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THE BILIARY ELIMINATION OF AMARANTH, INDOCYANINE GREEN AND NITRAZEPAM IN GERM-FREE RATS

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Abstract—In anaesthetised bile duct-cannulated rats the overall rate of bile flow was 15–50% lower in both male and female germ-free (GF) rats. Except in the case of amaranth in female GF rats, this was reflected in a lower rate of biliary excretion of the three test xenobiotics, of which two (amaranth and indocyanine green) are excreted unchanged and one (nitrazepam) is excreted solely as metabolites. It was also noted that compared with conventional (CV) rats the relative liver weights (g/kg body weight) were about 20% lower in GF animals. After the intravenous injection of ^{14}C-nitrazepam, thin-layer chromatographic separation of biliary nitrazepam-derived radioactivity revealed three loci (A, B and C in decreasing order of polarity). The relative proportions of A, B and C were similar in GF and CV rats, with C and B being the major and minor "metabolites" respectively. When ^{14}C-nitrazepam was given intragastrically to non-anaesthetised rats, by 9 days about 20% and 70% of the dose had been recovered in the urine and faeces respectively of both GF and CV rats. The rate of elimination of urinary radioactivity was similar in GF and CV rats. However, faecal elimination was much slower in GF animals, for example after 24 hr the respective amounts of radioactivity excreted in GF and CV rats corresponded to 13% and 52% of the dose. These findings indicate that the indigenous bacterial population of an animal may indirectly affect the disposition of a xenobiotic whether or not it is metabolised by the bacteria.

GF† rats can be useful in investigating the involvement of the gut flora in xenobiotic metabolism [1], as recently exemplified in the elucidation of the "methylthiolation" pathway [2]. However, GF rats exhibit certain differences in anatomy and physiology from CV rats [3], which may limit their usefulness where a quantitative assessment of the involvement of the flora is required. Of particular significance as far as drug disposition is concerned, is the reported 30% lower cardiac output [4] and a concomitant decrease in regional blood perfusion, featuring a 50% reduction in blood flow for the liver and intestines [3].

Since liver blood flow is likely to affect bile production, we decided to initiate drug-handling studies in GF rats by examining their ability to excrete xenobiotics in the bile. Three xenobiotics, amaranth, IG and nitrazepam, with differing characteristics as far as biliary elimination is concerned were chosen. Both amaranth and IG are polar anionic dyes that are excreted unchanged in the bile [5]. However, IG has a cholestatic effect and its biliary elimination is bile salt-dependent [6], while amaranth is not cholestatic and its elimination is bile salt-independent [7, 8]. In contrast, the nitrobenzodiazepine hypnotic nitrazepam is lipid-soluble and is excreted in the bile almost entirely as metabolites [9].

Using radiolabelled nitrazepam it was also proposed to compare its biliary metabolite profile in GF and CV rats, as well as to see if any observed differences in the excretion of radioactivity in the bile could be reflected in the overall elimination of radioactivity, particular in the faeces.

MATERIALS AND METHODS

Materials. The dyes amaranth and IG were purchased from Sigma London (Poole, Dorset, U.K.) and Hynson, Westcott and Dunning (Baltimore, MD) respectively. Nitrazepam, 3-hydroxynitrazepam, 7-aminotrazepam, 7-acetamidonitrazepam and 5-^{14}C-nitrazepam (52 μCi/mg) were gifts from F. Hoffmann-La Roche (Basle, Switzerland).

Animals. GF rats were bred and maintained in positive-pressure Trexler isolators [10]. Inbred GF rats of the BDIX and WAG/Rij strains were originally obtained as "breeding nuclei" from the Medical Research Council Experimental Embryology and Teratology Unit, Carshalton, Surrey.

The WAG/Rij rats were housed in wire-bottomed cages, fed a gamma-irradiated (25 kGy) sterilised standard breeding diet (Rat and Mouse No. 3, Special Diet Services, Witham, Essex, U.K.) and supplied with sterile drinking water supplemented with vitamin K_1 (2 mg/l Konakion, Roche Products Ltd, Welwyn Garden City, Hertfordshire U.K.). The BDIX rats were maintained similarly except that the animals used for the IG study had additional vitamins (vitamins A, B_1, B_2, B_6, B_{12}, C and E;

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† Abbreviations: GF, germ-free; CV, conventional; IG, indocyanine green.

pantothenyl alcohol and nicotinamide) added to their drinking water (4 ml/l Multibionta Infusion, E. Merck, Wokingham, Berks, U.K; 0.05 mg/l cyanocobalamin as Cytamen, Glaxo, Greenford, Middlesex U.K.) in an attempt to overcome breeding/lactation problems encountered at that time.

Sterility checks, described by Fuller [11], were performed on the GF rats, which remained free from microbial contamination.

CV rats were produced by removing recently-weaned GF rats (about 3 weeks old) from the isolators and giving them normal rat caecal contents via the oral and anal routes (about 0.5 ml of a 25% v/v suspension of caecal contents in physiological saline was given by each route). For all GF versus CV rat studies, littermate pairs were used, except for nitrazepam/bile duct cannulation experiments, where age-matched pairs were used. The CV animals were housed in an environment matched in conditions of lighting, humidity and temperature to those for the GF animals in the isolators. These animals were also fed the same irradiated diet and vitamin-supplemented drinking water given to the GF rats.

Biliary excretion studies. Littermate pairs of male (10 weeks old) and female (12 weeks old) GF vs CV BDIX rats were used for the amaranth and IG studies, while age-matched pairs of male GF and CV (11–22 weeks) WAG/Rij rats were used for those involving nitrazepam. In the latter case, the ages of the rats from each pair were within a week of each other. For comparison with their CV counterparts, the GF rats were removed from the isolators just prior to injection of anaesthetic. BDIX or WAG/Rij rats were anaesthetised with urethane (50% w/v, 1.5 g/kg i.m.) or pentobarbitone (45 mg/ml, 45 mg/kg s.c.) respectively. The time between removal of the rats from the isolators and subsequent administration of the test xenobiotic was not greater than one hour.

The surgical procedure, involving cannulation of a femoral vein and the bile duct, was essentially as described by Klaassen and Strom [5]. Rectal temperature was maintained at 37° throughout the experiment by means of a heat-lamp regulator device (Yellow Springs Instrument Co., Yellow Springs, OH). The drugs, amaranth (20 mg/ml, 20 mg/kg), IG (10 mg/ml, 10 mg/kg) or ^{14}C-nitrazepam (10 mg/ml in dimethylsulphoxide, 10 mg/kg, 11 μCi/kg), were injected via the cannulated femoral vein. Bile samples were collected every 10 min after the dyes or hourly after nitrazepam. Saline was given i.v. at 10 min or hourly intervals to replace the fluid lost as bile. Each bile sample was weighed and the volume determined, assuming a specific gravity of 1.0. Following the experiments using BDIX rats, the livers from each pair of animals were removed and weighed.

Amaranth and IG in the bile were quantitated spectrophotometrically after the addition of appropriate amounts of water [12, 13]. Total ^{14}C-nitrazepam-derived radioactivity in the bile was determined by liquid scintillation counting following direct addition of bile to NE 260 scintillant (New England Nuclear, Dreieich, F.R.G.).

^{14}C-Nitrazepam-derived radioactivity in the bile was further examined by thin-layer chromatography. Bile was applied directly to silica gel chromatography sheets (Eastman Chromagram 13174, with fluorescent indicator) and chromatographed using either a chloroform-based (chloroform: methanol: acetone; 90:5:6, by volume; Yanagi *et al.* [14] or a butanol-based (*n*-butanol: ethanol: NH_4OH (0.88): water; 40:10:1:9 by volume) solvent system. The chromatographic standards, comprising nitrazepam, and the putative nitrazepam metabolites 3-hydroxynitrazepam, 7-aminotrazepam and 7-acetamidonitrazepam were visualized under u.v. light (wavelength, 254 nm) using a "Chromatolite" lamp (Hanovia Ltd, Slough, Bucks, U.K.). The radioactive chromatographic loci were located with the aid of a spark chamber (Birchover Instruments Ltd, Hitchen, Herts, U.K.) and a radiothin-layer scanner (Panax Equipment Ltd, Redhill, Surrey, U.K.), cut out and transferred to glass vials containing 10 ml NE260 scintillant for liquid scintillation counting. R_f values were calculated by dividing the distance travelled by the "spot" (leading edge) by that travelled by the "solvent front".

Urinary and faecal excretion of ^{14}C-nitrazepam-derived radioactivity. Littermate pairs of male (11–17 weeks, 190–280 g) GF vs CV WAG/Rij rats were housed in plastic metabolism cages. For the GF rats, sterile cages were set up within isolators. ^{14}C-Nitrazepam was prepared as a suspension (10 mg/ml, the vehicle containing 2.5% w/v Compound Powder of Tragacanth B.P.). sterilized by gamma irradiation (25 kGy) and given intragastrically (20 mg/kg, 25 μCi/kg). This was followed by intragastric dosing with water (about 8 ml/kg) at 5 min, 2, 4, 6, 24 and 30 hr after drug administration to encourage urine production. Urine and faeces were collected for 9 days. After this study period, sterility tests on GF rat faecal samples confirmed the absence of microbial contamination.

Total radioactivity was determined in the faeces (after drying to constant weight at 60°) and urine by liquid scintillation counting [15].

Statistics. All experimental results are means ± S.E. and the data were analysed using Student's paired *t*-test. A probability of less than 0.05 was taken as significant.

RESULTS

Subsequent to injection, amaranth stimulated bile flow for about 1 hr both in GF and CV rats (Fig. 1a). However, the overall rate of bile flow was lower in GF rats by about 50 and 15% in male and female GF rats respectively. In male GF rats the greater reduction in bile flow was reflected in a lower amaranth excretion rate during the first 20 min (Fig. 1b). After 30 min, the excretion rate tended to be greater in the GF rats, presumably resulting from the greater proportion of the dose remaining to be excreted in these animals. Half the amaranth dose was excreted by 35 and 20 min in male GF and CV rats respectively. There was no marked difference in amaranth excretion between female GF and CV rats, 50% of the dose being excreted in 17 to 20 min.

Unlike amaranth, IG tended to reduce bile flow,

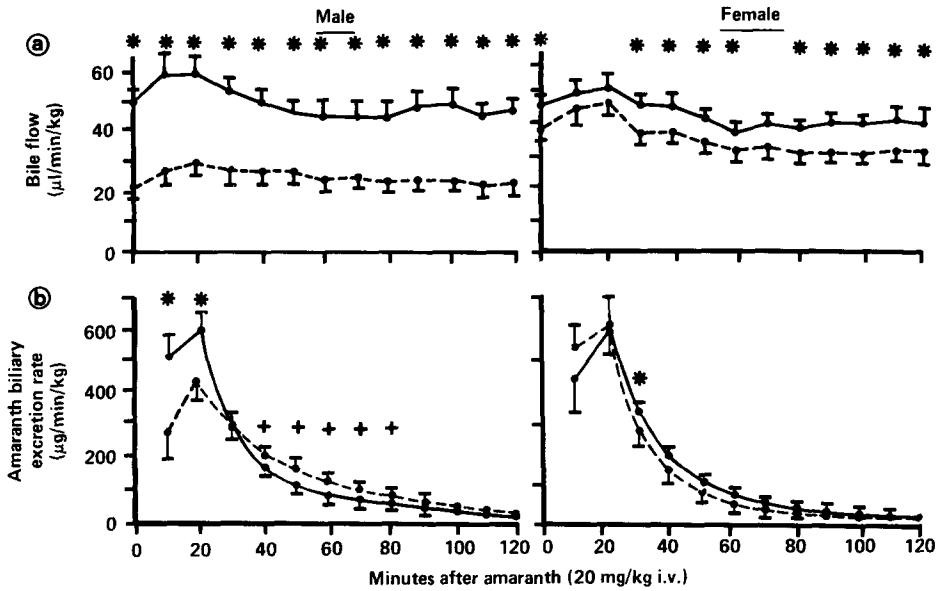

Fig 1. Bile flow and biliary amaranth excretion in GF and CV rats. The GF rat data are indicated by dashed lines. Seven littermate pairs (GF vs CV) of each sex were used. Means ± S.E. are given. Asterisks and crosses indicate CV > GF and GF > CV ($P < 0.05$) respectively, using Student's paired *t*-test.

this effect being more marked in female rats (Fig. 2a). The rate of bile flow was again lower in GF than CV rats; in this group of rats the rate was 30 to 40% lower in both sexes. With the more slowly excreted dye, the decreased bile flow was reflected by a generally lower IG excretion rate after 20 to 30 min in both male and female GF rats for a large part of the experimental period (Fig. 2b). The female rats tended to eliminate IG more rapidly; in GF and CV rats respectively, females eliminated half the dose in about 65 and 45 min, while in males the corresponding figures were about 85 and 60 min.

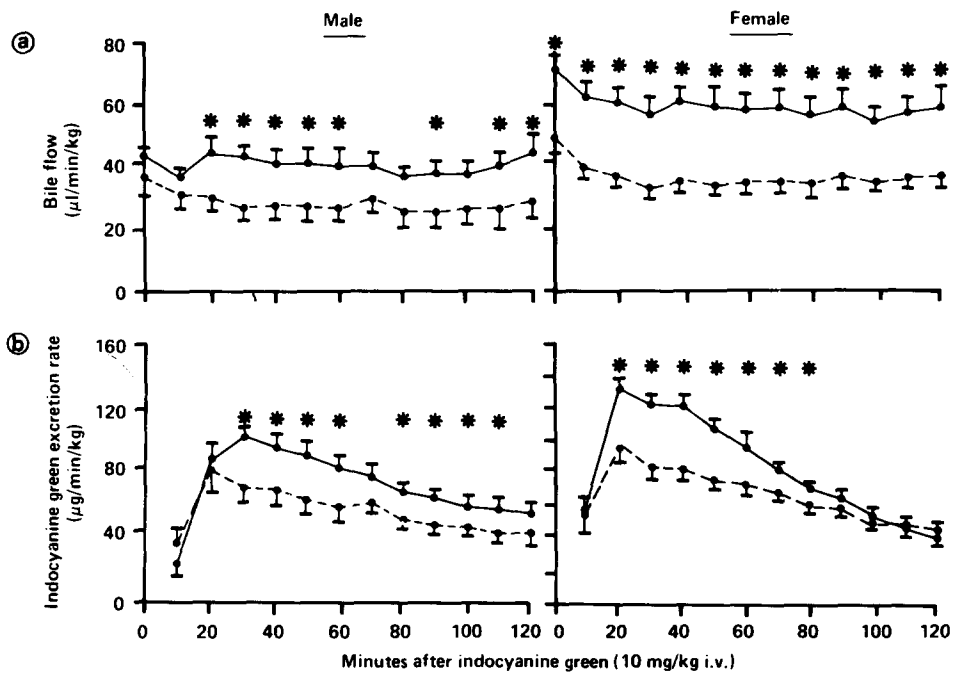

Fig. 2. Bile flow and biliary indocyanine green excretion in GF and CV rats. The GF data are indicated by dashed lines. Four and five littermate pairs (GF vs CV) of male and female rats respectively were used. Means ± S.E. are given. Asterisks indicate CV > GF ($P < 0.05$), using Student's paired *t*-test.

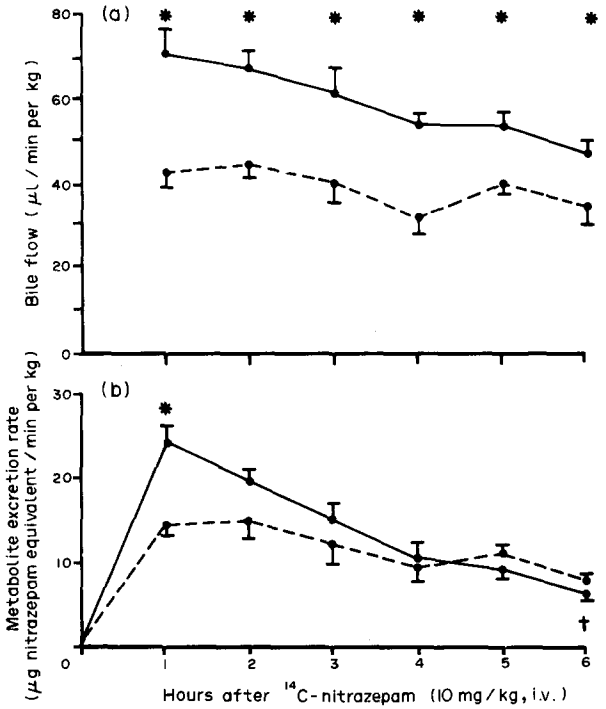

Fig. 3. Bile flow and biliary excretion of ^{14}C-nitrazepam-derived radioactivity in male germfree (GF) and conventional (CV) rats. The GF data are indicated by dashed lines. Five age-matched pairs (GF vs CV) were used. Means ± S.E. are given. Asterisks and the cross indicate CV > GF and GF > CV ($P < 0.05$) respectively, using Student's paired *t*-test.

The relative weights of GF rat livers were 15 to 30% lower ($P < 0.05$) than those for CV rats. In the "amaranth" experiments, the relative liver weights (g/kg body weight) for GF and CV rats respectively were 36.0 ± 1.8 and 47.4 ± 2.6 (males), 34.8 ± 1.4 and 48.0 ± 2.6 (females). In the "indocyanine green" experiments the corresponding values were 41.9 ± 2.4 and 48.7 ± 3.1 (males), and 42.7 ± 1.2 and 54.5 ± 1.9 (females). In the amaranth study the body weights of the male GF rats were lower (0.245 ± 0.0012 and 0.276 ± 0.006 kg) but those of the female GF rats were not significantly different from those of their CV counterparts (0.184 ± 0.007 and 0.194 ± 0.008 kg). With the IG study where the animals received additional vitamins, body weights of GF and CV rats were not significantly different; 0.247 ± 0.007 and 0.236 ± 0.0026 kg (GF and CV males respectively) and 0.16 ± 0.0065 and 0.152 ± 0.0046 kg (GF and CV females respectively).

As with the BDIX rats bile flow was lower (by 30–40%) in male GF WAG/Rij rats (Fig. 3a). The resultant rate of excretion of ^{14}C-nitrazepam-derived radioactivity was significantly less during the first hour after nitrazepam injection (Fig. 3b). At 4 hr about 33 and 45% of the dose had been excreted in the bile of GF and CV rats respectively, and by 6 hr the corresponding figures were 47 and 55%.

When bile from ^{14}C-nitrazepam-treated GF or CV rats was chromatographed using the chloroform-based solvent system which separates nitrazepam, 7-aminonitrazepam, 7-acetamidonitrazepam and 3-hydroxynitrazepam (respective R_f values; 0.52, 0.44, 0.37, 0.16) nearly all of the radioactivity was associated with unidentified polar material remaining near the origin, although there were possible traces of nitrazepam and acetamidonitrazepam. When the polar material was chromatographed using the more polar butanol-based solvent system, three radioactive components, A, B and C, were separated with R_f values of zero (remained on origin), 0.2 and 0.5 respectively. In this system the nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam reference compounds had an R_f value of 0.8, while that of 3-hydroxynitrazepam was 0.65.

The relative proportions of A, B and C in GF and CV rat bile were generally similar throughout the 6 hr experimental period, apart from somewhat higher and lower initial proportions of A and C respectively in GF rats (Fig. 4). In both cases during the first 4 hr, the proportion of A decreased while that of C increased.

After intragastric dosing with ^{14}C-nitrazepam, about 20 and 65% of the radioactivity was eventually eliminated in the urine and faeces respectively (Fig. 5). The rate of urinary elimination of radioactivity was similar in GF and CV rats, about 50% of the urinary radioactivity being eliminated in 8 hr. However, faecal elimination was much slower in GF rats; for example after 24 hr the respective amounts of radioactivity excreted in GF and CV rats corresponded to 13 and 52% of the dose. It can be seen from Fig. 5 that the time taken to excrete half the faecal radioactivity was twice as long in GF rats (about 40 hr vs 20 hr).

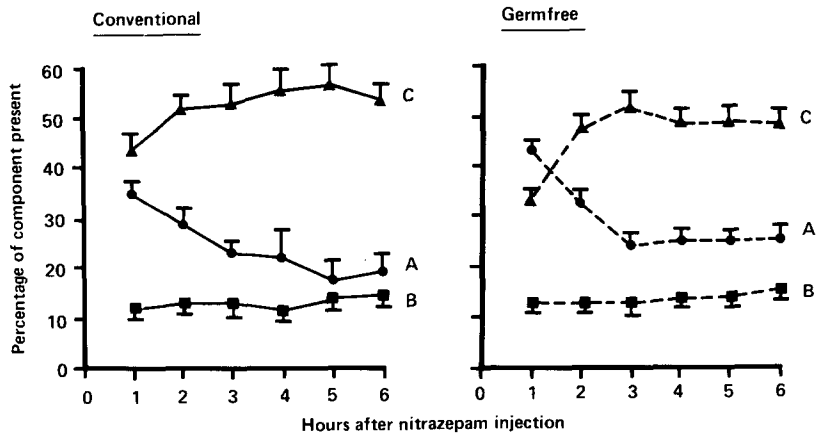

Fig. 4. The relative proportions of nitrazepam "metabolites" present in germfree and conventional rat bile at various times after nitrazepam injection. The bile came from the experiment illustrated in Fig. 3. Components A, B and C represent chromatographic loci (in decreasing order of polarity) separated using a butanol-based solvent system (see Materials and Methods)). Means ± S.E. are given (N = 4).

DISCUSSION

A consistent finding in the two strains of rat has been the lower rate of bile flow seen in the GF animals. This was associated with a reduction in xenobiotic excretion rate, except in the case of amaranth in female GF rats. In this particular group of rats it was noted that the reduction in bile flow was not as marked as that seen with other groups of GF rats. While the reason for this is not clear, it may partly explain why in this instance, a reduction in the excretion of the most rapidly eliminated of the three xenobiotics was not seen.

It seems that the reduction in biliary excretion of the three test xenobiotics in GF rats could be explained at least in part on the basis of a reduced liver blood flow as reported by Gordon [3]. An

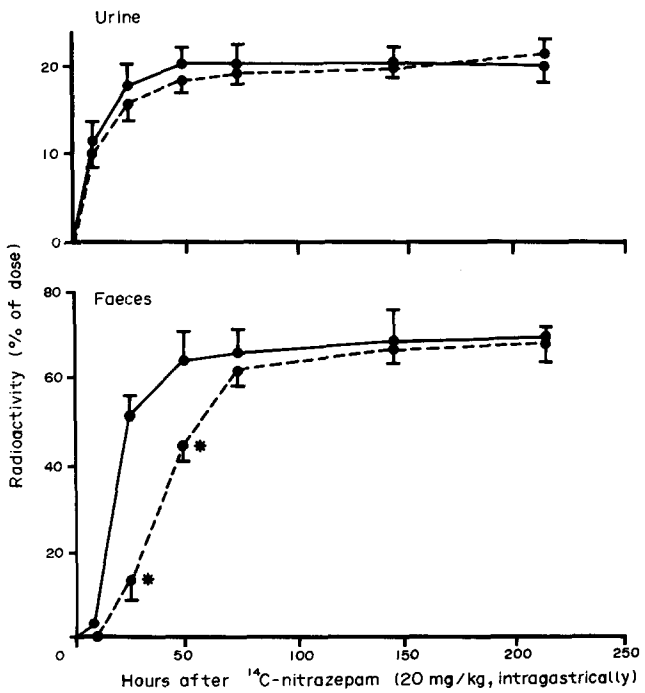

Fig. 5. The cumulative urinary and faecal elimination of ^{14}C-nitrazepam-derived radioactivity in male GF and CV rats. The GF data are indicated by dashed lines. Each point is the mean ± S.E. of four experiments. For each experiment, two littermate pairs (GF vs CV) were compared (there were two GF or two CV rats per metabolism cage). Asterisks indicate CV > GF ($P < 0.05$), using Student's paired *t*-test.

observation made during our experiments which could relate to a reduced blood supply was that GF rat livers were consistently paler in colour. Gordon [3] noted that blood distribution to organs or tissues which are normally in close association with the microbial flora (skin, lungs, intestinal tract and liver) tends to be reduced to a greater extent than that to more "remote" organs such as the kidney and thymus. It has therefore been suggested that in CV animals the microflora may be responsible for the relative hyperaemia by inducing a sort of "physiological inflammation" [16]. An alternative proposal is that the germfree rat caecum secretes into the systemic circulation a substance that is antagonistic to noradrenaline and which causes a resultant reduction in cardiac output and organ blood flow [17]. This explanation was originally suggested by the finding that the reduced cardiac output associated with GF animals could be largely prevented if they were caecectomised at weaning [18].

The reduced liver weight in GF rats noted in the present study has not always been a consistent finding by other workers [19]. However, when the GF and CV rats are carefully "matched" the difference is seen [20]. It has been suggested that the larger liver in CV rats is a response to a greater workload because of the additional need to metabolize bacterial end products [20]. This can be considered an extension of the "physiological inflammation" theory.

In the present study similar results were obtained to those reported by Tanayama *et al.* [19] using normal rats for ^{14}C-nitrazepam biliary excretion experiments. These workers found that after intravenous injection of ^{14}C-nitrazepam (5 mg/kg) radioactivity equivalent to 58% and 61% of the dose was excreted after 4 and 6 hr respectively. Furthermore as found by us, the radioactivity mainly comprised polar material that did not correspond chromatographically to nitrazepam, 3-hydroxynitrazepam, 7-aminonitrazepam or 7-acetamidonitrazepam.

Despite the fact that ^{14}C-nitrazepam was excreted more slowly in the bile of GF rats, the relative proportions in the bile of the "polar metabolites" A, B and C were generally similar in GF and CV rats throughout the experimental period. Although components A, B and C did not correspond to any of the chromatographic standards, it is possible that A, B and C could be polar conjugates of one or more of these compounds. In both GF and CV rat bile there was a characteristic fall during the first 3–4 hr in the proportion of component A and a corresponding rise in that of component C. This could suggest a link between the formation of these two products. For instance, conversion of component C to the more polar product A could be occurring, with less conversion occurring as more of the drug is eliminated.

It has been reported that the intestinal microflora can reduce the nitro group of nitrazepam to produce 7-aminonitrazepam [21]. However, during the present experiments in bile duct-cannulated rats in which nitrazepam was given intravenously, there was little chance for the drug to come in contact with the gut flora before its elimination. The lack of direct bacterial nitrazepam metabolism is indicated by the similar metabolite pattern seen in GF and CV rat bile.

With the same dose of ^{14}C-nitrazepam also given by the oral route, our results are similar to those of Yanagi *et al.* [14] using normal rats in that about one-third and two-thirds of the dose was excreted in the urine and faeces respectively. However, in GF rats, whereas urinary excretion of radioactivity was relatively unaffected, faecal elimination was markedly delayed. This differs somewhat from the findings of a preliminary investigation using GF rats [21] in which a significant reduction in urinary excretion was also reported. Although specific reasons for the discrepancy are not known, the results of the present work should be more reliable because of the use of "matched pairs" and other improvements in experimental design. In the earlier study the GF rats were "bought in" and used directly on arrival at the laboratory, with no period of "acclimatisation" being given. It is well established that GF rats suffer from a mild chronic diarrhoea [3] and it is likely that the GF animals used in our earlier study had become relatively dehydrated during transit, and thus were not adequately matched. Furthermore, it should be noted that the two investigations are not strictly comparable in that intraperitoneal rather than oral drug dosing was used for the first study.

Although the absence of gut bacterial metabolism of nitrazepam could in some way be responsible for the reduced faecal elimination, there are at least two probable additional contributory factors which do not involve bacterial drug metabolism. Firstly, the GF intestinal tract as well as exhibiting anatomical differences [3], also has a decreased motility as measured by its reduced ability to "propel" various non-absorbable test substances along its length [22, 23]. Secondly, it seems highly likely that the reduced biliary excretion of nitrazepam, as found in the present study, would contribute to its slower faecal elimination.

It is therefore clear that the presence of the intestinal microflora can indirectly affect the disposition of a drug whether or not it is metabolised by the bacteria. It would be interesting to see if such indirect effects on drug handling can be induced by antibacterial agents used clinically to remove the gut flora [24].

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