Liver snips

A simple, rapid and reproducible method for studying metabolism in small fragments of tissue, as applied to glucuronidation in rat liver

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(Received 22 September 1981/Accepted 30 October 1981)

A simple, rapid method is described of preparing intact cells as small (about 2 mm) pieces of organized tissue capable of performing synthetic metabolic functions. It has been applied to the study of glucuronidation in rat liver. In this process, snips appear less damaged, more versatile and more active than tissue slices and yield results of reproducibility comparable with those with homogenates. From a comparison with the literature, snips glucuronidate the substrates employed at a rate much the same as in perfused preparations and some 30% less than the rate in isolated-hepatocyte suspensions; the advantages they offer in certain situations over these two techniques are discussed.

It is important to extrapolate the activity of an enzyme assayed in broken-cell preparations to its presumed activity in the intact cell or tissue, particularly when the enzyme is membrane-bound and latent, and hence subject *in vivo* to a degree of constraint difficult to predict. Additional factors operating in the tissue are availabilities of substrates and activators, and competition from related pathways; all of these can change with development, diet and exposure to hormones, drugs or other inducers of the system. Parallel studies are therefore required with a range of preparations, which may extend from the pure enzyme through broken-cell preparations, cell suspensions, sliced tissue and perfused organ to the whole animal. Each preparation has its advantages and limitations. We describe here those of a new technique, the 'snip', which for liver should supersede slices and for some purposes may be more useful than perfusions or hepatocyte suspensions.

We have applied it to the study of glucuronidation in intact cells, a strictly aerobic, energy-dependent biosynthetic process which undergoes all the constraints mentioned above and whose final step employs microsomal UDP-glucuronyltransferase (EC 2.4.1.17) (see Dutton, 1980).

Methods

Preparation of snips

Excise the liver into a Petri dish of gassed bicarbonate-Ringer solution at 30–37°C (Krebs & Henseleit, 1932) (MgCl₂ may replace MgSO₄ to minimize competitive formation of sulphate conjugate; Storey, 1950). Hold the liver at the lobular junction and cut into constituent lobes (for rat) or similar sizes. Transfer each lobe to another dish of Ringer solution and snip round its undamaged periphery with fine-pointed scissors to produce 'snips' as uniform as possible and no larger than 4 mm long and 1 mm wide. Leave the snips in the dish of medium. Iridectomy scissors (17 mm from blade-tip to pivot: from any surgical supplier) are ideal. With very little practice 200 snips, enough for six flasks, can be cut single-handed within 20 min from excising the liver. Unless a particular area of liver is studied, randomly mix the snips.

Incubation mixture

Add 4.0 ml of the gassed Ringer solution to each of a series of 25 ml conical stoppered flasks. With 2-aminophenol (0.5 mM) as substrate: add 0.1 ml of a 20 mM stock solution (containing 40 mM-ascorbic acid if stored long frozen; see Dutton & Storey, 1962). With 1-naphthol (0.3 mM) as substrate: before adding the 4.0 ml of Ringer solution, add to the dry flask 30 µl of stock 40 mM ethanolic solution of 1-[¹⁴C]naphthol (The Radiochemical Centre, Amersham, Bucks., U.K.) and evaporate under N₂; shake well and check solution of the naphthol by counting

Abbreviations used: UDPGlcA, UDP-glucuronic acid; UDPGlcNAc, UDP-*N*-acetylglucosamine.

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radioactivity of a small sample from the flask: some 5×10^5 c.p.m. per flask is suitable.

Incubation of snips

Wash the snips by decanting with Ringer solution in the dish. With a spatula, place some 30 snips (about 10 mg of protein) in each flask. Gas the flasks with O_2/CO_2 (19:1) for 30 s, stopper, place in a bath at $37^\circ C$, equilibrate pressure, and incubate for up to 100 min, shaking sufficiently to ensure circulation of snips within the medium.

Assay of glucuronidation

Remove $125 \mu l$ samples every 20–25 min, re-gas and re-stopper. With 2-aminophenol as substrate, add the sample to $125 \mu l$ of 1 M-trichloroacetic acid on ice; with 1-naphthol, add it to $375 \mu l$ of a 1:1 (v/v) mixture of 0.5 M-trichloroacetic acid and 0.8 M-glycine, pH 2.2, on ice. Assay the conjugates and, when using new conditions, also the proportion specifically shown to be glucuronide, as described elsewhere (Dutton, 1980; Dutton *et al.*, 1981). After incubation, snips are homogenized or sonicated in the medium, and protein is assayed by Lowry's method. Alternatively, snips can be centrifuged down, washed and homogenized before protein assay; this also permits measurement of glucuronide present within the snips, for comparison with that excreted into the medium.

Glucuronidation by liver snips is quoted below as units of $nmol$ of glucuronide $\cdot min^{-1} \cdot mg^{-1}$ of liver protein for male Wistar 30–50-day-old rats. Assays were in triplicate, average values being employed for comparison between conditions of assay or between groups of animals.

Results and discussion

Reaction rate

After a short lag phase, the rate of appearance of glucuronide in the medium remained virtually linear up to 100 min (Fig. 1). Rates are therefore expressed for the 20–100 min period, during which the rate of acceptor substrate uptake, donor substrate (UDPGlcA) supply and product excretion presumably remain approximately constant. The lag phase (common in intact-cell preparations; e.g. Wiebkin *et al.*, 1978) depends on the substrate offered, as indicated in Fig. 1.

Glucuronide production remained linear with protein concentration (up to 20 mg of protein per flask) under these conditions, when using a uniform snip size (see below).

Effect of acceptor-substrate concentration

For 30–50-day-old rats, half-maximum velocity occurs at substrate concentrations of about 0.2 mM and 0.05 mM for 2-aminophenol and 1-naphthol

Fig. 1. Typical time course for the glucuronidation of 0.25 mM-2-aminophenol (●) and of 0.3 mM-1-naphthol (○) by liver snips

Incubation volumes were double those described in the Methods section, and $125 \mu l$ samples were removed more frequently. Dotted lines are extrapolations of the linear part of the time course. Separate pools of rat liver were used for each substrate. Snip size was 0.3 mg of protein.

Fig. 2. Effect of substrate concentration on the rate of glucuronidation by liver snips

Separate experiments were performed for 2-aminophenol (●) and 1-naphthol (○). Each point is the mean of triplicate determinations, of reproducibility as described in the text. Snip size was 0.3 mg of protein.

respectively (Fig. 2); the standard assay concentrations chosen, 0.5 mM and 0.3 mM respectively, are at or close to saturation for that age.

Effect of snip preparation

Snips prepared from lobe edges previously cut averaged 75% of the activity of those taken from the uncut lobe periphery. When snips are prepared from previously cut edges (e.g. sampling inner regions of the lobe), controls should allow for this finding.

The time taken to cut snips and their temperature before incubation seem not too critical. Snips cut from liver of 2- or 37-day-old rats, (a) used within 20 min of liver excision with Ringer solution maintained at 30–37°C, and (b) used after 1 h with the solution cooling to room temperature (20°C), possessed similar glucuronidation rates; loss of O₂/CO₂ from the dish of gassed medium during this period therefore seems unimportant.

Effect of snip size

Glucuronidation varies with snip size (i.e. weight; see Fig. 3). Choice of size is determined by three factors: smaller sizes give higher activities; smaller sizes require more snips to be cut; and a slight size variation between experiments should not incur large variation in specific activities. We choose a snip length of about 2 mm (0.3 mg of protein).

Snips are prepared much more rapidly by the McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) than by hand, but the liver must be firm, and their activity is less. We compared activities in snips from the same lobe cut by either method, with 2-aminophenol as

substrate. With 'manual' snips, average activity was 0.45 unit. With the instrument set to give cubes (i.e. chopping in two directions at right angles successively) activities at cube settings 1.0, 0.75 and 0.50 mm were 0.34, 0.21 and 0.19 unit respectively. Cubes at 0.75 mm setting, cut in only one direction, gave 0.30 unit. Thus, activity falls with decreasing cube size (unlike the manual procedure) and with increased number of cutting operations; from this we infer that the tissue chopper damages a higher proportion of cells than manual snipping, and so detracts from one of the major advantages of the technique.

Effect of gas phase

Gassing the flasks with air/CO₂ (19:1) instead of O₂/CO₂ (19:1) gave lower rates of glucuronidation, e.g., 0.31 and 0.47 unit respectively with 2-aminophenol, and 0.24 and 0.49 unit with 1-naphthol. Replacing the (25 mM) bicarbonate in the Ringer with Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] or phosphate buffer (10 mM, plus an additional 15 mM-NaCl) and gassing with 100% O₂ also diminished glucuronidation of 1-naphthol in liver snips, by 35% and 25% respectively, confirming that liver tissue glucuronidates better when buffered with bicarbonate/CO₂ than with phosphate (Storey, 1950).

Reproducibility

Reproducibility of the assay within a single batch of snips is high, being similar to that measured for UDP-glucuronyltransferase in cell-free homogenates, assayed as described by Dutton *et al.* (1981). In one typical batch of snips, the activity range was 10% of the average value, with a standard deviation (σ) of $\pm 6\%$ (10 determinations); with the same substrate (2-aminophenol) in homogenates, σ was $\pm 10.5\%$, $\pm 5\%$ and $\pm 6\%$ respectively for the latent enzyme and for preparations optimally activated by UDPGlcNAc and by digitonin. Reproducibility between batches from similarly aged animal groups is necessarily less, specific activities for the glucuronidation of 0.5 mM-2-aminophenol being 0.37 ± 0.06 unit ($\sigma = \pm 16\%$, 10 groups), and for 0.3 mM-1-naphthol 0.53 ± 0.09 unit ($\sigma = \pm 17\%$, 10 groups); nevertheless these standard deviations compare well with those for UDP-glucuronyltransferase in homogenates fully activated with digitonin, where $\sigma = \pm 17\%$ (12 groups) with 2-aminophenol, and $\pm 9\%$ (8 groups) with 1-naphthol.

Snip size for these experiments ranged from 225 to 360 μg of protein/snip, average 283 ± 41 ($\sigma = \pm 14.5\%$, 10 experiments). From Fig. 3, this range in size should itself account for a variation in 2-aminophenol glucuronidation of $\pm 7\%$ (range) or $\pm 5\%$ (σ). Fig. 3 can also correct for snip sizes off the norm.

Fig. 3. Effect of snip size, expressed as mg of protein per snip, on the rate of glucuronidation of 0.5 mM-2-aminophenol

Four batches of snips, graded in size, were prepared from a single pool of rat liver. For each batch, the averaged (○) and the three individual determinations (●) are shown.

Integrity of snips

The donor substrate UDPGlcA, and the specific activator UDPGlcNAc, of UDP-glucuronyltransferase (see Dutton, 1980) were added to the incubation medium to check that liver cells in snips remain impermeable to UDP-sugars, and to gauge the proportion of damaged (i.e. permeable) cells. In a typical experiment (0.5 mM-2-aminophenol as acceptor) addition of 2.4 mM-UDPGlcA had negligible effect on glucuronidation (0.35 unit without, 0.33 unit with), and further addition of 0.8 mM-UDPGlcNAc increased it only slightly (to 0.38 unit). Since the concentration of UDPGlcA in fresh liver from adult rats is reported as 0.12–0.30 mmol·kg⁻¹ fresh wt. of liver (Zhivkov, 1970; Keppler *et al.*, 1970; Bock & White, 1974; Moldéus *et al.*, 1978; M. R. Pollard & G. J. Dutton, unpublished work), and because UDP-glucuronyltransferase activity when assayed with the homogenized liver requires a UDPGlcA concentration of 2–3 mM for half $V_{\max}$ (depending on its state of activation; M. R. Pollard & G. J. Dutton, unpublished work), then the lack of rate enhancement on addition of 2.4 mM-UDPGlcA to incubations with snips suggests that the cells of the snips remain virtually impermeable to exogenous UDPGlcA. Further, UDP-glucuronyltransferase activity towards 0.5 mM-2-aminophenol in liver homogenates from this animal, in presence of 2.4 mM-UDPGlcA and 0.8 mM-UDPGlcNAc, was 0.40 unit. Thus the stimulation by 0.04 unit when these same concentrations of UDP-sugars are added to incubations with liver snips suggests little (<10%) cell rupture. In a parallel experiment with 0.3 mM-1-naphthol the rate of glucuronidation by snips, 0.44 unit, rose to 0.75 unit on addition of 3 mM-UDPGlcA + 3 mM-UDPGlcNAc. As the equivalent rate in UDPGlcNAc-activated liver homogenates was 3.15 units, the increase of 0.31 unit in the incubations with snips again suggests 10% cell rupture or less.

Comparison with other intact-cell preparations

Finally, we compare glucuronidation rates between snips and other intact cell preparations. As rates are expressed variously in the literature, we have standardized them as nmol of glucuronide·min⁻¹·mg⁻¹ of liver protein, employing the following conversion factors: 1 g of fresh liver is equivalent to 180 mg of liver protein (determined by the Lowry procedure: Bock *et al.*, 1976; Henderson, 1978; M. R. Pollard & G. J. Dutton, unpublished work), to 33 mg of microsomal protein (Bock & Fröhling, 1973; Bock *et al.*, 1976; Dutton *et al.*, 1981), and to 1.2×10^8 parenchymal cells (Bock *et al.*, 1976; Moldéus *et al.*, 1978). All comparisons are with weaned male rats, but, as they derive from both Wistar and Sprague–Dawley

strains and body weights of 100–450 g (i.e. from different ages), they remain approximate.

(a) *Liver slices.* The cutting of snips is easier to learn than the cutting of slices, and for certain uses, e.g. work with foetal, infant, fatty or other small or fragile livers, snips alone are practicable. Snips, because of their small size, also facilitate study of discrete interlobular areas. Snips possess greater reproducibility than slices prepared as described elsewhere (e.g. by Dutton, 1980). In three separate experiments, each employing multiple incubations from a pool of liver slices, standard deviations with 2-aminophenol glucuronidation were ± 20 , 22, and 33%, from an unskilled operator whose performance with snips gave a value of less than $\pm 10\%$. Snips have a consistent crescentic shape, are easily screened for selection and possess a useful control index (μ g of protein per snip) for comparing preparations from different experiments. Slices have two variables, thickness and surface area, not readily assessed. Ten snips contain about as much protein as an average slice, but more effectively 'randomized'.

Liver snips are more active in glucuronidation than are liver slices. In four separate experiments by reasonably-experienced operators directly comparing the two preparations, slices gave 45–70% the activity of snips. The reasons may include the greater cellular damage in slices, for addition of UDPGlcA + UDPGlcNAc to the incubation medium suggested a considerably higher proportion of ruptured cells in slices (35%) than in snips (<10%).

(b) *Liver perfusions.* Bock's group reports rate of glucuronidation for adult rat liver perfused with 0.5 mM-1-naphthol as 0.55 ± 0.22 unit (Bock & Fröhling, 1973; Bock & White, 1974), similar to our finding with that substrate for liver snips (0.53 ± 0.09 unit). Perfusion of liver from adult male rats with 0.5 mM-2-aminophenol gave a rate of 0.05 unit (Burchell & Bock, 1980), similar to earlier work with slices (Winsnes & Dutton, 1973), but less than we find with snips from our present adult (i.e. older than 30–50 days) rats (0.22 unit).

(c) *Suspensions of isolated hepatocytes.* Rates of glucuronidation are of the same order of magnitude in snips and hepatocyte suspensions, but slightly lower in snips. This is not surprising, because snips contain connective tissue and other cell types, and because the tissue organization of the cells may modify substrate access. If, in Fig. 2, we extrapolate snip size from our average in this study (283 μ g of protein/snip) to zero (i.e. towards an isolated hepatocyte) we gain approx. 50% activity. Experimentally, 1-naphthol (0.5 mM) is glucuronidated by isolated hepatocytes at a rate of 0.72 ± 0.07 unit (Bock & Fröhling, 1973), some 30% higher than we find (0.53 ± 0.09 unit) for snips with that substrate.

Moldéus' group (Moldéus *et al.*, 1978; Andersson *et al.*, 1978) noted similar maximum rates of glucuronidation by hepatocytes of 2-naphthol and other substrates (0.65–1.35 units).

In conclusion, snips possess the often important cell–cell interactions of perfused tissue and slices, and the high reproducibility of cell suspensions. Their preparation is simpler than these, and incurs neither digestion at cell interfaces, which may alter cell membranes (see Dutton, 1980), nor anaesthesia, which may also affect cell membranes and drastically change nucleotide concentrations (Eriksson & Stråth, 1981). Snips are particularly suitable for exploratory and topological work, or for studies such as those on perinatal development, where the need for large sample pools and the use of small fragile livers precludes perfusion and discourages 'slicing', and where age-dependent conditions for isolating minimally damaged cells complicate the use of hepatocytes.

We thank Mr. Donald Gardiner for excellent technical help, Dr. B. Burchell and Dr. J. E. A. Leakey for useful discussion and the Medical Research Council for a grant to G. J. D. We are indebted to Dr. Paul Skett, University of Glasgow, for pointing out the possibilities of the McIlwain Tissue Chopper in preparing snips.

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