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CONJUGATION AND EXCRETION OF METABOLITES OF 7-HYDROXYCOUMARIN IN THE SMALL INTESTINE OF RATS AND GUINEA-PIGS

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Abstract— 7-Hydroxycoumarin is conjugated with glucuronic acid and sulphate in intestinal preparations of both rats and guinea-pigs. The highest activity was found at the oral end of the small intestine and the lowest activity at the aboral end. Some distributional differences between the glucuronyl transferase(s) and sulphotransferase(s) were observed. In both gut sacs and *in situ* preparations a considerable secretion of conjugate into the lumen was found; the significance of this finding to chemical toxicity in the lower gut is discussed.

A number of investigators have studied the metabolism of steroids and xenobiotics by the intestinal wall [1–12]. The aim of these investigations was mainly to obtain information on the nature and extent of direct conjugation of these compounds. In the majority of cases an everted gut sac preparation was used [1–7, 9, 10, 12]. The incubation time was at least an hour in each study, and often as long as 3 hr. When everted gut sacs are used it is difficult to ascertain the viability of the preparation and since the cells will start to die as soon as the animal is killed, it is likely that most of the cells will not be viable after a 2 hr incubation period. The lack of an intact blood supply in the sacs could create an artificial barrier to absorption and excretion. Therefore, data obtained using gut sacs requires reinforcement by results obtained *in situ*, e.g. from an isolated *in situ* loop of intestine.

The intestinal wall is known to be very active in conjugation of xenobiotics, but little is known of the fate of the conjugates. Different parts of the small intestine may have different enzyme activities [17, 18]. It has been shown, using gut sacs from rats, that the conjugates generally pass into the serosal fluid [3, 4, 8–12], though in most instances small amounts of conjugates were also found in the mucosal fluid.

The fate of xenobiotic conjugates, formed in the intestinal wall, is not clear. We have chosen an easily measured xenobiotic, 7-hydroxycoumarin, and investigated the fate of its glucuronic acid and sulphate conjugates using short term incubations with everted gut sacs and *in situ* isolated loops from both guinea-pigs and rats. We have chosen the guinea-pig for particular attention because individual cells isolated from the guinea-pig intestine have been shown to have a much better viability than those derived from rat intestine. (J. R. Dawson and J. W. Bridges, Unpublished data). We have also examined the distribution of conjugating ability along the intestine using everted gut sacs prepared from different parts of the gut.

MATERIALS AND METHODS

Chemicals

7-Hydroxycoumarin was purchased from Sigma Chemicals Co. (Poole, Dorset, England). Leibovitz L-15 medium was purchased from Gibco Biocult (Paisley, Renfrewshire, Scotland). All other chemicals and solvents used were of Analar grade from British Drug Houses, (Poole, Dorset, England).

Animals

Male Gordon Hartley guinea-pigs, 300–400 g, and male Wistar albino rats, 250 g (bred in University of Surrey Animal Unit), allowed free access to food and water were used.

In situ *isolated loop experiments*

Animals were anaethetised, using pentobarbitone or urethane, and a longitudinal abdominal incision was made. The method used was essentially that of Doluisio *et al.* [13], except that a 10–15 cm long portion of the small intestine was used instead of the entire small and large intestine. The section of intestine used in the guinea-pig was between 90 and 110 cm away from the pylorus, this being the most accessible portion. In the rat the section used was between 3 and 20 cm below the entry of the bile duct. The intestinal loop was flushed out with approximately 20 ml of cell culture medium (Leibovitz L-15 medium [22]), to remove the intestinal contents. Substrate solution (between 4 and 5 ml of cell culture medium containing 100 μM 7-hydroxycoumarin), at 37°C, was introduced into the loop, and the loop made into a closed system by shutting the stopcocks at both ends. After the requisite incubation time, the entire contents of the loop were flushed out with air, by means of a syringe, into a collection syringe, and an aliquot of this solution was taken for determination of the conjugates produced. Each incubation was followed by a 5 min post-incubation 'wash', in order to obtain an

indication of the amount of conjugates left in the cells after such incubation. Fresh cell culture medium, which was substrate-free, was introduced into the intestinal loop, removed after 5 min and an aliquot taken for determination of 7-hydroxycoumarin conjugates.

Measurement of 7-hydroxycoumarin conjugates

A 0.2 ml portion of the incubation fluid was made up to 2 ml with fresh cell culture medium, and placed in a screw-cap tube. The unmetabolised 7-hydroxycoumarin was extracted into three 7 ml portions of ether containing 1.5% isoamyl alcohol (efficiency of extraction greater than 98 per cent), and discarded. The 7-hydroxycoumarin conjugates were hydrolysed and measured by the method of Shirkey *et al.* [14].

Everted sacs of guinea-pig small intestine

A length of small intestine was excised from an animal and flushed out with ice-cold saline. The intestine was then cut into six 10 cm lengths. Each length was everted over a metal rod, one end tied off, the sac was then filled to slight distension with cell culture medium, and the other end ligated. The sac was placed in a 50 ml open conical flask containing 5 ml cell culture medium. 7-Hydroxycoumarin was added in 5 μl dimethylformamide, to give a final concentration of 100 μM in the luminal fluid surrounding the sacs. In order to minimise variations between preparations in enzyme activity with distance from the stomach, a portion of intestine from the aboral end of the section used was paired with a portion from the oral end. The flasks were incubated in a shaking water bath (55 cycles/min) at 37°C for the required length of time. The reaction was stopped by plunging the flasks into ice-water. An aliquot (0.5 ml) of the luminal fluid was taken, made up to 2 ml with cell culture medium and used for determination of amounts of conjugates. The remainder of the luminal fluid was discarded and the flask and sac rinsed once with medium, which was also discarded. The sac was then cut open and a portion (0.5 ml) of the contents taken, made up to 2 ml with cell culture medium and used for the determination of conjugates, as before.

Determination of variation in conjugation ability with distance from the pylorus

A length of small intestine was removed from each guinea-pig and cut into eight 15 cm lengths. Each length was everted and filled with medium, as previously described. This was incubated in 5 ml cell culture medium, containing 100 μM 7-hydroxycoumarin, at 37°C for 20 min. The reaction was stopped by plunging the flasks into ice-water, and a portion was taken from each flask for determination of 7-hydroxycoumarin conjugates, as before.

RESULTS

7-Hydroxycoumarin conjugation by everted sacs of guinea-pig small intestine

The initial concentration of 7-hydroxycoumarin in the luminal fluid was 100 nmole/ml. After a 5 min incubation of the everted sacs, 10 per cent of the substrate had reappeared in the lumen in the form of glucuronic acid (4.5 per cent) and sulphate (5.5 per cent) conjugates (Fig. 1A), whilst only 4 per cent had appeared in the serosal fluid (Fig. 1B) as conjugates. After 30 min incubation, 28 per cent of the 7-hydroxycoumarin had reappeared in the lumen as glucuronic acid (10 per cent) and sulphate (18 per cent) conjugates, whilst only 5 per cent of the substrate had appeared as conjugates in the serosal fluid.

Metabolism of 7-hydroxycoumarin in situ

The results of the guinea-pig *in situ* loop experiments (Fig. 2) confirmed those obtained using the everted sacs. The mesenteric blood was not sampled, owing to the difficulty of assaying for 7-hydroxycoumarin and its conjugates in blood. Measurement of the levels of conjugates in the lumen of the intestine agreed quite well with the results of the everted sac experiments, the main difference being in the relative proportions of sulphate and glucuronic acid conjugates. After a 5 min incubation in the isolated loop, 13 per cent of the total 7-hydroxycoumarin added had emerged into the lumen, conjugated with sulphate (4 per cent) and glucuronic acid (9 per cent). After 30 min incubation 27 per cent of the total substrate had emerged into the lumen, conjugated to sulphate (9.5 per cent) and glucuronic acid (16.5 per cent).

The Wistar albino rat was used to determine if the appearance of conjugates in the lumen in such high concentrations was peculiar to the guinea-pig. The *in situ* isolated loop of rat small intestine gave a very similar picture to that seen with the guinea-pig, the main difference being that rat intestine had a linear rate of sulphate conjugation, which was proportionally lower than that found in guinea-pig. After 5 min incubation in rat, 4 per cent of the total 7-hydroxycoumarin added had emerged into the lumen (Fig. 3), conjugated with sulphate (0.5 per cent) and glucuronic acid (3.5 per cent). After 30 min incubation, 14 per cent had appeared in the lumen as sulphate (3.5 per cent) and glucuronic acid (10.5 per cent) conjugates.

Variation in conjugation of 7-hydroxycoumarin along the intestine

Consecutive lengths of guinea-pig small intestine were incubated as everted sacs in 100 μM 7-hydroxycoumarin to investigate the variation in conjugating ability in the small intestine. Glucuronic acid conjugation increased between 0 and 45 cm from the pylorus, before decreasing to half of the initial level at over 90 cm from the pylorus. Sulphate conjugation did not show any initial increase, but fell slightly between 0 and 60 cm from the pylorus, then fell to less than half the initial level at distances greater than 60 cm away. Although there was some variation between individual animals, the same distributional pattern was seen in each animal.

DISCUSSION

It has previously been postulated that glucuronidation in the small intestine might be an important mechanism for the transfer of steroids and xenobiotics from the lumen into the blood stream [1–6]. Using everted gut sacs we have shown that substantial amounts of 7-hydroxycoumarin conjugates are released back into the luminal fluid rather than being transferred to the serosal fluid. Previous workers using everted gut sac preparations have shown that exit of conjugates from the rat

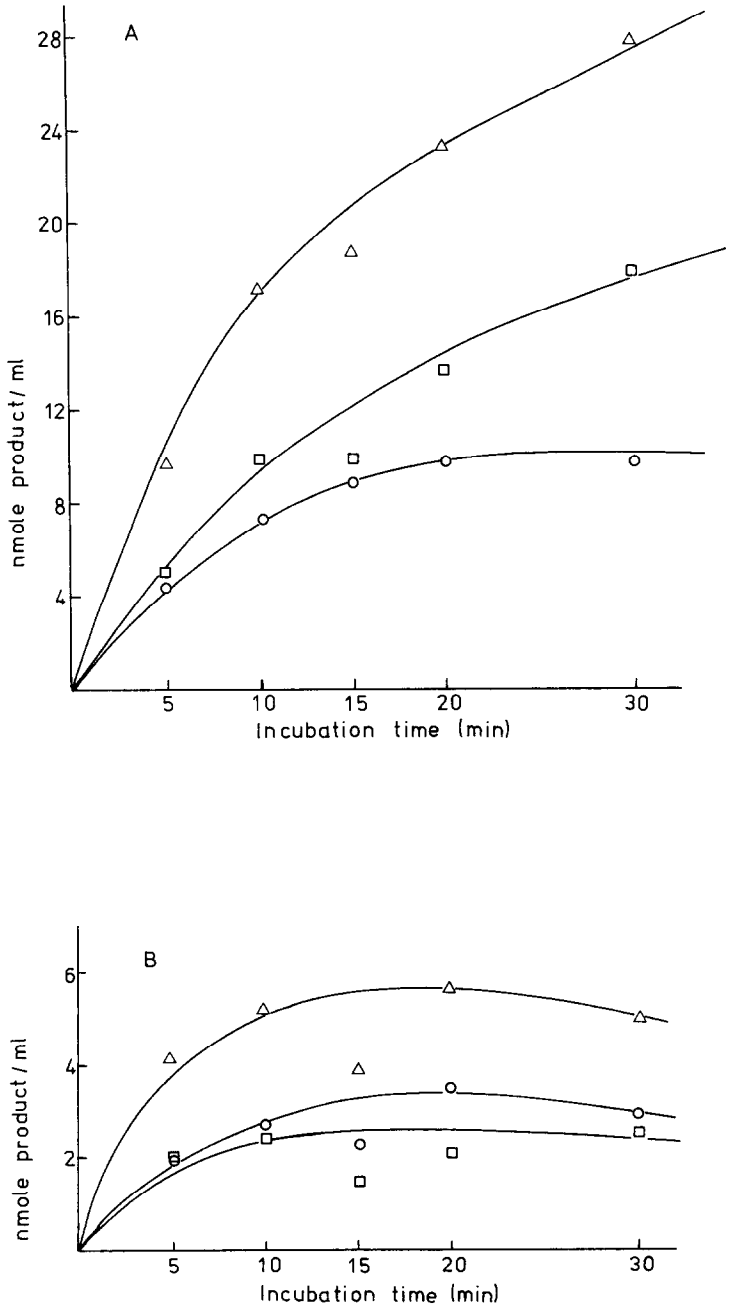

Fig. 1. Time course of the metabolism of 7-hydroxycoumarin in guinea-pig everted gut sacs. A–Conjugates released into the luminal fluid. B–Conjugates released into the serosal fluid.

Everted gut sacs were incubated with 100 μM 7-hydroxycoumarin at 37°C for periods of up to 30 min. At the times indicated, samples of the luminal and serosal fluid were taken. These samples were deconjugated, extracted with ether and the ether fraction extracted with glycine/NaOH buffer, pH 10.4. The various fractions obtained by this procedure were analysed fluorimetrically for 7-hydroxycoumarin. Results represent the mean of 3 determinations. △ total metabolites; ○ glucuronides; □ sulphates.

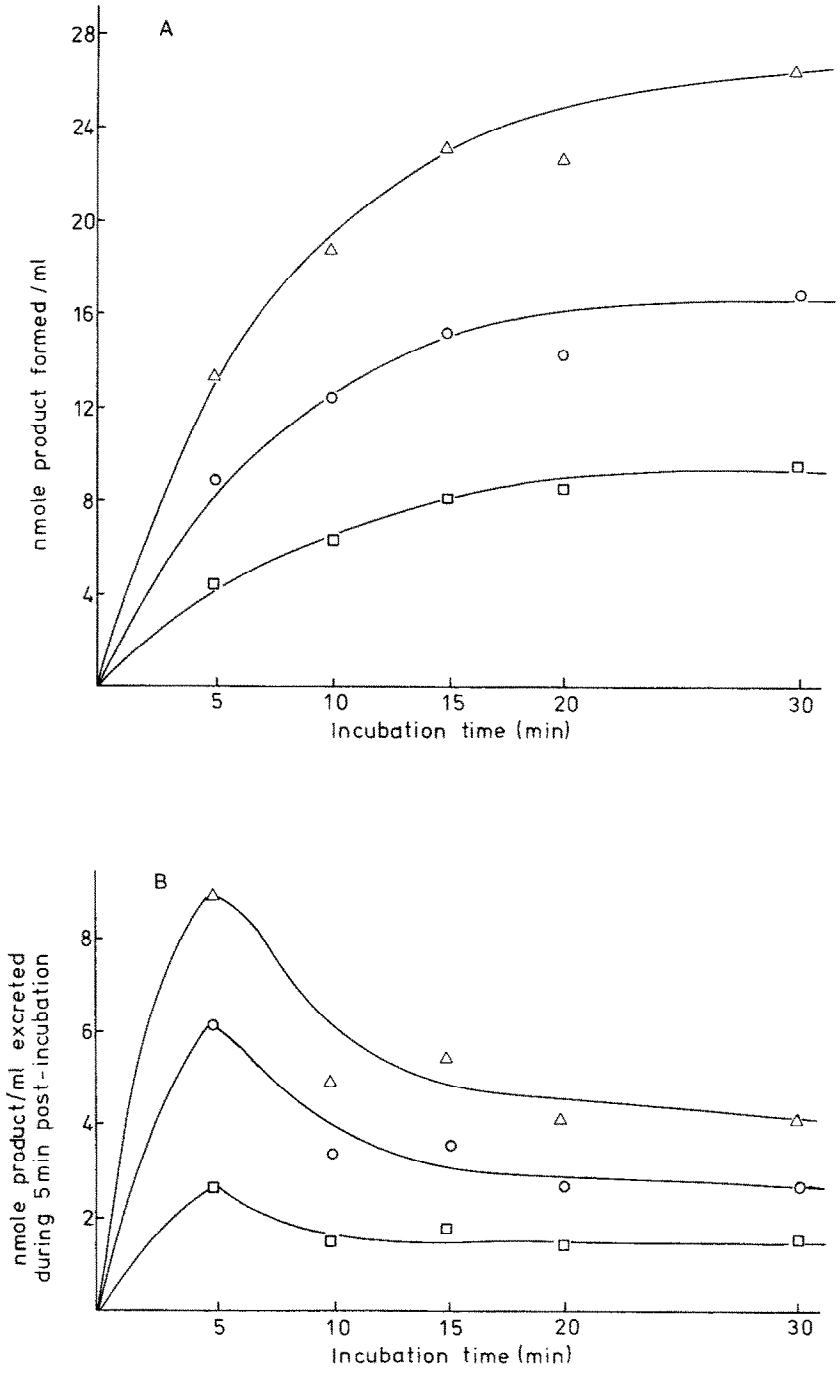

Fig. 2. Time course of the metabolism of 7-hydroxycoumarin by an isolated *in situ* loop of guinea-pig small intestine, showing A-conjugates released into luminal fluid during substrate incubation, and B-conjugates released into luminal fluid during a 5 min post-incubation 'wash'. 7-Hydroxycoumarin (100 μM) was incubated in an isolated *in situ* loop of guinea-pig small intestine for periods of up to 30 min. At the times indicated, the luminal fluid was withdrawn and an aliquot taken for deconjugation, extraction and measurement of the 7-hydroxycoumarin released. After each period of incubation the conjugates remaining in the epithelial cells were determined by a 5 min incubation with substrate-free medium and the conjugates released were measured as before—all other details as for Fig. 1.

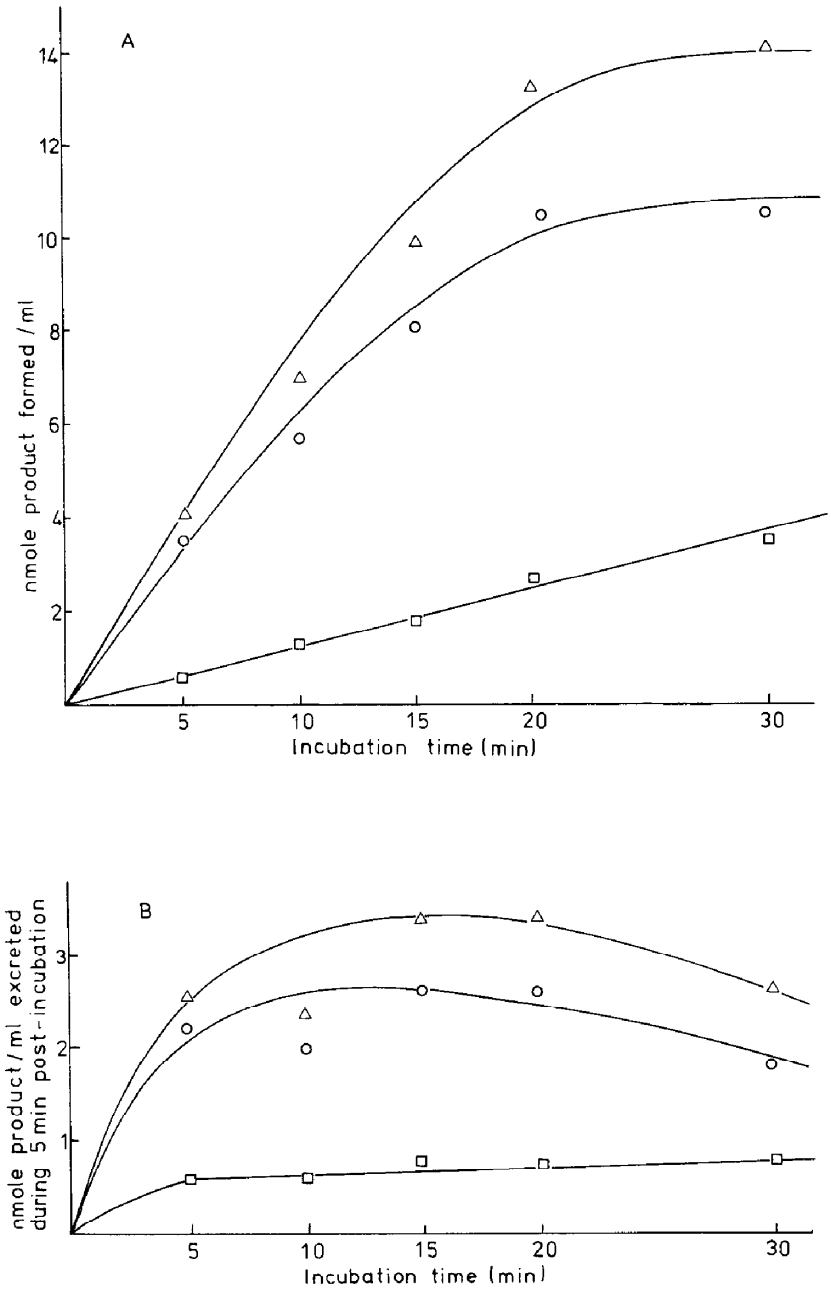

Fig. 3. Time course of the metabolism of 7-hydroxycoumarin by an isolated *in situ* loop of rat small intestine. A—Conjugates released into luminal fluid during incubation of substrate. B—Conjugates released into luminal fluid during 5 minute post-incubation 'wash'. Results represent the mean of four determinations—all other details as for Fig. 1.

intestinal mucosa into the lumen may occur [2–4, 6, 9, 10], though in most cases only trace amounts of conjugates were found in the lumen and little *in vivo* significance was ascribed to the results.

In the present investigation the gut sac results are supported by the finding that in *in situ* preparations of both guinea-pigs and rats substantial amounts of glucuronic acid and sulphate conjugates of 7-hydroxycoumarin are also found in the luminal fluid. In the present investigation, sulphate conjugation accounted for 21 per cent in rats, and 36 per cent in guinea-pigs, of the total conjugates produced *in situ*. When gut sacs were used relatively larger proportions of sulphate conjugates (~50 per cent) were formed along the entire length of the small intestine. Whether this discrepancy is due to some loss of viability of the gut sac preparations or to pharmacokinetic reasons, is difficult to ascertain. Sulphate conjugation of phenol by the intestine has been reported previously in one study [16], but the amount was only one twentieth of the extent of glucuronidation, whereas other investigators have failed to detect significant levels of sulphate conjugation [1–12].

Some workers [1, 3, 4, 9, 10] have shown an accu-

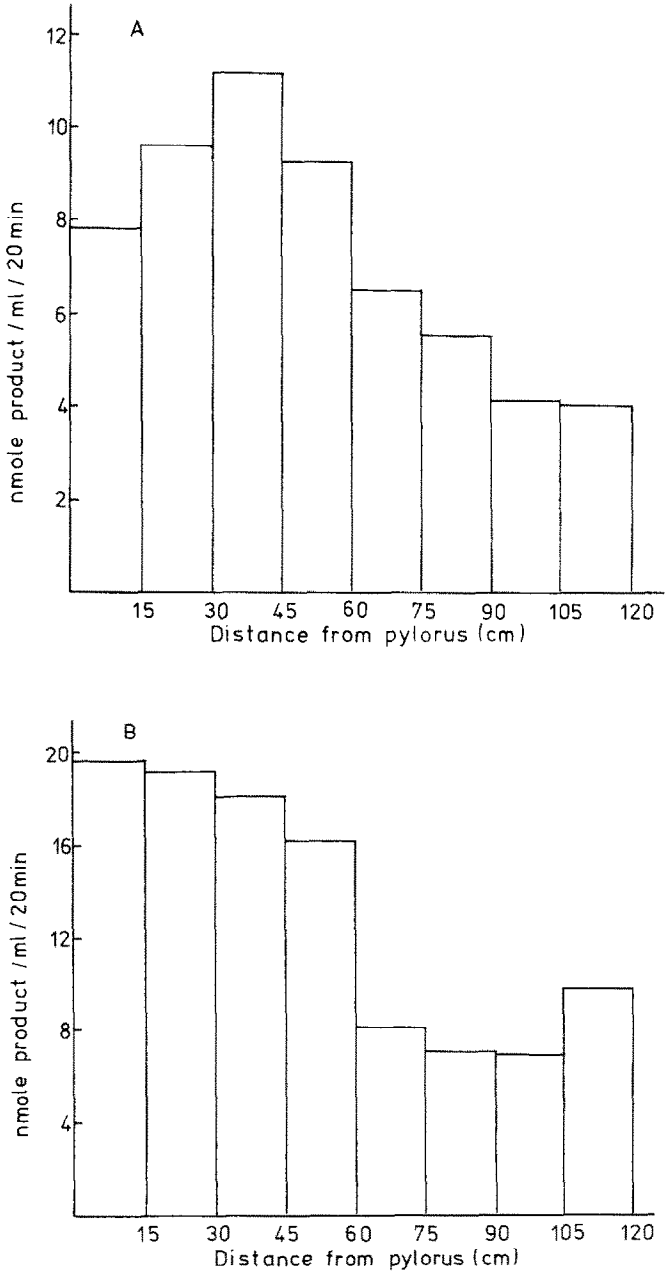

Fig. 4. Metabolism of 7-hydroxycoumarin along the small intestine of guinea-pig. Glucuronides (A) and sulphates (B) produced at different distances from the pylorus. Everted gut sacs of guinea-pig small intestine were incubated for 20 min and the conjugates in the luminal fluid determined as for Fig. 1. The results represent the mean of three determinations—all other details as for Fig. 1.

mulation of radioactivity in the intestinal wall during absorption of a radio labelled compound. This is particularly common with gut sac preparations [23]. The *in situ* experiments (Figs. 2B and 3B) show release of conjugates into the lumen of the intestine over a 5 min period, after removal of substrate from the lumen, accounting for 3–9 per cent of the 7-hydroxycoumarin added originally. This demonstrates that considerable amounts of substrate can accumulate in the epithelial cells prior to release of metabolites into the lumen.

The variation of UDP-glucuronyl transferase with distance from the pylorus, in the rat, has been demonstrated by Hanninen *et al.* [18], who found an exponential decrease in the oral half and a much less marked decrease in the aboral half. Scharf and Ullrich [17] investigated the variation in 7-ethoxycoumarin *O*-deethylase in mouse intestine, relative to distance from the pylorus. They found an initial increase in activity between 0 and 60 cm, followed by a decrease between 10 and 26 cm. In the present study, glucuronidation of 7-hydroxycoumarin in guinea-pig small intestine increased between 0 and 45 cm before gradually decreasing to less than half of the maximum value. In contrast, sulphate conjugation gave no initial increase, but fell to less than half of the highest value, after 60 cm. This decrease in conjugating enzymes as distance from the pylorus increases may be an adaptation to the level of dietary substrates encountered. The highest levels of orally ingested xenobiotics found will be nearest the stomach, progressively smaller amounts will be encountered in the lower part of the intestine because increasingly the xenobiotic will be absorbed and/or metabolised.

The release of conjugates formed in the intestinal epithelial cells into the intestinal lumen has important implications, especially in the case of low molecular weight compounds, such as phenol, which would not be excreted into the bile after hepatic metabolism. Glucuronides which are excreted into the lumen of the small intestine will pass into the large intestine, where significant amounts of bacterial β-glucuronidase are found [19–21]. The glucuronides excreted into the small intestine could be lysed in the lower gut and since there are much lower levels of conjugating enzymes in these regions the aglycones could cause damage to the cells of the gut in this region, leading to possible chronic toxic sequalae such as colonic cancer. Volp and Lage (1978) have shown that the major water-soluble metabolite of digitoxin, a glucuronide, was excreted in the bile, but only a few per cent of the amount excreted in the bile was recovered in the faeces and urine. This they found to be due to bacterial hydrolysis of the glucuronide in the caecum [24]. Significant levels of bacterial sulphatases in the colon have not been reported but may also occur.

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REFERENCES

1. R. Herz Jr., D. F. Tapley and J. E. Ross, *Biochim. biophys. Acta* **53**, 273 (1961).
2. M. J. Kreek, F. G. Guggenheim, J. E. Ross and D. F. Tapley, *Biochim. biophys. Acta* **74**, 418 (1963).
3. J. C. Pekas, *Am. J. Physiol.* **220**, 2008 (1971).
4. J. C. Pekas, *Toxicol. appl. Pharmac.* **29**, 404 (1974).
5. M. J. Rance and J. S. Shillingford, *Xenobiotica* **7**, 529 (1977).
6. F. R. Smith, D. F. Tapley and J. E. Ross, *Biochim. biophys. Acta* **69**, 68 (1963).
7. T. J. Stahl and D. F. Tapley, *Endocrinology* **73**, 271 (1963).
8. K. W. Bock and D. Winne, *Biochem. Pharmac.* **24**, 859 (1975).
9. J. C. Pekas, *Toxicol. appl. Pharmac.* **23**, 62 (1972).
10. N. R. Strahl and W. H. Barr, *J. Pharmaceut. Sci.* **60**, 278 (1971).
11. J. C. Turner, V. Shanks, W. J. Kelly and R. S. Green, *Gen. Pharmac.* **8**, 51 (1977).
12. W. H. Barr and S. Riegelman, *J. Pharmaceut. Sci.* **59**, 164 (1970).
13. J. T. Doluisio, N. F. Billups, L. W. Dittert, E. T. Sugita and J. V. Swintosky, *J. Pharmaceut. Sci.* **58**, 1196 (1969).
14. R. J. Shirkey, J. Kao, J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **28**, 1461–1466 (1979).
15. J. R. Dawson and J. W. Bridges, *Biochem. Pharmac.* **28**, 3299–3305 (1979).
16. G. M. Powell, J. J. Miller, A. H. Olavesen and C. G. Curtis, *Nature, Lond.* **252**, 234 (1974).
17. R. Scharf and V. Ullrich, *Biochem. Pharmac.* **23**, 2127 (1974).
18. O. Hanninen, A. Aitio and K. Hartiala, *Scand. J. Gastroenterol.* **3**, 461 (1968).
19. R. T. Williams, *Toxicol. appl. Pharmac.* **23**, 769 (1972).
20. R. R. Scheline, *J. Pharmaceut. Sci.* **57**, 2021 (1968).
21. R. R. Scheline, *Pharmac. Rev.* **25**, 450 (1973).
22. A. Leibovitz, *Am. J. Hyg.* **78**, 173 (1963).
23. J. B. Houston, D. G. Upshall and J. W. Bridges, *J. Pharmac. exp. Ther.* **189**, 244 (1974).
23. R. F. Volp and G. L. Lage, *Drug Metab. Dispos.* **6**, 418 (1978).