The Isolation of 2,3-Oxidosqualene from the Liver of Rats Treated with 1-Dodecylimidazole, a Novel Hypocholesterolaemic Agent

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1. Non-saponifiable lipid from the livers of rats treated with 1-dodecylimidazole contained an unidentified compound that was not present in the livers from untreated animals. 2. Treated rats had lower serum cholesterol concentrations than control rats. 3. 1-Dodecylimidazole, when added to rat liver slices, inhibited the incorporation of [$1-^{14}\text{C}$]acetate and [$2-^{14}\text{C}$]mevalonate into digitonin-precipitable sterols and resulted in the accumulation of a labelled compound, which was chromatographically identical with the unknown compound described in 1 above. 4. Rats treated with 1-dodecylimidazole incorporated less [^{14}C]mevalonate into liver digitonin-precipitable sterols than untreated animals and accumulated the unknown compound as a labelled intermediate. 5. The unknown intermediate had the same chromatographic properties, n.m.r. and mass spectra as authentic 2,3-oxidosqualene. 6. The identity of the intermediate as 2,3-oxidosqualene was further established by showing that it was incorporated into sterols by rat liver homogenates under anaerobic conditions. In addition, incubation of [^{14}C]squalene with rat liver homogenates resulted in trapping of the radioactivity by the added intermediate. 7. It is suggested that the hypocholesterolaemic activity of 1-dodecylimidazole results in part from the inhibition of cholesterol biosynthesis at the level of 2,3-oxidosqualene sterol cyclase.

The conversion of squalene into lanosterol *in vitro* involves the oxidation of squalene to 2,3-oxidosqualene (van Tamelen *et al.*, 1966; Corey *et al.*, 1966). van Tamelen *et al.* (1966) showed that incubation of racemic 2,3-oxido[^{14}C]squalene with rat liver homogenates yields 24,25-dihydrolanosterol as the major product, and Corey *et al.* (1966) have obtained 2,3-oxido[^{14}C]squalene after incubation of rat liver homogenates with [^{14}C]squalene and non-radioactive 2,3-oxidosqualene. Such observations indicate that 2,3-oxidosqualene is an essential intermediate in the biosynthesis of cholesterol. However, the occurrence of 2,3-oxidosqualene *in vivo* has not so far been demonstrated.

In the present paper we describe the isolation of 2,3-oxidosqualene (I) from the liver of rats treated with a novel hypocholesterolaemic agent, 1-dodecylimidazole.

Materials and Methods

Chemicals

All organic solvents were distilled before use. 1-Dodecylimidazole was prepared as described by Buchel & Falbe (1968) and 2,3-oxidosqualene as described by Willett *et al.* (1967).

Sodium [$1-^{14}\text{C}$]acetate and [$2-^{14}\text{C}$]mevalonolactone were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium [$2-^{14}\text{C}$]mevalonate was prepared from the lactone by the addition of NaOH until the pH remained slightly alkaline (pH 7-8). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Cholesterol was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and lanosterol from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Hepatic cholesterol synthesis *in vitro*

Liver slices. Slices approx. 0.5 mm thick were cut with a Stadie-Riggs microtome. The slices (250 mg) were incubated at 37°C in Erlenmeyer flasks for 3 h with 10 μmol (2 μCi) of sodium [$1-^{14}\text{C}$]acetate or for 2 h with 2.5 μmol (0.5 μCi) of DL-[$2-^{14}\text{C}$]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.

After incubation, the contents of each flask were saponified at 70°C with 5ml of 15% (w/v) KOH in 90% (v/v) ethanol for 30min. The non-saponifiable fraction was extracted three times into 20ml of light petroleum (b.p. 40–60°C) and the digitonin-precipitable sterols were isolated as described by De Matteis (1968). The digitonides were dissolved in methanol and a portion was taken for liquid-scintillation counting. Sterols, prenol pyrophosphates and prenoic acids were isolated from the non-saponifiable fraction by the method of Goodman & Popják (1960). Squalene, 2,3-oxidosqualene and oxo steroids were isolated by t.l.c.

Liver homogenates. Homogenates of the livers of male rats (180–200g) were prepared essentially by the method of Bucher & McGarrah (1956). All operations were done at 2–4°C and all equipment was pre-cooled in ice. The livers of three rats were passed through a stainless-steel mincer and homogenized by three passes in a Potter–Elvehjem homogenizer with a loose-fitting Teflon pestle. The homogenization was done in small batches with 2.5ml of buffered medium/g of tissue. The buffered medium was the same as that used for the liver slices. The coarse tissue homogenate was centrifuged at 18000g for 20min at 4°C to give the 18000g supernatant.

Incubations were for 2h, in a shaking incubator at 37°C, in Erlenmeyer flasks containing 3ml of the 18000g supernatant in either air or N₂, according to the requirements of the experiment. Substrates were added to the incubation flasks in solution in benzene with 100μl of propylene glycol before the addition of the other components. On evaporation of the benzene the added substrate was distributed as a thin film in solution in the propylene glycol. Water-soluble components were then added as aqueous solutions immediately before the incubation. The NADPH-generating system consisted of 2μmol of NADP, 5μmol of glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase, in a total volume of 0.5ml. The incubations were terminated by adding 3.5ml of 15% (w/v) KOH in aq. 90% (v/v) ethanol. The non-saponifiable fraction was isolated as described above.

Hepatic cholesterol synthesis in vivo

Male rats (80–100g) of the CFY strain were obtained from Carworth Europe, Alconbury, Hunts., U.K. The rats were fed with a stock pelleted diet (diet FFG, E. Dixon and Sons, Ware, Herts., U.K.) for 10 days. During that period each rat received a daily dose, by stomach tube, of either 1-dodecylimidazole (150mg/kg body wt.) dissolved in arachis oil (150mg/ml) or arachis oil alone (1ml/kg body wt.). Each rat was given 5μCi of sodium [2-¹⁴C]mevalonate (10.3μCi/μmol) in 0.1ml of 0.9% NaCl by intraperitoneal injection, 1h after the final oral dose of

1-dodecylimidazole. All rats were killed exactly 1h after receiving the mevalonate. Blood was taken from the jugular vein and the liver was removed and analysed for lipid as follows.

Liver samples were saponified, under reflux, with a mixture of 0.5ml of aq. 60% (w/v) KOH and 1.0ml of ethanolic 1% (w/v) pyrogallol/g of tissue. The non-saponifiable fraction was extracted four times into light petroleum (b.p. 40–60°C) and a portion of the solution was taken for liquid-scintillation counting.

Analytical techniques

Preparative chromatography of non-saponifiable lipids. Weighed non-saponifiable fractions were applied (in light petroleum solution) to columns of acid-washed alumina (Woelm), deactivated to Brockman grade III by the addition of water. Increasing concentrations of ether in light petroleum (b.p. 40–60°C) were used for elution (10ml/g of alumina). The fractions obtained were further purified by t.l.c.

Thin-layer chromatography. T.l.c. was done on glass plates coated with 0.4mm of silica gel G by using either 5% (v/v) ethyl acetate in hexane or 25% (v/v) ethyl acetate in hexane as the developing solvent. Plates were pre-run in developing solvent before use. Compounds were detected by spraying with anisaldehyde-sulphuric acid reagent (McSweeney, 1965; Dunphy *et al.*, 1966). Authentic 2,3-oxidosqualene gave a characteristic yellow-brown colour with this reagent. Zones corresponding to reference compounds were scraped off the plate and eluted with ether. A portion of each was taken for liquid-scintillation counting, when required.

Radioactivity and spectroscopy. Radioactivity measurements were performed with an Intertechnique ABAC SL40 liquid-scintillation spectrometer, the scintillation fluid consisting of 10ml 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. Correction for quenching was made by the external standard channels-ratio method.

N.m.r. spectra were recorded with a Varian A60 magnetic-resonance spectrometer with trimethylsilane as an internal reference. Mass spectrometry was carried out on an AEI MS9 mass spectrometer at an ionizing voltage of 70eV. The temperature of the ion source was 130°C.

Serum cholesterol. Total cholesterol in serum and liver were measured by the Technicon AutoAnalyzer method N24A (Technicon Instruments Corp., 1965) as modified by Seigel & Bowdoin (1971).

Results

Detection of unknown intermediate

T.l.c. of the non-saponifiable lipid from livers of rats treated with 1-dodecylimidazole showed the

presence of an unidentified compound that was not present in the livers of control animals. The R_F value for this compound did not correspond to those of any common intermediates of cholesterol biosynthesis. Further experiments were designed to identify this intermediate.

Hepatic biosynthesis of cholesterol

Effect of 1-dodecylimidazole in vitro. 1-Dodecylimidazole (0.1 mM) added to the liver slice preparation inhibited, by 90%, the incorporation of both [$1-^{14}C$]acetate and [$2-^{14}C$]mevalonate into digitonin-precipitable sterols. The incorporation of [$2-^{14}C$]mevalonate

into the different lipid classes is shown in Table 1. The distribution pattern shows that the addition of 1-dodecylimidazole to liver slices resulted in a large accumulation of radioactivity in the zone corresponding to authentic 2,3-oxidosqualene.

Effect of 1-dodecylimidazole in vivo. Rats dosed with 1-dodecylimidazole had significantly lower serum cholesterol concentrations than untreated animals (49 ± 3.2 mg/100 ml and 74 ± 0.3 mg/100 ml respectively; $P < 0.001$). Further, the incorporation of [$2-^{14}C$]mevalonate into digitonin-precipitable sterols *in vivo* was considerably decreased and, in contrast with the experiments *in vitro*, incorporation into the total non-saponifiable fraction was increased (Table

Table 1. *Effect of 1-dodecylimidazole on the incorporation of [$2-^{14}C$]mevalonate into liver lipid fractions in vitro*

Rat liver slices were incubated with potassium phosphate buffer (5 ml) and [$2-^{14}C$]mevalonate ($2.5 \mu\text{mol}$, $0.5 \mu\text{Ci}$) for 2 h at 37°C . 1-Dodecylimidazole was added to a final concentration of 0.1 mM. The different lipid classes were isolated as described in the text.

Lipid class	Incorporation of [$2-^{14}C$]mevalonate (%)	
	Control	With 1-dodecylimidazole
Total non-saponifiable material	16.50	11.00
Digitonin-precipitable sterols	12.25	0.85
Squalene	1.24	2.09
2,3-Oxidosqualene	0.17	3.64
α -Oxo steroids*	0.17	0.11
Prenol pyrophosphates	2.25	5.80
Prenoic acids	1.6	0.9

* Substances having the same R_F as marker 5α -cholestan-4-one.

Table 2. *Effect of 1-dodecylimidazole on incorporation of [$2-^{14}C$]mevalonate into liver lipids of rats*

Two groups of rats (ten rats/group) were fed with a stock pelleted diet for 10 days. During that period each rat received a daily dose, by stomach tube, of either 1-dodecylimidazole (150 mg/kg body wt.) dissolved in arachis oil (150 mg/ml) or arachis oil alone (1 ml/kg body wt.). Each rat was given $5 \mu\text{Ci}$ of sodium [$2-^{14}C$]mevalonate by intraperitoneal injection 1 h after the final oral dose. The animals were killed 1 h after receiving the mevalonate and liver lipids were isolated as described in the Materials and Methods section. Mean values are given $\pm$ S.E.M. * $P < 0.001$ (Student's t test for difference between control and drug-treated groups).

Lipid class	$10^{-3} \times$ Radioactivity (d.p.m./g of liver)	
	Control	With 1-dodecylimidazole
Non-saponifiable material	65.37 ± 3.54	$95.92 \pm 4.18^*$
Digitonin-precipitable sterols	51.77 ± 3.32	35.47 ± 5.01
Squalene	6.00 ± 1.11	7.88 ± 1.08
2,3-Oxidosqualene	0.24 ± 0.08	$27.21 \pm 4.12^*$
Lanosterol-containing	1.99 ± 0.18	$6.68 \pm 0.07^*$
α -Oxo steroids†	0.31 ± 0.07	$0.69 \pm 0.13^*$
Prenol pyrophosphates	0.48 ± 0.04	$0.67 \pm 0.02^*$
Prenoic acids	12.63 ± 0.97	12.19 ± 0.94

† Substances having the same R_F as marker 5α -cholestan-4-one.

2). T.l.c. of the non-saponifiable fraction from livers of treated rats showed an accumulation of a radioactive compound that was not present in the control animals. This compound had the same R_F value as authentic 2,3-oxidosqualene.

The non-saponifiable fraction from the livers of ten rats was chromatographed on columns of alumina. The column fractions were monitored by t.l.c. and the unknown intermediate was eluted with 1% (v/v) ether in light petroleum (b.p. 40–60°C). The fraction was rechromatographed on t.l.c. and yielded 2mg of unknown compound that was suitable for analysis.

The results from mass and n.m.r. spectra were in agreement with those reported by Willett *et al.* (1967) for 2,3-oxidosqualene. The mass spectrum showed a molecular-ion peak of $m/e = 426$, with major fragments of $m/e = 357, 203, 191, 189, 177, 175$ and 163. The n.m.r. spectrum showed peaks at 1.18, 1.21, 1.58, 1.65 and 1.97 p.p.m.

Further identification of the radioactive intermediate

[^{14}C]Squalene (5.14×10^4 d.p.m., 580 μg) and the ^{14}C -labelled intermediate (8.24×10^4 d.p.m., 214 μg) obtained from animals given 1-dodecylimidazole were incubated with the rat liver homogenates under both anaerobic and aerobic conditions. The products of these incubations were isolated by saponification and extraction with light petroleum (b.p. 40–60°C), followed by t.l.c. in 25% (v/v) ethyl acetate in hexane. The 2,3-oxidosqualene (R_F 0.7) and squalene (R_F 0.8) were clearly separated from lanosterol (R_F 0.44) and cholesterol (R_F 0.31). [^{14}C]Squalene was incorporated into sterols only under aerobic conditions whereas the ^{14}C -labelled intermediate was incorporated into sterols under both aerobic and anaerobic conditions (Table 3).

[^{14}C]Squalene (9.45×10^5 d.p.m., 100 μg), biosynthetically labelled from [$2\text{-}^{14}\text{C}$]mevalonic acid, was incubated with rat liver homogenates in the presence of an NADPH-generating system under an atmo-

sphere of air. Six replicate incubations were done both with and without the addition of 2mg of the unlabelled intermediate, which had been obtained from rats dosed with 1-dodecylimidazole as described above. The products of the incubations were isolated by saponification and extraction with light petroleum (b.p. 40–60°C) followed by t.l.c. in 5% (v/v) ethyl acetate in hexane. The bands corresponding to squalene (R_F 0.8), 2,3-oxidosqualene (R_F 0.6) and total sterols (R_F 0–0.1) were removed from the plates and assayed for radioactivity. Recoveries of 80% of the total initial radioactivity were achieved. The distribution of recovered activity, in the experiments in which 2mg of the unlabelled intermediate was present, was: squalene (73.9%), sterols (7.6%), 2,3-oxidosqualene (3.1%). In the incubations from which the unlabelled intermediate was omitted, there was a markedly lower incorporation of squalene into 2,3-oxidosqualene (0.4%) and an enhanced incorporation into the total sterol fraction (11.8%), whereas the recovery of the unchanged squalene was 61.7%. These values are in good agreement with those obtained by Willett *et al.* (1967) in a similar experiment with chemically prepared 2,3-oxidosqualene.

Discussion

Hypocholesterolaemic agents can act in a variety of ways, one of which is by inhibition of cholesterol biosynthesis. 1-Dodecylimidazole is a novel hypocholesterolaemic agent, which we have shown to be effective in the rat and other species (B. Morgan, unpublished work). It is a marked inhibitor of hepatic cholesterol synthesis both *in vivo* and *in vitro* and this may account for its biological activity. The results reported here have shown that 1-dodecylimidazole inhibits cholesterol biosynthesis at a stage later than mevalonic acid, resulting in an accumulation of an intermediate not observed in untreated animals. This compound, which has been shown on the basis of both

Table 3. *Incorporation of squalene and the unknown intermediate into sterols by rat liver homogenates in vitro*

Homogenate preparations (3ml) were incubated with either [^{14}C]squalene or ^{14}C -labelled unknown intermediate for 1 h at 37°C in a final volume of 3.5 ml. NADPH was generated with NADP $^+$ (2 μmol), glucose 6-phosphate (5 μmol) and glucose 6-phosphate dehydrogenase (1 unit). The gas phase was as indicated. The incorporation of ^{14}C into the lanosterol-containing region and cholesterol were measured as described in the text.

^{14}C -labelled substrate	Gas phase	Incorporation (d.p.m.)	
		Lanosterol-containing	Cholesterol
Squalene	N $_2$	340	258
Unknown intermediate	N $_2$	23588	838
Squalene	Air	2476	2274
Unknown intermediate	Air	7920	10035

physical and biochemical evidence to be 2,3-oxidosqualene, is a recognized intermediate of cholesterol biosynthesis (Yamamoto & Bloch, 1970). However, 2,3-oxidosqualene has not previously been found *in vivo*.

Many other compounds are known to inhibit cholesterol biosynthesis at different points in the pathway, e.g. AY 9944 [*trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] blocks the conversion of 7-dehydrocholesterol into cholesterol (Dvornik *et al.*, 1963) and triparanol [1-(4-diethylaminoethoxy)phenyl)-1-(*p*-chlorophenyl)-ethanol] inhibits the final step in cholesterol biosynthesis, demosterol to cholesterol (Avigan *et al.*, 1960). As far as we are aware no other compound has previously been shown to interfere with cholesterol biosynthesis at the level of 2,3-oxidosqualene. Possibly this action results from inhibition of 2,3-oxidosqualene cyclase and 1-dodecylimidazole may thus prove to be a useful tool in studying sterol biosynthesis.

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