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GUINEA-PIG INTESTINAL SULPHOTRANSFERASES: AN INVESTIGATION USING THE CYTOSOL FRACTION

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Abstract—A rat liver cytosol preparation fortified with a PAPS regenerating system has been used to study enzymically mediated sulpha conjugation of several phenols. In agreement with previous findings with isolated intestinal epithelial cells and hepatocytes from rats 2-hydroxybiphenyl was poorly sulphated compared with 4-hydroxybiphenyl and 7-hydroxycoumarin. The results are attributed to the involvement of different sulphotransferases in the conjugation of these substrates. Examination of the effects of changing pH and substrate concentration indicated that at least two sulphotransferases are probably involved in the sulphation of 2-hydroxybiphenyl and four sulphotransferases participate in the sulphation of 4-hydroxybiphenyl.

In general, sulphation of a xenobiotic will render it less toxic, more water soluble and thus more readily excreted. However, sulphation can also produce reactive intermediates (e.g. *N*-hydroxy-*N*-acetylaminofluorene-sulphate, *N*-hydroxy-phenacetin sulphate) which are more reactive than the parent compound and can bind covalently to cell constituents [1].

There is evidence in the liver of both rats [2–4] and guinea-pigs [5–7] for more than one sulphotransferase. At least three sulphotransferases have been found in guinea-pig liver [5–7]. One is specifically a phenol sulphotransferase, and the others are an androstenolone sulphotransferase (also catalysing cholesterol sulphation), and an oesterone sulphotransferase, both of which have some phenol sulphotransferase activity as well, either due to incomplete purification or overlapping substrate specificity of the individual enzymes.

The small intestine, which is the main site of absorption of xenobiotics ingested orally, has considerable capacity for conjugating xenobiotics [8–11]. It has been shown using guinea-pig isolated intestinal cells that considerable sulphate conjugation can occur in the intestine [12]. In the latter study the two structural analogues 2- and 4-hydroxybiphenyl gave quite different patterns of sulphation. A similar lack of sulphation of 2-hydroxybiphenyl has been observed using rat hepatocytes [13]. Sulphotransferase(s) is located in the soluble fraction of the cell (cytosol), as are the enzymes involved in generating 'active sulphate' (PAPS). To determine whether the low level of 2-hydroxybiphenyl sulphate formed by guinea-pig intestinal cells [12] was due to poor access of this substrate, to the sulphotransferases, the cytosol fraction is used in the present report for a further investigation of guinea-pig intestinal sulphotransferase(s). Evidence for more than one phenol sulphotransferase in guinea-pig intestine is also presented.

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (sodium salt) was purchased from Sigma Chemical Co. (Poole, U.K.). All other chemicals and reagents were of laboratory or Analar grade.

Animals. Male Gordon Hartley guinea-pigs were used (300–400 g). Housing conditions etc. were as described previously [12].

Preparation of cytosol fraction. Intestinal cells were removed from the submucosal tissue by vibration of three lengths of everted intestine and homogenates prepared as described previously [14,15].

The homogenate was immediately readjusted to isotonicity (by addition of 0.25 M Tris–KCl, pH 7.4) and centrifuged at 1000,000 g_{max} for an hour (MSE superspeed 50, 8 × 25 ml fixed angle rotor), to sediment all particles. The resultant supernatant was called the 'cytosol fraction', it was diluted with 0.25 M Tris–HCl buffer pH 7.4 and used as the enzyme source in subsequent assays.

Assay of sulphotransferase. The reaction mixture contained 20 mM Na_2SO_4 (0.2 ml), 50 mM $MgCl_2$ (0.2 ml), 50 mM ATP (0.2 ml), 0.2 ml of diluted cytosol fraction (0.45–0.55 mg protein), substrate was added in 2 μl dimethylformamide (DMF) and the volume made up to 2 ml by addition of 0.25 M Tris–HCl (1.2 ml), pH 7.4. The reaction was started by addition of the ATP. The incubations were carried out in open 25 ml conical flasks in a shaking water bath (55 c/min) at 37°. The reaction was stopped at the times specified by removal of the flasks from the water bath and addition of 7 ml of the appropriate solvent (ether for 7-hydroxycoumarin, or *n*-heptane containing 1.5 per cent iso-amyl alcohol for 2- and 4-hydroxybiphenyl). The unreacted substrate was extracted into the solvent, the aqueous solution deconjugated overnight with sulphatase, and the substrate released determined fluorimetrically, as described by Dawson and Bridges [12].

When the assay was carried out at varying pH values, $\beta\beta$-dimethyl glutaric acid–NaOH buffer (0.25 M) was used at pH 5–7.5 and the Tris–HCl buffer (0.25 M) was used at pH 7.0–9.5. There was no significant difference in enzyme activity between these two buffers at identical pH values. Samples were incubated for 10 min before stopping the reaction.

RESULTS

The sulphation of 7-hydroxycoumarin (100 μM) by the cytosol fraction of guinea-pig intestinal cells was linear with time between 5 and 60 min, however, there was an apparently quicker phase over the first 3–5 min. Almost 200 nmoles of substrate was sulphated per mg of cytosolic protein in an hour at this substrate concentration.

The sulphation of 2-hydroxybiphenyl (100 μM) was linear with time (Fig. 1) from zero time to 60 min but the rate was only 5% of that for 7-hydroxycoumarin.

The sulphation of 4-hydroxybiphenyl (100 μM) in common with 7-hydroxycoumarin occurred in two linear phases, in the first 10 min period the rate was most rapid. (Fig. 1) The amount of sulphate formed in this instance was intermediate between that formed with the other two substrates, being approximately 48 nmoles sulphate formed per mg of cytosolic protein in an hour. At 5 μM 4-hydroxybiphenyl sulphation was linear from zero time until 10 min after which virtually all of the substrate had been sulphated.

At a substrate concentration of 100 μM formation of 2-hydroxybiphenyl and 4-hydroxybiphenyl sulphate was linear with protein concentrations over the range studied i.e. between 0.2 and 1 mg of protein per 2 ml incubation with time. However, when substrate concentrations were varied, unexpected patterns of sulphation emerged. For 7-hydroxycoumarin a maximal sulphation rate of 2.55 nmoles/mg protein/min was observed at 150 μM substrate (Fig. 2). At higher substrate concentrations the rate of sulphate formed decreased gradually to

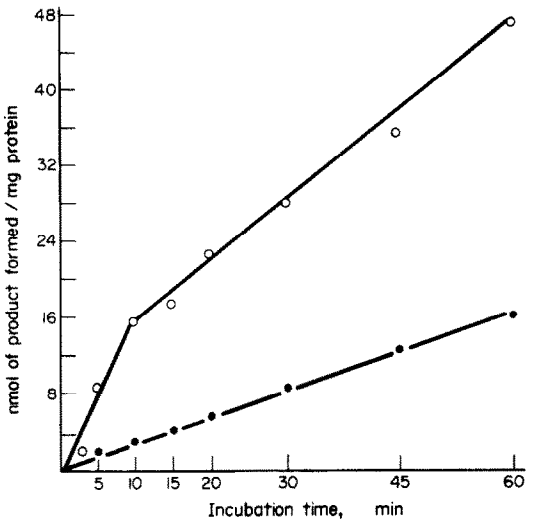

Fig. 1. Sulphation of 2- and 4-hydroxybiphenyl by cytosol fraction of guinea-pig intestinal cells. 2-Hydroxybiphenyl (● — ●) or 4-hydroxybiphenyl (○ — ○) (100 μM) was incubated with a PAPS generating system and 0.35–0.45 μg cytosolic protein in Tris–HCl buffer (0.25 M, pH 7.4) at 37°. At the times indicated the reaction was stopped and the unreacted phenol removed by extraction with *n*-heptane. The aqueous fraction remaining was deconjugated and the 2-hydroxybiphenyl or 4-hydroxybiphenyl released determined fluorimetrically. Results represent the mean of three duplicate determinations.

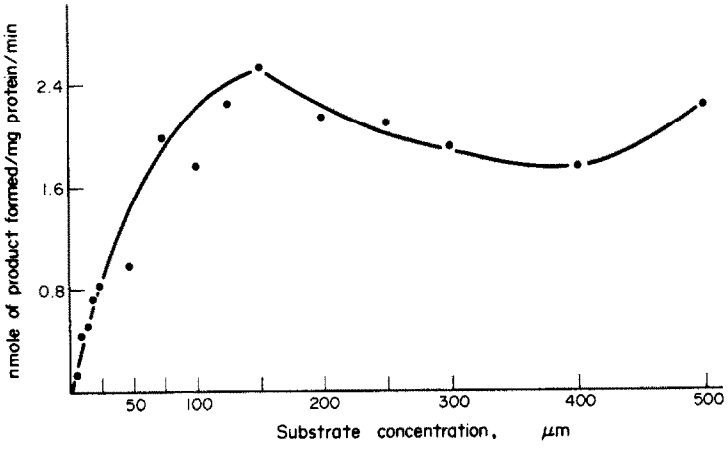

Fig. 2. Sulphation of 7-hydroxycoumarin by cytosol fraction of guinea-pig intestinal cells—variation with substrate concentration. 7-Hydroxycoumarin (5–500 μM) was incubated with a PAPS generating system and 0.35–0.45 mg cytosolic protein in Tris–HCl buffer (0.25 M, pH 7.4) at 37°. After 10 min the reaction was stopped and the unreacted 7-hydroxycoumarin removed by extraction with ether. The aqueous fraction remaining was deconjugated and the 7-hydroxycoumarin released determined fluorimetrically. Results represent the mean of four duplicate determinations.

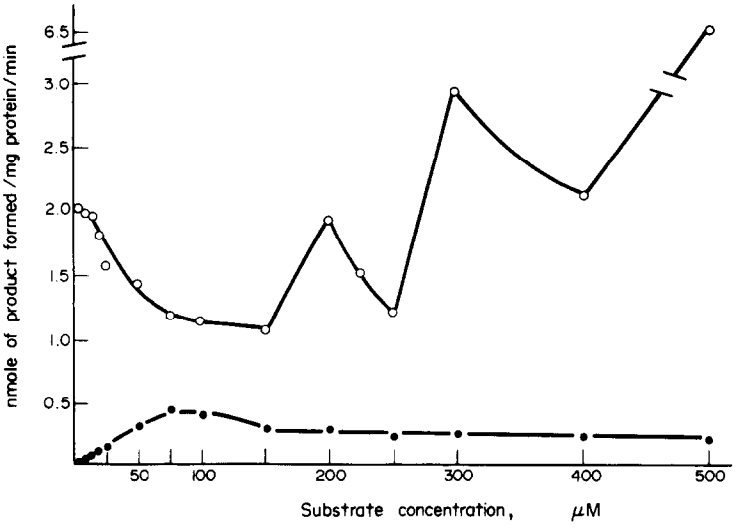

Fig. 3. Sulphation of 2- and 4-hydroxybiphenyl by cytosol fraction of guinea-pig intestinal cells—variation with substrate concentration. 2-Hydroxybiphenyl (● — ●) or 4-hydroxybiphenyl (○ — ○) (5–500 μM) was incubated with a PAPS generating system and 0.35–0.45 mg cytosolic protein in Tris–HCl buffer (0.25 M, pH 7.4) at 37°. After 10 min the reaction was stopped and the unreacted phenol removed by extraction by *n*-heptane. The aqueous fraction remaining was deconjugated, and the 2- or 4-hydroxybiphenyl released determined fluorimetrically. Results represent the mean of three duplicate determinations.

1.76 nmoles/protein/min formed at 400 μmM substrate then plateaued.

Sulphation of 2-hydroxybiphenyl reached a maximum at 75 μM substrate, then gradually decreased with further increase in substrate concentration (Fig. 3). The maximum value was 0.43 nmoles sulphate formed/mg cytosolic protein/min which decreased to under 0.20 nmoles formed/mg protein/min at 500 μM 2-hydroxybiphenyl.

The situation which arose by varying 4-hydroxybiphenyl concentration was more complex than with the other substrates. Four peak values of sulphate formation were evident over the hundred-fold range of substrate concentration investigated (5–500 μM) (Fig. 3). The first peak was at 5 μM 4-hydroxybiphenyl, and as substrate concentration was increased, there was a gradual decline in the amount of sulphate formed to a minimum of approximately 1.1 nmoles sulphate formed/mg protein/min at 150 μM 4-hydroxybiphenyl. The second peak occurred at 20 μM substrate, falling to approximately 1.2 nmoles/mg protein, formed in 1 min at 250 μM 4-hydroxybiphenyl. Another peak was present at 300 μM substrate, and at 400 μM 4-hydroxybiphenyl a smaller amount of sulphate was formed. At 500 μM 4-hydroxybiphenyl the maximum value of 6.5 nmoles product formed/mg protein/min was found.

The 'kinetics' of 4-hydroxybiphenyl sulphation by the guinea-pig intestinal cytosol fraction raised the possibility that more than one sulphotransferase was involved. This possibility was further investigated by using the two extremes of substrate concentration (5 and 500 μM), to investigate the pH optima of the enzyme(s) involved [Figs. 4(A) and (B)]. When 5 μM 4-hydroxybiphenyl was used, and a 10 min incubation, the pH optimum appeared at pH 7.5, with negligible sulphate conjugation produced below pH 6 [Fig. 4(A)]. In contrast, when 500 μM 4-hydroxybiphenyl was used, two pH optima appeared, at 7.0 and pH 8.5 [Fig. 4(B)]. Significant amounts of sulphate were produced at all pH values studied (pH 5–9.5).

DISCUSSION

In contrast to the liver [2,3,16–20] and brain sulphotransferases [19,21–23] intestinal sulphotransferases have received relatively little attention [10,24]. The present study demonstrates that considerable phenol sulphating activity is present in the cytosol of intestinal epithelia.

The use of a crude preparation of PAPS to investigate the properties of sulphation enzymes could be criticised on the grounds that PAP and ADP (strong inhibitors of sulphotransferases), might be present as contaminants [25]. An additional possible problem with the use of a PAPS generating system (ATP, SO_4^{2-}, Mg^{2+}, plus cytosol fraction as enzyme source) in kinetic studies is that with more than one reaction taking place, the rate-limiting step may not be the sulphotransferase. However, the results indicate that formation of PAPS was not rate-limiting in our studies, since the rate of the reaction was linear with K_m even at high concentrations of a rapidly sulphated substrate such as 7-hydroxycoumarin. The rate of 7-hydroxycoumarin and 4-hydroxybiphenyl sulphate formation agrees well with the rates observed when intact cells are used. This supports the contention that PAPS levels are not normally rate limiting for sulphation in the intact guinea pig intestine [12,26].

2-Hydroxybiphenyl was sulphated at only one third of the rate observed with 4-hydroxybiphenyl. It has been postulated that the specificity of *in vivo* sulpho-conjugation, in rabbit, is governed to some extent by the nature of the substituent groups, on the benzene ring of phenols [27], electrophilic ortho

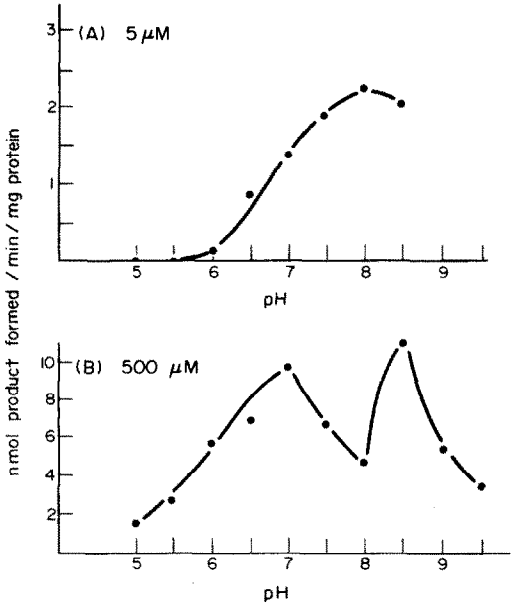

Fig. 4. Sulphation of 4-hydroxybiphenyl by cytosol fraction of guinea-pig intestinal cells—variation with pH at two substrate concentrations. 4-Hydroxybiphenyl [(A), 5 μM and (B) 500 μM] was incubated with a PAPS generating system and 0.35–0.45 mg cytosolic protein in dimethylglutaric acid–NaOH buffer (0.25 M, pH 5–7.5) or Tris–HCl buffer (0.25 M, pH 7.0–9.5) at 37°. After 10 min the reaction was stopped and the unreacted 4-hydroxybiphenyl removed by extraction with *n*-heptane. The aqueous fraction remaining was deconjugated, and the 4-hydroxybiphenyl released determined fluorimetrically. Results represent the mean of three duplicate determinations.

substituent groups tending to depress and nucleophilic ortho substituent groups to enhance sulphation. Meta substitutent groups had a similar, but lower effect while para substituents had relatively little effect. On these grounds it would be expected that 2-hydrobiphenyl would be less well sulphated than 4-hydroxybiphenyl. Alternatively, the poorer sulphation of 2-hydroxybiphenyl could be due to steric factors. The hydroxyl group of 2-hydroxybiphenyl, being angled towards the centre of the molecule, is in a fairly lipophilic environment, compared with the hydroxyl groups of the other two substrates. This could result in the hydroxyl group of 2-hydroxybiphenyl being less accessible to the sulphotransferase(s).

The amount of 2-hydroxybiphenyl sulphate formed, although low, was considerably higher (approx 16 fold) than would have been anticipated from results obtained using whole cells [12]. This indicates that the poor sulphation of 2-hydroxybiphenyl in intact cells, both of guinea-pig intestine [12] and rat liver [13] is probably at least partly due to factors other than the inherent activity of the appropriate sulphotransferase for 2-hydroxybiphenyl. Possibly the sulphotransferase(s) responsible for the sulphation of 2-hydroxybiphenyl but not that concerned with the sulphation of 4-hydroxybiphenyl and 7-hydroxycoumarin becomes activated or separated from relatively specific endogenous inhibitors on prepartion of the cytosol.

Determination of the rates of 2-hydroxybiphenyl and 7-hydroxycoumarin sulphation over a range of substrate concentrations indicates that more than one enzyme is probably involved for each substrate (Figs. 2 and 3).

The kinetics of 4-hydroxybiphenyl sulphation is even more complex. There appeared, in this instance, to be at least four rate maxima, followed in three of the cases, by a decrease in the amount of sulphate formed with increase in substrate concentration. These maxima occurred at 5, 200, 300 and 500 mM 4-hydroxybiphenyl. Such complex kinetics are most likely to occur if a multienzyme system is present, each enzyme showing substrate inhibition. Comparison of the pH profile for the sulphation of 4-hydroxybiphenyl at low and high substrate concentrations also supports the contention of several sulphotransferases acting on this substrate. At the lowest substrate concentration studied (5 μM) only one peak of activity was observed at pH 7.5, whilst at 500 μM 4-hydroxybiphenyl two peaks of activity, at pH 6.5 and 8.5 were found. Substrate inhibition has been reported for rat brain phenol sulphotransferase [27]. Sekura and Jakoby [4] studied a wide range of substrates for their rat liver sulphotransferase and found that marked substrate inhibition was evident with those compounds for which a low $K_m$was observed. From the limited data available it appears that substrate inhibition of the hepatic enzymes occurs mainly where the sulphotransferases have not been completely separated and with substrates having a low K_m. Banerjee and Roy [7] purified a phenol sulphotransferase from guinea-pig liver and reported no unusual kinetics (*p*-nitrophenol as substrate). Thus it would appear that substrate inhibition may become less dramatic as the sulphotransferases are separated and purified. However many sulphotransferases are very unstable during and after purification [2–5,17,20,27], and it is therefore quite possible that significant alterations in the *in vivo* characteristics of these enzymes may occur on purification. Four or more sulphotransferases appear to be involved in 4-hydroxybiphenyl sulphation (see Fig. 3). Such a system of sulphotransferases in the intestine would enable the body to quickly and effectively sulphate a wide range of concentrations of dietary phenolic compounds. Since each enzyme would have a different spectrum of substrate affinities, kinetics would be very complex.

In this regard it is worth noting that humans can ingest and detoxify up to 600 mg of phenolic material per day, mainly of plant origin [9,10], and of a very wide variety of structures [28].

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