A Model System for Investigating the Biliary Excretion of an Anion in the Rat

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(Received 4 November 1974)

1. The biliary excretion of phenolphthalein di[³⁵S]sulphate was studied in rats. 2. The conjugate was administered by continuous infusion at rates of 3, 4.5, 6, 9 and 12 µg/min, and kinetic analysis of the rate of biliary excretion was consistent with a two-compartment open-model system. 3. The results obtained after single injections of the ester were also consistent with the model. 4. An essential feature of the model is the presence of a compartment into which the ester may pass as an alternative to direct excretion via the bile. 5. It is suggested that such a compartment may be located within the liver.

Although the elimination of conjugates of various compounds via the biliary route is well documented, little information is available on the mechanisms by which such conjugates are transferred into the bile. We have applied a mechanistic approach to the problems of biliary excretion of certain conjugates in the rat (Powell *et al.*, 1973) and it has been shown that a number of anionic conjugates compete for the same mechanism(s) of biliary excretion. Kinetic evaluation of the data obtained after the constant infusion of a sulphuric acid conjugate (phenolphthalein di[³⁵S]sulphate) was consistent with the model system described by:

where K is the infusion rate, A is the amount of conjugate in the rat having direct access to the bile and to an alternative compartment, k_1 is the rate constant for the biliary excretion of the conjugate, B is the amount of conjugate in the bile, k_2 is the rate constant for entry into the alternative compartment and C represents the amount of conjugate in that compartment. The present study was carried out to test the validity of this model system.

Materials and Methods

Tripotassium phenolphthalein di[³⁵S]sulphate

Sulphation of phenolphthalein with ³⁵S-labelled chlorosulphonic acid (0.4 g; specific radioactivity

8.9 mCi/mmol; obtained from The Radiochemical Centre, Amersham, Bucks., U.K.) was carried out by the method of Whitehead *et al.* (1952). A mixture of dry pyridine (0.62 ml) and dry chloroform (0.62 ml) was cooled to 0°C in a centrifuge tube. Chloro[³⁵S]-sulphonic acid (0.245 ml) was added dropwise with stirring followed by phenolphthalein (400 mg) dissolved in dry chloroform (0.8 ml) to which sufficient dry pyridine to solubilize the phenolphthalein had been added. The mixture was allowed to attain room temperature (18°C), stirred for 2 h and stored at room temperature for 18 h. It was then cooled to 0°C, neutralized with 80% (w/v) KOH, stirred for 10 min and poured into ethanol (15 ml). The yellow precipitate was harvested by centrifuging and the solid extracted at 80°C with aq. 80% (v/v) ethanol (3 × 20 ml) while the mixture was maintained at alkaline pH.

The extracts were combined and cooled to 0°C; the ³⁵S-labelled phenolphthalein disulphate was precipitated by the addition of ethanol (100 ml) and harvested by centrifuging. The white precipitate was crystallized by dissolving in a minimum volume of water and the ester was salted out by addition, with vigorous stirring, of 80% (w/v) KOH. The ester was recrystallized from aqueous solution by the addition of ethanol. The yield was 217 mg. T.l.c. on silica gel G with butan-1-ol-acetic acid-water (4:1:1, by vol.) (R_F of the product 0.25) and benzene-butan-2-one-ethanol-water (1:1:2:1, by vol.) (R_F of the product 0.66) showed the product to be homogeneous and free from inorganic [³⁵S]sulphate. The product gave negative tests for phenolphthalein.

The ester was stored as a dilute frozen aqueous solution (5 mg/ml) to minimize degradation by self-irradiation.

Animals

Male MRC hooded rats (350–400 g body wt.) were used throughout. Animals were anaesthetized with ether, the right jugular vein was cannulated and pentobarbitone sodium (Nembutal; Abbot Laboratories Ltd., Queenborough, Kent, U.K.) was administered via the cannula at a dose of 10–20 mg/kg body wt. The common bile duct was cannulated through a mid-line abdominal incision. Animals were maintained under Nembutal anaesthesia throughout the experiments. Under these conditions normal body temperature is maintained. In some experiments the left jugular vein was cannulated also and constant infusions were introduced via this cannula.

Whole-body radioautography

Young male MRC hooded rats (30–50 g body wt.), under ether anaesthesia, each received an intravenous injection of phenolphthalein di[^{35}S]sulphate (1 mg dissolved in 0.2 ml of water). Rats were killed at suitable time-intervals (up to 2 h) and whole-body radioautograms were prepared by the method of Powell *et al.* (1967).

Measurement of radioactivity in bile

Samples of bile (10 μl) were added to 10 ml of scintillation 'cocktail' [toluene (1600 ml), methyloxitol (400 ml), diphenyloxazole (14 g)], and ^{35}S was determined by using a Tracerlab Corumatic 200 liquid-scintillation spectrometer.

Experimental and Results

Continuous-infusion experiments

Animals with bile-duct fistulae received continuous infusions of phenolphthalein di[^{35}S]sulphate at a rate of 15 $\mu\text{l}/\text{min}$ (Unita I Continuous Infusion Apparatus; B. Braun, Melsungen, Germany). The

concentrations of the solutions were adjusted so that the ester was infused at the following rates: 3.0, 4.5, 6.0, 9.0 and 12.0 $\mu\text{g}/\text{min}$. Bile samples were collected in tared tubes at regular intervals (usually 10 min) for 3–4 h after the beginning of the infusion. Bile weights were recorded and each bile sample was assayed for total radioactivity.

The results obtained (Fig. 1) demonstrated that when phenolphthalein di[^{35}S]sulphate was infused at a constant rate, the rate of excretion of the ester into bile increased with time, reaching a limiting value which was then maintained for the duration of each experiment. At each infusion rate this limiting value was considerably less than the corresponding infusion rate (mean of the five doses was $68 \pm 16\%$) showing that the excretory mechanism was not saturated and

Fig. 1. Biliary excretion of phenolphthalein di[^{35}S]sulphate in rats receiving constant infusions of the ester

The ester was infused at the following rates ($\mu\text{g}/\text{min}$): ●, 3; ■, 4.5; □, 9; ○, 12. The points are experimental and the curves are calculated from eqn. (1) (see the text) by using the values for slope (k_1/α) and lag (α) shown in Table 2.

Table 1. Biliary excretion of phenolphthalein di[^{35}S]sulphate administered to rats by continuous intravenous infusion

Lag period was determined by extrapolation of the linear portions of the curves in Fig. 1, to intercept the time axis. For further details see the text.

Infusion rate ($\mu\text{g}/\text{min}$)	Bile flow ($\mu\text{l}/\text{min}$)	Lag period (min)	Rate of excretion during the steady state	
			($\mu\text{g}/\text{min}$)	(% of infusion rate)
3.0	17	25	2.1	71
4.5	24	15	3.6	80
*6.0	11	30	2.5	42
9.0	17	22	5.7	63
12.0	22	18	10.0	83

* The results of this experiment are not included in Fig. 1 owing to the exceptionally slow bile flow which affects the rate of excretion during the steady state and these values are not adjusted for variations in bile flow. For details see the text.

Fig. 2. Steady-state excretion of phenolphthalein di[³⁵S]sulphate

This figure shows the relationship between the rate of production of bile and the proportion of administered ester which is excreted over the same time-interval during the steady-state periods shown in Fig. 1. The points are experimental and the line is the best fit obtained by linear-regression analysis.

Fig. 3. Relationship between the rate of infusion of phenolphthalein di[³⁵S]sulphate and the steady-state rate of its excretion in bile

(a) Before and (b) after corrections for variations in bile flow. For details see the text.

the value must be considered to represent a steady state.

The progress curves shown in Fig. 1 are each characterized by a lag period before the steady state is established. This lag period, determined for each experiment by extrapolating the linear portion of each curve to meet the time axis, showed no correlation with the infusion rate (Table 1). The mean value for the lag period from five experiments was 22.2 ± 5.9 min. Table 1 also shows that there was no positive correlation between bile production and the rate of infusion, although a correlation exists between the rate of bile formation and the proportion of the infused material excreted in the bile (Fig. 2). Accordingly, the positive correlation between the rate of excretion of phenolphthalein di[³⁵S]sulphate during the steady-state period and the rate of infusion of the compound is improved if allowance is made for the variation in bile flow (Fig. 3).

The results obtained in the constant-infusion studies are compatible with the simple model system represented by Scheme 1.

The amount of phenolphthalein di[³⁵S]sulphate in the bile at any time is given by

$$B = \frac{k_1 K t}{\alpha} - \frac{k_1 K}{\alpha^2} (1 - e^{-\alpha t}) \quad (1)$$

Where $\alpha = (k_1 + k_2)$.

When t is large this expression reduces to

$$B = \frac{k_1 K t}{\alpha} - \frac{k_1 K}{\alpha^2}$$

which describes the steady-state portion of the experimental curves in Fig. 1. The slopes of these lines are given by $k_1 K / \alpha$ and the extrapolated value for the lag gives $1/\alpha$. Hence the slope of the steady state should give a linear plot against the infusion rate. This correlation is improved if allowance is made for variation in bile flow (Fig. 3), since k_1 is also included in the expression describing bile flow. Theoretical values for B were calculated from eqn. (1) by using adjusted values for k_1 and α (estimated from the slope and lag of the experimental curves). These

Scheme 1. Simple model system representing fate of infused phenolphthalein di[³⁵S]sulphate

A, represents the amount of phenolphthalein di[³⁵S]sulphate contained by a volume (V_a) in the animal having direct access to a second volume (V_c) in the animal and to the bile. B and C represent the amount of phenolphthalein di[³⁵S]sulphate in bile and in the compartment (V_c) respectively. K is the infusion rate of phenolphthalein di[³⁵S]sulphate ($\mu\text{g} \cdot \text{min}^{-1}$) and k_1 and k_2 are pseudo-first-order rate constants describing the removal of phenolphthalein di[³⁵S]sulphate from V_a into bile and into V_c respectively.

theoretical values for B were used to draw the lines in Fig. 1, and the points in Fig. 1 are experimental. The close agreement of theoretical and experimental curves provides strong evidence for the validity of the model system described for continuous-infusion experiments. The question arises as to whether this model is equally valid for the biliary excretion of phenolphthalein di[³⁵S]sulphate when the ester is administered as a pulse dose.

Single-dose experiments

Animals with bile-duct fistulae received aqueous solutions of phenolphthalein di[³⁵S]sulphate at doses ranging from 1.4 to 16 mg/animal via the jugular-vein cannula. Bile was collected and assayed for total radioactivity. The results obtained were subjected to analysis in the light of the model system proposed above. After a single dose the amount of phenolphthalein di[³⁵S]sulphate excreted in the bile at any time is given by

$$B = \frac{k_1 A_0}{\alpha} (1 - e^{-\alpha t}) \quad (2)$$

where k_1 and α are defined as above and A_0 is the amount of phenolphthalein di[³⁵S]sulphate administered. Eqn. (2) predicts that a portion (k_1/α) of the original dose will be excreted in the bile by a first-order process with a rate constant of $\alpha \text{ min}^{-1}$. Some typical excretion patterns after single doses of phenolphthalein di[³⁵S]sulphate are shown in Fig. 4 and the mean

values for α and k_1/α from seven such experiments are collected in Table 2. These values are in good agreement with those calculated from experiments using constant infusions of phenolphthalein di[³⁵S]sulphate, illustrating that the model system may also be applied to single doses of the ester.

One of the essential features of the model is the presence of compartment C, into which phenolphthalein di[³⁵S]sulphate may pass as an alternative to direct excretion via the bile. However, the model makes no prediction as to the location of compartment C, which may be anywhere in the animal. Attempts were made to locate the possible sites of compartment C by whole-body radioautography.

Whole-body radioautography

Each animal received an aqueous solution (1 mg of phenolphthalein di[³⁵S]sulphate dissolved in 0.2 ml of water) intravenously. Animals

Fig. 4. Biliary excretion of phenolphthalein di[³⁵S]sulphate in rats receiving single intravenous injections of the ester

(a) Amounts of the ester excreted in the bile after intravenous administration of (●) 1.4 mg, (■) 10 mg, (○) 16 mg of ester. (b) The data in (a) were replotted according to the first-order eqn. (2). A'_0 is the total amount of the ester expected to be excreted by this process and B is the amount of the ester excreted at any time. The best estimate of A'_0 was used in each case to give a straight line. $A'_0 = k_1 \cdot A_0 / \alpha$.

Table 2. *Kinetic parameters for the biliary excretion of phenolphthalein di[³⁵S]sulphate*

Phenolphthalein di[³⁵S]sulphate was administered intravenously to rats either by continuous infusion or as a single pulse dose

Method of administration	No. of experiments	Amount administered	k_1/α (mean $\pm$ s.d.)	α (min ⁻¹) (mean $\pm$ s.d.)
Pulse dose	7	1.4–16 (mg/animal)	0.68 $\pm$ 0.11	0.036 $\pm$ 0.010
Continuous infusion	5	3.0–12.0 (μ g/min)	0.68 $\pm$ 0.16	0.048 $\pm$ 0.012

were killed at time-intervals ranging from 10 min to 2 h after injection and whole-body radioautograms were prepared. All radioautograms were characterized by accumulation of radioactivity in liver, bile canaliculi and gut contents. At no other site was the concentration of radioactivity higher than that of the circulating blood. The results raise the possibility that compartment C is located within the liver itself.

Discussion

Many substances are secreted in the bile in conjugated form, and although the significance of conjugation, in relation to biliary excretion, is not understood it is consistent with the certain degree of polarity which is apparently necessary for secretion via the biliary route. Thus intrahepatic conjugation with such polar substances as glucuronic acid and H₂SO₄ appears to be a prerequisite for the biliary excretion of a number of compounds. Various attempts have been made to investigate the secretory mechanism and, in particular, a number of reports have suggested that cholephilic substances compete with one another at several stages in their transport from plasma to bile. Other studies have resulted in the availability of much information on the biliary excretion of individual compounds, for example bromosulphthalein. The secretion of the dye has been subjected to kinetic analysis, but interpretation of the data is complicated by the intrahepatic conjugation of the dye (Richards, 1965). The data presented here on phenolphthalein disulphate obviate this difficulty, for the conjugate appears in the bile unchanged (Millburn, 1970).

The model used here to describe the distribution and excretion of phenolphthalein disulphate is analogous to one that is commonly used in pharmacokinetics and termed the 'two-compartment open model' (Wagner & Pernarowski, 1971). However, in the case described here, it is not necessary to consider any significant removal of material from

compartment C either into compartment A or into bile. It must be stressed that, for the single injection of phenolphthalein disulphate into rats, all of the administered dose is eventually excreted in the bile, but the second excretory phase is considerably slower than the initial one and becomes apparent approx. 90 min after injection. In this respect the data resemble those predicted by the two-compartment open model. However, within the limits of the experiments using constant infusions of phenolphthalein disulphate, there can be only insignificant removal of material from compartment C into compartment A or into bile. If this were not true then a steady state of excretion would mean that either (a) the rate of excretion is equal to the rate of infusion or (b) the excretory mechanism is saturated. Clearly, neither of these conditions apply with phenolphthalein disulphate, since the rate of excretion is directly proportional to, but lower than, the rate of infusion. Compartment C therefore receives a constantly accumulating amount of phenolphthalein disulphate during a constant infusion of the compound, and the question arises as to the location of compartment C. Since whole-body radioautography shows significant accumulation of material in the liver only, it appears likely that compartment C is located in the liver.

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