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UPTAKE OF ACETAMINOPHEN (PARACETAMOL) BY ISOLATED RAT LIVER CELLS

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Abstract—The characteristics of the uptake of acetaminophen (*N*-acetyl-*p*-aminophenol or paracetamol, APAP) in incubations of isolated rat liver cells were consistent with diffusion of the drug being the predominant mechanism of APAP influx in these cells at concentrations above 0.5 mM. At lower substrate concentrations (below 0.5 mM) a saturable component was apparent. Both uptake processes could have a role in the control of the metabolism of APAP, because, at low concentrations, there was no intracellular accumulation of unconjugated drug, all the APAP entering the cell being converted to sulphate and glucuronide. After addition of drug, there was a lag phase of approximately 5 min before APAP–glucuronide and APAP–sulphate appeared in the incubation medium; during this time both conjugates accumulated inside the cells. These results have implications for our understanding of the mechanisms of APAP transport, and indicate how these processes may affect the drug's overall metabolism.

Glucuronidation and sulphation are phase II conjugation reactions of major importance in the detoxification of many endogenous and exogenous compounds [1]. Membrane-enclosed UDP-glucuronyltransferases and the cytoplasmic sulphotransferases catalyse these reactions and require the co-substrates UDP-glucuronic acid and 3′-phosphoadenosine 5′-phosphosulphate, respectively.

The commonly used analgesic drug, acetaminophen (*N*-acetyl-*p*-aminophenol or paracetamol, APAP), is a substrate for both glucuronidation and sulphation [2]. The conjugates, more water-soluble than the parent compound, are eliminated from the body in the urine or bile.

Metabolism of APAP involves three distinct steps: (i) transport across the plasma membrane into the liver cell, (ii) conjugation of the drug within the cell and (iii) transport of the conjugates out of the cell. In the past, transport into the liver cell, and the impact of this step on the further metabolism of APAP (and of drugs in general), have been virtually ignored [3]. In intermediary metabolism, however, membrane transport can be important in the regulation of metabolic flux (e.g. in the metabolism of aromatic amino acids [4] and of glutamine [5, 6]).

We have investigated the transport of APAP into isolated rat liver cells to identify the transport mechanisms involved, and studied the kinetics of both APAP metabolism within the cell and the appearance of conjugates in the medium. Comparison of the rates of uptake and of metabolism of APAP has provided insight into the cellular control mechanisms associated with drug detoxification.

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MATERIALS AND METHODS

Animals. Male Wistar rats (6–10 weeks old; 150–250 g) were used throughout. Animals were allowed free access to standard laboratory rat chow and water. Anaesthesia was induced by intraperitoneal injection (60 mg/kg) of sodium pentobarbitone (Sagatal).

Chemicals. All radiolabelled compounds were purchased from Amersham International (Amersham, U.K.). Collagenase H (from *Clostridium histolyticum*) was purchased from either Boehringer Mannheim (U.K.) or the Sigma Chemical Co. (Poole, U.K.). The "silicone oil" used was a 2:1 (v/v) mixture of Dow Corning 550 and dinonyl phthalate supplied by BDH Chemicals (Poole, U.K.). All other chemicals used were of analytical or higher grade and were from BDH Chemicals, Sigma (including unlabelled APAP) or the Aldrich Chemical Co. Ltd (Poole, U.K.), unless otherwise stated.

Separation of conjugates. Separation involved a modification of the method of Adriaenssens and Prescott [7], with a reverse-phase HPLC column (Ultratechsphere 5 ODS, 25 cm × 4.6 mm). The mobile phase consisted of 0.1 M phosphate (Na^+) buffer (pH 4.5):isopropanol:98% (v/v) formic acid (100:1.7:0.1) at a flow rate of 1 mL/min. APAP and its glucuronide and sulphate conjugates were detected by absorbance at 254 nm (Beckman System Gold Scanning Detector, Model 167) and quantified using APAP standards, kindly donated by Dr R. Anderson, Sterling Laboratories.

Preparation and incubation of cells. Isolated liver cells were prepared using a modification [8] of the

method of Seglen [9] except that perfusions were commenced at any time from 09:30 to 14:00 hr, and the perfusion medium contained 20 mM D-glucose. Viability of cells was determined by measurement of ATP contents [10].

Suspensions of liver cells (1–2 mL; 5–10 mg dry wt) were incubated in Krebs–Henseleit buffer [11] (without albumin but supplemented with 10 mM D-glucose) with additions of 10–20 μL of APAP solution in dimethyl sulphoxide [final concentration of solvent 1% (v/v)], under an atmosphere of O_2/CO_2 (19:1). Incubations were at 37° in a reciprocating water bath (100 cycles/min). Reactions were stopped by removal of 0.8 mL samples which were centrifuged in an "Eppendorf-type" centrifuge (Sarstedt, Leicester, U.K.) for 30 sec at 10,000 *g* to pellet the liver cells. The resulting supernatants were removed and heated in a boiling water bath for 5 min to precipitate protein, before being centrifuged again (30 sec in a benchtop centrifuge); the final supernatant was analysed by HPLC.

Measurement of APAP uptake and export of conjugates. Suspensions of liver cells (0.2 mL; 3 mg dry wt) were preincubated for 10 min at 37° under an atmosphere of O_2/CO_2 (19:1) in a shaking water bath (120 oscillations/min) before addition of 4 μL of [^{3}H]APAP in 25% dimethyl sulphoxide. Reactions were stopped by addition of 1 mL of ice-cold 0.9% saline. After mixing, 1 mL samples were removed and centrifuged through 500 μL of "silicone oil" for 30 sec at 12,000 *g*, the isolated liver cells moving through the oil into 40 μL of 12% (v/v) $HClO_4$. Supernatants (0.9 mL) were removed and heated in a boiling water bath for 5 min to precipitate protein, and were then centrifuged again. Samples (100 μL) of the resulting supernatants were injected onto a reverse-phase HPLC column. The isolated liver cells under the oil were resuspended in a total of 240 μL of 12% (v/v) $HClO_4$ to disrupt cell membranes and centrifuged for 30 sec at 10,000 *g*, and the radioactivity in 50 μL of supernatant, mixed with 5 mL of scintillant, was determined by liquid scintillation counting. A further 100 μL of this supernatant were injected onto the HPLC column. After an initial 5 min, 0.5 mL fractions were collected from the column over the next 20 min; radioactivity was determined as above. The intracellular and contaminating extracellular spaces were determined simultaneously by measurement of the ^3H_2O- and [U-^{14}C]sucrose-permeable spaces from incorporation of label into cell pellets; ^3H_2O penetrates both intra- and extra-cellular spaces, while [U-^{14}C]sucrose does not enter cells so that its accumulation in pellets gives a measure of the extracellular space alone. In a typical transport experiment the intracellular volume was found to be 1.6 ± 0.4 μL/mg dry wt and the extracellular volume (1.5 ± 0.3 μL/mg dry wt (means ± SD, N = 3). These volumes were calculated for each transport experiment.

Kinetic parameters were obtained using a Basic Programme (L. Wilkinson, *Systat: the Systems for Statistics*, Systat Inc., Evanston, IL, U.S.A.).

RESULTS AND DISCUSSION

Time course of labelled APAP uptake

The mechanism of APAP uptake into isolated liver cells was studied by monitoring the time courses of the uptake of radiolabelled APAP at various substrate concentrations. Uptake of 0.1 and 5.0 mM APAP into cells is shown in Fig. 1. At both these concentrations, uptake of radioactivity was linear from zero time to 30 sec, approaching a plateau for later time points. This plateau corresponded to an equilibrium of APAP entry, metabolism and exit, both as unmodified drug and as conjugates. The concentrations of radiolabelled APAP metabolites, both within the cells and in the extracellular medium, were also measured, as shown for 0.1 mM APAP in Fig. 2. At this concentration (see below), the

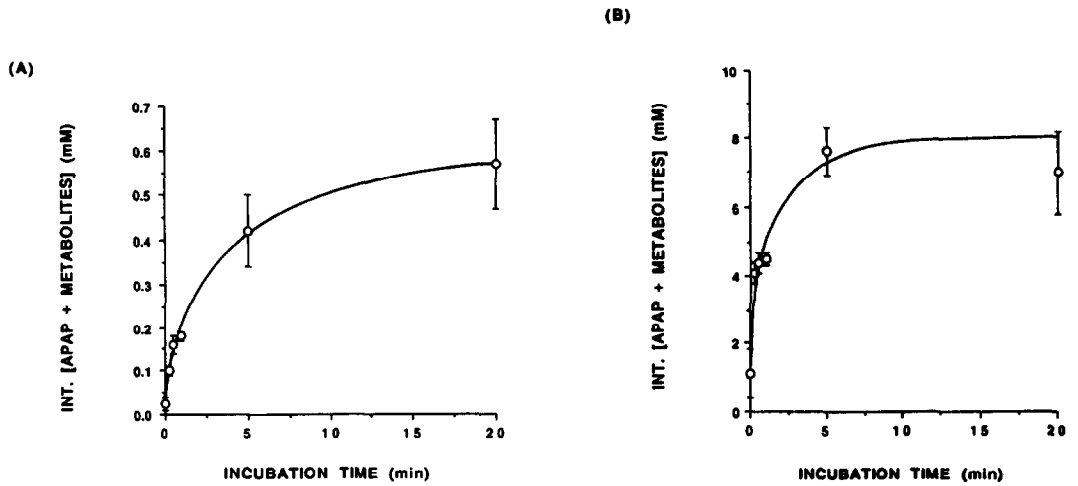

Fig. 1. Time-course of the uptake of [^{3}H]APAP by rat liver cells: (A) 0.1 mM APAP; (B) 5.0 mM APAP. Incubations (0.2 mL of approximately 15 mg dry wt cells/mL) were at 37°. Procedures were as described in Materials and Methods. Results are means ± SEM for three independent observations.

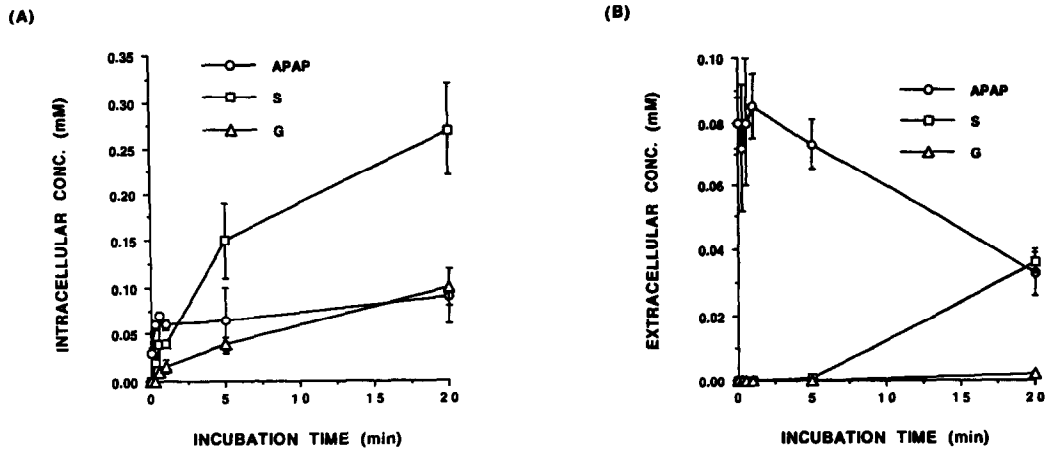

Fig. 2. Time-courses of the concentrations of APAP and its conjugates in incubations of 0.1 mM [^{3}H]-APAP with rat liver cells: (A) intracellular; (B) extracellular. Incubations (0.2 mL of approximately 15 mg dry wt cells/mL) were at 37°. Procedures were as described in Materials and Methods. Results are means ± SD for three determinations.

predominant intracellular conjugate of [^{3}H]APAP was the sulphate, the glucuronide being present at approximately 30% of this concentration. The concentration of radiolabelled APAP outside the cells steadily decreased over the 20 min period, reaching approximately 0.03 mM APAP at 20 min (Fig. 2B); at this time the intracellular concentration of the drug was approximately three times that of the incubation medium. This could be due to a mechanism for concentration in the cell, but is more likely attributable to binding to a specific protein or proteins [12].

There was a lag phase before the appearance of both conjugates in the medium of approximately 5 min; other workers have found a similar lag phase [3]. As uptake of APAP is relatively rapid, substrate limitation cannot be an explanation for this. It is of interest that the lag applies to both conjugates even though glucuronidation occurs in the endoplasmic reticulum whereas sulphation is a cytosolic process. The lag may therefore be associated with a post-conjugation event, such as release from the cell.

After the first 5 min, both conjugates were released linearly with time (results not shown). It was also observed that, at steady-state, there was a higher rate of export of APAP–sulphate than glucuronide, even when the relative intracellular concentrations were taken into account (resulting in a 20-fold ratio in the medium as opposed to 3-fold in the cells). This could be due to faster transport of the sulphate or the presence of more sulphate than glucuronide transporters. Sundheimer and Brendel [13] found only a single carrier process for harmol glucuronide, but both a high and low affinity carrier for harmol sulphate. The presence of different transporters for each conjugate is also supported by experiments with 20 μM verapamil (a calcium channel blocker which interacts with the P-glycoprotein multidrug transporter [14]) that had the effect of decreasing the rate of appearance in the medium of APAP further possibility is that export of the glucuronide is limited by the need for such conjugates to escape from the endoplasmic reticulum before crossing the plasma membrane, a step not affecting sulphate release because sulphotransferases are cytosolic.

Initial rate of APAP uptake

Preliminary experiments indicated that initial rates of transport could be measured over the first 30 sec of incubation. The near linearity of APAP transport with concentration up to 10 mM (Fig. 3A) indicated that APAP transport into the cell either required a transporter with a very high K_m or was diffusion dependent. Close examination of rates at the lower APAP concentrations showed, however, that behaviour departed from linearity below 0.5 mM (Fig. 3B). This is consistent with the presence of a high affinity saturable process ($K_m = 0.066$ mM, $V_{max} = 0.33$ nmol/mg per min) together with a second component ($K_d = 0.71$ nmol mg^{-1}min^{-1}mM^{-1}) which may be diffusion or involve a transporter with a K_m well above 10 mM. The saturable process, although playing only a small role at 10 mM APAP, may be important at concentrations encountered *in vivo*.

Effects of temperature on APAP uptake

Initial rates of uptake of 1 mM APAP were measured at 37°, 30° and 0°. Changes of temperature from 37° to 30° or to 0° decreased uptake by 28% and 50%, respectively (Table 1). Exclusively carrier-mediated transport would have been expected to fall by ≥38% and ≥92%, respectively (assuming a Q_{10} of ≥2). From the kinetic constants above, one would predict that approximately 70% of uptake from 1 mM APAP is attributable to the unsaturated process and approximately 30% to the high affinity transporter. On the assumption that the latter has a Q_{10} of ≥2, it can be calculated that the rate of the unsaturated process would have decreased by ≥31%

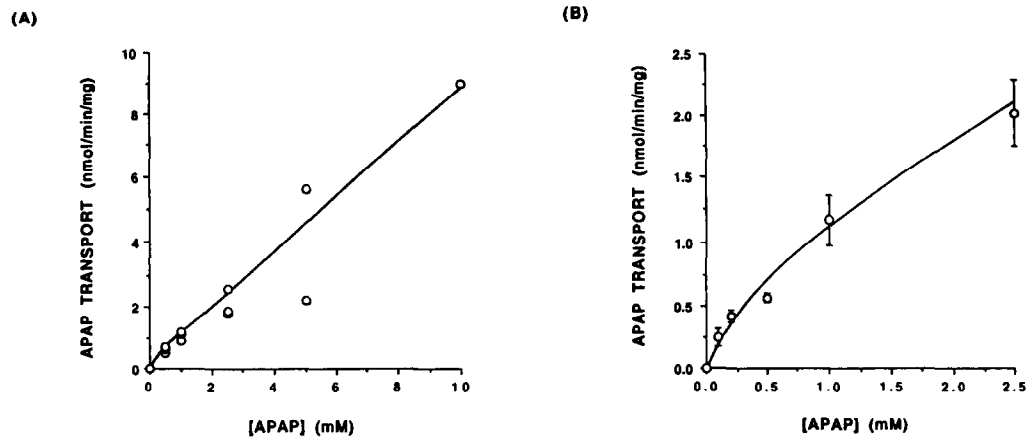

Fig. 3. Concentration dependence of the uptake of [^{3}H]APAP by rat liver cells. Incubations (0.2 mL of approximately 15 mg dry wt cells/mL) were for 30 sec at 37°. Procedures were as described in Materials and Methods. (A) Initial rates between 0 and 10 mM; (B) expanded graph to show rates for the lower concentrations. The equation of the curve is:

$$v = (0.328 \times x)/(0.0657 + x) + (0.709 \times x)$$

where $v = (V_{max} \times x)/(K_m + x) + (K_{diffusion} \times x)$. Points represent single determinations (A) or means ± SEM for three independent observations (B).

Table 1. Effect of temperature on the initial rate of uptake of 1 mM [^{3}H]APAP by rat liver cells

Temperature (°C)	Measured total rate of uptake	Calculated rate of saturable process (pmol/sec)	Calculated rate of non-saturable process
37	60 ± 2.5	18	42
30	43 ± 1.0 (72 ± 5)	11 (62)	32 (76)
0	30 ± 2.0 (50 ± 5)	1 (8)	29 (69)

Incubations (0.2 mL of approximately 15 mg dry wt cells/mL) were for 30 sec at 0°, 30° and 37°. Other procedures were as described in Materials and Methods. The proportions of the saturable and non-saturable components at 37° were calculated from the equation given in the legend to Fig. 3. The rates of the saturable process at 30° and 0° were calculated from the assumption that $Q_{10} = 2$; rates of the non-saturable process were then obtained by subtraction.

Rates are means ± range from two individual experiments; values in parentheses are percentages of the corresponding rates at 37°.

(Table 1) as the temperature fell from 37° to 0°. This implies a Q_{10} of <0.1, incompatible with carrier-mediated transport but consistent with simple diffusion across the plasma membrane.

Relationship between APAP transport and metabolism

The rates of synthesis of glucuronide and sulphate from APAP are shown in Table 2. At the lowest concentrations the main conjugate is the sulphate. As the substrate concentration increases, however, the glucuronide increases as a proportion of the total conjugates, as shown previously elsewhere [15]. When these rates are compared with those for APAP uptake in Fig. 3 (as shown in Fig. 4), it can be seen that, at the lowest substrate concentrations, all the APAP taken up was metabolized, predominantly to the sulphate. This suggests that, under these conditions, membrane transport could exert significant control over the whole pathway of APAP metabolism; at higher concentrations, control shifts increasingly to the conjugation reactions themselves.

General discussion

The full pathway for drug metabolism in the liver involves several transport steps. Thus, drugs must first cross the plasma membrane before metabolism that may include both the cytosolic and endoplasmic reticular compartments. Although conjugation with sulphate is cytosolic, conjugation with glucuronic acid requires the transport of both drug (or its metabolite) and UDP-glucuronic acid [16] into the endoplasmic reticulum. The glucuronide so formed then must recross the intracellular membrane to return to the cytosol (or, conceivably, pass directly to the extracellular space). Both conjugates are

Table 2. Effect of concentration on rates of conjugation of APAP in rat liver cells

[APAP] (mM)	Rate of synthesis (nmol/mg dry wt cells per min)	
	Glucuronide	Sulphate
0.025	—	0.14 ± 0.02
0.05	0.01 ± 0.01	0.23 ± 0.02
0.10	0.02 ± 0.01	0.31 ± 0.02
0.20	0.06 ± 0.01	0.44 ± 0.03
0.50	0.12 ± 0.01	0.44 ± 0.03
1.00	0.18 ± 0.02	0.45 ± 0.05
2.50	0.22 ± 0.03	0.39 ± 0.02
5.00	0.31 ± 0.02	0.37 ± 0.00

Incubations (2 mL of approximately 10 mg dry wt cells/mL) were for 40 or 60 min at 37°. Procedures were as described in Materials and Methods.
Results are means ± SEM for three independent observations.

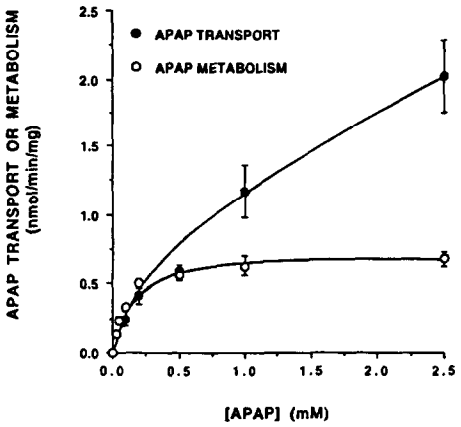

Fig. 4. Effect of concentration on the uptake and conjugation of APAP by rat liver cells. Incubations (2 mL of approximately 10 mg dry wt cells/mL) were for 40 or 60 min at 37°. Procedures were as described in Materials and Methods. Metabolism represents the sum of the accumulation of glucuronide and sulphate conjugates. Results are means ± SEM for three independent observations.

charged and water-soluble, again suggesting a role for a carrier-mediated process for export from the cell. Such a system has been shown for harmol conjugates [21].

APAP is a moderately water- and lipid-soluble weak organic acid with a pK_a of 9.5, and is as a result largely uncharged at physiological pH values. It is reasonable to expect that APAP should be able to cross the cell membrane by simple diffusion alone; the presence of a carrier-mediated system with possible regulatory significance is, therefore, of particular interest. Although we have no evidence regarding the substrate specificity of this transport system, it is likely that it could be important at concentrations of xenobiotics encountered *in vivo*.

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