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THE *IN VIVO* METABOLISM OF CHOLESTEROL BY GUT BACTERIA IN THE RAT AND GUINEA-PIG

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SUMMARY

Cholesterol introduced into the colon of guinea-pigs is metabolized to oestradiol, which is then excreted in the urine. This conversion is also carried out by gut bacteria *in vitro*; the *in vivo* reaction is abolished by pre-treatment of the animals with antibiotics which suppress the gut bacterial flora. When cholesterol is introduced into the caecum of rats very little is converted to metabolites excreted in the urine, virtually all of the metabolites being faecally excreted. Lithocholic and iso-lithocholic acids accounted for 6% of the excreted ^{14}C. It is concluded that in the guinea-pig the gut bacterial flora completely removes the cholesterol side chain whereas in the rat only a 3-carbon unit is removed.

INTRODUCTION

We have demonstrated that cholesterol can be modified by gut bacteria *in vitro*, the three types of modification being (a) nuclear hydrogenation to give coprostanol [1] or dehydrogenation to give a phenolic steroid [2] (b) oxidation of the hydroxyl to an oxo group (and also the reverse reaction), and (c) side-chain cleavage to yield C-24 acid steroids and ultimately complete removal of the side-chain [3]. It is widely accepted that reactions (a) and (b) take place *in vivo* but side-chain cleavage by gut bacteria has still to be established.

It has been demonstrated that cholesterol and related steroids are not absorbed from the large intestine [4] and consequently analysis of the metabolites produced following the introduction of labelled cholesterol into the caecum of an animal should indicate whether such side-chain cleavage reactions take place *in vivo*.

In this paper we describe such experiments in the rat and guinea-pig. Since the products of side-chain cleavage may be absorbed from the gut the urinary metabolites were examined, as was the effect of antibiotic treatment.

MATERIALS AND METHODS

Materials

[C_4-^{14}C]-Cholesterol was obtained from the Radiochemical Centre, Amersham, Bucks; all other steroids were supplied by Koch–Light Ltd., Colnbrook, Bucks. The labelled cholesterol was tested for purity by t.l.c. as described below. It ran as a single spot and therefore contained no coprostanol, coprostanone, 4-cholesten-3-one, acid steroids or oestrogens. The solvent systems used would not have revealed the presence of any cholest-5α-an-3β-ol. The enzymes sulphatase (ex. *Helix Pometia*) *and* β-glucuronidase (type 1, ex. *Escheria. coli*) were from Sigma Chemicals London. The α-naphthylamine, 2,5-diphenyloxazole (PPO) and 1,4-di-(2,5-phenyloxazoyl)-benzene (POPOP) used in scintillation studies were obtained from Koch–Light Ltd., whilst the ethanediol and dioxan were analytical grade from British Drug Houses, Poole, Dorset. Insta-gel and Soluene-100 were from Packard Instruments Ltd., Warrenville, Illinois. All other reagents and solvents were of reagent grade unless otherwise stated.

Analytical methods

Thin-layer chromatography (*t.l.c.*). Polygram Sil G/UV_{254} prepoured t.l.c. plates (Camlab, Cambridge) were activated at 110°C for 30 min. prior to development with solvent systems of benzene–dioxan–water (100:10:1 by vol.) to separate phenolic steroids, hexane–chloroform–acetone (10:9:1 by vol.) to separate neutral steroids, and the solvents S1 and S10 of Eneroth[5] to separate acid steroids. These contain benzene–dioxan–acetic acid (75:20:2 by vol.) and trimethylpentane–iso-propanol–acetic acid (60:20:0·5 by vol.) respectively. The purity of the labelled cholesterol was determined using the solvent systems benzene–dioxan–acetic acid (100:10:1 by vol.) and chloroform–methanol–hexane (90:1:1 by vol.). The steroids were visualized using 10% phosphomolybdic acid in ethanol (a non-specific spray) or potassium ferricyanide reagent (for phenolic steroids) whilst labelled products were

located using a Panax radioactive scanner fitted with a propane argon detector.

Spectrophotometric analysis

Ultraviolet absorption spectra of solutions in ethanol (B.D.H "Aristar" grade) were obtained using a Unicam SP 8000 recording spectrophotometer. Infrared spectra of solutions in carbon disulphide (B.D.H "Aristar" grade) were measured in potassium bromide cells (path length 1 mm) using a Unicam SP 1200 recording spectrophotometer.

Mass spectra

Mass spectra from products purified by preparative t.l.c. were obtained using a Varian CH5 mass spectrometer at an operating temperature ranging from 80–150°C and with an ionizing energy of 70 eV.

Liquid scintillation

Liquid scintillation counting was carried out using a Packard TriCarb liquid scintillation counter (Packard Instruments Co., Warrenville, Illinois) and corrected for quenching by the internal channels ratio method of Bush[6]. All samples were dissolved or suspended in Bray's scintillation fluid [7] unless otherwise specified.

Animal experiments

Guinea-pigs (300 g) or Sprague–Dawley rats (250 g) were anaesthetized with ether and a short incision was made longitudinally in the lower middleright part of the abdomen to expose the caecum. Labelled cholesterol, at a dose of 2–10 μCi and dispersed in sterile saline, was injected into the proximal end of the caecum. The incision was closed and the animals allowed to recover then placed in "Metabowl" metabolic cages (Jencons, Hemel Hempstead). Urine and faeces were collected for 72 h. and the animals were then sacrificed and the liver, gall bladder of the guinea-pig (together with the bile) and the contents of the caecum, colon and rectum were removed. A sample of heart blood was also taken.

Antibiotic treated guinea-pigs were given a mixture of clindamycin (10 mg), gentamycin (2 mg) and nystatin (2 mg) in 1 ml distilled water by gastric intubation 72 h. and 24 h. before the introduction of labelled cholesterol into the caecum.

Analysis of samples

Intestinal contents. The steroids present in the faecal, caecal and colonic contents were extracted and separated into acid and neutral fractions as described by Hill and Aries[8]. Both fractions were redissolved in 10 ml choroform and a sample (0·5 ml of the acid steroid fraction and 0·1 ml of the neutral steroid fraction) was transferred to a liquid scintillation vial. The solvent was removed by evaporation (since it has high quenching properties in liquid scintillation) and the residue redissolved in 15 ml Bray's scintillation fluid then counted.

Urine

Urine samples were counted directly following the addition of 0·5 ml urine to 15 ml scintillation fluid. In addition, urine samples were hydrolysed by boiling with an equal volume of 2 N NaOH for 1 h. acidified with HCl, then extracted with 1 vol. of chloroform–methanol (2:1 v/v) followed by 1 vol. of chloroform. The extracts were pooled, evaporated to dryness then redissolved in 15 ml scintillation fluid and counted.

Urinary steroid conjugates were hydrolysed with sulphatase and β-glucuronidase at pH 5·5. The sample dissolved in 0·01 M phosphate buffer solution, pH 5·5, was incubated with β-glucuronidase at 37°C, and the release of free steroid determined by thin-layer chromatography followed by radioactive scanning as described above. Incubation was continued until no further free steroid was released. Sulphatase was then added and the mixture was again incubated until no further conjugate was hydrolysed.

Bile

Bile was counted directly following the addition of 15 ml scintillation fluid to the whole contents of the gall-bladder.

Blood

A blood sample (0·1 ml) was mixed with 1·5 ml Soluene-100–isopropanol mixture (1:1 v/v) then oxidised by the addition of 0·5 ml of 30% H_2O_2. After gently shaking for 1 h. to remove the colour 15 ml of a 0·5 N HCl–Insta-gel mixture (12:88 v/v) was added, the mixture gently shaken and counted.

Liver

After homogenization in 10 vol. of water using a Citenco homogenizer (M.S.E.), 0·4 ml of homogenate together with 0·8 ml water and 10 ml Insta-gel were shaken together gently then counted.

RESULTS

The fate of [^{14}C]-cholesterol introduced into the caecum

[^{14}C]-cholesterol (5 μCi) was introduced into the caecum of rats and guinea-pigs and the faeces and urine collected for 72 h. In all 94% of the total dose was recovered from the rats and 90% from the guinea-pigs (Table 1), the majority being excreted in the faeces in

Table 1. The percentage distribution of ^{14}C in the faeces and urine after the introduction of labelled cholesterol into the caecum of 7 rats and 5 guinea-pigs

	Rats (%)	Guinea-pigs (%)
No. of animals	7	5
Faeces neutral steroids	87·7 ± 4·6	88·2 ± 4·4
Acid steroids	6·3 ± 4·2	0·8 ± 0·3
Urine	0·3 ± 1·8	1·7 ± 0·4
Total recovered	94·3 ± 2·0	90·7 ± 0·4

the neutral steroid fraction. In the rat there is also considerable metabolism to acid steroids whilst in the guinea-pigs although there was only a small acid steroid fraction there was a relatively large amount of ^{14}C excreted in the urine.

More than 90% of the recovered activity was excreted in the first 48 h, whilst animals kept longer than 72 h excreted negligible further amounts of label. At 72 h the amount of ^{14}C in the caecum and colon was negligible.

Analysis of the neutral steroid fraction

The neutral steroid fractions were analysed by t.l.c. and the components quantitated by integrating the peaks obtained from the radioactive scans (Table 2). There were three major components in the faecal neutral steroids (mobility relative to cholesterol = 0·00, 1·00 and 1·28) and two minor components (relative mobility 1·81 and 2·09).

The two minor components co-chromatographed with authentic samples of 5β-cholestan-3-one and 4-cholesten-3-one respectively; the latter component had a UV absorption maximum at 244 nm (E = 15,000) corresponding to a 4-en-3-one.

The component of relative mobility 1·28 co-chromatographed with 5β-cholestan-3β-ol(coprostanol). When purified by preparative t.l.c. the isolated compound had a melting point of 99–101°C, identical to that of authentic coprostanol and to the mixed melting point. The metabolite had an I.R. spectrum identical to that of authentic coprostanol, with a triplet of sharp peaks at 3·25, 3·30 and 3·35 μm and two broader peaks at 3·40 and 3·60 μm.

Table 2. Percentage composition of the neutral steroid fraction

	Rats (%)	Guinea-pigs (%)
5-Cholesten-3β-ol (cholesterol)	35·0	26·7
5β-Cholestan-3β-ol (coprostanol)	57·3	69·0
5β-Cholestan-3-one (coprostanone)	2·0	0·0
4-Cholesten-3-one	5·7	4·3

The component with relative mobility 1·00 was unchanged cholesterol (melting point 147–150°C); that which remained at the origin was not identified but appeared to be a non-steroidal product of cholesterol metabolism accounting for as much as 24% of the total "neutral steroid" fraction in one case, although it was absent from guinea-pig faeces.

The neutral steroid fraction from guinea-pig faeces contained a higher proportion of coprostanol than did rat faeces but contained no coprostanone.

Analysis of the faecal acid steroids

The rat faecal acid steroid fraction, when analysed by t.l.c. using solvent S1, contained a single component remaining at the origin. When incubated with sulphatase two metabolites were released, referred to as A and B; metabolite A was present at roughly half the concentration of B and their relative mobilities in S1 were 1·00 and 1·15 respectively relative to lithocholic acid. The two metabolites were identified as lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) and isolithocholic acid (3β-hydroxy-5β-cholan-24-oic acid) by their t.l.c. behaviour in solvents S1 and S10 and the mass spectra of their methyl esters.

Quantitative t.l.c. (from the areas under the peaks of the radio-chromatogram scans) indicated that of the original dose of cholesterol introduced into the caecum 1·8% was excreted as lithocholic acid sulphate and 3·5% as isolithocholic acid sulphate. About 15% of the total acid steroid fraction was resistant to sulphatase and β-glucuronidase and remained at the origin on t.l.c.; this material was not identified.

Very little acid steroid metabolite was excreted by the guinea-pigs (Table 1) and this was not identified.

Analysis of the urinary metabolites

The radioactive material in rat urine could not be extracted into chloroform even after extensive hydrolysis with acid and with enzymes. Due to the low level of activity in the urine no attempt was made to identify the metabolites of cholesterol present.

Incubation of guinea-pig urine with sulphatase and β-glucuronidase followed by acidification and extraction with chloroform removed between 30–45% of the activity. Analysis of this extract by t.l.c. revealed the presence of two components with the mobility of oestradiol and oestrone. Both reacted with the phenol-specific ferricyanide spray reagent and their identity was confirmed by mass spectrometry in comparison with authentic compounds. Both metabolites were

present in approximately equal amounts and each represented less than 1% of the total dose.

Effect of antibiotics

Two guinea-pigs were treated with antibiotics before the introduction of 5 μCi of labelled cholesterol into the caecum. Urine and faeces were then collected for 72 h. Two control guinea-pigs were treated identically except that the antibiotic cocktail was replaced by distilled water. Antibiotic treatment resulted in a 5-fold reduction in the urinary excretion of ^{14}C.

Enterohepatic circulation of ^{14}C following introduction of labelled cholesterol into the caecum

Two guinea-pigs were sacrificed 24 h. after the introduction of 10 μCi labelled cholesterol into their caecum. The amount of label in the urine and faeces accounted for 55% of the dose and a further 35% of the activity was recovered from the caecum and large intestine. Negligable counts were obtained in the blood and bile but the liver contained small amounts of activity accounting for about 0·5% of the total dose.

DISCUSSION

In this paper we report the isolation of two known products of microbial cholesterol side-chain degradation—the bile acids lithocholic and isolithocholic acid in the rat and the oestrogens oestrone and oestradiol in the guinea-pig.

In the guinea-pig urinary oestrogens derived from caecal cholesterol represented 1·7% of the administered dose. This could not be due to the action of mammalian enzymes because: (a) cholesterol is very poorly absorbed, if at all, from the large bowel [4]. The very low concentrations of ^{14}C in the serum, bile and liver support this conclusion; (b) only a minute proportion of the serum cholesterol pool is metabolized to urinary oestrogens in a 72 h. period. Thus even if all of the cholesterol not recovered from the faeces (i.e. 9·3% of the dose) had been absorbed undegraded it is inconceivable that 18% of this should then be converted to oestrogens and excreted in the urine so rapidly. We conclude, therefore that the oestrogens were produced by the action of the gut bacteria and were then absorbed, conjugated by hepatic enzymes and subsequently excreted in the urine. In support of this conclusion, it has already been demonstrated that the gut flora can metabolize cholesterol to oestradiol and oestrone *in vitro* [2]. Treatment of the animals with an antibiotic mixture to suppress the gut flora resulted in a dramatic reduction in the urinary excretion of oestrogens derived from caecal cholesterol.

Very little radioactive material was found in the rat urine; any oestrogens produced by microbial action would in any case be excreted in the faeces since this is the route of their excretion in the rat [9]. However in the rat there is other evidence of microbial side-chain cleavage of cholesterol in the form of the sulphates of lithocholic and isolithocholic acid. The arguments in favour of these being the products of microbial action rather than of mammalian enzymes are the same as those used above for the production of urinary oestrogens in the guinea-pig. Norman and Palmer[10] have shown the presence of lithocholic acid sulphate in human faeces and Baulieu[11] has presented evidence that sterol sulphation may occur in the intestinal mucosa. Since lithocholic acid is so poorly absorbed from the large bowel we consider it likely that the sulphates reported here were produced by mucosal rather than hepatic enzymes. The sequence would then be for the flora to remove a 3-carbon unit from coprostanol or cholesterol to yield iso-lithocholic acid which then undergoes partial inversion of the 3-hydroxyl group to give lithocholic acid: both acids would then be sulphated prior to excretion.

In these experiments we have evidence that the gut flora can cleave or remove the cholesterol side-chain *in vitro* and that in the guinea-pig this is accompanied by, or followed by, aromatization of ring A. However there is a clear species difference between the two animals studied and consequently these results give no guidance on whether these reactions take place in the human large bowel.

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