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INTESTINAL MICROSOMAL DRUG METABOLISM

A COMPARISON OF RAT AND GUINEA-PIG ENZYMES, AND OF RAT CRYPT AND VILLOUS TIP CELL ENZYMES

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Abstract—A comparison was made of the properties of microsomes prepared from the small intestines of guinea pigs and rats. The $NADPH_2$ cytochrome *c* reductase activity and cytochrome b_5 and cytochrome P-450 content in rat microsomes was 42, 47 and 64% of that in the guinea pig, ethoxycoumarin deethylase activity was comparable, while arylesterase activity was twice as active in rats as guinea pigs. Investigation of the distribution of these and other parameters in rat intestinal epithelia revealed a preferential location of cytochrome P-450 in the villous tip while other parameters showed a more similar distribution between microsomes prepared from the villous tip and crypt.

Scraping the mucosal cells off the intestine, followed by homogenisation and a single pre-microsomal centrifugation step is an unreliable method for preparing intestinal microsomal systems with a stable P-450 [1–6] because intestinal cytochrome P-450 is readily destroyed by autolytic enzymes which are released from damaged cells on scraping. Moreover scraping tends to yield irreproducible proportions of villous tip and crypt cells. The method developed by Shirkey *et al.* [7], involving a high frequency vibration of everted intestine in order to obtain epithelial cells devoid of non-mucosal contamination, followed by a low speed centrifugation to sediment nuclei and brush borders, has resulted in an easily reproducible method for obtaining rat intestinal microsomes with high levels of cytochrome P-450 and other drug metabolising enzymes. This method is equally applicable in principle to other species and to examining the distribution of drug metabolising enzymes between villous tip and crypt. In the present report intestinal preparations from guinea-pigs are compared with those of rats as we have previously studied drug metabolism in an isolated intestinal epithelial cell suspension of the guinea-pig since it has a high initial viability and relatively long useful lifetime. In contrast, isolated intestinal cells from rats are prone to mucus contamination which results in clumping of the cells and loss of viability [8].

Intestinal cells in the crypts of Lieberkuhn are immature villous cells. After a number of divisions in the lower half of the crypt, the epithelial cell differentiates during a 12 hr period, while migrating along the upper half of the crypt. After entering the functional compartment of the villus the cells migrate for about 36 hr toward the tip of the villus where they are extruded into the intestinal lumen [9]. Many workers have compared enzyme levels in the crypt cells with those of the villous cells [10–19]. The most commonly studied enzymes in these cases were those concerned with digestion, which are mainly located in the brush border of the cells. Only two studies have compared the level of drug metabolising enzymes in crypt and villous tip cells [20, 21]. Methods which have been used for separating crypt and villous tip cells include cryostat sectioning [10, 11, 15, 16], differential scraping [17, 20], sequential washing [13, 14], and vibration and dilation [19, 21, 22]. The Webster and Harrison [19] method slightly modified, is used in the present investigation to compare the levels of some microsomal enzymes involved in drug metabolism in the crypt and villous tip cells of rat small intestine.

MATERIALS AND METHODS

Chemicals and equipment. The vibromixer, Model E1, A. G. Chemap, Zurich, was obtained through Shandon Southern, Camberley, U.K.; the variable speed laboratory motor, Model S63C, from Camlab, Cambridge, U.K. Sodium sulphite 2,4,6-trinitrobenzene sulphonic acid, 1-naphthol were obtained from BDH Laboratories Ltd, Poole, U.K.; nicotinamide adenine dinucleotide phosphate reduced form (NADPH), bovine serum albumin (fraction V), indoxylacetate, cytochrome *c* (type III), Triton X-100, Brij '35', umbelliferone, UDP-glucuronic acid ammonium salt (UDPGA), 4-nitrophenol were obtained from Sigma Chemicals Co., Poole, U.K.; 1-naphthyl glucuronide sodium salt was purchased from Koch Light Laboratories, Colnbrook, U.K. 7-Ethoxycoumarin was prepared according to the method of Ullrich and Weber [23].

Animals. Male Wistar albino rats 200–250 g, and male Gordon Hartley guinea-pigs 300–400 g were used. The animals were fed standard laboratory diets (Spillers No. 1 Laboratory Diet, Spillers Ltd, Croydon, for the rats, and FD1 diet, Christopher Hill Gp. Ltd., Poole, U.K., for guinea-pigs), *ad lib.* and were also allowed free access to tap water.

Tissue and microsomal preparation. Animals were killed at approximately the same time each day (8.30–9.30 a.m.) by cervical dislocation. The abdo-

men was opened, an incision made in the small intestine immediately below the pyloric sphincter and a polythene cannula inserted. The intestine was filled with cold (0–4°) 0.9 per cent NaCl (w/v), pH 7.4, from a 20 ml plastic syringe attached to the cannula. The cannula was withdrawn and the gut clamped with artery forceps to retain the saline solution while the mesentry were cut and stripped away. The intestine was cut through at the aboral end, the contents flushed out, and the intestine was placed in ice-cold saline. All subsequent procedures were carried out in a cold room at 0–4°.

(i) *Mixed villous tip and crypt preparation.* This was prepared using a modification of the method of Shirkey *et al* [7]. Three 20 cm lengths of intestine were inverted over metal rods which were then attached to a vibromixer and vibrated vertically at high frequency (100 Hz) and low amplitude (1.5 mm) for one minute at 4° in 0.9 per cent NaCl, pH 7.4 This solution, which contained sloughed cells and acellular material in strands of mucus, was discarded. The intestine was vibrated for a further 30 min in 0.9 per cent NaCl containing 5 mM EDTA (pH 7.4). The cells were harvested by centrifugation (1000 g_{max} for 1 min in a MSE minor bench-top centrifuge).

The pellet of isolated cells was weighed in a tared centrifuge tube and homogenised in hypotonic sucrose (4 ml of 75 mM sucrose, pH 7.4, for 1 g wet wt cells), in a Potter–Elvehjem Teflon glass homogeniser (size B). Eight up and down strokes were used and a variable speed laboratory motor (max. speed 12,000 rpm, low torque) was used to drive the pestle. The homogenate was then immediately readjusted to isotonicity by the addition of an equal volume of 0.5 M sucrose (pH 7.4), and diluted to 1 g cells in 16 ml solution by addition of 0.3 M sucrose buffer (pH 7.4). The diluted homogenate was the centrifuged at 1000 g_{max} at 4° (MSE Minor, swing out 4 × 50 ml rotor), then the pellet was washed twice with 10 ml 0.3 M sucrose and the supernatants combined. The pellet contained the nuclei and brush borders of the cells. The combined supernatants were centrifuged at 14,000 g_{max} for 10 min (MSE High Speed 18 centrifuge, fixed angle 8 × 50 ml rotor at 11,000 rpm). The pellet from this spin contained the mitochondria and lysosomes. This supernatant was centrifuged at 100,000 g_{max} for 1 hr (MSE Superspeed 50 centrifuge, fixed angle 8 × 25 ml rotor at 40,000 rpm) to produce a particle-free supernatant and a microsomal pellet. The microsomal pellet was resuspended (1 g wet wt cells to 4 ml solution) in 0.1 M Tris–HCl, pH 7.4

(ii) *Preparation of separate crypt and villous tip cell microsomes.* Cells from the villous tips of Lieberkuhn were separated by a modification of the method of Harrison and Webster [22]. The intestine was washed and removed from the animal as described above, and three 20 cm lengths of gut were everted over dialysis tubing which covered hollow metal rods which had holes drilled out along their lengths, in order that buffer could be infused into the rods to dilate the dialysis tubing. The rods were vibrated, as above, for 1 min in 0.9 per cent NaCl to remove acellular material and sloughed cells, then for 30 min in 0.9 per cent NaCl containing 5 mM EDTA (pH 7.4). The cells from this half-hour vibration were harvested as before and called the 'tip cells'. Buffer (0.9 per cent NaCl containing 5 mM EDTA, pH 7.4) was then infused into the rods, causing the dialysis tubing, and thus the intestine, to dilate. The rods were vibrated for a further 20 min in saline/EDTA solution and these cells again harvested by centrifugation and were termed the 'crypt cells'. The intestine remaining after the two vibrations was of a smooth, shiny appearance and transparent. Microsomes were then prepared from both cell populations as described above.

Assay methods. Alkaline phosphatase (EC 3.1.3.1) was assayed by a modification [24] of the method described by Bergmeyer [25]. The *O*-deethylation of 7-ethoxycoumarin was assayed according to the method of Ullrich and Weber [23].

NADPH cytochrome *c* reductase (EC 1.6.2.4) activity was measured by recording the appearance of reduced cytochrome *c* at 550 nm in a Pye Unicam SP1800 UV split beam spectrophotometer [26, 27].

Arylesterase (EC 3.1.1.2) was assayed by modification of the method described by Shepherd and Hubscher [28] using indoxylacetate as substrate. The reaction mixture consisted of 50 mM potassium phosphate buffer pH 6.8, 1 mM EDTA, Triton X-100 (0.1 per cent) and 20–100 μg of microsomal protein. After a 2 min preincubation at 25°, the reaction was started by the addition of 50 μl indoxylacetate (50 mM in methanol) and followed spectrophotometrically at 386 nm for up to 5 min. A molar extinction coefficient of E_{386}^{1cm} 3.1×10^3 mole^{-1} cm^{-1} was used to calculate initial rates. The reaction was followed using 1 cm^2 cuvettes in a Pye Unicam SP1800 split beam recording spectrophotometer with constant temperature cell compartments. A blank without enzyme was run simultaneously to correct for spontaneous substrate hydrolysis.

Glucuronidation of 1-naphthol was determined by a modification of the method of Bock [29] for the fluorimetric determination of 1-naphthyl glucuronide. Contained in a 0.5 ml volume of Tris–HCl (0.1 M, pH 7.6) was UDPGA 5 mM, 1-naphthol (4×10^{-5} to 4×10^{-4} M final concentrations, added in 100 μl distilled water), $MgCl_2$ 5 mM final concentration Brij '35' 0.1 per cent and tissue equivalent to 0.2 mg of microsomal protein. The solutions were incubated in a water bath at 37°. After 4 min, the reaction was stopped by the addition of 1 ml of ice-cold glycine–trichloroacetic acid buffer (0.5 M, pH 2.2) and the tubes were placed in ice water. UDPGAwas without effect on the fluorimetric assay and could be omitted from control and standard incubations. A standard of 1-naphthyl glucuronide sodium (1–20 nmoles used as a 1 mM aqueous solution) was added to the appropriate tubes. The incubates were then rotary extracted for 10 min (30 c/min) with chloroform (6 ml) and centrifuged (2000 rpm for 15 min) to remove the unreacted naphthol and precipitate the protein solubilised by the detergent. An aliquot of the aqueous layer (200 μl–1 ml) was adjusted to 1.5 ml with glycine–sodium hydroxide buffer (1.0 M, pH 10.6) and the fluorescence read at λ303 nm excitation and 334 nm emission.

Microsomal cytochrome b_5 concentration was determined in the presence of sodium dithionite by

Table 1. A comparison of rat and guinea-pig intestinal microsomes

	Rat	Guinea-pig
Total yield of cells (g)	1.94 ± 0.07	2.9 ± 0.78
Total microsomal protein (mg)	27.2 ± 2.5	26.3 ± 7.6
Cytochrome P-450 (nmole/mg)	0.16 ± 0.017	0.21 ± 0.038
Cytochrome b_5 (nmole/mg)	0.18 ± 0.034	0.38 ± 0.058
Arylesterase (μmole/min/mg)	3.9 ± 1.1	1.8 ± 0.63
NADPH cytochrome *c* reductase (nmole/min/mg)	93.0 ± 11.7	219.0 ± 50.7
7-Ethoxycoumarin *O*-deethylase (nmole/min/mg)	0.113 ± 0.017	0.124 ± 0.036

Values are the means of 6 (rat) or 8 (guinea-pig) determinations ±S.D.

the reduced minus oxidised difference spectrum method of Omura and Sato [30] using a differential extinction coefficient of 17 $mM^{-1}\,cm^{-1}$ for the 423 nm (peak) minus 406 nm (trough) wavelength pair.

Cytochrome P-450 was similarly estimated from the sodium dithionite reduced versus carbon monoxide difference spectrum using the differential extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the wavelength pair 450 nm minus 490 nm, using a Pye Unicam SP 1800 recording u.v. spectrophotometer.

Total protein was determined by the method of Goodwin and Choi [31].

RESULTS

A comparison of some properties of rat and guinea-pig intestinal microsomes is shown in Table 1. The yield of cells from guinea-pig intestine was 50 per cent greater than from rat intestine, but the total microsomal protein from the two species was roughly the same. The greater weight of cells obtained from guinea-pig intestine is probably due to a larger diameter of gut giving a greater surface area than rat intestine. Cytochrome P-450 content of rat intestine was found to be 75 per cent of the levels found in guinea-pig intestine. Interestingly, guinea-pig liver also has a higher cytochrome P-450 content than rat liver. Cytochrome b_5 content of rat intestine was very similar to the cytochrome P-450 content, on a molar basis. In guinea-pig intestinal microsomes the cytochrome b_5 content was almost twice that of cytochrome P-450 and more than double the amount of cytochrome b_5 observed in rat intestine. Arylesterase activity in rat intestine was more than double that of guinea-pig, and was as active as the rat liver esterase. The NADPH cytochrome *c* reductase of guinea-pig intestine (219 nmoles/min/mg) was more than twice as active as that of rat intestine (93 nmoles/min/mg). The ethoxycoumarin *O*-deethylase (at 100 μM substrate conc.) was roughly the same in both species, in spite of the differences in cytochrome P-450 content.

The glucuronidation of 1-naphthol by rat intestinal microsomes gave a value of 1.25×10^{-4} M for the K_m and 15 nmoles/min/mg protein for V_{max} for the activated enzyme. The same reaction with guinea-pig microsomes produced a maximum rate of

Table 2. A comparison of rat intestinal crypt and villous tip cell microsomes

	Crypt	Tip	Crypt/tip per cent
Yield of cells (g)	0.78 ± 0.20	1.95 ± 0.12	40
Total microsomal protein (mg)	7.6 ± 1.04	22.4 ± 2.82	34
Cytochrome P-450 (nmole/mg)	*0.053	0.16 ± 0.012	40
Cytochrome b_5 (nmole/mg)	*0.11	0.18 ± 0.007	73
Arylesterase (μmole/min/mg)	3.11 ± 0.58	4.13 ± 0.41	75
NADPH cytochrome *c* reductase (nmole/min/mg)	70.0 ± 9.8	101.0 ± 13.6	69
7-Ethoxycoumarin *O*-deethylase (nmole/min/mg)	0.07 ± 0.03	0.15 ± 0.06	47

Values are the means of seven determinations except * only one determination possible through insufficient yield of microsomes.

Rat intestinal crypt and tip cell microsomes were prepared as described in Materials and Methods.

±S.D.

Table 3. Validation of the separation of rat intestinal crypt and villous tip cells

Cell Fraction	Crypt	Tip	Crypt/tip per cent
(A) Acid phosphatase (nmole/min/mg)			
Nuclear/brush border	11.0 ± 0.86	11.0 ± 2.05	100
Mitochondria/lysosomes	7.5 ± 1.42	7.1 ± 0.61	106
Cytosol	8.0 ± 1.06	7.0 ± 0.81	114
Microsomes	4.9 ± 0.42	5.6 ± 0.64	87
(B) Alkaline phosphatase (nmole/min/mg)			
Nuclear/brush border	359.0 ± 22	693.0 ± 87	52
Mitochondria/lysosomes	73.0 ± 13	117.0 ± 13	63
Cytosol	5.8 ± 1.2	13.5 ± 2.0	43
Microsomes	77.0 ± 11	142.0 ± 12	54

Results are the means of 4 duplicate determinations ±S.D.

8 nmoles product/mg microsomal protein/min at 120 μM substrate, but with a higher substrate concentration the velocity of the reaction decreased. This meant that it was not possible to obtain meaningful values for the K_m or V_{max} of the reaction.

Cryostat sectioning [15] has shown that the acid phosphatase is found at roughly the same concentration in crypt cells as in villous tip cells, whereas alkaline phosphatase is at a much lower level in crypt cells than in tip cells. Table 3 demonstrates that acid phosphatase was as active in all cell fractions of the crypt cell population as in the corresponding villous tip cell fractions. Alkaline phosphatase was only half as active in the crypt cells as it was in the tip cells.

DISCUSSION

Modification of the method of Shirkey *et al.* [7] gave consistently cytochrome P-450 values which are considerably higher than those previously reported in the literature. The key alterations in the method of Shirkey *et al.* [7] which resulted in such a marked increase in both yield and specific activities of the microsomal enzymes are the omission of two of the washings, one at 1000 g_{max} and one at the 14,000 g_{max} spin, and the use of a different homogeniser motor. The decreased number of washings decreases the amount of non-microsomal protein sedimented with the microsomes, while the change from a Stanley drill (speed 4000–5000 rpm, high torque) to a variable speed laboratory motor (max speed 12,000 rpm, low torque) (Camlab, Cambridge) gave a gentler homogenisation, which caused less disruption of the intracellular organelles and thus did not release the autolytic enzymes from the lysosomes. In both rat and guinea-pig intestinal microsomes (Table 1) the ratio of NADPH cytochrome *c* reductase and cytochrome b_5 to cytochrome P-450 is much greater than the corresponding liver values [1, 31]. However the rate of 7-ethoxycoumarin deethylase per nmole of P-450 is much lower despite these apparently more favourable conditions for the transfer of electrons from NADPH to cytochrome P-450. The physiological significance of the relatively high levels of cytochrome b_5 and cytochrome *c* reductase in the intestine is uncertain. Cytochrome b_5 is known to be important in the desaturation of fatty acyl CoA's, phospholipid, alkylacylglycerophosphoryl-ethanolamine and in the reduction of *N*-hydroxylamines, therefore it is possible that these functions of cytochrome b_5 are quantitively more important in the intestinal epithelial cells, since these cells play a vital role in the digestion of food, including lipids. As well as being reduced by NADPH cytochrome b_5 reductase, cytochrome b_5 can also be reduced by NADPH cytochrome *c* reductase. Indeed, NADPH cytochrome *c* reductase may act in concert with cytochrome b_5.

Although rat intestine contained cytochrome P-450 at 25 per cent of the levels found in rat liver, the specific activity of 7-ethoxycoumarin *O*-deethylase (a P-450 catalysed reaction) was only 7 per cent of that found in rat liver. It is possible that the cytochrome P-450 of the intestine has relatively greater importance for the metabolism of endogenous compounds such as lipids, steroids, cholesterol, than that of liver.

In contrast to cytochrome P-450 mediated metabolism the arylesterase (indoxylacetate as substrate) of guinea-pig intestine was found to be less than half as active as that of rat intestine (Table 1). Inoue *et al.* [32] has recently compared human, rat, mouse, guinea-pig, rabbit and dog intestinal esterases and they too found that rat intestine has a more active esterase than guinea-pig intestine, the guinea-pig values being more like those in man. The amount of esterase activity present in the small intestine may be an important consideration in the drug design, since penicillin esters, for example, administered orally could be extensively cleaved following absorption by the intestinal epithelial cells. Guinea-pig intestinal glucuronyl transferase has a higher affinity for 1-naphthol than rat intestine does. This is in agreement with the findings [33] that guinea-pig liver glucuronyltransferase has a higher (ten times as high) affinity for *o*-aminophenol.

As well as differences in their anatomical location, crypt and tip cells differ in a number of biochemical properties (see Introduction). It has been reported [20, 21] that much lower levels of microsomal drug metabolising enzymes exist in the crypt cells than in

the tip cells. However, the method used by Hoensch *et al.* [34] to separate the different cell types entailed 'differential scraping' of lengths of gut, which is bound to result in more non-mucosal cell protein being found in the crypt cell fraction, and contamination of all cell populations with sub-mucosal tissue. Schiller and Lucier [21] although using a somewhat similar method to that reported here for separating the cell types, did not prepare microsomes from the cells they obtained, but used the cells in suspension. Their isolated cells had a poor viability (70 per cent) even at the start of their assays, and this might have had an important influence on the results they obtained. In the present method negligible sub-mucosal contamination of the cell populations occurred and because microsomes were prepared from the cells obtained, a direct and reliable comparison of enzyme activity from crypt and tip cells under well defined conditions can be made. From Table 2 it can be seen that, as might be expected, yield of cells and microsomal protein was much less from the crypts than from the villi (40 per cent). Both the amount and the 7-ethoxycoumarin deethylase activity of cytochrome P-450 is much lower (40–50 per cent) in the crypt cells than in the villous cells. It is probable that the cell population termed 'crypt cells' in the present investigation also contains some cells from the villi, but because of the nature of the isolation method and the fact that the cells obtained are less than one third of the total cell weight, the proportion of contaminating villous cells is probably small. The differential distribution of alkaline phosphatase confirms the validity of the tip and crypt cell separation procedure used in the present experiments. When expressed in terms of total enzyme in the epithelial cells the levels of alkaline phosphatase in the crypt cells was only 20 per cent of that in the tip cells.

Cytochrome b_5 and arylesterase were both observed to have about three-quarters of the activity in the crypt cells that they had in the villous tip cells. NADPH cytochrome *c* reductase was found at relatively high levels in the crypt cells too (Table 2), (~70 per cent of the tip cell activity). Although two previous reports [11, 19] have found much greater levels of esterase in the tip than the crypt cells neither of these reports expressed enzyme activities per milligram of microsomal protein; this could account for the discrepancies with our findings.

The data support the view [20] that intestinal monooxygenase activity is localised mainly in the most mature, villous tip cells and that cytochrome P-450 is synthesised in maturing mucosal cells as they migrate from the crypts to the tops of the villi. Our data shows that in contrast, other enzymes also associated with drug metabolism, namely cytochrome b_5, arylesterase, NADPH cytochrome *c* reductase and glucuronyl transferase show more similar distribution between crypt and tip cells. The fact that the ratio of P-450 to glucuronyl transferase increases from the crypt to the tip lends further support to the view that there is no tight coupling between these two enzymes [35]. The influence of various inducing agents on the activity of these enzymes in crypt and tip cells is currently under investigation.

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