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RENAL TUBULAR TRANSPORT OF PARACETAMOL AND ITS CONJUGATES IN THE DOG

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1 The renal tubular transport of paracetamol and its conjugates was investigated with renal clearance and stop flow studies in the dog. Paracetamol is sparingly bound to plasma proteins and therefore undergoes glomerular filtration. It is reabsorbed in the renal tubules by simple diffusion.

2 The conjugates of paracetamol, the sulphate and the glucuronide, both undergo glomerular filtration being weakly protein bound. At low concentrations in plasma both compounds are secreted by an active transport process. At higher concentrations both compounds are reabsorbed. Clearances are not dependent on urinary pH or flow rate. It is concluded that reabsorption is not a passive process but that there is an active bidirectional transport of the conjugates.

3 Net.tubular secretion of the sulphate, but not the glucuronide, conjugate was inhibited by the administration of probenecid.

Introduction

The metabolism and toxicity of paracetamol have recently undergone intensive study for two reasons. First, the drug and its metabolites form approximately 80% of the metabolites of phenacetin (Brodie & Axelrod, 1949), a drug which has been strongly implicated in the pathogenesis of analgesic nephropathy (Dubach, Levy, Ehrensperger, Baumeler, Muller, Peier & Rosner, 1974). Secondly, paracetamol itself has gained considerable popularity within recent years as an antipyretic and analgesic and its wider availability has led to its use in attempted suicides with resultant hepatic necrosis (Clark, Thompson, Borirakchanyavat, Widdop, Davidson, Goulding & Williams, 1973).

Analgesic nephropathy is a unique type of renal injury in that the medulla is primarily affected and the cortical lesions appear secondary to medullary damage (Lindeneg, Fisher, Pedersen & Nissen, 1959). This implies that in order to produce damage either a drug or its metabolites must be selectively concentrated within the medulla, or that this region of the kidney has unique metabolic features which are affected by the drug, or that both factors are operative.

There have been a number of studies on the renal excretion of paracetamol (Barraclough, 1972; Prescott & Wright, 1973; Silberbusch, Lenstra, Leynse & Gerbrandy, 1974), but there have been no comprehensive studies on the mechanisms underlying the tubular transport of either the parent compound or its metabolites. In the present study we have investigated these problems with clearance and stop flow techniques.

Methods

Animal experiments

Sixteen female mongrel dogs were used, each weighing between 10 and 15 kg. Anaesthesia was induced with intravenous pentobarbitone 30 mg/kg and maintained with small intravenous doses as necessary. A midline lower abdominal incision was made and the ureters were cannulated at the level of the pelvic brim. Blood samples were taken from a femoral artery and blood pressure was monitored with an intra-arterial cannula in either a femoral or a carotid artery using 16 gauge polyethylene cannulae and a Sanborn 964 polygraph and pressure transducer. Intravenous infusions were administered with a Harvard peristaltic pump for larger volumes and a Braun syringe for smaller ones. In 10 dogs, paracetamol was given intravenously in an initial dose of 40 mg/kg followed by a 1% solution in isotonic saline at 4 mg/min; in 6 dogs the initial dose was 100 mg/kg followed by 10 mg/minute. Inulin was prepared as a 1.5% solution in isotonic saline to

which 20 μCi of [^{14}C]-inulin (New England Nuclear) was added per 100 ml. After an initial injection of 20 ml, this was infused at 0.9 ml/minute. Urine for all clearance periods was collected for 15 min with a mid-point blood sample. Urine pH was recorded immediately with glass electrode and Leeds Northrup pH meter. A single experiment was done with the stop-flow technique according to the procedure previously presented in detail (Mudge, Cucchi, Platts, O'Connell & Berndt, 1968).

Due to the restriction of water intake for the preceding 18 h, most animals were hydropenic and excreted urine of high osmolality. To vary the rate of urine flow, mannitol was infused in a 5% solution. Urine pH was varied by administering 0.2 g/kg body weight of ammonium chloride in the diet for 1-2 days before the experiment and an additional 0.1 g/kg intravenously at the start of the experiment, subsequently followed by an infusion of 1% sodium bicarbonate (2 experiments) or the more rapid injection of a 10% solution (2 experiments). In those experiments in which the plasma concentration of paracetamol was varied, this was accomplished by increasing the infusion rate of the drug. Probenecid was administered intravenously in a dose of 40 mg/kg. All results are summarized as mean ± standard error of the mean. Statistical evaluations were made with Student's *t* test for unpaired data, regression analysis by the method of least squares and analysis of covariance (Snedecor, 1956). *P* values of less than 0.05 are regarded as significant. Clearance ratios refer to the clearance of a compound divided by the clearance of inulin.

Chemical methods

Paracetamol was estimated in duplicate by a modification of the method of Brodie & Axelrod (1949). A volume of plasma or urine containing 5-50 μg of paracetamol was diluted with distilled water to 2 ml. Sodium chloride (1 g) was added and the paracetamol was extracted into 20 ml diethyl ether U.S.P. grade, containing 1.5% iso-amyl alcohol. Of the ether phase 15 ml was transferred and the compound re-extracted into 3.2 ml of 0.1 N NaOH. Of the NaOH solution, 3 ml was then boiled for 30 min after the addition of 0.8 ml 12 N HCl in a 16 x 150 mm test-tube covered with a glass marble. The solution was then cooled, 0.1 ml 1% sodium nitrite was added, mixed, allowed to stand at room temperature for 20 min, following which 0.1 ml of 5% ammonium sulphamate was added, mixed, and allowed to stand for 3 minutes. Of a freshly prepared solution of 12% α-naphthol (Eastman Kodak) 0.1 ml was then added, shaken briefly, and then 2 ml of 6 N NaOH was immediately added, mixed on a Vortex Mixer for 3 s and then the tube was placed in crushed ice. Optical density was read on a Gilford spectrophotometer at 510 mu. Internal standards, reagent blank and biological blank were run simultaneously. Results with this procedure were similar to those of Brodie & Axelrod (1949) and Routh, Shane, Arredondo & Paul (1968), with the advantage that a larger number of samples could be processed at one time. The timing after the addition of the α-naphthol is critical as the dye formed is sensitive to temperature and time with deterioration after the times specified. Recoveries of paracetamol added to plasma and urine were 100 ± 1.58%. The maximum biological blank value was 1.2 μg/ml in urine and was always negligible in plasma. Eastman Kodak α-naphthol does not require sublimation but other suppliers' samples may be oxidized and hence inactive. Samples of diethyl ether need to be water saturated prior to use. If there is excess ethyl alcohol present in the particular batch then conjugates can be extracted and interfere with the estimation.

The conjugates of paracetamol (paracetamol-conj) were estimated in duplicate by incubating 0.01-1 ml of plasma or urine, with 1 ml of acetate buffer at pH 5 containing 5 mg/ml of β-glucuronidase and aryl sulphatase from *Helix pomatia* (Sigma type H 1) for 24-48 h at 37°C. The solution was then assayed for paracetamol as above, and paracetamol-conj calculated as the difference before and after enzymatic hydrolysis. The results were expressed as μg of paracetamol. Sample hydrolysis is complete in 16-24 h as determined by thin layer chromatography using the system outlined below.

Paracetamol sulphate (paracetamol-SO_4) and paracetamol glucuronide (paracetamol-gluc) were determined in duplicate on ultrafiltrates of 2-3 ml of plasma prepared by the method of Toribara, Terepka & Dewey (1957). Of the ultrafiltrate 200 μl was streaked on a silica gel (100 μm) thin layer chromatogram containing an ultraviolet phosphor (Eastman Kodak). Urine containing 50-100 μg of total paracetamol was chromatographed without pre-treatment. The chromatographs were developed using System III of Buch, Pfleger, Rummel, Ullrich, Hey & Staudinger (1967) consisting of acetone, butanol, water, 50 : 40 : 10. The sites of the conjugates were identified with ultraviolet light. In this system the R_F values were: paracetamol, 0.91; paracetamol-SO_4, 0.68; and paracetamol-gluc, 0.20. The area of silica gel containing the conjugate was then removed and added to 2 ml of β-glucuronidase and aryl sulphatase (as above), and the procedure completed as previously described. Internal standards and reagent blanks were carried through the

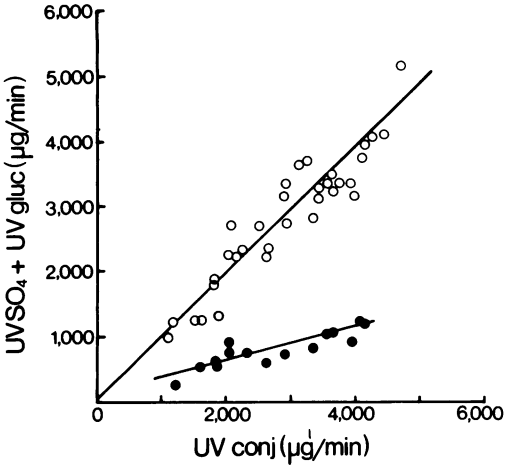

Figure 1 A comparison of two methods of determining paracetamol conjugates in urine. A regression analysis by the method of least squares (Snedecor, 1956) of the excretion rate of the total conjugates (○) vs the sum of the individual conjugates gave $y = 0.99x + 29.4$; $r = 0.926$ and the slope is significantly different from 0 at $P < 0.001$. Paracetamol-SO_4 (●) excretion rate alone (excluding studies with probenecid) showing the increase relative to the total conjugate.

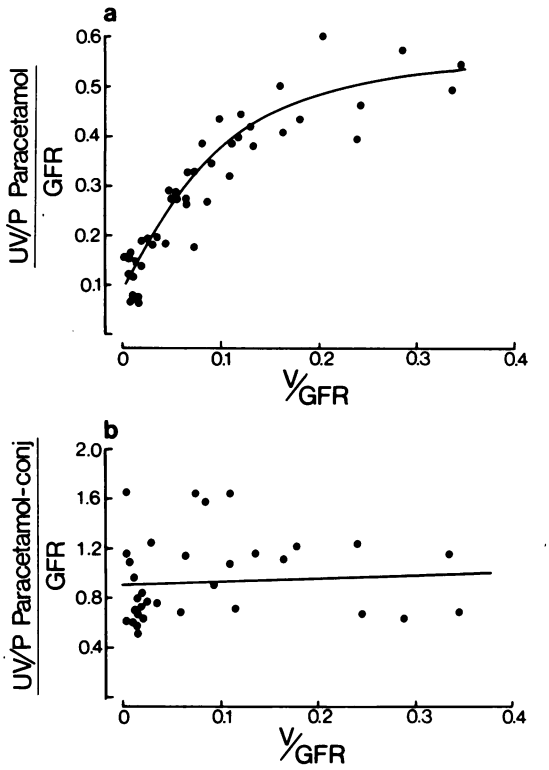

Figure 2 (a) Effect of fractional excretion of water on paracetamol clearance ratio. Summary of six experiments. Plasma paracetamol level was kept constant for each experiment with a range from 20 to 200 μg/ml for all animals. Line drawn by inspection. (b) Effect of fractional excretion of water on paracetamol-conj clearance ratio. Data from same experiments as Figure 2a. Plasma paracetamol-conj ranged from 12-70 μg/ml but was essentially constant for each experiment. $Y = 0.35x + 0.91$; $r = 0.2$. Slope is not significantly different from zero, $P = 0.50$.

entire procedure. Recovery of individual conjugates is complete when compared to the method for total conjugates (Figure 1). The plasma assay estimates the non-protein bound conjugate.

Inulin-[^{14}C] (New England Nuclear) was determined using 0.1-0.2 ml of plasma or urine with P.C.S. Solubilizer (Amersham-Searle) added to a final volume of 10 ml. This was counted in a Packard Tricarb 3310 Liquid Scintillation counter using external automatic standard for quench correction. Concentrated urine samples were diluted and alkaline urine samples were acidified to maintain stable counts. Counts remained stable over a 48 h period. In some experiments, inulin was determined by the anthrone method (Fuhr, Kaczmarczyk & Kruttgen, 1955).

Results

Protein binding

The amount of paracetamol bound to plasma protein was estimated by the method of Toribara *et al.* (1956), in ten samples from three dogs. The plasma concentration ranged from 24 μg/ml to 94 μg/ml. Binding was estimated to be 11.0% ± 1.2% and was independent of plasma concentration over the range studied. Paracetamol clearances have been calculated without correction for plasma binding.

From the total paracetamol-conj estimated in plasma and the sum of the paracetamol-SO_4 and paracetamol-gluc estimated in plasma ultrafiltrate, the degree of binding is 13%. Clearances from the individual conjugates have been estimated on the basis of the observed concentrations in ultrafiltrates.

Effect of rate of urine flow

The clearance ratio of paracetamol increased with the fractional excretion of water (Figure 2a) but the paracetamol-conj ratio remained unchanged (Figure 2b). The individual conjugates (paracetamol-SO_4 and paracetamol-gluc) showed no

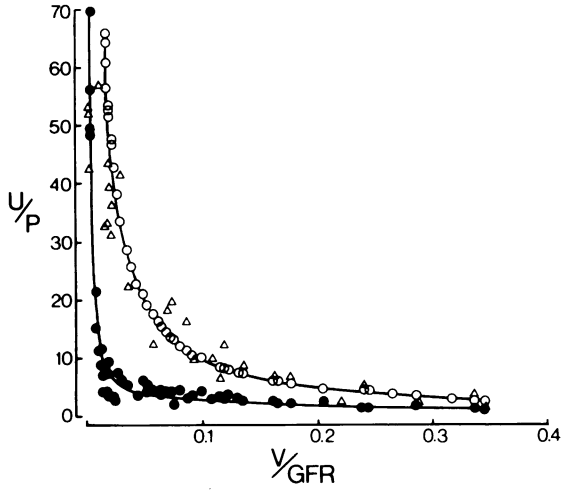

Figure 3 Urine/plasma concentration ratio for paracetamol, paracetamol-conj and inulin plotted against the fractional excretion of water. Lines are drawn by inspection; (•) paracetamol; (△) paracetamol-conj; (○) inulin (lines for inulin and paracetamol-conj are co-incidental).

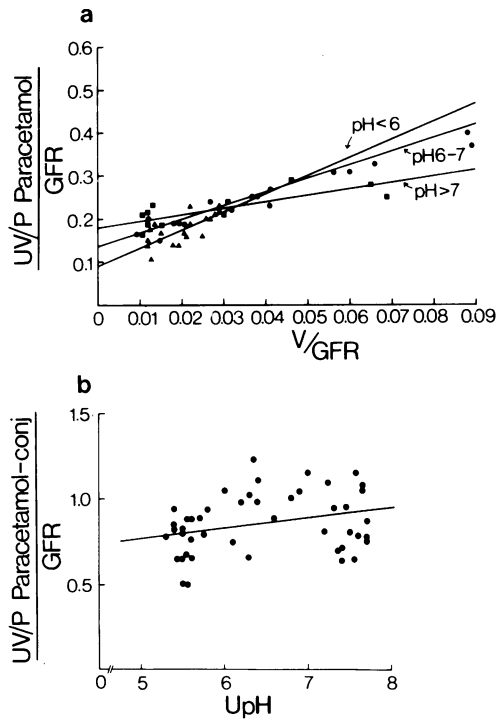

Figure 4 (a) Effect of urine pH on clearance ratio of paracetamol. Results of four experiments in which a small increase in urine flow rate occurred as a result of bicarbonate administration. At pH > 7 (○) $y = 3.853x + 0.099$, $r = 0.68$; for pH < 6 (△) $y = 2.83x + 0.139$, $r = 0.97$. By analysis of covariance these slopes are not significantly different ($P = 0.56$) from each other, but are significantly different from zero $P = 0.002$ and $P < 0.001$, respectively. At pH 6-7, slope is not different from pH < 6 or pH > 7. (b) Effect of urine pH on the clearance ratio of paracetamol-conj. Same experiments as above. $y = 0.57x + 0.49$, $r = 0.28$. The slope is not significantly different from zero ($P = 0.06$).

significant change in their clearances as a result of the rate of urine flow. From Figure 3, the urine/plasma (U/P) concentration ratio of paracetamol is highest at the lowest rates of urine flow. The U/P ratio falls dramatically with a slight increase in the fractional excretion of water. The U/P ratios for inulin and paracetamol-conj fall less steeply. For paracetamol, the highest U/P ratio occurs at the lowest clearance.

Effect of urine pH

The clearance ratio of paracetamol remained unchanged over the entire range of urine pH values. In Figure 4a this ratio is shown as a function of the fractional excretion of water with regression estimates for three groups of pH values. The slight slope of the line is a result of a small diuresis from sodium bicarbonate infusion. The increase in clearance is as would be predicted from a change in urine flow rate alone. Clearance ratios of paracetamol conjugates (Figure 4b) did not vary with urine pH. Individual conjugates were estimated in two dogs over ten clearance periods. Paracetamol-SO_4 between pH 5 and 6 had a mean clearance ratio of 1.03 ± 0.14 and between 7 and 8 of 1.37 ± 0.10 ($P = 0.21$). For the same pH ranges paracetamol-gluc clearance ratios were 0.77 ± 0.04 and 0.75 ± 0.04 respectively ($P = 0.89$).

Effect of plasma level on drug

The clearance ratio of paracetamol remained unchanged over a wide range of plasma drug concentration in two dogs, and in a third there was a minimal increase in clearance at the higher drug concentrations (Figure 5a). The difference in the clearance ratios in these three dogs is due to the difference in the spontaneous rates of urine flow. Taking the results from all three animals together, the plasma concentration of paracetamol had no significant effect on its clearance. However, the clearance ratio of paracetamol-conj showed a significant decrease with increasing plasma con-

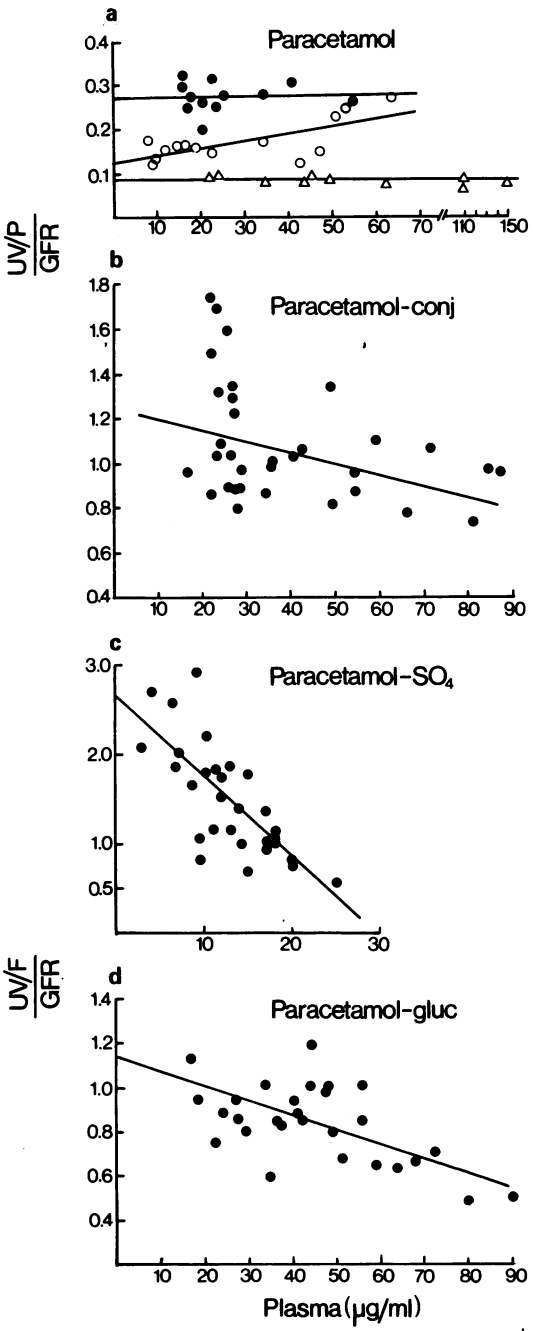

Figure 5 (a) Effect of plasma drug concentration on clearance ratio of paracetamol. Results from three experiments in which rate of urine flow was constant for each but differed from one to another, (•) > (○) > (△). Urine pH ranged from 6 to 7. Slope is not significantly different from zero for (•) and (△), but is different for (○) ($r = 0.694$, $P = 0.006$). For all three experiments together, over the comparable plasma range from 10-70 μg/ml, the slope is not significantly different from zero ($P = 0.68$). (b) Effect of drug plasma level on clearance ratio of paracetamol-conj. Same animals as Figure 4a. By regression analysis, $y = 1.259 - 0.004x$, $r = -0.45$, $P = 0.006$. (c) Effect of drug concentration of plasma ultrafiltrate on clearance ratio of paracetamol-SO_4. Summary of all data from seven experiments in each of which the rate of urine flow and urine pH were essentially constant. By regression analysis, $y = 2.66 - 0.09x$, $r = -0.71$, $P < 0.001$. (d),Effect of drug concentration of plasma ultrafiltrate on clearance ratio of paracetamol-gluc. Same experiments as Figure 4c. By regression analysis, $y = 1.158 - 0.007x$, $r = -0.62$, $P < 0.001$.

centrations (Figure 5b). The same phenomenon is seen when the individual conjugates are measured separately (Figures 5c and 5d).

Effect of probenecid

This agent was given in a dose known to inhibit the renal tubular transport of many organic acids (Weiner, 1973). It had no effect on the clearance ratio of paracetamol, paracetamol-conj, or paracetamol-gluc (Table 1). However, probenecid significantly decreased the paracetamol-SO_4 clearance ratio, the mean fall being 44% of the control.

Stop-flow study

A single stop-flow experiment was undertaken to define the tubular localization of the transport of paracetamol and its conjugates and to examine certain mechanisms of transport (see discussion section) suggested by the results from conventional clearances. As previously reported with the identical stop-flow technique (Mudge *et al.*, 1968) the static column of fluid which had remained in contact with the distal nephron is represented by the nadir of urinary chloride concentration (samples 4-8, Figure 6), while urine previously in contact with the proximal tubule is represented by sample numbers about twice that great. The results shown in Figure 6 indicate that the reabsorption of paracetamol is accentuated in the distal area and that this is unaffected by probenecid. Paracetamol-SO_4 undergoes net proximal secretion which is probenecid-sensitive. With paracetamol-gluc there is net reabsorption without direct evidence of its tubular localization and with no apparent effect of probenecid.

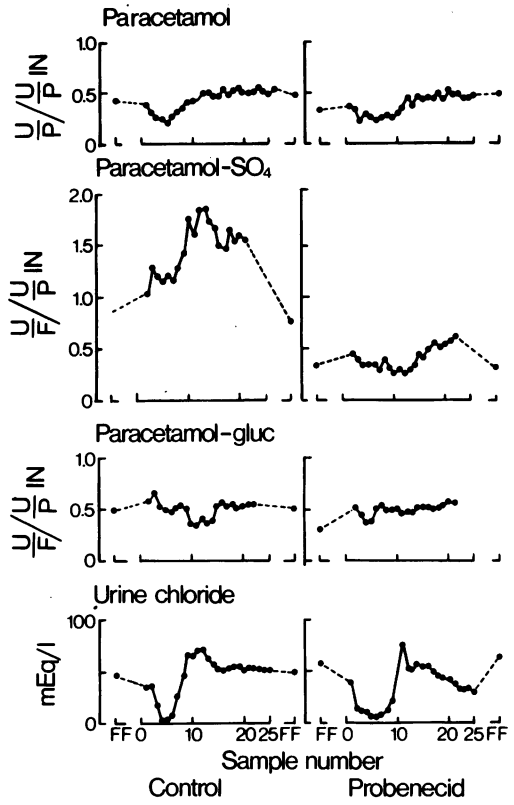

Figure 6 Stop-flow analysis of tubular transport of paracetamol and its conjugates, before and after probenecid. Flow was stopped for 6 min following which urine collections were 0.6 ml each. FF = free-flow. Probenecid given intravenously in dose of 40 mg/kg. Plasma drug concentrations rose between first and second stop-flow procedures; the range for the entire experiment was paracetamol 88-142, paracetamol-SO_4 20-39, and paracetamol-gluc 80-145 μg/ml. Control U/P inulin ratios were 5 and 8.

Discussion

The renal excretory mechanisms for paracetamol appear to involve filtration and reabsorption by passive diffusion of the non-ionic form. The relevant evidence includes the following: 1. paracetamol/inulin clearance ratios far less than unity; 2. the lipid solubility of paracetamol; 3. the curvi-linear positive correlation between clearance and the rate of urine flow; 4. the fact that clearance is independent of the plasma concentration and hence also of the amount reabsorbed; and 5. the absence of any effect of probenecid. Since paracetamol is a weak acid with a pKa of 9.5 (Prescott & Wright, 1973) one would not anticipate that its clearance would be influenced by changes in urinary pH within the achievable physiological range (Weiner, 1973). Stop-flow analysis failed to localize a unique reabsorptive site and reabsorption throughout the entire nephron may be inferred. The above interpretation would not be changed if the data were corrected for the slight degree of binding to plasma proteins. Thus the excretory pattern of paracetamol is somewhat similar to that of urea and its analogues.

The excretory mechanisms for the conjugates are different from those of the parent compound, and the excretory pattern of sulphate and glucuronide conjugates are somewhat different from each other. For both, clearance is not affected by urine pH or the rate of urine flow, but is strongly influenced by the concentration of the conjugate in the plasma (or ultrafiltrate). Clearance, corrected for plasma binding, shows net tubular secretion at low plasma levels and net reabsorption at high. Thus, each conjugate undergoes bidirectional tubular transport. The secretory and reabsorptive mechanisms may be considered separately. For paracetamol-SO_4 the relationship between net secretion and the plasma levels suggests that secretion is a rate limited

Table 1 Effect of probenecid on the clearance ratio (clearance/G.F.R.) of paracetamol and its conjugates

Compound	*Plasma conc. range (μg/ml)*	*Mean clearance ratio Control*	*Mean clearance ratio Probenecid*	P*
Paracetamol	74-142	0.234 ± 0.05	0.227 ± 0.04	0.92 NS
Paracetamol-conj	54-121	0.79 ± 0.05	0.725 ± 0.03	0.65 NS
Paracetamol-SO_4	9-39	1.18 ± 0.15	0.57 ± 0.016	0.005
Paracetamol-gluc	18-145	0.78 ± 0.08	0.81 ± 0.13	0.84 NS

* *P* = significance probability from Student's *t* test on unpaired data.
NS = not significant.
Values are based on a total of 18 clearance periods from three dog experiments.

process. By stop-flow analysis this transport system is in the proximal nephron. It is also probenecid-sensitive. Paracetamol-SO_4 secretion thus shares many features in common with other organic acids (Weiner, 1973). Paracetamol-gluc behaves in a similar manner. However, its secretion appears not to be inhibited by probenecid, nor has the tubular site of secretion been positively identified. In the presence of bidirectional transport, the lack of an effect of probenecid could be due to an insensitivity of the secretory mechanism to this agent or to approximately equal sensitivities of both the secretory and reabsorptive mechanisms. Precedent for drug inhibition of transport in both directions has been provided in the case of uric acid transport (Weiner & Tinker, 1972). The present data do not permit one to distinguish between the above possibilities. It should be emphasized that at all doses of paracetamol the concentration of paracetamol-gluc is approximately three times greater than that of paracetamol-SO_4 in both plasma and urine. Therefore, under all conditions each conjugate is undergoing active secretion in the presence of the other. The degree to which this might result in competitive inhibition of secretion can not be evaluated.

Prescott & Wright (1973) reported that the renal clearance of paracetamol in man depended on the rate of urine flow but was unrelated to urine pH, and that the clearance of the conjugates was independent of both flow rate and pH. The renal mechanisms in the dog thus appear to be similar to those in man.

Non-ionic diffusion is the most common mechanism regulating the renal tubular reabsorption of drugs and their metabolites (Weiner, 1973). The sulphate and glucuronide conjugates of paracetamol have not been characterized as to their pKa or solubility in different lipid-like solvents. For both, lipid solubility less than the parent compound is apparent from the analytical method. It may be presumed that, like other glucuronides, the pKa of paracetamol-gluc is in a range in which a change in urinary pH would effect excretion if the glucuronide were sufficiently lipid soluble (Weiner, 1973). In the case of paracetamol-SO_4, by analogy to other sulphate conjugates, the probable pKa may be so low that excretion would not be pH-dependent (Weiner, 1973). Since both conjugates appear relatively lipid insoluble and since for neither is excretion pH-dependent, it is highly unlikely that reabsorption occurs by non-ionic diffusion. Thus, both conjugates resemble *m*-hydroxybenzoate for which carrier mediated reabsorption and secretion have been demonstrated (May & Weiner, 1970).

The total conjugates are equal to the sum of the two individual conjugates. Since the glucuronide forms approximately 70% of the total, the behaviour of the total conjugates more closely resembles that of the glucuronide rather than that of the sulphate. This would explain the apparent absence of any effect of probenecid on total conjugates.

The tubular transport of these compounds has obvious implications for the pathogenesis of analgesic nephropathy. Paracetamol itself is relatively lipid soluble and undergoes reabsorption throughout the nephron. The conjugates, on the other hand, are probably transported in the proximal tubule. This suggests the possibility that paracetamol might be reabsorbed in the medulla, particularly in the collecting duct, at a high concentration. Previous work has suggested that N-hydroxy metabolites may be cytotoxic (Weisburger & Weisburger, 1973). Oxidative reactions are known to occur within the kidney in the case of phenacetin (Uehleke & Schnitger, 1969), but have not been studied for paracetamol. In the rat it has been shown that dehydration is a necessary prerequisite for the development of papillary necrosis (Saker & Kincaid-Smith, 1969).

In their entirety, the above relationships suggest that the following sequence of events may underly this type of nephropathy. Oliguria leads to maximal concentrations of paracetamol within the tubular fluid of the collecting duct. The reabsorption of paracetamol at this site might lead to a high intracellular level which, as the substrate for the oxidative reaction, would enhance the formation of the more cytotoxic metabolites. Critical to this mechanism is the concentration of paracetamol within the tissue of the medulla. Bluemle & Goldberg (1968) have previously proposed that paracetamol is concentrated within the medulla. However, their data fail to distinguish between the drug within the distal tubular fluid and that which might be present either within the cells or in the interstitial fluid. Although both paracetamol and its conjugates may give rise to high medullary concentrations as determined by analysis of whole tissue, from a consideration of the mechanisms of their transport it is unlikely that they are distributed in a similar manner between the several tissue compartments. Additional experiments are in progress in an attempt to provide a definitive answer. The mechanisms by which phenacetin is transported by the renal tubule are also under study.

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