantly greater than adjuvant-treated rats given the same diet ($P = 0.014$)

Table 4. *Effect of carbon disulphide on cholesterol synthesis and drug metabolism*

Rats (180–200 g) were given orally either CS_2 (1 ml/kg body wt.) or arachis oil and were killed 24 h later. Livers were removed and assays were carried out as described in the text. Results are given as means $\pm$ S.E.M. of four values.

Treatment	Wt. of liver as a percentage of body wt.	Cholesterol synthesis (nmol of [^{14}C]acetate incorporated/h per g of liver)	Aminopyrine demethylase (μmol of formaldehyde formed/h per g of liver)	Cytochrome <i>P</i> -450 in liver (nmol/mg of protein)
Arachis oil	4.4 ± 0.5	6.56 ± 3.80	7.6 ± 0.8	0.46 ± 0.05
CS_2	4.6 ± 0.2	13.85 ± 1.82	$2.4 \pm 0.5^*$	$0.14 \pm 0.04^*$

* Significantly less than value from rats not given CS_2 ($P < 0.01$)

[1-¹⁴C]acetate into digitonin-precipitable sterols was greater in liver slices from CS₂-treated rats than those from controls, but the effect was not significant (Table 4).

Discussion

The possibility that cholesterol synthesis may proceed by processes closely related to those involved in drug hydroxylation is suggested by a number of pieces of circumstantial evidence. First, many substances have been found to inhibit both processes (Brodie, 1956; Holmes & Bentz, 1960; Kato *et al.*, 1963; Marton & Chappel, 1964). Secondly, the potent inducer of drug-metabolizing-enzyme activity, phenobarbitone, increases cholesterol biogenesis (Jones & Armstrong, 1965; Kato *et al.*, 1968; F. Wada *et al.*, 1969a; O. Wada *et al.*, 1969). Finally, CO, which binds reversibly to cytochrome P-450, inhibits cholesterol biogenesis *in vitro* (F. Wada *et al.*, 1967a,b, 1969a).

We consider that, if cytochrome P-450 is involved in cholesterol biogenesis, and if its concentration is rate-limiting, then the livers of rats that have been experimentally depleted of cytochrome P-450 should have a decreased capacity to synthesize cholesterol. Two techniques, CS₂ treatment (Bond & De Matteis, 1969) and adjuvant-induced arthritis (Morton & Chatfield, 1970), are known to produce a decrease in drug-metabolizing-enzyme activity, with little other hepatotoxicity. In our experiments, both treatments produced a marked decrease in drug-metabolizing-enzyme activity and cytochrome P-450, but had no effect on cholesterol in the serum. Also, the incorporation *in vivo* and *in vitro* of [¹⁴C]acetate or [¹⁴C]mevalonate into digitonin-precipitable [¹⁴C]-sterols was not inhibited by either treatment. Indeed, in one experiment, arthritic rats synthesized significantly more digitonin-precipitable [¹⁴C]-sterols than the controls did.

These results suggest that the normal hepatic concentration of cytochrome P-450 is not rate-limiting for the overall hepatic biogenesis of cholesterol, as it is for the metabolism of drugs. However, this must be reconciled with the finding of F. Wada *et al.* (1967a, 1969a) that CO inhibits cholesterol synthesis *in vitro*. It is possible, therefore, that only a small amount of the normal cytochrome P-450 is rate-limiting in cholesterol synthesis, or, alternatively, a special form of cytochrome P-450 is required for cholesterol synthesis and this is not affected by either of the experimental treatments. Some evidence for the latter view was found by Omura *et al.* (1965), in that the affinity of CO for the drug-metabolizing-enzyme system is different from its affinity for the cholesterol-synthesizing system (F. Wada *et al.*, 1969a). Further, we have found (S. D. Atkin, unpublished work) that *n*-propyl gallate, piperonyl butoxide- $\{\alpha$ -[2-(2-butoxy-

ethoxy)ethoxy]-4,5 - methylenedioxy - 2 - propyl - toluene} and ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) have no effect, when added at 0.1 mM concentration, on the biogenesis of cholesterol *in vitro*, although at this concentration they are inhibitors of drug hydroxylation.

The evidence for the involvement of cytochrome P-450 in 7 α -hydroxylation of cholesterol is, at present, also somewhat equivocal. In particular, there are contradictory views on the effect of phenobarbitone on this process (Boyd *et al.*, 1969; Einarsson & Johansson, 1968; Mosbach, 1969).

Our present experiments have confirmed that cholestyramine increases the activity of cholesterol 7 α -hydroxylase without affecting either the activity of the drug-metabolizing enzyme, aminopyrine demethylase, or the concentration of cytochrome P-450. Moreover, depletion of hepatic cytochrome P-450 by treatment of the rats with adjuvant did not result in any significant change in cholesterol catabolism.

The results can be interpreted as indicating that the processes of cholesterol 7 α -hydroxylation and drug metabolism involve two distinct enzyme systems. Certainly, as with cholesterol synthesis, the normal hepatic concentration of cytochrome P-450 is not rate-limiting for cholesterol 7 α -hydroxylation. However, the reversibility of the CO inhibition of cholesterol 7 α -hydroxylase by monochromatic light (450nm) (Boyd *et al.*, 1971) suggests that a small amount of cytochrome P-450 is an essential component of that enzyme. Possibly 'cholesterol-hydroxylating enzyme, cytochrome P-450', is distinct from 'drug-hydroxylating enzyme, cytochrome P-450', in that it is not inducible by phenobarbitone and is not destroyed during adjuvant-induced arthritis.

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