A relationship between the activities of hepatic lanosterol 14 α -demethylase and 3-hydroxy-3-methylglutaryl-CoA reductase

Carmen MARCO DE LA CALLE,* William HWANG,† Clive R. PULLINGER‡ and Geoffrey F. GIBBONS
Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

At 1–2 h after intragastric administration of ketoconazole, a cytochrome *P*-450 inhibitor, to rats, there was a 50–60 % decrease in the activity of hepatic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Inhibition reached a maximum at 6–12 h after the drug was given, but after 24 h enzyme activity was stimulated by 60 %. The rates of synthesis of hepatic non-saponifiable lipids *in vivo* showed a similar time-dependent pattern of change. During the first few hours after drug administration, the hepatic cytochrome *P*-450-dependent metabolism of lanosterol was suppressed *in vivo*. However, 24 h after treatment, this activity was stimulated, an effect which was also observed by pre-treatment of the rats with the drug for several days. Suppression of hepatic HMG-CoA reductase and lanosterol 14 α -demethylase activities was accompanied by a relative increase in the accumulation of labelled polar sterols in the liver *in vivo*. In the intestine, ketoconazole also resulted in a rapid decline in the rate of synthesis of non-saponifiable lipids and an inhibition of lanosterol 14 α -demethylation *in vivo*. However, in contrast with the liver, there was no stimulation of non-saponifiable lipid synthesis after 24 h.

INTRODUCTION

It has been known for some time that certain oxygenated derivatives of cholesterol are powerful inhibitors of HMG-CoA reductase and sterol synthesis in all cell types studied (Kandutsch & Chen, 1973; Kandutsch *et al.*, 1978; Schroepfer, 1981; Gibbons, 1983a). Several of these inhibitory sterols are precursors or metabolites of cholesterol, and there is substantial evidence to suggest that one or more sterols of this type are involved in the complex sequence of events linking changes in cellular cholesterol balance with compensatory changes in the activity of HMG-CoA reductase (Kandutsch *et al.*, 1978; Gibbons, 1983b; Taylor *et al.*, 1986). Conclusive evidence has, however, remained elusive, partly owing to the difficulty of demonstrating a causal relationship between changes in the rate of enzymic formation of identifiable oxysterols and changes in HMG-CoA reductase activity. Cytochrome *P*-450 is involved in the conversion of lanosterol into C₃₂ oxysterol derivatives (Gibbons & Mitropoulos, 1973a; Gibbons *et al.*, 1979), which are obligatory cholesterol precursors (Gibbons *et al.*, 1976; Akhtar *et al.*, 1978; Trzaskos *et al.*, 1984; Shafiee *et al.*, 1986). These substances, when added to cell cultures, suppress HMG-CoA reductase (Gibbons *et al.*, 1980). An alternative approach to investigating the role of oxygenated cholesterol precursors has involved studying the effects of cytochrome *P*-450 inhibitors on the regulation of cholesterol biosynthesis *in vitro* (Trzaskos *et al.*, 1986; Gupta *et al.*, 1986; Panini *et al.*, 1986; Boogaard *et al.*, 1987). Under these conditions, the biosynthetic formation of oxysterols is prevented. One of the objectives of the present work was to investigate the relationship between the rates of

cytochrome *P*-450-mediated oxidation of lanosterol, the activity of hepatic HMG-CoA reductase, and the rate of hepatic sterol synthesis *in vivo*. This was achieved by intragastric administration of the cytochrome *P*-450 inhibitor, ketoconazole (Van den Bossche *et al.*, 1983). Rudney and his colleagues (Gupta *et al.*, 1986) have shown that the detailed effects of ketoconazole on HMG-CoA reductase activity in rat intestinal epithelial cells is dependent on its concentration in the incubation medium: an inhibition was observed at low concentrations, which gave way to an increased activity as the drug concentration increased. These changes were inversely related to the rate at which labelled mevalonate was converted into sterols more polar than cholesterol. Another aim of the present work was to investigate the detailed time- and concentration-dependent effects of ketoconazole on sterol biosynthesis *de novo* in the rat intestine *in vivo*, and to relate these changes to effects on the cytochrome *P*-450-mediated demethylation of lanosterol in this organ.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats were housed in a windowless room artificially lit with tungsten bulbs (2 × 60 W) between 16:00 h and 04:00 h. Rats were fed on a commercially available pelleted diet (Diet PRM; E. Dixon and Sons, Ware, Herts., U.K.), which contained (w/w) 47.5% carbohydrate (mainly starch), 2.7% fat and 18.7% protein. Food and water were available *ad libitum*. Animals weighed 250–300 g at the time of the experiment. In some cases ketoconazole (21 mg/100 g) was added to

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

* Present address: Department of Biochemistry and Molecular Biology, University of Granada, Granada, Spain.

† Present address: Goldman Sachs (Japan) Corp., Minato-Ku, Tokyo 107, Japan.

‡ Present address: Molecular Medicine Research Group, Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

the food. This was done by grinding the pellets and adding a solution of ketoconazole in 0.1 M-HCl (21 mg/ml) which had been diluted with 100 ml of distilled water. Sufficient additional water was added to give a stiff paste, and the pellets were then reconstituted. The pellets were dried overnight in a stream of air at room temperature. In these experiments, the control diet was prepared in a similar way, except that 0.1 M-HCl only was added. Where appropriate, ketoconazole was administered intragastrically as a solution in 0.1 M-HCl (4 mg/ml). The drug was administered at different times (i.e. 1 h, 6 h, 12 h, 17 h and 24 h) before injection of $^3\text{H}_2\text{O}$ (0.2 ml, 4 mCi) intraperitoneally between 10:00 h and 11:00 h (i.e. the mid-point of the dark phase of the cycle).

Measurement of $^3\text{H}_2\text{O}$ incorporation into non-saponifiable lipids *in vivo*: isolation of labelled sterols and sterol precursors

At 1 h after injection of $^3\text{H}_2\text{O}$, the animals were anaesthetized by intraperitoneal injection of a solution of sodium pentobarbital in water (60 mg/kg body wt.). A portion of the liver was rapidly excised and chilled on ice. This was used for measurement of HMG-CoA reductase activity. Another portion of the liver was removed and frozen in liquid N_2 . Portions of the ileal and jejunal portions of the intestine (10 cm of each) were removed and the contents rinsed out with ice-cold 0.9% NaCl. Each ileal and jejunal portion was combined and frozen in liquid N_2 . The frozen tissues of liver and intestine were ground to a fine powder under liquid N_2 , and about 1 g of each was weighed out and saponified for 1 h at 70 °C with 10.0 ml of a 7.5% (w/v) solution of KOH in ethanol. Before saponification, a known amount of [^{14}C]cholesterol was added to each sample as an internal standard. Non-radioactive lanosterol (1.0 mg) and squalene (1.0 mg) were also added at this stage. The non-saponifiable lipid fraction was isolated as described previously (Gibbons *et al.*, 1986), and a sample was removed for measurement of radioactivity. Fractions containing labelled cholesterol, lanosterol and squalene were isolated from the remaining non-saponifiable lipid by t.l.c. (Gibbons & Mitropoulos, 1972). Labelled polar sterols were obtained by scraping the region of the plate extending from the origin to the cholesterol band. Where appropriate, cholesterol was separated from 5α -cholest-7-en- 3β -ol by argentation chromatography of the acetate derivatives (Gibbons *et al.*, 1979).

Measurement of rates of [$2\text{-}^{14}\text{C}$]mevalonate conversion into sterols *in vitro*

Livers from animals fed on the ketoconazole-supplemented and the control diets were removed, chilled on ice, and minced through an ice-cold stainless-steel tissue mincer into 3 vol. of a solution of EDTA (1 mM), nicotinamide (30 mM), GSH (10 mM), MgCl_2 (4 mM) and sucrose (125 mM) in 0.1 M-potassium phosphate buffer (pH 7.4) (Bucher *et al.*, 1959). The suspension was homogenized by ten passes of a Teflon/glass homogenizer (Jencons, Leighton Buzzard, Beds., U.K.), and the homogenate was centrifuged at 800 g for 15 min. The supernatant was re-centrifuged at 16000 g for 20 min. Portions of this 16000 g supernatant (2.0 ml) were used in the subsequent incubation, containing [$2\text{-}^{14}\text{C}$]mevalonate (1 μCi ; 54 mCi/mmol), fructose 1,6-bisphosphate (16 mM) and NAD^+ (1.6 mM) in a total volume of 2.5 ml. Incubations were carried out for 1 h at

37 °C and, after addition of unlabelled lanosterol (1.0 mg) and squalene (1.0 mg), the labelled non-saponifiable lipid fraction was isolated (see above). The individual components of the non-saponifiable lipid were isolated by t.l.c. (Gibbons & Mitropoulos, 1972).

Isolation of liver microsomal fractions

Ice-cold liver was passed through a chilled tissue mincer into 10 vol. of a solution of sucrose (250 mM) and NaF (50 mM). The liver was homogenized by ten passes of a Teflon/glass homogenizer (Jencons). The resulting homogenate was centrifuged at 800 g for 15 min, followed by re-centrifugation of the supernatant at 16000 g_{av} for 20 min. This last centrifugation step was repeated in order to remove completely any remaining lysosomes. The top half of the supernatant was removed and centrifuged at 100000 g_{av} to sediment the microsomal pellet. This was used either for measurement of HMG-CoA reductase activity or for assay of the rate of [^{14}C]lanosterol metabolism.

Measurement of HMG-CoA reductase activity

The microsomal pellet derived from 1.3 g of liver was suspended in 1.5 ml of 20 mM-imidazole/HCl buffer (pH 7.4) containing dithiothreitol (5 mM) (buffer A), with a 23-gauge needle and a 1.0 ml syringe. All the operations were carried out at 0–4 °C. A portion of the microsomal suspension (20 μl) was incubated in the presence or absence of alkaline phosphatase (5 units in 20 μl of buffer A) for 30 min at 37 °C (Brown *et al.*, 1979). The pre-incubated microsomes were then used to determine HMG-CoA reductase activity using the method of Brown *et al.* (1979). Activity observed after incubation in the absence of phosphatase represents the 'expressed' activity that is present in the liver *in vivo* (i.e. the dephosphorylated form of the enzyme). Activity observed after incubation with phosphatase represents 'total' enzyme activity (i.e. the sum of activities that were originally present as the phosphorylated and non-phosphorylated forms of the enzyme). The labelled product, mevalonic acid, was isolated as mevalonolactone by t.l.c. (Cavenee *et al.*, 1981).

Other analytical methods

Protein was determined by the method of Gieger & Bessman (1972). For determination of the specific radioactivity of the plasma water, a sample of blood (1.0 ml) was taken from the descending vena cava and the plasma was obtained by centrifugation.

Materials

Except for [^{14}C]lanosterol, all radiochemicals were obtained from Amersham International, Little Chalfont, Bucks., U.K. [^{14}C]Lanosterol was prepared biosynthetically from [^{14}C]mevalonate as described previously (Gibbons & Mitropoulos, 1973b). Ketoconazole was given by Mr. N. Blatchford, Janssen Pharmaceuticals, Grove, Wantage, Berks., U.K. Enzymes and cofactors were obtained from Sigma, Poole, Dorset, U.K. All organic solvents were redistilled before use.

Statistical treatment of results

All values are presented as means $\pm$ S.E.M. for several independent observations. The significance of any observed difference was tested by Student's *t* test.

Fig. 1. Time course of the effects of intragastric ketoconazole administration on hepatic HMG-CoA reductase activity and non-saponifiable lipid formation

Rats were treated with a solution of ketoconazole (6 mg) in 0.1 M-HCl (1.5 ml) or with 0.1 M-HCl only, and were killed at various times later (see the Materials and methods section). Each treated rat was paired with a control animal, and the results are expressed in terms of the effect of ketoconazole in the treated animal as a percentage of that observed in the paired control. Each point represents the mean $\pm$ S.E.M. of eight pairs of animals, except at 6 h, 12 h and 17 h when two pairs were used. At 10:00 h, after 2 h treatment, the control values for the expressed and total forms of HMG-CoA reductase were 68.1 ± 14.1 and 178.9 ± 28.9 pmol/min per mg of protein. The control value for non-saponifiable lipid synthesis was 46.1 ± 5.9 nmol of $^3\text{H}_2\text{O}$ /min per g of liver. The corresponding control values at the same time of day, 24 h after treatment with 0.1 M-HCl alone, were 66.1 ± 15.2 and 218.4 ± 47.4 pmol/min per mg and 47.2 ± 7.2 nmol/min per g respectively. These values were not significantly different from the 2 h controls. The control values corresponding to the other lengths of treatment time were also similar to the above. Points marked * and ** were significantly different from the controls at $P < 0.05$ and $P < 0.001$ respectively. ●, Expressed activity of HMG-CoA reductase; ▲, total activity of HMG-CoA reductase; ■, $^3\text{H}_2\text{O}$ incorporation into non-saponifiable lipid.

RESULTS

Time-dependent effects of ketoconazole on hepatic non-saponifiable lipid synthesis and HMG-CoA reductase activity

At 2 h after intragastric administration of ketoconazole (24 mg/kg), there was a 50–60% decrease in the activity of hepatic HMG-CoA reductase (Fig. 1). Ketoconazole had no direct effect when present at 200 μM in the HMG-CoA reductase assay (results not shown). The decrease in the active (dephosphorylated) form of the enzyme was similar to that of the total (the sum of the phosphorylated and dephosphorylated forms) (Fig. 1). These changes were accompanied by a decrease in the rate of carbon flux through the enzyme *in vivo*, as

Fig. 2. Effect of ketoconazole treatment on the distribution of label among the components of the non-saponifiable lipid fraction synthesized from $^3\text{H}_2\text{O}$ in liver

The non-saponifiable lipid fraction was obtained from rats treated with ketoconazole for different periods of time as described in the legend to Fig. 1. Fractions containing labelled cholesterol (■), lanosterol (●) and polar sterols (▲) were obtained by t.l.c. The amount of radioactivity in each fraction is expressed as a percentage of the radioactivity of the non-saponifiable lipid from which it was derived. Each point represents the mean $\pm$ S.E.M. of eight independent measurements, except at 6 h, 12 h and 17 h, at which two measurements were made.

measured by the incorporation of label from $^3\text{H}_2\text{O}$ into the total non-saponifiable lipid fraction. At 6–12 h after ketoconazole administration, HMG-CoA reductase remained depressed to a similar degree to that observed after 2 h. However, after 12 h, enzyme activity began to increase, a recovery which culminated in a 60% stimulation of enzyme activity 24 h after drug administration ($P < 0.05$). Carbon flux through the enzyme *in vivo* was also significantly stimulated ($P < 0.05$) at this time, as determined by $^3\text{H}_2\text{O}$ incorporation into the non-saponifiable fraction. However, it should be noted that the ketoconazole-mediated oscillation in the maximum capacity of HMG-CoA reductase, measured under substrate-saturating conditions, was greater than the corresponding changes in the actual rate of carbon flux through the enzyme *in vivo* (Fig. 1).

Effect of ketoconazole on the rates of formation of hepatic cholesterol and its precursors *in vivo*

Isolation of the individual constituents of the labelled non-saponifiable lipid fraction from the previous experiment revealed the distribution of radioactivity shown in Fig. 2. At 1–2 h after administration of ketoconazole, over 50% of the decreased amount of label in the non-saponifiable lipid fraction was associated with lanosterol, compared with 4.5% in the corresponding control animals which had not been treated with the drug. That this reflected a direct inhibitory effect of ketoconazole on lanosterol 14 α -demethylation was shown by experiments

Table 1. Effects of pre-treatment of animals with ketoconazole on the acute effects of ketoconazole on hepatic sterol metabolism *in vivo*

A group of rats received two doses of ketoconazole in 0.1 M-HCl intragastrically at 24 h intervals. Another group received 0.1 M-HCl only. At 24 h after the last treatment, both groups received a solution of ketoconazole in 0.1 M-HCl, and $^3\text{H}_2\text{O}$ (0.2 ml, 4 mCi) was injected intraperitoneally 1 h later. After a further 1 h, the animals were anaesthetized and samples of the liver were removed and placed in liquid N_2 . The labelled lanosterol and cholesterol fractions were isolated from both groups of animals. Each value represents the mean $\pm$ S.E.M. for four animals in each group. Values marked *, ** and *** are significantly different from the corresponding values in the group not receiving drug pre-treatment at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. There were no significant differences between the respective control groups (i.e. between those animals receiving three doses of 0.1 M-HCl and those receiving only one dose of 0.1 M-HCl).

Sterol	Pre-treatment (three doses total)		No pre-treatment (one dose only)	
	Incorporation of $^3\text{H}_2\text{O}$ (nmol/g)	% of non- saponifiable lipid	Incorporation of $^3\text{H}_2\text{O}$ (nmol/g)	% of non- saponifiable lipid
Cholesterol	$13.5 \pm 1.7^{**}$	$56.5 \pm 3.5^{***}$	7.0 ± 0.6	22.6 ± 4.0
Lanosterol	$5.2 \pm 0.4^{**}$	$22.7 \pm 3.1^*$	14.0 ± 2.3	51.0 ± 10.8

Table 2. Effect of dietary ketoconazole on the accumulation of labelled lanosterol from $[2-^{14}\text{C}]$ mevalonate by rat liver homogenates

Groups of rats were fed on either the standard diet (control) or a similar diet supplemented with ketoconazole (21 mg/100 g) for 24 h. During this period the control rats each consumed 37.3 ± 4.3 g of chow, and those fed on the ketoconazole-supplemented diet consumed 36.0 ± 5.5 g. The rats were killed at 10:00 h, the livers were removed and 16000 g supernatants were prepared. Each supernatant fraction (2.0 ml) was incubated with $[2-^{14}\text{C}]$ mevalonate (1 μCi ; 51.4 mCi/mmol) for 1 h in the presence of potassium phosphate buffer (0.1 M, pH 7.4) containing EDTA (1 mM), nicotinamide (30 mM), sucrose (125 mM), MgCl_2 (4 mM), GSH (10 mM), fructose 1,6-bisphosphate (16 mM) and NAD^+ (1.6 mM) in a total volume of 2.5 ml. Each value represents the mean $\pm$ S.E.M. of five animals in each group. Values marked ** are significantly different from the controls at $P < 0.01$.

Dietary treatment	$[2-^{14}\text{C}]$ Mevalonate conversion into non-saponifiable lipid (pmol/ min per mg of protein)	$[^{14}\text{C}]$ Lanosterol (% of non- saponifiables)	Relative effect on lanosterol accumulation (%)
Control	43.4 ± 10.2	14.8 ± 2.8	100
Ketoconazole	54.7 ± 9.6	8.9 ± 2.8	$53.4 \pm 9.5^{**}$

using cell-free preparations of liver. In these experiments, the drug at 10 μM almost completely blocked cholesterol synthesis from $[^{14}\text{C}]$ mevalonate and, under these conditions, $[^{14}\text{C}]$ lanosterol accumulated (results not shown).

In the experiments *in vivo* during the next 22 h period after ketoconazole treatment, the proportion of ^3H label in lanosterol declined. The pattern of incorporation of label into cholesterol was the mirror image of that for lanosterol, suggesting that ketoconazole blocked the normally active oxidative 14α -demethylation of this compound. This resulted in a decrease in cholesterol production. The blockage of lanosterol demethylation was accompanied by the appearance of labelled substances more polar than cholesterol. As a proportion of the non-saponifiable lipid, these substances, which have the chromatographic properties expected of oxygenated cholesterol precursors, reached a maximum 2–6 h after administration of ketoconazole. There were no changes in the proportion of label associated with squalene in the livers of rats treated with ketoconazole at any of the time points studied (results not shown).

Induction of hepatic lanosterol 14α -demethylation by longer-term treatment with ketoconazole

In some experiments, rats were divided into two groups. One group of animals was given three doses of ketoconazole intragastrically (24 mg/kg) at 24 h intervals and were then injected with $^3\text{H}_2\text{O}$ 1 h after the last dose. The other group received only a single dose of ketoconazole, and $^3\text{H}_2\text{O}$ was injected 1 h later. In both groups the distribution of ^3H label among the cholesterol and lanosterol fractions of the non-saponifiable lipid was determined. In the rats pre-treated with ketoconazole, there was significantly less ($P < 0.01$) lanosterol and more cholesterol 1 h after the last dose than in the rats which had not been pre-treated (Table 1). This indicated that the pre-treated animals were less susceptible to the acute inhibitory effects of ketoconazole on lanosterol 14α -demethylation than were the untreated animals.

In another experiment, animals were fed on either a normal diet or a diet containing ketoconazole (21 mg/100 g of food) for 24 h. Subcellular fractions of rat liver (16000 g supernatant) obtained from animals in each

group were then incubated with [2-¹⁴C]mevalonate. Extraction of the non-saponifiable lipid, followed by isolation of the labelled lanosterol, showed that there was considerably less accumulation of this material in

Table 3. Liver microsomal metabolism of [¹⁴C]lanosterol in rats treated with ketoconazole 24 h previously

Animals were given a solution of ketoconazole (1.5 ml; 4.0 mg/ml) in 0.1 M-HCl intragastrically. Control animals received the same amount of 0.1 M-HCl only. At 24 h later, liver microsomal fractions were isolated from animals in each group, and incubated in 1.0 ml of 0.1 M-potassium phosphate buffer (pH 7.4) at 37 °C for 45 min with [¹⁴C]-lanosterol (50000 d.p.m., 3.26 d.p.m./pmol) which was previously emulsified with Tween 80 (0.5 mg). The incubation medium contained GSH (10.0 mM), nicotinamide (30 mM), NAD⁺ (0.8 mM), NADH (1.3 mM), ATP (1.3 mM), MgCl₂ (4.0 mM), NADP⁺ (2.0 mM), glucose 6-phosphate (12.0 mM), 1.0 unit of glucose-6-phosphate dehydrogenase, and 0.1 M-potassium phosphate buffer (pH 7.4). The total volume of the incubation was 1.35 ml, containing 3–4 mg of microsomal protein. After 45 min, non-radioactive lanosterol (1.0 mg), 5 α -cholest-7-enol (0.3 mg) and [³H]cholesterol (22600 d.p.m.) were added and the non-saponifiable lipid fraction was extracted. Labelled lanosterol, cholesterol and 5 α -cholest-7-enol were obtained by t.l.c. (see the Materials and methods section). Each value represents the mean $\pm$ S.E.M. of four animals in each group. Values marked * are significantly different at $P < 0.05$ from the controls.

Type of animal	5 α -Cholest-7-enol (pmol/mg of protein)	Cholesterol (pmol/mg of protein)
Control	103.6 $\pm$ 19.6	41.4 $\pm$ 9.6
Ketoconazole-treated	179.1 $\pm$ 23.5	89.9 $\pm$ 13.0*

the animals which had undergone continuous ingestion of ketoconazole over the previous 24 h period (Table 2).

Finally, microsomal fractions were obtained from rats which had been treated intragastrically either with a solution of ketoconazole in HCl or with HCl alone (controls) 24 h previously. The microsomal fraction was incubated with [¹⁴C]lanosterol for 45 min, and the non-saponifiable lipid fraction was extracted. The rate of metabolism of [¹⁴C]lanosterol was determined by measuring its rate of incorporation into cholesterol and into a cholesterol precursor, 5 α -cholest-7-en-3 β -ol. The results are presented in Table 3. This shows that, in the rats treated with ketoconazole 24 h previously, the rates of lanosterol conversion into cholesterol and 5 α -cholest-7-en-3 β -ol were greater than in the control animals.

Effects of ketoconazole on sterol metabolism in the intestine

Between 1 and 2 h after administration of ketoconazole, the rate of non-saponifiable lipid synthesis in the intestine *in vivo* decreased to less than 60% of the control value. The rate remained suppressed to approximately the same extent for at least a further 11 h after the drug was given. During the next 5 h, the rate of formation of the non-saponifiable lipid *in vivo* increased, so that 17 h after drug administration there was no difference between the rates of non-saponifiable lipid synthesis in the control and drug-treated animals. In contrast with the liver, there was no stimulation of non-saponifiable lipid synthesis in the intestine 24 h after administration of ketoconazole (Fig. 3). Increasing the concentration of ketoconazole from 24 to 240 mg/kg body wt. had no further effect on non-saponifiable lipid synthesis. Two animals treated in this way incorporated 22.8 and 18.9 nmol of ³H₂O/h per g of tissue, compared with 23.0

Fig. 3. Time course of the effect of ketoconazole on the synthesis of non-saponifiable lipids and sterols from ³H₂O in the intestine

Animals were treated either with a solution of ketoconazole in 0.1 M-HCl or with 0.1 M-HCl alone as described in the legend to Fig. 1. In (a), the effects of ketoconazole on intestinal non-saponifiable lipid synthesis are expressed as a percentage of the label incorporated into paired controls as described in Fig. 1. Each value represents the mean $\pm$ S.E.M. of four pairs, except at 6 h, 12 h and 17 h, at which times two pairs were used. Points marked * are significantly different from the controls at $P < 0.001$. In (b), fractions corresponding to cholesterol (■), lanosterol (●) and polar sterols (▲) were obtained from the labelled non-saponifiable lipid by t.l.c. The amount of radioactivity in each fraction is expressed as a percentage of the radioactivity in the non-saponifiable lipid from which it was derived. Each point represents the mean $\pm$ S.E.M. of four animals, except at 6 h, 12 h and 17 h, at which two animals were used.

and 21.3 nmol/h per g in animals given the lower concentration of the drug.

Isolation of the labelled cholesterol and its precursors from the non-saponifiable fraction obtained at different times after giving ketoconazole (24 mg/kg body wt.) showed that the accumulation of labelled lanosterol was maximal after 1–2 h, when it accounted for 65 % of the radioactivity of the non-saponifiable lipid (Fig. 3). This proportion gradually decreased with time until, after 24 h, there was no difference between the drug-treated and the control animals. Unlike the liver, the accumulation of labelled lanosterol in the intestine was not accompanied by any significant increase in the proportion of label associated with the polar sterol fraction. The proportion of labelled cholesterol in the non-saponifiable fraction showed an inverse relationship with that of labelled lanosterol (Fig. 3), reaching a minimum value 1–2 h after administering the drug.

DISCUSSION

Relationship between the formation *de novo* of polar sterols and carbon flux through HMG-CoA reductase

Substituted imidazoles such as ketoconazole are powerful inhibitors of lanosterol 14 α -demethylase (Van den Bossche *et al.*, 1983). This effect is probably mediated through an interaction with cytochrome *P*-450, an isoenzyme of which is an essential component of the microsomal system responsible for the oxidative removal of the lanosterol 14 α -methyl group (Gibbons & Mitropoulos, 1973a; Gibbons *et al.*, 1979). This is an obligatory step in the transformation of lanosterol into cholesterol, and inhibition of this enzyme gives rise to an accumulation of lanosterol. During sterol biosynthesis from labelled precursors by certain mammalian tissues *in vitro*, this inhibition is accompanied by an increase in the concentration of labelled polar sterols (Trzaskos *et al.*, 1986; Gupta *et al.*, 1986; Boogaard *et al.*, 1987). In the liver, two of these sterols have been identified as 5 α -lanost-8-ene-3 β ,32-diol and 3 β -hydroxy-5 α -lanost-8-en-32-al (Trzaskos *et al.*, 1986), both of which are intermediates in the oxidative removal of the lanosterol 14 α -methyl group (Gibbons *et al.*, 1976; Akhtar *et al.*, 1978; Trzaskos *et al.*, 1984). Rudney and his colleagues have demonstrated an inverse relationship between the appearance of labelled polar sterols and the activity of HMG-CoA reductase when intestinal epithelial cells were incubated with ketoconazole *in vitro*. This observation is consistent with the hypothesis, first proposed by Kandutsch & Chen (1973, 1974) and for which abundant evidence has subsequently emerged [e.g. see Gibbons (1983a) for a review] that cholesterol itself is not the regulatory molecule which is directly responsible for the regulation of HMG-CoA reductase activity at the molecular level. In the present work, during the period in which ketoconazole gave rise to an increase in the relative concentration of newly synthesized polar sterols in the liver *in vivo*, HMG-CoA reductase activity was suppressed. Even though the enzyme was completely activated by dephosphorylation *in vitro* (Brown *et al.*, 1979), only 45 % of the activity remained 2 h after ketoconazole treatment compared with that observed after dephosphorylation *in vitro* of the enzyme obtained from control animals. Although ketoconazole did not appear to result in any selective decrease in the proportion of

active (i.e. expressed, or dephosphorylated) enzyme present *in vivo*, we cannot completely rule out the possibility that any such change may have been obscured by rapid, non-physiological, phosphorylation of active enzyme during excision of the liver (Easom & Zammit, 1984a,b). Nevertheless, we tried to minimize such an effect by rapidly excising small pieces of liver and quickly chilling them on ice. We also tried to eliminate stress as far as possible by anaesthetizing the animals rather than by stunning them with a blow to the head.

In contrast with the liver, in the intestine the decrease in actual carbon flux through HMG-CoA reductase *in vivo*, as measured by the incorporation of $^3\text{H}_2\text{O}$ into the non-saponifiable lipid fraction, was not accompanied by any detectable increase in the proportion of label associated with sterols more polar than cholesterol. Recent work with cultured intestinal epithelial cells *in vitro* has shown that the decreased HMG-CoA reductase activity observed when low concentrations of ketoconazole were present in the medium was accompanied by a relative increase in the concentration of labelled polar sterols when [2- ^{14}C]mevalonate was the sterol precursor. Furthermore, higher concentrations of ketoconazole eliminated the accumulation of labelled polar sterols and, under these conditions, the inhibition of HMG-CoA reductase was reversed (Gupta *et al.*, 1986). In the present work, we did not observe any changes in the rate of formation of non-saponifiable lipids at high, compared with low, concentrations of ketoconazole. However, it should be noted that, under certain conditions, the maximum capacity of HMG-CoA reductase measured under substrate-saturating conditions in subcellular fractions may change without any similar changes in actual carbon flux through the enzyme in the intact cell (Gibbons *et al.*, 1984). Nevertheless, the inability of ketoconazole to effect any increase in intestinal non-saponifiable lipid synthesis, in contrast with the liver, reflects a difference in the chronic effects of this drug on the control of sterol synthesis in these organs.

Induction of lanosterol 14 α -demethylase activity, cholesterologenesis and HMG-CoA reductase activity

Longer-term treatment of rats with ketoconazole gave rise to an increase in the rate of cytochrome-*P*-450-mediated lanosterol 14 α -demethylation in the liver. The evidence for this came from three types of experiment. First, liver microsomal fractions from rats treated with the drug 24 h previously had an increased capacity for metabolizing lanosterol (Table 3), the first step of which requires cytochrome *P*-450 (Gibbons & Mitropoulos, 1973a; Gibbons *et al.*, 1979). Second, during sterol biosynthesis from [2- ^{14}C]mevalonate in liver homogenates from the livers of animals which had consumed ketoconazole in the diet for the previous 24 h, there was significantly less accumulation of [^{14}C]lanosterol than in the controls (Table 2). Finally, ketoconazole was not so effective in blocking the metabolism of lanosterol *in vivo* in the liver of animals which had been pre-treated with the drug for 2–3 days, compared with those which had not received this type of pre-treatment (Table 1).

It was of interest that 24 h after a single intragastric dose of ketoconazole, when the cytochrome-*P*-450-requiring lanosterol 14 α -demethylase activity was high (Table 3), there was also an increase in the rate of formation of hepatic non-saponifiable lipids *in vivo*

compared with that observed in control animals (Fig. 1). Most of this increase could be accounted for by an increase in the rate of cholesterol synthesis ($138.0 \pm 18.5\%$ of the control; $n = 8$; $P < 0.05$). This was accompanied by increases in both the expressed and the total activities of HMG-CoA reductase (Fig. 1). It appears therefore that there is a positive relationship between the activity of the isoenzyme of cytochrome *P*-450 required for lanosterol demethylation (cytochrome *P*-450_{LDM}) and the rate of carbon flux through HMG-CoA reductase. For instance, both these activities were low 2–12 h after ketoconazole administration, and both became elevated after 24 h (Fig. 1). It may be worth noting that, when rats were fed on diets containing 0.1% cholesterol or 0.1% 7-oxocholesterol for periods longer than 18 h, the original decrease in hepatic HMG-CoA reductase activity was reversed, and this recovery was associated with an increase in hepatic cytochrome *P*-450 (Erickson *et al.*, 1977). Other investigators have also reported increases in hepatic and intestinal microsomal mixed-function-oxidase activity (Reif *et al.*, 1954) and cytochrome *P*-450 (Hietanen & Laitinen, 1978) after cholesterol feeding. It may also be of relevance that, after feeding 7-oxocholesterol to mice for 8 days, conditions under which, on the basis of the above evidence, hepatic cytochrome *P*-450 might be expected to increase, there was a 10-fold increase in the rate of hepatic cholesterol synthesis (Kandutsch *et al.*, 1977).

We thank Mrs. M. Barber for typing the manuscript. Mr. N. Blatchford, Janssen Pharmaceuticals, Grove, Wantage, U.K., kindly provided a sample of ketoconazole. This work was supported by a grant from the Medical Research Council (M.R.C.). G.F.G. is a member of the External Staff of the M.R.C.

REFERENCES

- Akhtar, M., Alexander, K., Boar, R. B., McGhie, J. F. & Barton, D. H. R. (1978) *Biochem. J.* **169**, 449–463
- Boogaard, A., Griffioen, M. & Cohen, L. H. (1987) *Biochem. J.* **241**, 345–351
- Brown, M. S., Goldstein, J. L. & Dietschy, J. M. (1979) *J. Biol. Chem.* **254**, 5144–5149
- Bucher, N. L. R., McGarrah, K., Gould, E. & Loud, A. V. (1959) *J. Biol. Chem.* **234**, 262–267
- Cavenee, W. K., Chen, H. W. & Kandutsch, A. A. (1981) *J. Biol. Chem.* **256**, 2675–2681
- Easom, R. A. & Zammit, V. A. (1984a) *Biochem. J.* **220**, 733–738
- Easom, R. A. & Zammit, V. A. (1984b) *Biochem. J.* **220**, 739–745
- Erickson, S. K., Cooper, A. D., Matsui, S. M. & Gould, R. G. (1977) *J. Biol. Chem.* **252**, 5186–5193
- Gibbons, G. F. (1983a) in *3-Hydroxy-3-methylglutaryl Co-enzyme A Reductase* (Sabine, J. R., ed.), pp. 153–168, CRC Press, Boca Raton, FL
- Gibbons, G. F. (1983b) *Biochem. Soc. Trans.* **11**, 649–651
- Gibbons, G. F. & Mitropoulos, K. A. (1972) *Biochem. J.* **127**, 315–317
- Gibbons, G. F. & Mitropoulos, K. A. (1973a) *Eur. J. Biochem.* **40**, 267–273
- Gibbons, G. F. & Mitropoulos, K. A. (1973b) *Biochem. J.* **132**, 439–448
- Gibbons, G. F., Mitropoulos, K. A. & Pullinger, C. R. (1976) *Biochem. Biophys. Res. Commun.* **69**, 781–789
- Gibbons, G. F., Pullinger, C. R. & Mitropoulos, K. A. (1979) *Biochem. J.* **183**, 309–315
- Gibbons, G. F., Pullinger, C. R., Chen, H. W., Cavenee, W. K. & Kandutsch, A. A. (1980) *J. Biol. Chem.* **255**, 395–400
- Gibbons, G. F., Björnsson, O. G. & Pullinger, C. R. (1984) *J. Biol. Chem.* **259**, 14399–14405
- Gibbons, G. F., Attwell Thomas, C. P. & Pullinger, C. R. (1986) *Biochem. J.* **235**, 19–24
- Gieger, P. J. & Bessman, S. P. (1972) *Anal. Biochem.* **49**, 467–473
- Gupta, A., Sexton, R. C. & Rudney, H. (1986) *J. Biol. Chem.* **261**, 8348–8356
- Hietanen, E. & Laitinen, M. (1978) *Biochem. Pharmacol.* **27**, 1095–1097
- Kandutsch, A. A. & Chen, H. W. (1973) *J. Biol. Chem.* **248**, 8408–8417
- Kandutsch, A. A. & Chen, H. W. (1974) *J. Biol. Chem.* **249**, 6057–6061
- Kandutsch, A. A., Heiniger, H.-J. & Chen, H. W. (1977) *Biochim. Biophys. Acta* **486**, 260–272
- Kandutsch, A. A., Chen, H. W. & Heiniger, H.-J. (1978) *Science* **201**, 498–501
- Panini, S. R., Sexton, R. C., Gupta, A. K., Parish, E. J., Chitrakorn, S. & Rudney, H. (1986) *J. Lipid Res.* **27**, 1190–1204
- Reif, A. E., Brown, R. R., Potter, V. R., Miller, E. C. & Miller, J. A. (1954) *J. Biol. Chem.* **209**, 223–226
- Schroepfer, G. J., Jr. (1981) *Annu. Rev. Biochem.* **50**, 585–621
- Shafiee, A., Trzaskos, J. M., Paik, Y.-K. & Gaylor, J. L. (1986) *J. Lipid Res.* **27**, 1–10
- Taylor, F. R., Kandutsch, A. A., Gayen, A. K., Nelson, J. A., Steckbeck Nelson, S., Phirwa, S. & Spencer, T. A. (1986) *J. Biol. Chem.* **261**, 15039–15044
- Trzaskos, J. M., Bowen, W. D., Shafiee, A., Fischer, R. T. & Gaylor, J. L. (1984) *J. Biol. Chem.* **259**, 13402–13412
- Trzaskos, J. M., Fischer, R. T. & Favata, M. F. (1986) *J. Biol. Chem.* **261**, 16937–16942
- Van den Bossche, H., Willemsens, G., Cools, W., Marichal, P. & Lauwers, W. (1983) *Biochem. Soc. Trans.* **11**, 665–667

Received 20 July 1987/22 September 1987; accepted 6 October 1987