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XENOBIOTIC METABOLISM BY ISOLATED INTESTINAL EPITHELIAL CELLS FROM GUINEA-PIGS

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Abstract—A method is described for the preparation of viable isolated intestinal epithelial cells from guinea-pigs. The preparation has been demonstrated to metabolise a number of model drug metabolism substrates. For ethoxycoumarin the rate of de-ethylation has been demonstrated to be the rate limiting step in its conversion to 7-hydroxycoumarin sulphate and 7-hydroxycoumarin glucuronide. On increasing the substrate concentration glucuronidation was saturatable whereas sulphation was not. In the case of 4-hydroxybiphenyl sulphation and glucuronidation rates were comparable, whereas for 2-hydroxybiphenyl glucuronidation was the predominant and sulphation the minor pathway. Stereo selectivity in the sulphatransferases is postulated as the explanation of this observation.

The contribution of the wall of the small intestine to the metabolism of xenobiotics is an important consideration for orally ingested compounds. Metabolism of xenobiotics by the intestine has been studied using microsomal systems, gut sac preparations and *in situ* isolated loops of intestine [1–4]. Microsomal systems require the addition of cofactors, substrate concentrations used are usually much higher than would be encountered *in vivo*, and the media used are non-physiological. When gut sac preparations are used, the lack of an intact blood supply may present an artificial barrier to diffusion, and substrate tends to accumulate in the tissue [1, 5]. In isolated *in situ* loops of small intestine it is difficult to control the pH of the perfusate, the 'dead space' (i.e. the regions where the perfusate is not exposed to the intestinal wall) will give artificially low levels of metabolites, and since these experiments are carried out under anaesthetic, the anaesthetic may influence the results obtained.

Only very limited use has been made of isolated intestinal cell preparations for studying drug metabolism [6, 7]. Such preparations have a number of potential advantages, for example no added cofactors are required, there is no artefactual barrier to the diffusion of substrate and metabolites into and out of the cells, and incubations can be carried out under carefully controlled conditions, yet the system remains a relatively simple one. Various means have been used to isolate intestinal cells, including mechanical vibration, chelation, scraping and enzymic methods [8–15].

In this paper we report a method for the isolation of viable cells from guinea-pig small intestine and their use to study *O*-de-ethylation of 7-ethoxycoumarin and conjugation of 7-hydroxycoumarin, 4-hydroxybiphenyl and 2-hydroxybiphenyl. Guinea-pigs were chosen for this study because it was possible to obtain a high yield of single, viable cells from guinea-pig intestine, with minimal interference from mucus, which is prevalent in the rat.

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MATERIALS AND METHODS

Chemicals

Protease (type VII), arylsulphatase (type HI), umbelliferone and bovine serum albumin (fraction V) were purchased from Sigma Chemicals Co. Ltd. (Poole, Dorset, England). Ketodase (sulphatase-free, ox liver β-glucuronidase) was purchased from W. R. Warner & Co. Ltd. (Eastleigh, Hampshire). Leibovitz L-I5 medium with glutamine, and foetal calf serum were purchased from Gibco Biocult (Paisley, Renfrewshire, Scotland). HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethane sulphonic acid) was purchased from Boehringer Corp. (Lewes, Sussex). 7-Ethoxycoumarin was synthesized according to the method of Ullrich and Weber [16]. All other solvents and chemicals used were of Analar grade from British Drug Houses (Poole, Dorset, England).

Method for isolation of intestinal epithelial cells

Male Gordon Hartley guinea-pigs, 300–400 g were used. The animals were allowed free access to food and water, and they were killed by cervical dislocation between 8.30 and 9.15 a.m. each day.

Intestinal epithelial cells were isolated from the upper 60 cm of small intestine. This section was excised from the animal and flushed with ice-cold saline (0.9% NaCl, w/v), pH7.4. Three 20 cm lengths of intestine were then everted over a metal rod, removed from the rod and one end ligated. Each length was then filled to slight distension with a Krebs phosphate buffer, which was calcium and magnesium free and contained 0.5% (w/v) bovine serum albumin, 5 mM glucose and 20 mM Hepes [Krebs CMF buffer (pH7.4)]. The other end was tied off and the lengths of intestine were then placed in a 250 ml conical flask containing 40 ml of the Krebs CMF buffer, with the addition of protease (1 mg/ml) and EDTA (1 mM). The flask was incubated in a shaking water bath (55 cycles/min) at 37°C for 20 min. The lengths of intestine were then transferred to another flask which contained 40 ml of the Krebs CMF buffer, without enzyme or EDTA, and incubation continued for a further 10 min.

The cells from both flasks were filtered through bolting cloth (150 μm pore size; Henry Simon Ltd., Cheadle Heath, Stockport), the filtrates were made up to 100 ml with ice-cold buffer and centrifuged (M.S.E. Minor) at approximately 200 g_{av} for 5 min. The cells were washed twice with 50 ml of the ice-cold Krebs CMF buffer and finally resuspended at a concentration of approximately 6×10^6 cells/ml in Leibovitz L-15 medium [17], containing 10% foetal calf serum (v/v). Cells were counted in an improved Neubauer Chamber (Gelman Hawksley, Lancing, Sussex) and viability assessed by the ability of the cells to exclude trypan blue.

Metabolism of 7-ethoxycoumarin and 7-hydroxycoumarin by isolated intestinal epithelial cells from guinea-pigs

Intestinal epithelial cell suspensions (2 ml) were incubated with substrate (100 μM) in 25 ml open conical flasks in a shaking water bath (50 cycles/min) at 37°C for the required length of time. The reaction was terminated by the addition of ice-cold ether (7 ml) containing 1.5% (v/v) isoamyl alcohol. Under these conditions the highly fluorescent, unconjugated 7-hydroxycoumarin was quantitatively extracted into the ether. The ether extracts (5 ml) were back extracted into 0.2 M glycine/sodium hydroxide buffer, pH 10.4 (3.5 ml). The fluorescence of the aqueous layer was determined at λex 370 nm, λfl 450 nm, using a Perkin-Elmer MPF-3 spectrofluorimeter and compared with appropriate standards and controls. The rest of the method is the same as that used by Shirkey *et al.* [7].

Metabolism of 4- and 2-hydroxybiphenyls by isolated intestinal epithelial cells from guinea-pigs

The cell suspension was divided into duplicate portions (2 ml) in 25 ml open conical flasks on ice. The substrate was added in 2 μl dimethylformamide (DMF) to give a final concentration of 100 μM. The flasks were incubated in a shaking water bath (50 cycles/min) at 37°C. The reaction was stopped at the appropriate time, by placing the flasks on ice. The unmetabolised substrate was removed by extraction into three 7 ml portions of n-heptane, containing 1.5% isoamyl alcohol, and discarded. The aqueous phase was then subjected to deconjugation by the addition of 1ml 0.2 M acetate buffer, pH 5.0 and 1 ml ketodase (β-glucuronidase in the same buffer), followed by overnight incubation at 37°C. The hydroxybiphenyl released was extracted into 7 ml n-heptane and 2 ml of the organic layer was back extracted into 5 ml of 0.2 M NaOH. The hydroxybiphenyls were measured fluorimetrically, by the technique of Creaven *et al.* [18]. To the aqueous phase remaining after deconjugation and extraction of the aglycones, was added 1 mg/ml of arylsulphatase and the deconjugation, extraction and measurement procedure was repeated. Appropriate standards and controls were also measured.

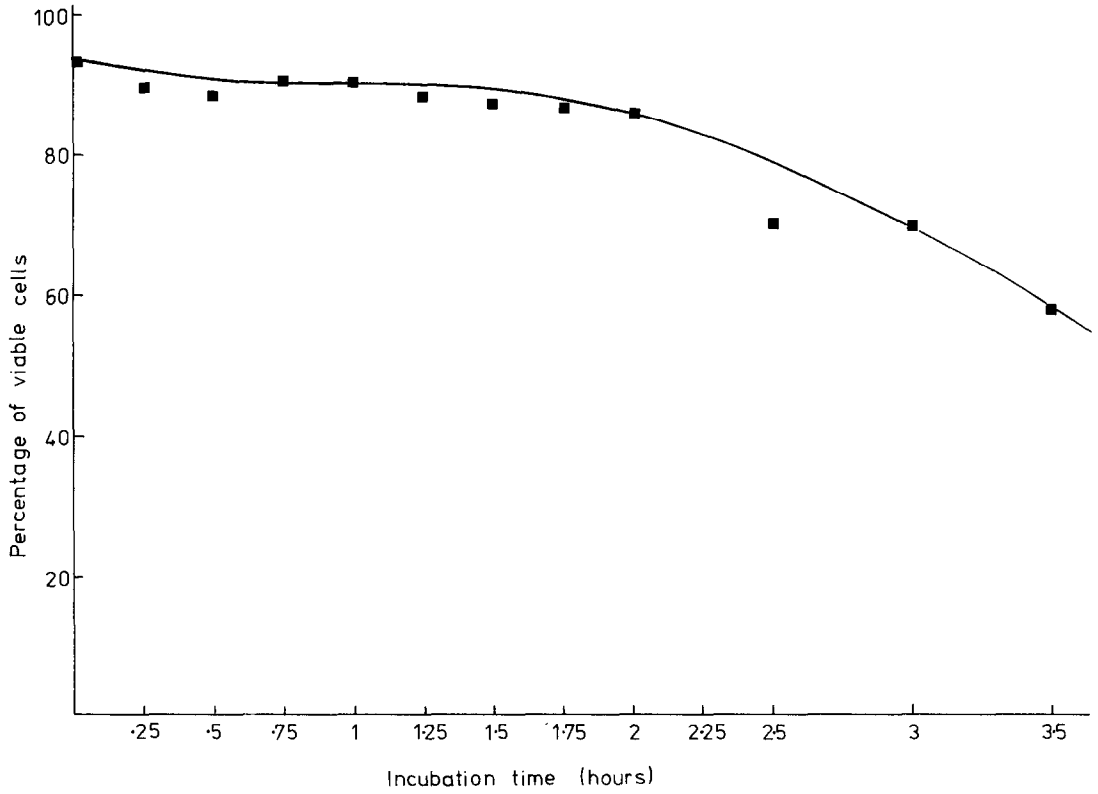

Fig. 1. Percentage viability of isolated intestinal epithelial cells from guinea-pigs, during incubation at 37°C. A suspension of isolated intestinal cells in cell culture medium (Leibovitz L-15), containing 10 per cent foetal calf serum (v/v), was incubated at 37°C in a shaking water bath (55 cycles/min). Aliquots (0.25 ml) of the cell suspension were withdrawn at the times indicated, for assessment of the percentage viability.

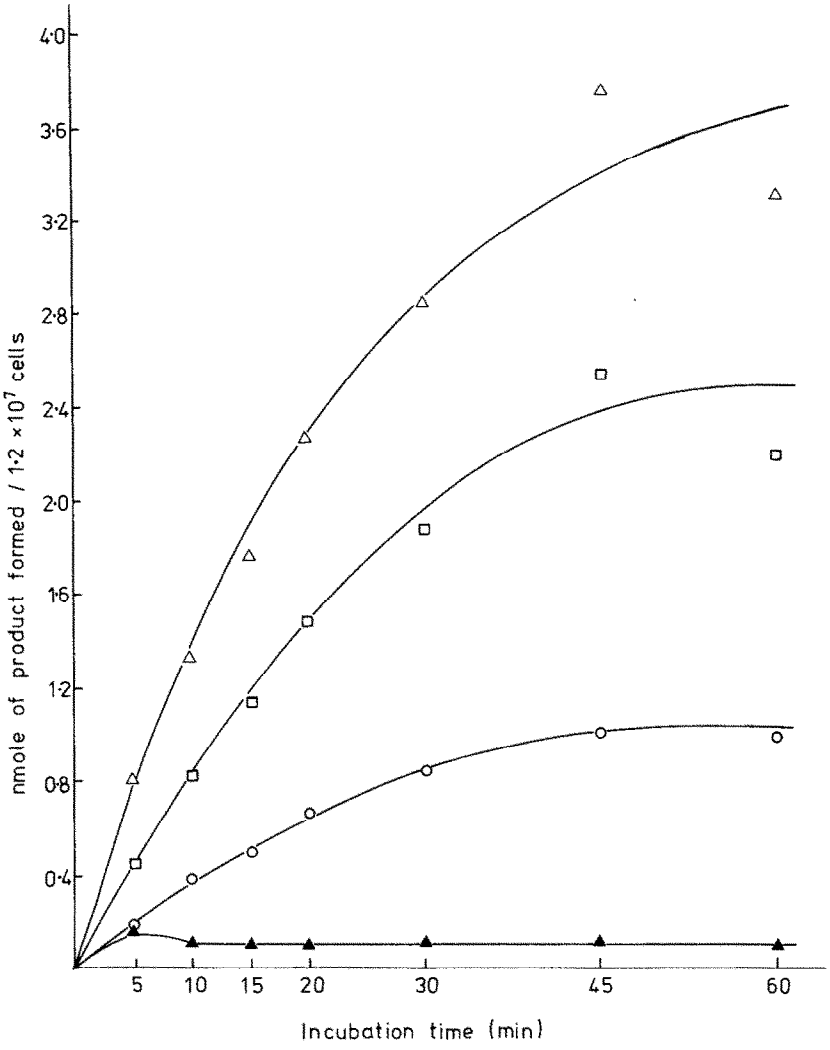

Fig. 2. Time course of the metabolism of 7-ethoxycoumarin in suspension of intestinal epithelial cells from guinea-pigs. Viable intestinal epithelial cells (approx. 1.2×10^7 cells in a 2 ml vol.) were incubated with 100 μM 7-ethoxycoumarin at 37°C in a shaking water bath (50 cycles/min), for periods of up to 60 min. At the times indicated, the suspensions 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. Cells were pooled from 2 animals. Results represent the mean of three determinations. △ total metabolites; ○ glucuronides; □ sulphates; ▲ free metabolites.

RESULTS

A suspension of isolated cells was incubated at 37°C in a shaking water bath (55 cycles/min) in order to assess the suitability of the cells for studies of drug metabolism. Portions (0.25 ml) of the cell suspension were withdrawn at the times indicated in Fig. 1, and the viability measured by the ability of the cells to exclude the dye trypan blue. The percentage of cells which were viable remained close to 90 per cent for up to 2 hr, before gradually falling to 60 per cent after 3.5 hr incubation. The cells were thus deemed suitable for drug metabolism studies, where the incubation time was always less than 1.5 hr.

The *O*-de-ethylation of 7-ethoxycoumarin resulted in very little free 7-hydroxycoumarin (3 per cent), most of the metabolites being sulphate (67.5 per cent) and glucuronic acid (29.5 per cent) conjugates of 7-hydroxycoumarin (Fig. 2). The total amount of 7-ethoxycoumarin found as metabolites, after a 1 hr incubation, was less than 2 per cent of the substrate added (i.e. < 4 nmoles/1.2×10^7 cells). When 7-hydroxycoumarin was the added substrate (at the same concentration as that used when 7-ethoxycoumarin was substrate), over 75 per cent of the substrate was metabolised in 1 hr (Fig. 3). The metabolites in this instance were glucuronic acid (23 per cent) and sulphate (77 per cent) conjugates.

Sulphation of 4-hydroxybiphenyl was linear over the time period studied (one hour), whilst glucuronidation was non-linear (Fig. 4). At the earlier time points (< 30 min) there was a greater proportion of glucuronides produced than sulphates, but sulphate conjugation dominated when the incubation time was greater than

45 min. Approximately 25 per cent of the substrate added was found as conjugates after 1 hr incubation (i.e. 50 nmoles/1.2 × 10^7 cells).

2-Hydroxybiphenyl produced a very different pattern of conjugates (Fig. 5). The rate of sulphate conjugation was linear, as for 4-hydroxybiphenyl, but the amount of sulphates produced was very low (approx. 1 per cent of the substrate added). Glucuronic acid conjugation accounted for 94.5 per cent of the metabolites produced (i.e. 18 nmoles/1.2 × 10^7 cells) in an hour (Fig. 5).

DISCUSSION

The percentage viability of the isolated intestinal epithelial cells was always between 88 and 98 per cent immediately after isolation. On incubation of the cells at 37°C in a shaking water bath (55 cycles/min), in the absence of substrate, the viability remained close to 90 per cent for up to 2 hr (Fig. 1). All drug metabolism studies were conducted on the cell suspension within 1½ hr of their isolation. Incubation for an hour with 100 μM of any of the substrates used resulted in a drop in percentage viability of not more than 5 per cent [i.e. viability never fell below 83 per cent (results not shown)].

7-Ethoxycoumarin *O*-de-ethylation and subsequent conjugation (Fig. 2) was very much slower (< 4 nmoles/1.2 × 10^7 cells/hr) than conjugation alone, determined by using 7-hydroxycoumarin as substrate (< 150 nmoles/1.2 × 10^7 cells/hr) (Fig. 3). Thus it appears that in guinea-pigs when 7-ethoxycoumarin is the substrate, the phase I reaction is the rate-limiting step, conjugation being forty times faster. With intestinal cells isolated from rats [7], conjugation of 7-hydroxycoumarin was only seven times faster than *O*-de-ethylation of 7-ethoxycoumarin. The finding that the oxidation rate of xenobiotics is rather low in the intestine compared with the liver is supported by findings using microsomes from both organs [19].

Sulphate conjugation accounted for over two-thirds of the metabolites in guinea-pigs regardless of whether 7-ethoxycoumarin or 7-hydroxycoumarin was the substrate (Figs. 2 and 3). In contrast, in rat intestinal cell suspensions [7], conjugation with glucuronic acid gave approximately 85 per cent of the total metabolites, for both 7-ethoxycoumarin and 7-hydroxycoumarin. A low level of sulphate conjugation in rat intestine has

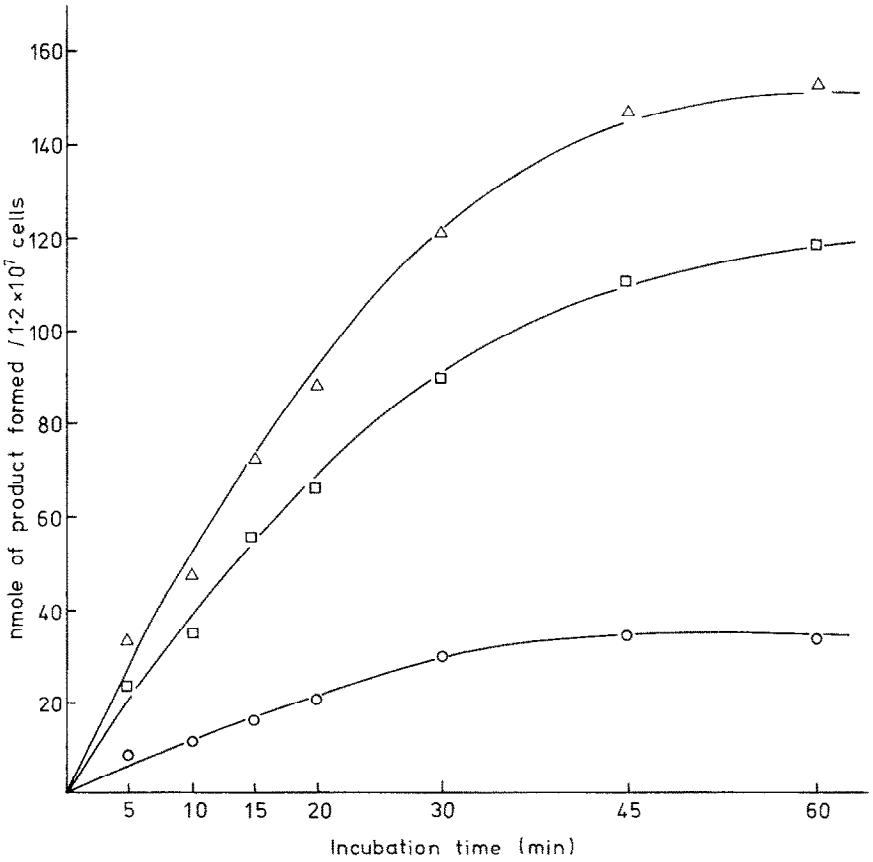

Fig. 3. Time course of the metabolism of 7-hydroxycoumarin in suspensions of intestinal epithelial cells from guinea-pigs. Viable intestinal epithelial cells (approx. 1.2 × 10^7 cells in a 2 ml vol.) were incubated with 100 μM 7-hydroxycoumarin at 37°C in a shaking water bath (50 cycles/min), for periods of up to 60 min. At the times indicated the suspensions 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. Cells were pooled from 2 animals. Results represent the mean of 3 determinations. △ total metabolites; ○ glucuronides; □ sulphates.

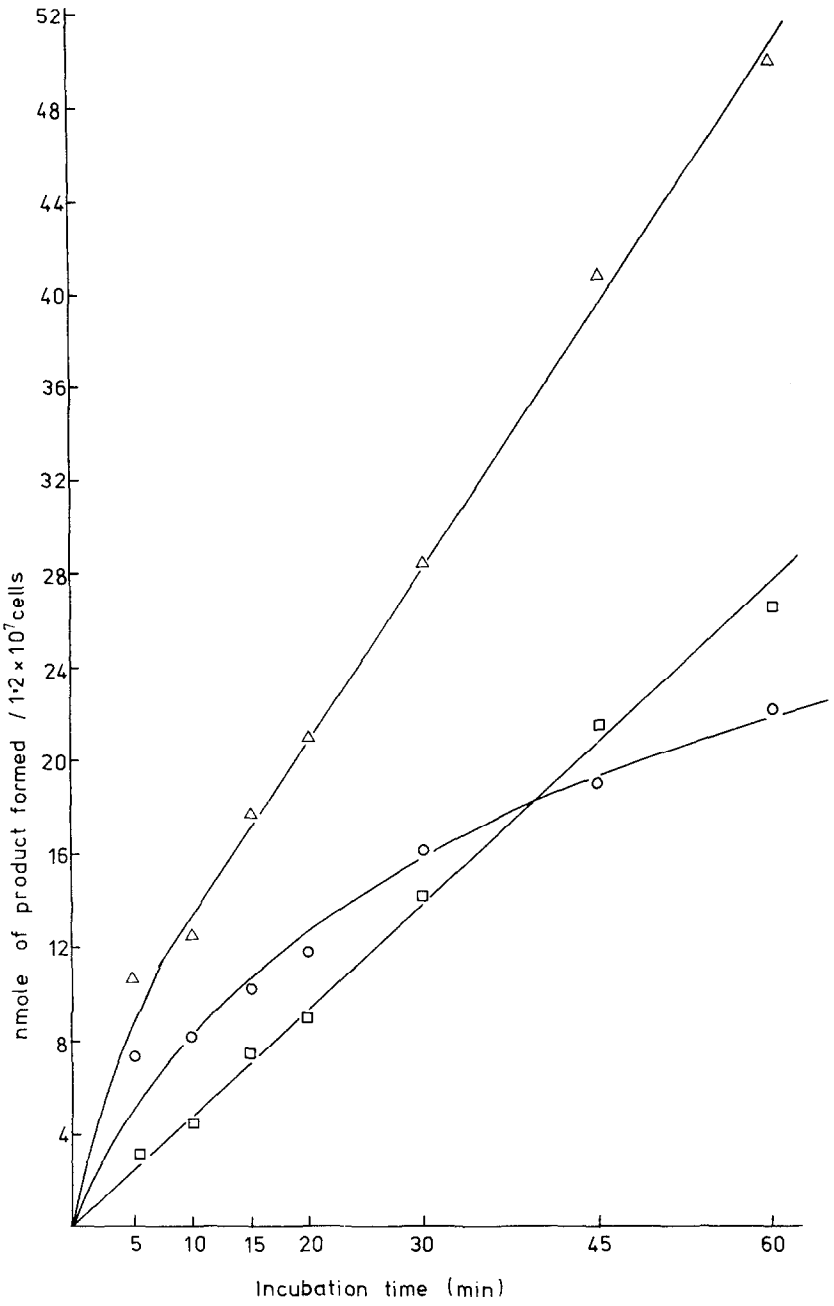

Fig. 4. Time course of the metabolism of 4-hydroxybiphenyl in suspensions of intestinal epithelial cells from guinea-pigs. Viable intestinal epithelial cells (approx. 1.2×10^7 cells in a 2 ml vol.) were incubated with 100 μM 4-hydroxybiphenyl at 37°C in a shaking water bath (50 cycles/min), for periods of up to 60 min. At the times indicated the suspensions were deconjugated, extracted with n-heptane and the n-heptane fraction extracted with NaOH (0.2 M). The various fractions obtained by this procedure were analysed fluorimetrically for 4-hydroxybiphenyl. Cells were pooled from 2 animals. Results represent the mean of 3 determinations. △ total metabolites; ○ glucuronides; □ sulphates.

been demonstrated in a number of studies using various intestinal preparations [3–7, 20]; however, no similar studies have previously been reported using guinea-pig intestine. The results of the present investigation have been confirmed using gut sacs and isolated *in situ* loops of guinea-pig small intestine (J. R. Dawson and J. W. Bridges, submitted for publication).

The pattern of conjugation seen with 4-hydroxybiphenyl as substrate (Fig. 4) contrasts with that obtained when 2-hydroxybiphenyl was the substrate (Fig. 5). Although sulphate conjugation in both instances was linear, 2-hydroxybiphenyl resulted in very few (< 6 per cent of the total metabolites) sulphate conjugates, whereas 4-hydroxybiphenyl resulted in almost equal

proportions of sulphate and glucuronic acid conjugates. The amount of glucuronides formed was roughly the same for both hydroxybiphenyl substrates. The only difference in the metabolism of 2-hydroxybiphenyl and 4-hydroxybiphenyl was in the amount of sulphate conjugates formed. The same phenomenon was observed *in vivo* and *in vitro* in rat liver with harmol and harmalol, which differ from each other only in the saturation of a double bond. *In vivo* harmol was excreted mostly as harmol-sulphate (70 per cent), with 30 per cent of the excretion products being harmol-glucuronide [21]. Harmalol, however, was excreted mainly as the glucuronide (70 per cent), with only a trace (<3 per cent), present as harmalol sulphate. Another similarity with the hydroxybiphenyls is that harmol and harmalol were glucuronidated by UDP glucuronyltransferase at comparable rates. This indicates that steric features may influence sulphation to a much greater extent than glucuronidation. Whether this effect occurs with other pairs of phenols is currently under investigation.

It has been suggested that sulphate conjugation of phenols by the intestine is easily saturated [22–24], whilst glucuronic acid conjugation is not [3, 6, 22, 23]. The results of the present investigation show that in guinea-pig intestine glucuronic acid conjugation appears to be saturable (Figs. 3–5), whilst sulphate conjugation is not. When 7-hydroxycoumarin was administered to a suspension of isolated intestinal cells from guinea-pigs, at concentrations of up to 300 μM (10^7 cells in a 2 ml vol.) no evidence of sulphate conjugation saturation was observed, whilst conjugation with glucuronic acid was maximal at 100 μM substrate, and no further increase in glucuronide formation was observed on increasing the substrate concentration (unpublished data).

In principle the cell isolation technique described above can be applied to other mammalian species. It is certainly applicable to the rat though the rat preparation tends to have lower viability because of the effects of mucus. This isolated cell system could be useful for the preliminary examination for the possibility of metabolism prior to studying absorption of new drug

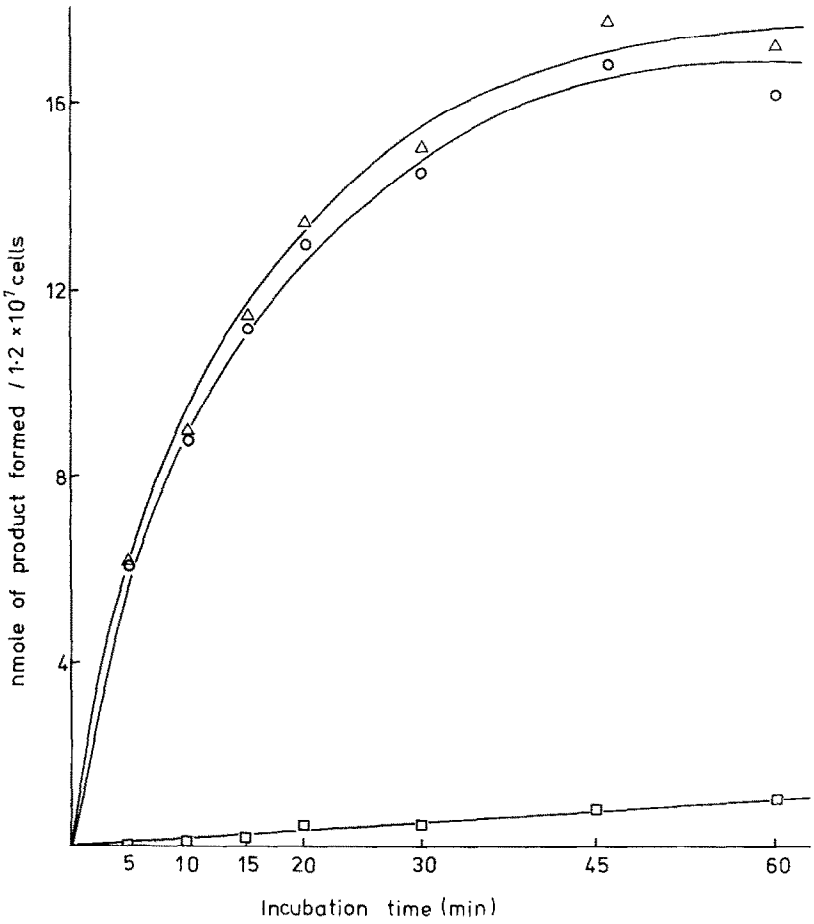

Fig. 5. Time course of the metabolism of 2-hydroxybiphenyl in suspensions of intestinal epithelial cells from guinea-pigs. Viable intestinal epithelial cells (approx. 1.2×10^7 cells in a 2 ml vol.) were incubated with 100 μM 2-hydroxybiphenyl at 37°C in a shaking water bath (50 cycles/min) for periods of up to 60 min. At the times indicated the suspensions were deconjugated, extracted with n-heptane and the n-heptane fraction extracted with NaOH (0.2 M). The various fractions obtained by this procedure were analysed fluorimetrically for 2-hydroxybiphenyl. Cells were pooled from 2 animals. Results represent the mean of 3 determinations. △ total metabolites; ○ glucuronides; □ sulphates.

candidates. It may be especially pertinent to use this model for those drugs given at low levels which contain molecular groups which can be directly conjugated or hydrolysed.

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