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EFFECTS OF NEOMYCIN ON THE BILIARY EXCRETION AND ENTEROHEPATIC CIRCULATION OF MESTRANOL AND 17β-OESTRADIOL

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Abstract—The biliary excretion of oestradiol and mestranol in the female rat is rapid. The continued circulation of the free steroids depends on their reabsorption from the gut following the hydrolysis of biliary conjugates. The antibiotic neomycin markedly inhibits this enterohepatic recirculation by directly affecting the viability of the gut microflora which are partly responsible for deconjugation, and also by inhibiting reabsorption of the free steroid. These effects may be important in considering the half-life of oestrogenic components of the contraceptive pill.

Mestranol (17α-ethynyloestradiol-3-methyl ether) has found wide application as the oestrogenic factor in many combination type contraceptive formulations. Its metabolism has been studied by Bolt and Remmer [1–3] and by Fotherby [4].

Ethynyloestradiol (the active metabolite of mestranol) undergoes extensive enterohepatic circulation [5], which is probably important in determining its effectiveness. Consequently factors affecting the hydrolysis and subsequent reabsorption of biliary conjugates in the gut must be equally important. The latter seems to have received little attention.

The gut microflora undoubtedly contribute to the hydrolytic enzymes present in the gut [6] and therefore suppression of the flora may modify the degree of enterohepatic circulation and consequently impair the efficacy of the oral contraceptive. This report describes the effect of neomycin on the enterohepatic circulation of mestranol and 17β-oestradiol in an attempt to assess the importance of the gastrointestinal microflora in the metabolism of these oestrogens.

MATERIALS AND METHODS

Mestranol (4-^{14}C), sp. act. 59.8 mCi/m-mole, was obtained from New England Nuclear, Boston, U.S.A. Oestradiol (4-^{14}C), sp. act. 56.7 mCi/m-mole was obtained from the Radiochemical Centre, Amersham, England. Neomycin sulphate was obtained from the Sigma Chemical Co., and Ketodase from Warner-Chilcott, Eastleigh, England.

Female Wistar albino rats (220–240 g) were used in all experiments. During surgery the bile duct of each animal was cannulated using a standard length (50 cm) or Portex PP25 tubing (Portex Ltd., Hythe, England). Animals receiving labelled steroids (0.25 μCi) or labelled bile intraduodenally also had the duodenum fitted with a cannula connected to a dosing syringe. All cannulae were exteriorised and bile collection commenced after suturing. Bile was collected as half hourly samples, and aliquots suspended in 4 ml Synperonic NXP/toluene/PPO scintillant for counting [7]. Anaesthesia was both achieved and maintained with Nembutal.

For monitoring the hydrolysis, reabsorption and subsequent biliary excretion of mestranol and oestradiol biliary conjugates, the following procedure was adopted. The 30–90 min bile samples collected from animals given labelled mestranol or oestradiol by i.p. injection were separately combined and diluted with 0.9% saline such that 1 ml contained approximately 50,000 dis/min. These aliquots were then administered to new subjects via an i.d. cannula followed by 0.25 ml 0.9% saline. Bile from animals dosed in this manner was collected as previously described. Radioactive content of this bile was taken as direct evidence of enterohepatic recirculation. The administered bile was checked before use for its free steroid content by extraction with diethyl ether (3 × 8 vol.) at pH 5.0. This process readily extracts free steroids. It was found in all experiments that the bile samples always contained >98% of the radioactivity in conjugated form.

For experiments involving neomycin, rats received oral neomycin sulphate (100 mg in 0.2 ml water) twice daily for 4 days and once on the fifth day 1–2 hr prior to the experiment.

For *in vitro* studies designed to assess the ability of rat gastrointestinal microorganisms to deconjugate mestranol and oestradiol conjugates, incubations with samples of rat caecal contents were performed by methods similar to those used by Scheline [8]. Thirty to 90 min oestradiol and mestranol bile samples were pooled as described earlier. Incubates consisted of labelled bile (200 μl), 10% (v/v) suspension of rat caecal contents (100 μl), and incubation medium (10 ml) containing 0.6% yeast extract, 0.6% peptone and 0.6% D-glucose in 0.1 M phosphate buffer, pH 6.9. The volume of caecal contents was minimised since extracellular enzymes were likely to be present [9]. Incubations were carried out under anaerobic conditions in 25 ml Thunberg tubes for 40 hr at 37°. Incubates were

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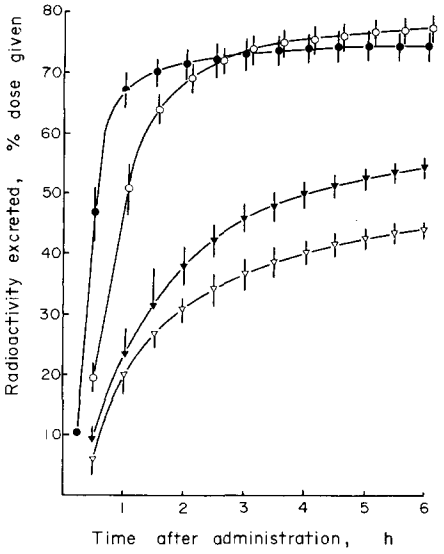

Fig. 1. Biliary excretion of radioactivity after i.p. and i.d. administration of [4-^{14}C]mestranol (0.25 μCi) or [4-^{14}C]-oestradiol (0.25 μCi) to female rats. Results are expressed as percentage of administered dose. ●—Intraduodenal oestradiol; ○—Intraduodenal mestranol; ▼—Intraperitoneal oestradiol; ▽—Intraperitoneal mestranol. Points represent the mean of 3 or 4 experiments (S.E.M. shown). Mean bile flow rates (±S.E.M.) were: intraduodenal oestradiol 763 ± 85 μl/hr; intraduodenal mestranol 738 ± 62 μl/hr; intraperitoneal oestradiol 585 ± 26 μl/hr; intraperitoneal mestranol 593 ± 185 μl/hr.

then adjusted to pH 5.0 with 1N HCl and deconjugated material determined in the ether extractable fraction (3 × 10 ml extractions).

Incubations of bile with β-glucuronidase (Ketodase) were performed to determine the proportion of glucuronides present. Appropriate controls were run with each incubation to determine the amount of background hydrolysis.

RESULTS

Biliary excretion of mestranol and 17β-oestradiol. The biliary excretion of [4-^{14}C]mestranol and [4-^{14}C]-17β-oestradiol by intraduodenal (i.d.) and intraperitoneal (i.p.) routes is shown in Fig. 1. Mestranol is cleared slightly slower following both routes of administration. More striking than this is the considerable difference in the rate of excretion of both steroids between i.d. and i.p. administration. Ninety min after dosing with the radiolabelled steroids the amount of radioactivity recovered in the bile was 25–35% for the i.p. route and 65–70% for the i.d. route.

Effect of neomycin pretreatment on the enterohepatic recirculation of mestranol and oestradiol. As can be seen in Figs. 2 and 3 a very significant reduction in the degree of enterohepatic circulation was observed for oestradiol and mestranol after neomycin pretreatment. After 8 hr there was approximately a 50 per cent reduction for both steroids. It is interesting to note that for both steroids the rate of excretion over the first 3 hr is similar in both untreated and neomycin-treated animals.

Hydrolysis of steroid conjugates by rat caecal microorganisms in vitro. The ability of rat caecal microorganisms to hydrolyse the biliary conjugates of mestranol and oestradiol is shown in Table 1. The degree of hydrolysis may be taken as the difference between the steroids extractable from untreated bile at pH 5.0 and that extractable at pH 5.0 after *in vitro* incubation with rat caecal microorganisms. Incubations after the microorganisms had been destroyed by boiling brought the degree of hydrolysis down to the levels of controls (no caecal contents added). The presence of neomycin also brought about considerable inhibition of hydrolysis. Hydrolysis of the biliary conjugates with pure β-glucuronidase showed that glucuronide conjugates were significant metabolites.

Effect of neomycin on the biliary excretion of mestranol and oestradiol. Neomycin pretreated animals showed a 15–20 per cent reduction in the biliary excretion of i.d. administered oestradiol and mestranol after 6 hr. The decrease in excretion was, however, smaller than the reductive effect of neomycin on enterohepatic recirculation (see Figs. 2 and 3).

DISCUSSION

It is probable that an efficient enterohepatic circulation of oestrogenic steroids and therefore an

Table 1. *In vitro* hydrolysis of the biliary conjugates of mestranol and *17β*-oestradiol by rat caecal microorganisms

% Free steroid in untreated bile†	% Free steroid after incubation with rat caecal microorganisms	% Free steroid after incubation: (i) with culture medium only	(ii) with prior boiling of incubate‡	(iii) in presence of neomycin (250 μg/ml)	% Free steroid after incubation with β-glucuronidase (10,000 units)
Oestradiol conjugates					
(a)* 1.0	72.5	5.7	7.3	—	42.6
(b)* 1.1	80.0	5.9	—	24.2	40.6
Mestranol conjugates					
(c)* 0.7	58.0	9.3	8.9	—	31.0
(d)* 0.7	56.0	5.1	—	10.5	33.9

Figures represent the percentage of radioactivity in the original bile (200 μl) which could be extracted by diethyl ether (3 × 10 ml) at pH 5.0 after the treatment indicated.

† bile diluted with an equal vol. of water before extraction.

‡ rat caecal extract plus medium boiled for 3 min before bile addition and subsequent incubation.

* Individual experiments with bile from separate animals.

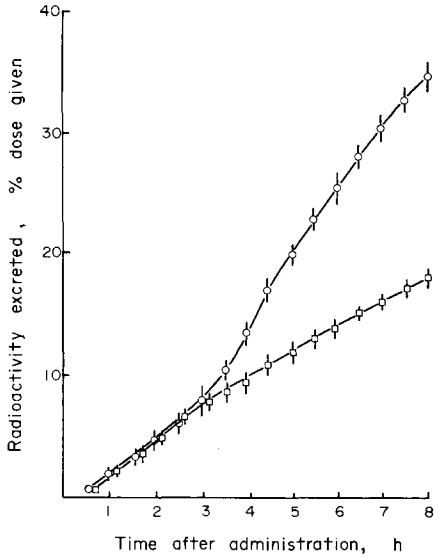

Fig. 2. Biliary excretion of radioactivity following i.d. administration of labelled biliary conjugates of mestranol to neomycin pretreated female rats. Results are expressed as a percentage of the administered dose of labelled bile. ○—control rats; □—neomycin pretreated rats. Points represent the mean of 3 or 4 experiments (S.E.M. shown). Mean bile flow rates: controls 672 ± 54 μl/hr; neomycin treated 553 ± 44 μl/hr.

extended half-life in the body is important in determining the effectiveness of these compounds with respect to their oestrogenic action. In order to reduce side effects many combination type contraceptive formulations contain the oestrogen mestranol at levels close to the minimum effective dose. Clearly, factors which affect the hydrolysis and reabsorption of the biliary conjugates may be important in determining its efficacy as an orally active oestrogen. One such important factor is the hydrolytic capacity of the gastrointestinal microflora.

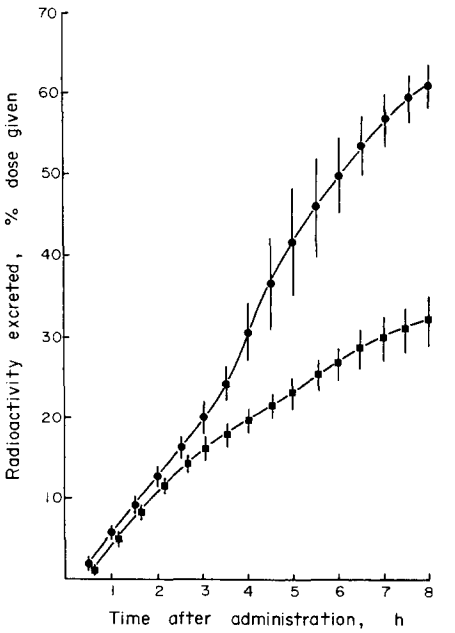

Fig. 3. Biliary excretion of radioactivity following i.d. administration of labelled biliary conjugates of oestradiol to neomycin pretreated rats. Results are expressed as a percentage of the administered dose of labelled bile. ●—control rats; ■—neomycin pretreated rats. Points represent the mean of 3 or 4 experiments (S.E.M. shown). Mean bile flow rates: controls 809 ± 30 μl/hr; neomycin treated 608 ± 42 μl/hr.

That oestradiol and mestranol are efficiently biliary excreted and undergo enterohepatic circulation has been clearly demonstrated. The degree of biliary excretion and recirculation is larger than that quoted for ethynyloestradiol [11]. Using a method involving intraduodenal administration of bile containing labelled metabolites to normal rats and rats in which the gastrointestinal microflora had been suppressed by neomycin the degree of enterohepatic circulation was studied. In neomycin treated rats recirculation was impaired by up to 50 per cent. The deconjugation of mestranol and oestradiol biliary conjugates has been demonstrated *in vitro* upon incubation with rat caecal microorganisms as has the inhibition of such hydrolysis by neomycin.

The results have also shown that neomycin pretreatment reduced the biliary excretion of mestranol and oestradiol after intraduodenal administration. This could be a result of the malabsorption syndrome of neomycin [10]. This reduction is, however, not as great as the reduction of enterohepatic recirculation by neomycin. It is likely that suppression of the gut flora is still a major factor in the impairment of enterohepatic circulation by neomycin and possibly other antibiotics. Complete suppression of enterohepatic recirculation by neomycin would not be expected as this antibiotic does not suppress the entire flora. The Bacteroides, a major group of intestinal microorganisms are largely unaffected [12].

In conclusion it has been shown that neomycin reduces the enterohepatic circulation of mestranol and oestradiol metabolites. This reduction is due to its bacteriocidal and malabsorption effects and may be important in considering 'low level' contraceptive formulations. The effect of antibiotics with a wider antibacterial spectrum may be even more important. Although apparently via a different action it is interesting to note that the antibiotic rifampicin has been shown to cause failures of the oral contraceptive [13].

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