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USE OF ISOLATED KIDNEY CELLS FOR STUDY OF DRUG METABOLISM

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Abstract—Isolated kidney cells were prepared from rat kidneys using a recirculating perfusion system with collagenase. The preparation was rapid and provided a high yield of intact metabolically active kidney cells predominantly of tubular origin. The respiration rate was 2.7 μmol O_2/hr per 10^6 cells and was not stimulated by ADP. GSH content was 28.9 nmol/10^6 cells and did not decline during 2 hr of incubation. Cytochrome P450 content was 0.064 nmol/10^6 cells. The cells were characterized for their drug metabolizing activity using paracetamol as substrate. The rate of formation of the glucuronide and sulfate derivatives was linear for 2 hr, but slower than previously reported for rat liver cells. In contrast to incubations with liver cells, no glutathione conjugate was detected. However, formation of both cysteine and *N*-acetylcysteine derivatives was observed. The rate of formation of total sulfhydryl conjugates was about 50 per cent of that reported for liver cells when expressed on a cytochrome P450 basis.

These studies establish the reliability and utility of this cell preparation as a model system for the study of drug metabolism by the kidney.

The kidneys play an important role in drug metabolism, functioning in oxidation and conjugation reactions [1–4], in further metabolism of conjugates formed in other tissues [3–6], and in excretion of drugs and drug metabolites [2, 7]. Although studies of drug excretion in general require maintenance of supracellular organization, the oxidation, conjugation and other metabolic reactions can be readily studied at the cellular and subcellular levels.

During recent years, isolated hepatocytes have become well established as an *in vitro* system for study of processes involving multiple subcellular components [8–14]. Following our experience with the use of this system for study of drug metabolism [11–14] we have developed a similar preparation for study of drug metabolism in the kidney. In this report, we describe this preparation, characterize its metabolic integrity, and analyze its function in drug metabolism. For this study, we have employed paracetamol *, a commonly used analgesic, as substrate. This substrate is known to be metabolized to a variety of products, including sulfate, glucuronide, glutathione, cysteine and *N*-acetylcysteine derivatives [10, 15–18]. The development of convenient analytical methods using high pressure liquid chromatography [10, 16, 17, 19] has allowed separation and quantitation of these metabolites in order to assess the function of isolated kidney cells in drug metabolism.

MATERIALS AND METHODS

Materials. Collagenase (Grade II) and GSH were obtained from Boehringer/Mannheim GmbH, Mannheim, Germany. Antimycin A, bovine serum albumin (Fraction V), NADH, and ADP were obtained from Sigma. All other chemicals were at least of reagent grade and purchased locally. Distilled, deionized water was used throughout.

Medium for cell preparation was a modified Hanks buffer, pH 7.4 (NaCl 8.0 g, KCl 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.2g, $NaHPO_4 \cdot 2H_2O$ 0.06 g, KH_2PO_4 0.06 g, and $NaHCO_2$ 2.19 g in 1 liter H_2O) with additions as described.

Preparation of kidney cells. Male Sprague–Dawley rats (200–250 g) were anesthetized with ether, and heparin (1000 IE) was injected into the tail vein. The peritoneal cavity was opened by a midventral incision and the aorta was freed below and above the renal arteries. A ligature was placed just below the renal arteries, and a second one was placed as high up in the abdomen as possible. The coeliac artery was ligated to avoid leakage of perfusion fluid. An oblique incision was made in the aorta below the upper ligature and a conical tipped probe cannula (1.5 × 80 mm, 2R2, Switzerland) was immediately inserted and secured with a new ligature (Fig. 1, insert). The perfusion was started with the kidneys *in situ* using modified Hanks Buffer containing Hepes (25 mM), EGTA (0.5 mM) and bovine serum albumin (2% w/v). The buffer was oxygenated (95% 0_2, 5% CO_2) and maintained at 37° (Fig. 1). To avoid gas embolism, the tubes and cannula were filled with fluid, and the fluid was allowed to drip out of the cannula during insertion. The flow rate was adjusted to a pump pressure of about 1 m H_2O, and within about one minute, both kidneys became pale. In some instances a satisfactory perfusion of both kidneys was not achieved, presumably due to the asymmetric branching of the renal arteries. Care was required to prevent insertion of the cannula below the level of the right renal artery.

The kidneys were excised from the posterior abdominal wall by cutting from the lateral aspect to the median line. The renal capsule and some connective tissue were

* Abbreviations and trivial names include: paracetamol, *N*-acetyl *p*-aminophenol (acetaminophen); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethanedioxybis (ethylamine) tetraacetate; TCA, trichloroacetic acid.

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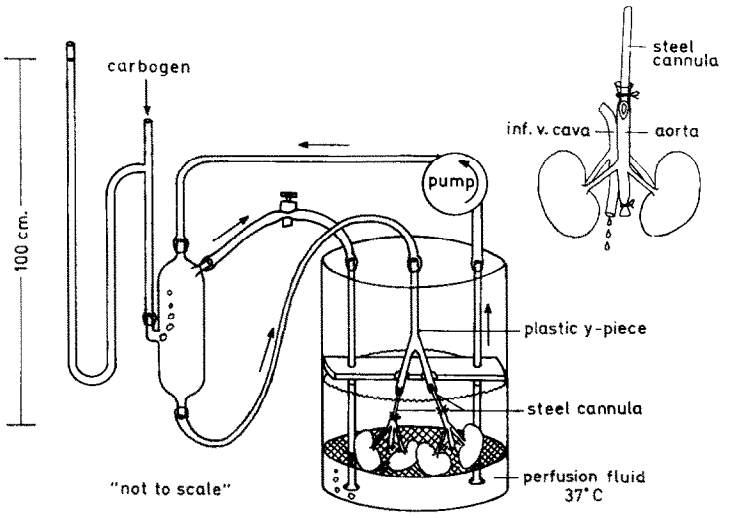

Fig. 1. Schematic drawing of apparatus for preparation of isolated kidney cells. "Carbogen" mixture was 95% O_2, 5% CO_2.

left in place. To avoid excessive hemorrhage, the vena cava was cut last. The kidneys were transferred to the beaker from which the perfusate was withdrawn to provide a recirculating system (Fig. 1).

Usually cells were prepared from two rats. Kidneys from the first rat were continually perfused during the second cannulation (usually about 6 min). After perfusion of the second pair of kidneys for 5 min, the perfusion assembly containing the four kidneys was transferred to another beaker with modified Hanks buffer containing $CaCl_2$ (4 mM) and collagenase(0.12% w/v). This solution was circulated through the kidneys under constant pressure for 10–13 min. Kidneys that were adequately perfused were slightly swollen and pale. The kidneys were removed from the perfusion apparatus, fat and connective tissue were gently removed and the kidney cells were dispersed with a pair of forceps in Krebs–Henseleit buffer containing Hepes (25 mM) and albumin (2% w/v).

The dispersed cells were filtered through nylon mesh to remove connective tissue and larger tissue fragments. The cells were allowed to settle for 2–3 min at ambient temperature and the excess medium was aspirated.

Cell concentration was estimated using a Bürker Chamber and trypan blue exclusion was determined in the presence of trypan blue (0.18% w/v). The yield of cells was increased 50–100 per cent by dispersing the tissue retained in the nylon mesh sieve in the medium containing collagenase and incubating at 37° for 4–5 min before transfer of cells to the Krebs–Henseleit buffer.

Incubations were performed in rotating round bottom flasks [11] at 37° under 95% O_2, 5% CO_2. Krebs–Henseleit buffer containing Hepes (25 mM) and bovine serum albumin (2% w/v) was used except as indicated.

Assays. 3 Methylcholanthrene pretreated rats received daily oral doses (25 mg/kg in corn oil (10 mg/ml)) and subcutaneous injections (50 mg/kg in corn oil) for 3 days followed by a day with no treatment prior to use. NADH penetration was assayed as described by Moldéus *et al.* [11]. Wet cell volume was measured using standard hematocrit tubes. Oxygen consumption was measured polarographically with a Clark electrode (Yellow Springs Instrument Co.), calibrated with air-saturated H_2O at 37°. Sodium dithionite was added to obtain an experimental zero oxygen concentration. Cytochrome P450 content was measured by the method of Orrenius *et al.* [20] after dispersing the cells in Krebs–Henseleit buffer containing Hepes (25 mM), glycerol (20% v/v) and sodium cholate (5 mg/ml), using 102 $cm^{-1}mM^{-1}$ as the extinction coefficient for 450 minus 490 nm [1]. GSH was estimated as total free sulfhydryl [21] in the cellular pellet following centrifugation (80 *g*), removal of supernatant, and acidification (6.5% TCA) to remove protein.

Paracetamol metabolites were separated and quantitated by high pressure liquid chromatography by the procedure of Howie *et al.* [16] as modified by Moldéus [10]. Metabolites were measured in the total incubation mixture following acidification with perchloric acid (3N, 0.1 ml/0.2 ml incubation volume) and centrifugation to remove protein.

RESULTS

Kidney cell preparation. The perfusion with collagenase provided rapid and gentle dispersal of kidney into small tissue components composed primarily of single cells. The preparation was examined by light microscopy and contained mainly spherical cells of uniform size which appeared to be derived from tubular epithelium. Smaller cells, of endothelial and reticuloendothelial origin, were also seen, but constituted a small fraction of the total cell yield and were not counted. Large fragments, recognizable as tubular and glomerular fragments, were present when perfusion with collagenase was performed for less than 10 min. Consequently, all data presented here were obtained from preparations in which perfusion time was longer than 10 min. Average cell yield was about 32×10^6 cells per preparation using 2 rats (Table 1) or about 9×10^6 cells per kidney after correction for poorly perfused kidneys. Preparations which were incubated after dispersal for 4–5 min in medium containing collagenase

Table 1. Characteristics of isolated kidney cell preparation. Data are given as mean ± S.E.M. with number of cell preparations given in parentheses

Cellular Parameters			
Yield per preparation using kidneys from two rats	32.5	± 6.4 × 10^6 cells	(22)
Packed volume per 10^6 cells*	14.3	± 1.2 μl	(16)
Trypan blue exclusion	86.1	± 1.4%	(18)
NADH penetration	15.4	± 1.4%	(12)
O_2 consumption per 10^6 cells†	2.71	± 0.27 μmol/hr	(11)
Inhibition of respiration by Antimycin A (0.5 μM)	81.3	± 1.3%	(10)
Stimulation of respiration by ADP (0.5 mM)		0%	(10)
GSH content	28.9	± 2.7 nmol/10^6 cells	(16)
Cytochrome P450 content		0.064 ± 0.004 nmol/10^6 cells	(8)

*Cells were sedimented in hematocrit tubes at 150 *g* for 3 min.
† Incubation medium was Krebs–Henseleit buffer containing Hepes (25 mM) and bovine serum albumin (2% w/v).

had yields as high as 20 × 10^6 cells/kidney. The packed cell volume was about 14 μl/10^6 cells (Table 1). Using the initial kidney weight of about 1 g/kidney, the packed cell volume (14 μl ≃ 14 mg), the cell yield (20 × 10^6/kidney), and assuming cell volume is 60–70 per cent of packed cell volume, about 17–20 per cent of the kidney was recovered as isolated tubular cells. This indicates a high recovery when non-cellular spaces such as vasculature and luminal areas are considered.

Isolated cells have been characterized by a variety of methods to establish whether the metabolic functions are intact [8, 11, 13, 22]. Simple and reliable methods include those which measure the integrity of the cellular permeability barrier by determining either the cellular permeability to intermediate molecular weight compounds or by measuring the retention of compounds readily lost upon cellular damage. The isolated kidney cells largely excluded the vital dye, trypan blue, as seen in the data from our first 18 preparations from control rats (Table 1). Although the percentage exclusion increased somewhat as the method became routine and is occasionally comparable to the data for our liver cell preparations (95–99 per cent), typical exclusion values remain 85–90 per cent. The NADH penetration assay which measures the increase in lactic dehydrogenase activity following the disruption of cells with detergent [23] indicates that most of the cells are intact (Table 1), since they do not leak LDH and are not permeable to NADH without disruption. The cells retain this property during 2 hr incubations (Fig. 3) which indicates that the cells remain intact during this incubation period.

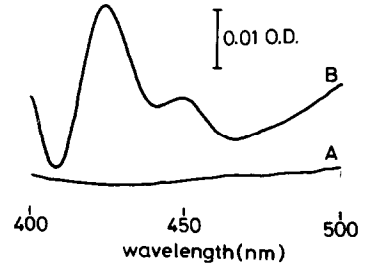

Fig. 2. Cytochrome P450 in isolated kidney cells. Isolated kidney cells were suspended (0.86 × 10^6 cells/ml) in Krebs–Henseleit buffer, pH 7.4, containing Hepes (25 mM), glycerol (20% v/v) and sodium cholate (5 mg/ml). Following addition of succinate (10 mM), the suspension was bubbled with CO for 2 min and incubated at room temperature for 5 min. The suspension was pipetted into sample and reference cuvettes, and a baseline was recorded (A). A few grains of dithionite were then added to the sample cuvette and after 2 min the spectrum (B) was recorded.

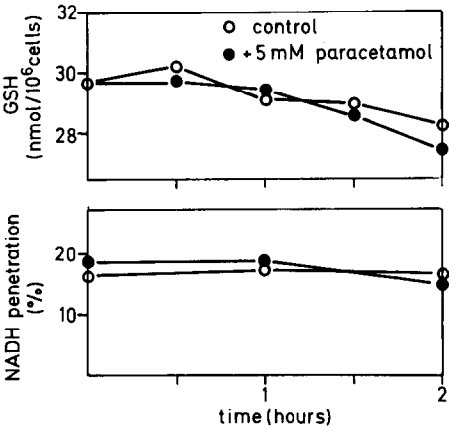

Fig. 3. GSH content and NADH penetration of isolated kidney cells as a function of incubation time. Kidney cells (2 × 10^6/ml) were incubated in Krebs–Henseleit buffer (pH 7.4, containing 25 mM Hepes) in rotating round bottom flasks [11]. For GSH assay, cells were removed from the suspending medium by centrifugation and GSH estimated as the total free sulfhydryl remaining after protein precipitation with trichloroacetic acid (6.5%). One experiment which is typical of nine.

Table 2. Rates of formation of paracetamol metabolites by isolated kidney cells from control and 3-methylcholanthrene pretreated rats

Paracetamol conjugates	Rate of conjugate formation (nmol/hr · 10^6 cells^{-1})	
	Control	3-MC
Glucuronide	2.81 ± 0.16	4.16 ± 0.73
Sulfate	1.67 ± 0.15	1.76 ± 0.16
Cysteine	0.41 ± 0.06	0.72 ± 0.13
N-acetylcysteine	0.36 ± 0.06	1.28 ± 0.18
Glutathione	0.02 ± 0.02	0.19 ± 0.19
Total sulfhydryl conjugates	0.79	2.19
Total conjugates	5.27	8.11

Rates are given as mean ± S.E.M. for at least 3 cell preparations. Initial paracetamol concentration was 5 mM.

The maintenance of a normal GSH concentration is also a valuable indicator of kidney cell integrity, since GSH released from the cell is likely to be oxidized [6] or to act as a substrate for γ-glutamyltranspeptidase [24]. The cells as isolated retained a GSH content (28.9 nmol/10^6 cells) comparable to fresh kidney cortex [25] when expressed in similar units (assuming 60–70 per cent of the packed cell volume to be cells). The GSH content declined minimally during 2 hr incubations (Fig. 3), even without added amino acids, which further suggested that the cells maintained metabolic function during this time.

The rate of O_2 consumption (2.71 μmol/hr per 10^6 cells) in the presence of bovine serum albumin (2%, w/v) is comparable to that obtained for intact kidney [26] assuming that the cell volume is 60–70 per cent of the packed cell volume. In the absence of bovine serum albumin, the O_2 consumption rate was 1.49 ± 0.12 μmol/hr per 10^6 cells ($n = 7$). This rate is only 55% of the rate with albumin and suggests that the crude albumin fraction contains substances which act as substrates for cellular respiration. Addition of succinate (1 mM) to the isolated cells with and without albumin further supported this interpretation since stimulation in the absence of albumin was 96 ± 10 per cent ($n = 7$) whereas stimulation in the presence of albumin was only 28 ± 9 per cent ($n = 7$). Addition of ADP (0.5 mM) had no effect on respiratory rate, either with or without additions of albumin or succinate. Thus, the cellular ADP pool appears to be unaffected by exogenous ADP and therefore the cellular permeability to succinate appears to be unrelated to altered membranal characteristics. Respiratory inhibition by antimycin A is 81.3 per cent which is a greater percentage inhibition than seen with isolated hepatocytes (unpublished data). Perhaps this difference indicates that a higher fraction of O_2 is consumed by mitochondrial respiration in the kidney than in the liver.

The cell preparation was examined by optical spectroscopy to measure hemoglobin contamination and cytochrome P450 content. The preparation was free from detectable hemoglobin as measured by the carbon monoxyhemoglobin minus oxyhemoglobin absorbance difference at 418 nm [27]. Direct measurement of cytochrome P450 in intact cells as used for isolated hepatocytes [13] was not possible due to the interference by carbon monoxycytochrome oxidase. To eliminate this problem, the cells were solubilized with detergent, and the selective reduction technique of Orrenius *et al.* [20] was used for visualization of cytochrome P450 (Fig. 2). The cytochrome P450 content (Table 1) is substantially less than that found in hepatocytes [13] which is in agreement with concentration differences previously reported for microsomes [20] and the differences in proportional volume of endoplasmic reticulum between liver and kidney.

Paracetamol metabolism. Incubation of kidney cells in the presence of paracetamol had no effect on the cell viability and resulted in only a small decrease in GSH content (Fig. 3). With 5 mM paracetamol, the rates of formation of paracetamol glucuronide and paracetamol sulfate were substantially higher than the rate of formation of sulfhydryl conjugates (Table 2). Cysteine and *N*-acetylcysteine derivatives were present at similar concentrations, while the glutathione derivative was not detected.

To determine the suitability of this preparation for drug metabolism studies and to determine whether incubation conditions, developed for study of drug metabolism in isolated liver cells, were adequate for similar studies in kidney cells, paracetamol metabolism was measured with variations in cell concentration, paracetamol concentration and time. The formation of glucuronide, sulfate and total sulfhydryl conjugates was linear up to 2 hr (Fig. 4A). However, the proportion of total sulfhydryl conjugates present as the *N*-acetylcysteine derivative increased with time (Fig. 4B). Presumably the rate of acetylation increased as the concentration of the cysteine derivatives increased and resulted in an increased rate of formation of the *N*-acetylcysteine derivative.

A similar phenomenon was observed when metabolite formation was measured as a function of cell concentration (Fig. 5). Formation of sulfate, glucuronide and total sulfhydryl derivatives was linear up to 2.8 × 10^6 cells/ml. However, the proportion of the total sulfhydryl conjugates present as the *N*-acetylcysteine derivative increased with increased cell concentration.

A study of the concentration dependence of metabolite formation demonstrated that the different conjuga-

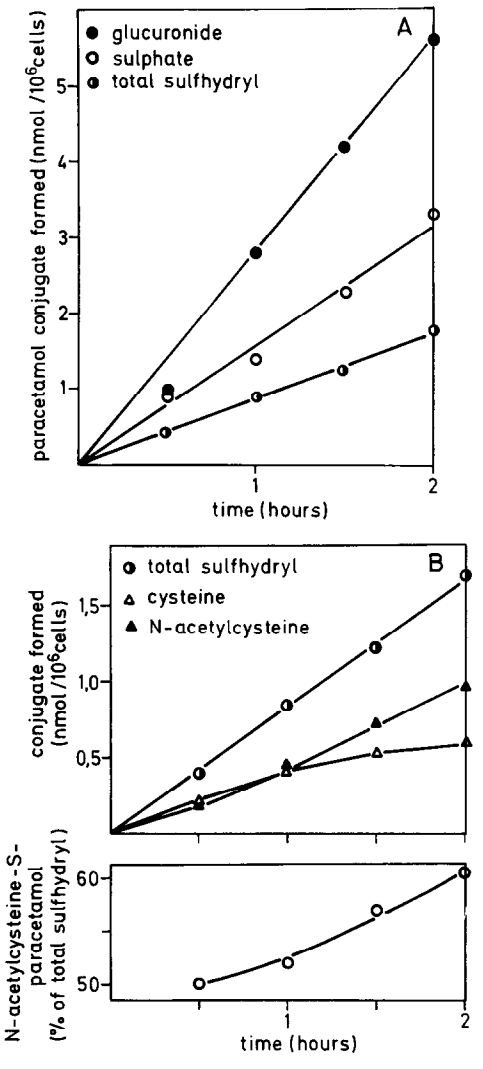

Fig. 4. Formation of paracetamol metabolites as a function of time. Cells were incubated as described in Fig. 3 in the presence of paracetamol (5 mM). Metabolites were quantitated by high pressure liquid chromatography. One experiment which is typical of nine.

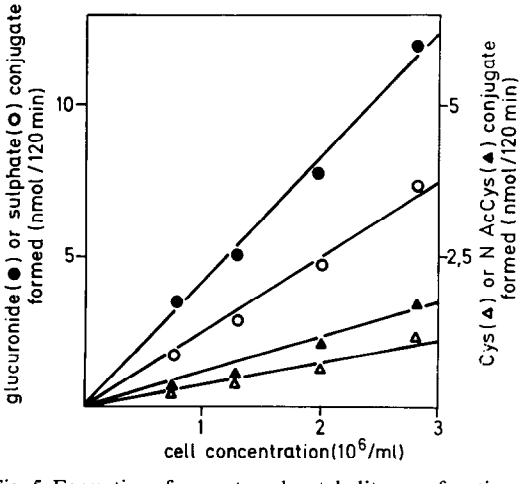

Fig. 5. Formation of paracetamol metabolites as a function of cell concentration. Incubations and assays were performed as in Fig. 4. One experiment which is typical of three.

tion systems in kidney have different apparent affinities for paracetamol (Fig. 6). The pattern of these affinities for the different reaction pathways is very similarly to that seen with hepatocytes [10]. The rate of formation of the sulfate conjugate is maximal above 2 mM paracetamol, and the rate of formation of the glucuronide begins to plateau before 7 mM. However, formation of sulfhydryl conjugates shows no decrease from linearity even at 10 mM paracetamol.

Pretreatment with 3-methylcholanthrene. The yield and viability characteristics of kidney cells prepared from rats pretreated with 3-methylcholanthrene were similar to cells from control rats. Incubations without paracetamol had stable GSH content and showed only a small increase in NADH permeability as seen for control cells (Fig. 3). Similarly, addition of paracetamol (5 mM) had no effect on NADH penetration and resulted in only a small decrease in GSH content. The rate of formation of sulfhydryl conjugates of paracetamol was more than doubled, while there was a smaller increase in the formation of the glucuronide and no change in the formation of the sulfate (Table 2).

DISCUSSION

In the present studies we have developed a model system using isolated kidney cells for the study of drug metabolism in the kidney. Unlike other kidney cell or tubule preparations [28–31], this preparation utilizes a recirculating perfusion system and does not involve either mincing or slicing the tissue. The entire preparation takes about 40 min and provides a cell yield adequate for a variety of metabolic studies. The cells were comparable to isolated liver cells[11, 13] with regard to exclusion of both trypan blue and NADH.

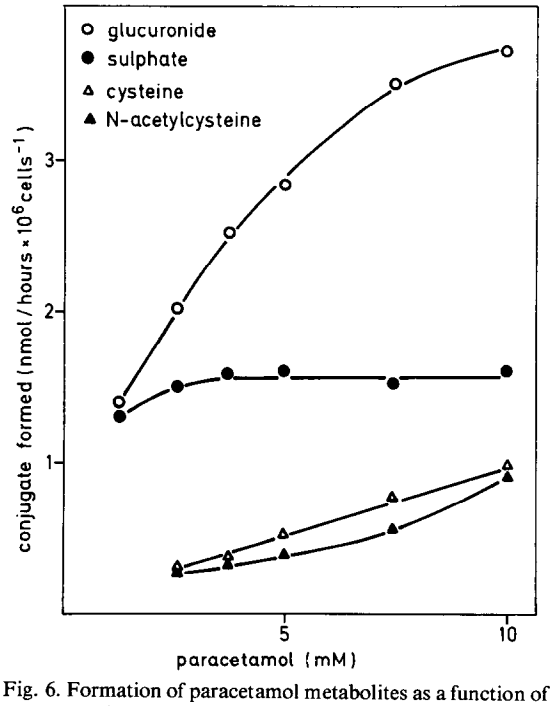

Fig. 6. Formation of paracetamol metabolites as a function of paracetamol concentration. Incubations were performed as described in Fig. 3. One experiment which is typical of five.

and lack of respiratory stimulation by ADP. The O_2 consumption rate and GSH content are comparable to other kidney preparations and the cells retain metabolic integrity during 2 hr incubations.

A variety of drug substrates are known to be metabolized by oxidation and conjunction pathways in the kidney. These reactions include (1) oxidation catalyzed by cytochrome P450[1, 20, 32], (2) conjugation of electrophilic oxidation products with glutathione, catalyzed by glutathione-*S*-transferase [33–34] or occurring spontaneously, (3) conjugation with sulfate, catalyzed by sulfotransferases [2, 35], (4) conjugation with UDP-glucuronic acid, catalyzed by UDP-glucuronosyl transferase [36], and (5) conjugation with amino acids (for review, see [37]). In addition, the kidney contains γ-glutamyltransferase, an enzyme which is thought to be involved in conversion of glutathione-*S*-conjugates to cysteine-*S*-conjugates [24], a particulate peptidase [38] which also may be involved in this conversion, and *N*-acetyltransferase, an enzyme which catalyzes the *N*-acetylation of cysteine conjugates [39]. The metabolites of paracetamol formed by kidney cells and detected by high pressure liquid chromatography include glucuronide, sulfate, cysteine and *N*-acetylcysteine derivatives. At present, we have no data to suggest that either the cysteine or the *N*-acetylcysteine derivative is formed directly by interaction of the cysteine or *N*-acetylcysteine with an oxidation product of paracetamol. With isolated kidney microsomes as with liver microsomes, these are relatively poor substrates for formation of sulfhydryl conjugates of paracetamol when compared to glutathione (unpublished results). We have found in subsequent experiments [6] that added glutathione-*S*-paracetamol is rapidly and quantitatively converted by isolated kidney cells to cysteine-*S*-paracetamol and *N*-acetylcysteine-*S*-paracetamol. Perhaps the cysteine and *N*-acetylcysteine derivatives in kidney cells are formed from glutathione-*S*-paracetamol by a reaction sequence including (1) γ-glutamyl transferase catalyzed removal of the glutamyl residue, (2) dipeptidase catalyzed hydrolysis of the glycyl residue and (3) *N*-acetyltransferase catalyzed acetylation of the cysteine derivative. If this occurs, then the absence of detectable glutathione-*S*-paracetamol upon addition of paracetamol to kidney cells suggests that the initial reactions in conversion of this derivative to the cysteine derivative are rapid; whereas, the nearly equimolar concentrations of cysteine and *N*-acetylcysteine derivatives suggest that the rate of *N*-acetylation under these conditions is about half the rate of *S*-conjugation. However, this difference may be somewhat dependent upon the use of an isolated cellular system rather than an intact system since it may be necessary for the glutathione-*S*-paracetamol to diffuse out of the cells to become accessible to enzymes involved in conversion to the cysteine derivative and then subsequently diffuse back into the cell to undergo *N*-acetylation. The flow characteristics in an intact kidney may facilitate this type of a sequential process, while in a cell suspension the diffusion processes would appear to occur randomly.

Fry *et al.* [28] examined drug metabolism by isolated rat kidney tubules and found that they had very low oxidative activity relative to liver with 7-ethoxycoumarin, biphenyl and benzo(α)pyrene as substrates. On the other hand, the conjugation activities (formation of glucuronide, sulfate or glycine derivatives) were 8–60 per cent of liver values with 7-ethoxycoumarin, 4-methylumbelliferone or benzoic acid as substrates. With paracetamol as substrate, we found that kidney cell activities for formation of glucuronide and sulfate derivatives are about 5 per cent of those for liver cells (compare Table 2 with data of Moldéus [10]); whereas, the rate of formation of sulfhydryl derivatives by control kidney cells is nearly 10 per cent of the rate by liver cells. The similarity of the ratio of sulfate and glucuronide conjugates formed in the kidney and liver cell preparations is consistent with the observation that liver and kidney cells demonstrate similar conjugation metabolite patterns [28]. However, the observation that formation of sulfhydryl derivatives is proportionally higher in kidney cells with paracetamol as substrate illustrates that the relationship between the metabolic processes is both organ specific and substrate specific.

The cytochrome P450 content of this cell preparation is about 20–25 per cent of that found in control liver cells [11,13]. However, unlike the liver cells, a large portion of the kidney cytochrome P450 appears to be present in mitochondria [40]. Assuming that the formation of sulfhydryl conjugates reflects the activity of cytochrome P450 in oxidation of paracetamol [41] the comparison of sulfhydryl conjugate formation on a cytochrome P450 basis by isolated liver cells (about 7.8 nmol/10^6 cells·hr^{-1} [10] and 0.26 nmol P450/10^6 cells) and by isolated kidney cells (0.79 nmol/10^6 cells·hr^{-1} and 0.064 nmol P450/10^6 cells) indicate that kidney cytochrome P450 may be only 40 per cent as active in the generation of sulfhydryl binding species as liver cytochrome P450. No data are presently available on which of the forms of kidney or liver cytochrome P450 are involved in metabolism of paracetamol.

The increased rate of formation of sulfhydryl conjugates in kidney cells from 3-methylcholanthrene pretreated rats demonstrates that in kidney, as in liver, the reaction processes involved in generation of reactive electrophiles may be increased by agents which increase cytochrome P450 content. Since this process is thought to be involved in generation of reactive species involved in cell damage [41], this mechanism may also be important in drug induced kidney damage (for example, see [42]) as well as liver damage. The relatively slow rate of formation of sulfhydryl conjugates and slow loss of cellular glutathione in rat kidney cells suggests that to elicit toxicity reactions by this mechanism in kidney due to paracetamol, additional factors such as enzyme induction or co-administration of other substances, which may be metabolized similarly, would be required.

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