Biochemical Pharmacology, Vol. 24, pp. 1855–1858. Pergamon Press. 1975. Printed in Great Britain.

EVIDENCE THAT D-GLUCARO-1,4-LACTONE SHORTENS THE PHARMACOLOGICAL ACTION OF DRUGS BEING DISPOSED VIA THE BILE AS GLUCURONIDES

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(*Received* 27 *January* 1975)

Abstract—Depression of locomotor activity caused by phenobarbital (80 mg/kg, i.p.) or progesterone (160 mg/kg, i.p.) was shortened in male rats pretreated with D-glucaro-1,4-lactone (1·2 g/kg, p.o.), which did not exert any effect when given alone. The duration of the zoxazolamine paralysis (75 mg/kg, i.p.) was not affected, however, by the presence of D-glucaro-1,4-lactone. After oral administration of D-glucaro-1,4-lactone, β-glucuronidase was greatly inhibited within 6 hr, when measured in luminal contents throughout the intestinal tract, at pH values of 6·5, 7·8 and 7·0, which were those found to exist in the jejunum, the ileum and the colon respectively. D-Glucaro-1,4-lactone itself, added *in vitro*, inhibited β-glucuronidase from intestinal epithelial cells and luminal contents at the forementioned physiological pH values. The results suggest that inhibition by D-glucaro-1,4-lactone of the intestinal bacterial β-glucuronidase could enhance the elimination of substances excreted in the bile as D-glucuronic acid conjugates. This is probably mediated by suppressing the enterohepatic circulation of the pharmacologically active aglycones.

Many endogenous and exogenous compounds are excreted into bile, either conjugated or not; conjugation is mainly with glucuronic acid. The contribution of intestinal microflora to the metabolism of such compounds has recently provoked attention, because of the changes in toxicity which may ensue [1]. β-Glucuronidase activity occurs in the intestinal lumen, where it arises both from the microflora and from sloughed intestinal epithelial cells [2]. This enzyme, by hydrolysing biliary excreted glucuronides, would allow reabsorption of liberated aglycones into the enterohepatic circulation [3]. The extended enterohepatic circulation of morphine [4] may be an example of such an effect. If the lumen β-glucuronidase could be inhibited *in vivo*, then excretion of glucuronidated aglycones by this route should become more efficient.

D-Glucaro-1,4-lactone, produced during D-glucuronic acid metabolism [5] is, *in vitro* and at an acid pH, a potent inhibitor of mammalian β-glucuronidase [6, 7], and also inhibits the bacterial enzyme under these conditions [6]. Its addition to an intraduodenal infusion of stilbestrol monoglucuronide notably decreased the absorption of free stilbestrol [8], consistent with its inhibition of β-glucuronidase in the intestine. However, the effect of D-glucaro-1,4-lactone on intestinal β-glucuronidase has not so far been investigated in animals given the compound.

The present work examines the result of oral administration of D-glucaro-1,4-lactone (glucarolactone) both on the β-glucuronidase activity of intestinal contents at physiological pH values, and on the biological activity of glucuronidogenic drugs excreted in the bile or urine. Inhibition by glucarolactone itself of β-glucuronidase activity from intestinal mucosa and luminal contents has also been examined *in vitro* at a physiological pH.

MATERIALS AND METHODS

Animals. Male albino rats of the Wistar/Af/Han/Mol/(Han 67) strain, were used for the work *in vivo*. They were purchased from Møllegaard Avlslaboratorier A/S (Denmark), and represent the fourth generation outbred with the rotational mating system in the Laboratory Animal Center of the University of Kuopio. The animals weighed 300 ± 20 g, and had free access to water and pelleted rat food (Hankkija Ltd, Finland). They were kept in artificial diurnal lighting, with 14 hr light and 10 hr darkness (from 9 p.m. to 7.00 a.m.). Mice (male ASH/TO stock) and Wistar rats were used for studies on the inhibition *in vitro* of β-glucuronidase by glucarolactone in the intestinal mucosa and luminal contents.

β-Glucuronidase activity in the intestinal contents. Glucarolactone (D-saccharo-1,4-lactone, Pfizer) was administered in two divided doses one hour apart (1·2 g/kg, orally). Controls received the same vol of water. Rats were killed with a blow on the head 3 or 6 hr after the second administration. About 0·5 g of the intestinal content was collected from the jejunum (about 10 cm from the pylorus), the ileum (5 cm before the ileo–caecal valve) and the colon (5 cm from the ileo–caecal valve).

Samples were homogenized with a mechanically driven Potter–Elvehjem homogenizer in four vol (w/v) of 0·1% Triton X-100 solution. The homogenates were centrifuged at 2000 *g* for 15 min and enzyme assays were carried out on the supernatants. With phenolphthalein-β-glucuronide (Sigma Chemical Co., St. Louis, U.S.A.) as substrate (1·5 mM), incubation was at 38° in phosphate buffer (50 mM) in a total vol of 1 ml for 1 hr. The pH of the reaction was 6·5, 7·8 and 7·0 for samples from the jejunum, the ileum and the colon respectively, and corresponded to the values we found

under physiological conditions in the above regions of the intestinal lumen. A tube containing only substrate and buffer was taken as the blank. The reaction was stopped by 0·5 ml 10% trichloroacetic acid; after centrifugation, 1 ml sodium bicarbonate buffer was added (1 M, pH 10·5). Liberated phenolphthalein was measured at 540 nm [9].

In vitro inhibition studies were with *p*-nitrophenyl-β-glucuronide (Sigma) as substrate, and β-glucuronidase assay was that of Hétu and Gianetto [10]. Bovine liver β-glucuronidase was Sigma Type B-1 preparation; bacterial enzyme was Sigma Type 1 powder from *E. coli* culture fluid. Colonic and jejunal contents were gently expressed from segments of mouse and rat intestine; mucosal preparations were scrapings of epithelia from similar saline-washed segments.

Protein determination was by the method of Lowry *et al.* [11] using bovine serum albumin (Sigma) as the standard. Statistical analysis was by Student's *t*-test or as indicated.

Pharmacological action of drugs. Phenobarbital (5-ethyl-5-phenylbarbituric acid) and progesterone (pregnen-4-dion-3,20) (both from E. Merck, Darmstadt, Germany) were used as model compounds which are excreted as glucuronides into the bile, and exert a pharmacological effect in depressing the CNS. Phenobarbital was dissolved (20 mg/ml) in 0·5 *N* NaOH and the pH was adjusted to 8·6 by 1 *N* HCl. Progesterone was dissolved (40 mg/ml) in olive oil (Fisher Co., New Jersey, U.S.A.). Phenobarbital (80 mg/kg, i.p.) or progesterone (160 mg/kg, i.p.) were administered to both experimental and control animals at 11 a.m. Experimental animals received glucarolactone 1·2 g/kg orally in two divided doses. The first was given 1 hr before and the second just before administration of the tested drug. The control animals received the same vol of water (2 ml).

Locomotor activity was registered for 24 hr on a double-channel ANIMEX® (LKB, Sweden), counts being taken at 5 min intervals from control and experimental recorder in succession. This permits an almost simultaneous recording of the control and experimental animals and excludes possible external interference in the counts. Each experiment was performed with a pair of animals of similar body wt. Locomotor activity of the experimental animal was expressed as a percentage of the control. Statistical analysis was performed with the test of Wilcoxon [12] for pair differences.

Zoxazolamine (2-amino-5-chlorobenzoxazole, McNeil Laboratories, Washington, U.S.A.) was dissolved in 0·2 *N* HCl (20 mg/ml), and was administered i.p. (75 mg/kg). The paralysis induced was estimated in animals pretreated with or without glucarolactone by measuring the duration of loss of the righting reflex. The endpoint of the experiment was considered the ability of the animal to right itself during two successive trials.

RESULTS

Activity of β-glucuronidase measured in the intestinal contents was found to increase aborally in rat gut, being very high in the colon. When glucarolactone was given orally it had after 3 hr apparently inhibited the activity in samples from jejunum and ileum, but not in that from colon. Only traces of activity could be detected in jejunum; in ileum a 66 per cent inhibition occurred. When samples were taken 6 hr after the administration of glucarolactone, β-glucuronidase was inhibited in all parts of the intestinal tract tested, the inhibition remaining more pronounced in jejunum. Enzyme activity was inhibited by 82 per cent in jejunum, 40 per cent in ileum and 30 per cent in colon (Table 1).

Further experiments demonstrated that this inhibition could be due to glucarolactone. At corresponding physiological pH values, the lactone, added *in vitro*, inhibited β-glucuronidase in both mucosal and luminal preparations from jejunum and colon of mice (Table 2). Ratios of enzyme activities at pH 4·5 and 7·0 were compared from various sources (Table 2). As expected [13] these ratios differed between mammalian and bacterial sources. Ratios from luminal preparations, especially those from colon, tended to resemble those of bacterial rather than of mucosal origin. A quantitative comparison is nevertheless difficult, because glucarolactone is subject to bacterial breakdown and unstable in non-acid solution [13]. Experiments using Wistar rats and *p*-nitrophenyl or phenophthalein glucuronide gave similar results to those for mice.

Table 1. Activity of β-glucuronidase in the contents of jejunum, ileum and colon 3 and 6 hr after an intragastric administration of D-glucaro-1,4-lactone (1·2 g/kg)

	Enzyme activity (nmoles phenolphthalein liberated/min per mg protein)		
	Control (8)	D-glucaro-1,4-lactone (8)	% Inhibition
	3 hr after administration		
Jejunum	1·9 ± 0·4	traces	100
Ileum	3·0 ± 0·5	1·4 ± 0·2†	66
Colon	66·2 ± 18·3	46·4 ± 31·7	—
	6 hr after administration		
Jejunum	2·1 ± 0·2	0·4 ± 0·1†	82
Ileum	4·0 ± 0·7	2·4 ± 0·7†	40
Colon	49·4 ± 3·4	33·8 ± 5·0*	30

The mean values and S.D. are quoted. The number of animals is given in parentheses. Statistical significance has been calculated in respect to the controls treated with the same volume of water (*$P < 0.05$, †$P < 0.005$).

Table 2. Effect of D-glucaro-1,4-lactone added *in vitro* to β-glucuronidase preparations (bovine liver: Sigma B-1 and bacteria: Sigma Type 1).

	Enzyme source					
	Bovine liver	Bacteria	Jejunal mucosa	Jejunal contents	Colonic mucosa	Colonic contents
(a) % Inhibition by glucarolactone at pH 7·0	84·2 ± 5·3 (5)	61·5 ± 8·1 (5)	—	—	70·0 ± 14·6 (5)	36·5 ± 12·3 (6)
(b) % Inhibition by glucarolactone at pH 6·5	90·0 ± 3·8 (5)	58·1 ± 6·0 (5)	75·1 ± 20·0 (5)	60·1 ± 18·1 (6)	—	—
(c) Mean ratio of enzyme activities at pH 7·0 : pH 4·5	0·1 (3)	3·0 (3)	0·5 (2)	1·0 (3)	0·3 (2)	2·1 (3)

Incubation was for 1 hr with or without 0·01 M glucarolactone at pH values indicated. For (a) and (b) concentrations of enzyme from the various sources were adjusted to give comparable uninhibited activity at pH 6·0. For (c) inhibitor was present, parallel assays being made at pH 4·5 and pH 7·0. Number of experiments in parentheses; S.D. quoted for (a) and (b).

The effect of glucarolactone on duration of drug action was then investigated. A single administration of phenobarbital to rats caused sedation for a few hours and a long depression of locomotor activity. Progesterone had a much shorter sedative effect, but also severely depressed locomotor activity. For each pair of animals tested, the cumulative locomotor activity was calculated at the 15th and 24th hr after administration of the drug. This also permitted an estimation of the drug effect up to the first half of the dark period, during which time rats are normally very active. Animals receiving glucarolactone in combination with the tested drug were found to be less sedated than the controls. When administered alone, glucarolactone had no effect on the locomotor activity (Fig. 1).

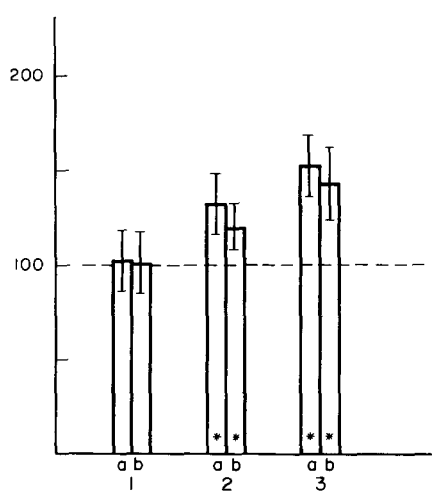

Fig. 1. Effect of D-glucaro-1,4-lactone (1), and phenobarbital (2) or progesterone (3) in combination with D-glucaro-1,4-lactone on the locomotor activity of male rats. Values have been expressed as percentage of the locomotor activity of the respective controls, 15 (a) and 24 hr (b) after the administration of the tested drug. Controls were animals treated with water (1), water and phenobarbital (2) and water and progesterone (3). In each series of experiments ten pairs of animals were used. The bars indicate S.D. Data have been calculated statistically according to the test of Wilcoxon [12] for pair differences (*$P < 0.005$).

The paralysis caused by zoxazolamine was not found to be affected by a simultaneous administration of glucarolactone. The duration of the loss of the righting reflex was 279 ± 44 (SD) (n = 10) and 262 ± 45 (SD) (n = 10) min for the zoxazolamine and zoxazolamine plus glucarolactone treated animals respectively.

DISCUSSION

Several workers have used or recommended glucarolactone to reduce toxic effects of administered compounds, by virtue of its presumed inhibition of β-glucuronidase *in vivo* [14–16]. Although the compound is readily absorbed from the intestine [17] and inhibition of β-glucuronidase is subsequently noted in mouse liver or kidney [17] and in bacteria of human bile [18], no attempt seems to have been made to determine how far β-glucuronidase is inhibited in the intestine under these conditions.

The above results indicate that, 6 hr after feeding glucarolactone, β-glucuronidase activity is significantly inhibited in the intestinal contents at their physiological pH values. Moreover, the lactone is able *in vitro* at this pH to inhibit intestinal β-glucuronidase to a comparable extent. The β-glucuronidase inhibition in the lumen observed after feeding glucarolactone is therefore likely to be due to the compound itself.

This inhibition could be expected to reduce enterohepatic circulation of biliary-excreted glucuronidogenic drugs. Both phenobarbital and progesterone qualify as such drugs. Some 10 per cent of labelled carbon from injected [^{14}C]phenobarbital is excreted in rat bile within 6 hr [19] and the compound is glucuronidogenic [20]. Progesterone also gives rise to biliary glucuronidated metabolites [21], and in rat 73 per cent of the labelled dose is excreted by this route [22]. There is no evidence in the literature, that phenobarbital and progesterone glucuronides may enter as such the enterohepatic circulation or may possess a pharmacological action.

Zoxazolamine appears to be excreted into urine either unchanged, as chlorzoxazone or as 6-hydroxyzoxazolamine [23]. The latter is glucuronidated but is not a muscle-relaxant [23], so that even if it were excreted into bile, hydrolysis of its conjugate by β-glucuronidase would not prolong the pharmacological effect of the parent zoxazolamine. This could explain the observed lack of effect of fed glucarolactone on zoxazolamine action.

It is therefore likely that the significant shortening of the pharmacological activity of phenobarbital and progesterone, demonstrated in the rats fed glucarolactone, resulted from inhibition of β-glucuronidase. The lactone by itself did not alter locomotor activity.

Inhibition in the intestine probably played the major role in this ameliorative effect. Glucuronidation may increase after glucarolactone administration [24–26], but much evidence suggests such increases are not significant *in vivo* [27, 28]. Rat intestinal β-glucuronidase derives from mucosa and bacteria [29]. Probably inhibition of the bacterial enzyme was largely responsible. Total β-glucuronidase activity of rat colonic contents is large and being preponderantly of bacterial origin [12] is operating near its optimal pH. Further up the tract much β-glucuronidase may be derived from sloughed mucosal cells, but it is operating at a pH far from optimal for the mammalian enzyme. The results reported above might suggest a bacterial, rather than mucosal, source of the enzyme.

Whatever the origin or site of operation of luminal β-glucuronidase, it appears to be inhibited by orally-administered glucarolactone. The lactone administered chronically to mice [30] or acutely to rats, as in this investigation, has no observable toxic effects. β-Glucuronidase occurs in human intestinal lumen and, allowing for differences in microflora among species or individuals [31–33], oral administration of glucarolactone may well be effective in ameliorating toxicity of compounds known to be excreted as glucuronides in human bile. Although not conclusive, this study strongly suggests that glucarolactone has the ability to enhance the excretion of glucuronidogenic compounds. A more direct pharmacokinetical approach would be beneficial in understanding this phenomenon in depth.

Acknowledgements—This study has been partly supported by a grant from the U.S. Public Health Service (AM-6018-13) and the M.R.C., London. Professor K. Hartiala (Turku, Finland) has generously supplied us with glucarolactone. Zoxazolamine was a kind donation of McNeil Laboratories, Inc. We are grateful to Miss M. Luukkainen for her able assistance.

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