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EFFECT OF POLY rI:rC TREATMENT UPON THE METABOLISM OF [^{14}C]-PARACETAMOL IN THE BALB/cJ MOUSE

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Abstract—The effect of the immunomodulator, poly rI:rC, upon the *in vivo* metabolism of [^{14}C]-paracetamol has been investigated in male BALB/cJ mice. In both poly rI:rC treated and control groups of mice the major part of the dose was excreted in the 0–24 hr urine and the major urinary metabolites were the glucuronic acid and sulphate conjugates. The urinary excretion of these two conjugates and of free paracetamol was not significantly altered following poly rI:rC treatment. Following enzymic hydrolysis of glucuronides and sulphates, the 3-cysteine, 3-mercapturate, 3-thiomethyl and 3-methylsulphoxide metabolites of paracetamol were all identified in the 0–24 hr urine together with very small amounts of 3-methoxy paracetamol. Although poly rI:rC treatment reduced the proportional urinary excretion of each of the thio adducts the individual differences were not significant. However, total thio adduct excretion, an indirect estimate of the metabolic activation of paracetamol, was significantly lower following poly rI:rC treatment. This depression in the urinary excretion of thio adducts following poly rI:rC treatment is discussed in relation to possible implications for paracetamol toxicity.

Immune system perturbation in experimental animals has been associated with depressed activity of the hepatic mixed-function oxidase (MFO) system that is responsible for the metabolic oxidation of drugs and many other xenobiotics. This loss in activity has been attributed to a reversible loss of cytochrome P-450 (for review see ref. 1). Mechanistically this relationship is poorly understood although the loss in cytochrome P-450 that follows the administration of a large number of immunomodulatory agents has been associated with an induction of interferon synthesis [2–4].

Compared to oxidative (Phase I) drug metabolism there has been little research into the effects of immunomodulation upon the Phase II conjugation reactions of xenobiotics. As part of an investigation into this area we have shown previously [5] that the metabolic conjugation of acetylsalicylic acid with glycine or glucuronic acid was little affected in mice by either of the two immunomodulators and interferon inducers, polyriboinosinic polyribocytidylic acid (poly rI:rC) or Newcastle Disease Virus. In contrast the oxidation of salicylate to gentisic acid was significantly depressed, but only in those strains of mice in which interferon synthesis was induced.

The use of minor analgesics in children during viral infections is presently under review due to the finding of an apparent association between aspirin therapy and the serious childhood disorder Reye's syndrome [6]. As a result paracetamol has been recommended as an effective alternative [7]. Consequently we have further studied the relationship between immunomodulation and *in vivo* drug metabolism by investigating the effect of poly rI:rC administration upon the metabolism of [*ring*-U-^{14}C]-paracetamol in the mouse.

Paracetamol is extensively excreted in the urine of man and experimental animals principally in the form of conjugates with either glucuronic acid or sulphate. While paracetamol is remarkably non-toxic at normal therapeutic doses, hepatotoxicity is seen following higher doses in both man and experimental animals [8, 9]. An electrophilic intermediate, which is known to bind to tissue macromolecules and to deplete cellular glutathione, has been implicated in this toxicity [10–13]. This reactive intermediate is considered to be the two electron oxidation product of paracetamol, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [14], and much indirect evidence implicates the cytochrome P-450 system as being responsible for its formation *in vivo* [15–17].

This reactive intermediate is inactivated under normal circumstances by conjugation with glutathione, the product of which is further transformed and excreted in the urine as the cysteine and/or mercapturic acid conjugates. The minor sulphur-containing metabolites, 3-thiomethylparacetamol and its sulphoxide, have also been identified [18–20]. Their formation is believed to involve C–S cleavage of the cysteine conjugate by the enzyme cysteine conjugate β-lyase to release the free thiol which is then methylated by *S*-methyl transferase in a reaction sequence known as the thiomethyl shunt [21, 22]. Warrender *et al.* [22] have further shown that in the hamster these minor thio containing metabolites do indeed derive from a common glutathione conjugate precursor. Several workers [19, 23] have proposed that the total 24 hr urinary excretion of these various thio adducts of paracetamol may be used as an index of NAPQI formation and thus indicate the susceptibility of an animal species, strain or individual to paracetamol toxicity. In addition to these thio

adducts, the catechol, 3-hydroxyparacetamol, and its 3-methoxy derivative have also been identified as minor metabolites in man [18] and the hamster [19].

Renton and Dickson [24] have studied the effect of poly rI:rC pretreatment upon paracetamol-induced hepatotoxicity in the mouse. These workers demonstrated that the pretreatment of Swiss mice with poly rI:rC depressed hepatic levels of both cytochrome P-450 and glutathione, protected mice against high dose paracetamol-induced hepatic necrosis and also reduced the covalent binding of ^{3}H-paracetamol to liver protein. We report here the effect of poly rI:rC treatment upon the *in vivo* conjugation of paracetamol with glucuronic acid and sulphate and also upon the 24 hr urinary excretion of thio adducts in the mouse. These findings are discussed in relation to the toxicity of paracetamol.

MATERIALS AND METHODS

[*ring*-U-^{14}C]-Paracetamol, specific activity was 19.5 mCi/mmol was obtained from Amersham International (Amersham, Bucks, U.K.). HPLC analysis revealed the radiochemical purity to be >99.8%. Polyriboinosinic polyribocytidylic acid (poly rI:rC, Na salt), paracetamol (4-hydroxyacetanilide), β-glucuronidase ("Glucurase", ex bovine liver, 5000 units/ml) and sulfatase (type H1 from *Helix pomantia*) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Ammonium acetate and orthophosphoric acid (both A.R. grade) and all other reagent grade chemicals were from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Methanol and tetrahydrofuran, both HPLC grade, were from Fisons (Loughborough, U.K.). Paracetamol sulphate, paracetamol glucuronide, paracetamol-3-cysteine, paracetamol-3-mercapturate, 3-thiomethylparacetamol and paracetamol-3-methylsulphoxide were kindly supplied by Dr D. I. Smith, Sterling Winthrop Research, Alnwick, Northumberland, U.K., and 3-methoxyparacetamol was the kind gift of Dr L. Notorianni, University of Bath, U.K.

Animals

Male BALB/cJ mice, 25–30 g b.w. and 8–12 weeks old, were from a colony maintained by the Animal Department of St. Mary's Hospital Medical School.

Dosing procedure

Poly rI:rC, dissolved at 1 mg/ml in sterile saline, was administered to each of five mice, i.p., at a dose of 10 mg/kg. Five control animals received saline vehicle only. All mice received [*ring*-U-^{14}C]-paracetamol, 100 mg/kg and 3 μCi/animal p.o. dissolved in 75% polyethylene glycol (PEG 400) 24 hr later. Mice were housed individually in glass metabolism cages ("Mini-Metabowls", Jencons Scientific Ltd, Leighton Buzzard, U.K.), food (Labsure CRM pellets, K. & K. Greef Ltd, Croydon, U.K.) and water were freely provided, and urine and faeces were collected daily for three days. Urine collection involved rinsing each metabowl with distilled water which was pooled with the urine to give a final volume of 10 ml. Following centrifugation to remove any solid matter aliquots of each diluted urine were stored at −20° until analysis.

Hydrolysis of conjugated metabolites

For selective hydrolysis 100 μl aliquots of 0–24 hr urine samples were treated with either 500 units of β-glucuronidase, or with 50 units of sulphatase in an acetate buffer, pH 5.0, containing glucurolactone (15 mM) to inhibit β-glucuronidase activity. Hydrolysis of both glucuronic acid and sulphate conjugates used a mixture of both enzymes in an acetate buffer, pH 5.0. All incubations were carried out at 37° for 24 hr.

High pressure liquid chromatography

HPLC was carried out using a Shimadzu LC4A liquid chromatograph with an integral u.v. detector, model SPD-2AS, monitoring the eluent at a wavelength of 254 nm, and a Shimadzu integrator, model CR3A (all supplied by Dyson Instruments, Houghton-le-Spring, Tyne and Wear, U.K.). The mobile phase was pumped at 1 ml/min through a 25 cm × 4.6 mm column packed with 5 μm Hypersil ODS (Shandon Southern Products Ltd, Runcorn, U.K.).

Two systems were developed for the separation of urinary paracetamol metabolites and were as follows.

System A. Solvent A—0.05 M ammonium acetate adjusted to pH 5.5 with orthophosphoric acid; solvent B—Methanol.

Gradient Program (all steps linear): Start–2.5 min 5–10% methanol: 2.5–10 min at 10% methanol: 10–12 min 10–40% methanol: 15–17 min 40–70% methanol: 17–25 min at 70% methanol.

System B. Solvent A—0.05 M ammonium acetate adjusted to pH 5.5 with orthophosphoric acid and containing 1.5% tetrahydrofuran; solvent B—Methanol.

Gradient program (all steps linear): Start—7 min at 5% methanol: 7–8 min 5–40% methanol: 8–20 min at 40% methanol.

System A was used for quantitative analysis of 0–24 hr urine samples and also for the qualitative analysis of corresponding urine samples before and after selective enzyme hydrolysis. System B was used for the quantitative analysis of urine samples following enzyme hydrolysis of both glucuronic acid and sulphate conjugates.

Following filtration through a 0.46 μm PTFE filter (Acro LC13; Anachem, Luton, U.K.) a 20 μl aliquot of each urine sample was injected on to the HPLC column and consecutive 0.2 ml fractions of the eluent were collected (LKB Redirac 2112 Fraction Collector, LKB Instruments, South Croydon, Surrey, U.K.). Their ^{14}C content was determined and a radiochromatogram plotted. Identification of radioactive peaks was achieved by comparison of retention times with those of authentic standards in both HPLC systems and by comparison of the metabolic profiles before and after selective enzyme hydrolysis. The proportion of each metabolite present in the sample was taken to be directly represented by the proportion of radioactivity that eluted with the appropriate chromatographic peak.

Radiochemical techniques

The ^{14}C content of dose solutions, excreta and HPLC eluent fractions were determined as previously described [5] by liquid scintillation spectrometry using Cocktail T scintillation fluid (BDH Chemicals Ltd, Poole, U.K.) with a Packard TriCarb instrument, Model 4640 (Packard Instrument Co., Caversham, Reading, U.K.). Counting efficiency was assessed by reference to an external standard, using a regularly determined quench correction curve.

Statistical methods

Significance testing between control and poly rI:rC treated groups used the unpaired Student's *t*-test with a probability level of <0.05 considered to be significant.

RESULTS

Chromatography

System A resolved all the available paracetamol metabolites with the exceptions of 3-methoxyparacetamol and paracetamol-3-methylsulphoxide which coeluted. The mobile phase of system B included 1.5% tetrahydrofuran and the increased selectivity afforded by this organic modifier enabled resolution of these two metabolites. Retention times of authentic standards of paracetamol metabolites in both systems are shown in Table 1. Figures 1 and 2 show typical u.v. traces for a mixture of metabolite standards and a typical 0–24 hr urine from control mice with its associated radiochromatogram, run in system A, for untreated urine, and in system B, following hydrolysis, respectively.

Effect of poly rI:rC upon the urinary excretion of [ring-U-^{14}C]-paracetamol metabolites

Urinary recovery of ^{14}C was greater than 80% in both groups in the first 24 hr period and greater than 85% after 72 hr. There were no significant differences between these recoveries. Faecal excretion of ^{14}C was not determined.

Table 2 shows the metabolic profiles of 0–24 hr urine for control and poly rI:rC treated mice. The major urinary metabolite in both groups was the glucuronide which accounted for some 60% of the recovered dose in both groups. The sulphate conjugate and free paracetamol each accounted for about 10% of the dose. There were no significant differences between the two groups in the urinary excretion of these compounds. The glucuronic acid and sulphate conjugates were not detected in urine samples following the appropriate selective enzyme hydrolysis.

A number of minor, early running radiolabelled peaks and one larger, later running peak, Rt 20.0 min in system A, did not co-chromatograph in system A with any of the available metabolite standards. Following enzyme hydrolysis these early running radiolabelled peaks were absent from the radiochromatogram and were therefore assumed to be conjugates of other minor metabolites. The later running peak disappeared after selective sulphatase treatment, and, as an equal amount of radioactivity migrated to the peak that co-chromatographed with 3-thiomethyl paracetamol, it was presumed to be the sulphate conjugate of this thio adduct.

Following complete enzyme hydrolysis of the 0–24 hr urine samples and quantitative analysis in system B it was found that paracetamol accounted for significantly less of the recovered radioactivity in control mice (74%) than in poly rI:rC treated mice (80%, $P < 0.05$). The mean proportion of the recovered dose excreted as paracetamol-3-cysteine was slightly greater in the control group than in the poly rI:rC treated group although this difference did not reach statistical significance. 3-thiomethylparacetamol accounted for 5.7% of the recovered radioactivity in control mice and 4.2% in poly rI:rC treated mice. Similarly paracetamol-3-mercapturate and paracetamol-3-methylsulphoxide accounted for approximately 1.5% and 1.0% of the dose respectively in control mice, while these proportions were again reduced in poly rI:rC treated mice. An unidentified radiolabelled peak, retention time 5.0 min, which accounted for 1.0% of the dose in the control group was significantly reduced ($P < 0.001$) to 0.4%, following poly rI:rC treatment. A minor radioactive peak, accounting for less than 0.1% of the dose in each group, co-eluted with the authentic standard of 3-methoxyparacetamol.

The total thio adduct formation (Σ-3-SR) was determined by totalling the 0–24 hr urinary excretion

Table 1. Retention times for authentic standards of paracetamol metabolites

Metabolite	Retention time (min)	
	System A	System B
Paracetamol glucuronide	4.8	—
Paracetamol sulphate	10.5	—
Paracetamol-3-cysteine	14.0	6.5
Paracetamol	16.0	9.7
Paracetamol-3-mercapturate	17.5	7.8
Paracetamol-3-methylsulphoxide	21.2	11.0
3-Thiomethylparacetamol	22.2	15.5
3-Methoxyparacetamol	21.2	12.5

For details of mobile phase composition, of gradient programs and other chromatographic details of each system see Materials and Methods.

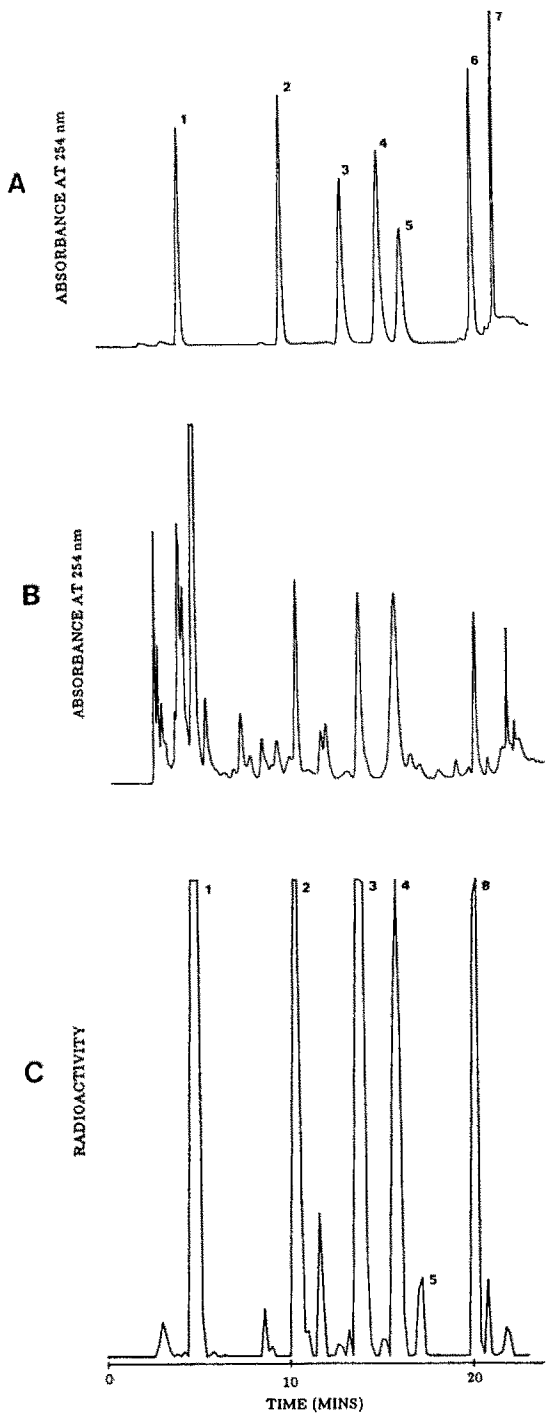

Fig. 1. Typical HPLC traces run in HPLC System A: (A) paracetamol metabolites—standard mixture; (B) a 0–24 hr untreated urine sample; (C) the associated radiochromatogram. 1, paracetamol glucuronide; 2, paracetamol sulphate; 3, paracetamol-3-cysteine; 4, paracetamol; 5, paracetamol-3-mercapturate; 6, paracetamol-3-methylsulphoxide; 7, 3-thiomethylparacetamol; 8, 3-thiomethylparacetamol sulphate. A 20 μl aliquot of each filtered 0–24 hr urine sample was injected onto the HPLC and consecutive 0.2 ml fractions of the eluent collected and counted for ^{14}C to derive the associated radiochromatogram. Details of mobile phase composition, the gradient program and other chromatographic details are in Materials and Methods.

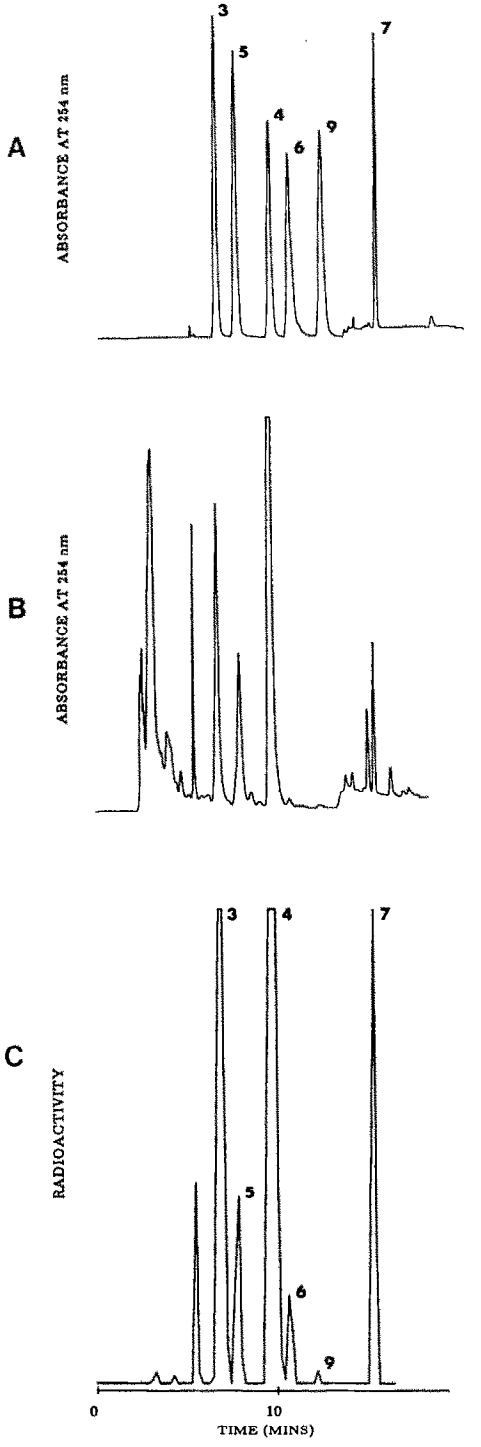

Fig. 2. Typical HPLC traces run in HPLC system B: (A) paracetamol metabolites—standard mixture; (B) A 0–24 hr urine sample after enzyme hydrolysis; (C) the associated radiochromatogram. 3, paracetamol-3-cysteine; 5, paracetamol-3-mercapturate; 4, paracetamol; 6, paracetamol-3-methylsulphoxide; 9, 3-methoxyparacetamol; 7, 3-thiomethylparacetamol. Following enzyme hydrolysis a 20 μl aliquot of the filtered 0–24 hr urine sample was injected onto the HPLC and consecutive 0.2 ml fractions of the eluent collected and counted for ^{14}C to derive the associated radiochromatogram. Details of mobile phase composition, the gradient program and other chromatographic details are in Materials and Methods.

Table 2. 0–24 hr Urinary metabolite profiles following [^{14}C]-paracetamol administration to control mice and mice pretreated with poly rI:rC

Metabolite	HPLC system[a]	% in 0–24 hr urine as recovered dose	
		Treatment	
		Control	Poly rI:rC
Paracetamol glucuronide	A	55.6 ± 4.1	60.1 ± 1.7
Paracetamol sulphate	A	9.0 ± 0.7	10.2 ± 0.3
Paracetamol	A	10.3 ± 2.8	10.3 ± 2.5
Unknown metabolite[b]	B	1.1 ± 0.2	0.4 ± 0.08**
Paracetamol-3-cysteine	B	16.5 ± 3.5	13.2 ± 0.6
Paracetamol-3-mercapturate	B	1.4 ± 0.7	0.9 ± 0.3
Paracetamol-3-methylsulphoxide	B	1.0 ± 0.1	0.7 ± 0.3
3-Thiomethylparacetamol	B	5.7 ± 0.9	4.2 ± 1.2
3-Methoxyparacetamol	B	<0.1	<0.1
Paracetamol	B	74.1 ± 4.8	80.2 ± 1.5*
Σ-3-SR	B	24.5 ± 4.7	19.0 ± 1.4*

Mice were treated with either saline (0.1 ml i.p.) or poly rI:rC (10 mg/kg i.p.) as appropriate, 24 hr later all mice received [*ring*-U-^{14}C]-paracetamol orally at a dose of 100 mg/kg b.w. Urine and faeces were collected daily for 3 days and 0–24 hr urinary metabolites assayed by radio-HPLC.

[a] Paracetamol, paracetamol glucuronide and paracetamol sulphate were assayed using chromatographic system A. All other metabolites were assayed as deconjugated forms following enzymic hydrolysis using chromatographic system B. Details of both systems in Materials and Methods.

[b] Unknown metabolite of retention time 5.0 min in system B.

Σ-3-SR refers to total thio adducts.

Values are means ± SD (N = 5). * $P < 0.05$, ** $P < 0.01$.

of paracetamol-3-cysteine, paracetamol-3-mercapturate, 3-thiomethylparacetamol and paracetamol-3-methylsulphoxide. For the control group the mean Σ-3-SR value was 24.5 ± 4.7% of the recovered dose while following poly rI:rC treatment the mean Σ-3-SR was significantly reduced to 19.0 ± 1.4% (x ± SD, $P < 0.05$).

DISCUSSION

The results reported here confirm our previous finding [5] that treatment with the immunomodulator, poly rI:rC, appears to have little effect upon xenobiotic glucuronidation *in vivo* in the mouse. Further to this, sulphation, another important conjugation mechanism, also appears to be unaffected by prior treatment with this agent. Consequently, as the major part of any therapeutic dose of paracetamol is eliminated as one or both of these highly polar conjugates, the clearance of this drug would not be greatly altered by prior treatment with poly rI:rC.

Chromatographic evidence presented here shows that the 3-methoxy metabolite of paracetamol, previously identified in man [18] and hamster [19], is also a product of paracetamol metabolism in the mouse. It is reasonable to suggest that this derives from the catechol precursor, 3-hydroxyparacetamol, produced by Phase I metabolism of paracetamol. Unfortunately no authentic standard for this metabolite was available. However, it is of interest to note that the unidentified radiolabelled peak seen following analysis of enzyme treated urine had a retention time of only 5.0 min in system B, indicating it to be a polar molecule. Further evidence to suggest that this early eluting peak is an oxidation product of paracetamol is provided by the finding that poly rI:rC significantly reduced its excretion from just over 1.0% of the recovered dose to less than 0.4%. This is in accord with our earlier observation [5] that the aromatic hydroxylation of acetylsalicylic acid is significantly depressed, *in vivo*, following poly rI:rC treatment. Similarly other workers [25] have reported that the hydroxylation of benzo[*a*]pyrene is depressed, *in vitro*, following interferon induction in the host animal.

As suggested by others [19, 23] we have used the index Σ-3-SR as an indirect measure of the *in vivo* formation of the reactive intermediate of paracetamol. Our results reveal that poly rI:rC pretreatment significantly reduced Σ-3-SR. The known ability of many immunomodulators and interferon inducers, such as poly rI:rC, to depress hepatic levels of cytochrome P-450 in experimental animals [2, 26, 27] strongly suggests that the reduction in the urinary excretion of Σ-3-SR reported here results from the decreased oxidation of paracetamol to its reactive intermediate. It is possible, however, that poly rI:rC treatment may have reduced Σ-3-SR by exerting an effect at the level of glutathione conjugation either by lowering available glutathione levels or by decreasing the activity of glutathione-*S*-transferases. Renton and Dickson [24] demonstrated that poly rI:rC treatment significantly reduced hepatic glutathione levels in mice whereas Balkwill *et al.* [28] have reported that direct administration of murine interferon was able to alter basal levels of glutathione-*S*-transferase in athymic nude mice. However, the results indicated a differential effect among different isozymes, the levels of some forms

being apparently increased while others were depressed.

The results reported here are in accordance with, and are complementary to, the earlier findings of Renton and Dickson [24]. Taken together both sets of observations prompt the speculation that during infection the concurrent activation of the immune system may indirectly confer a degree of protection, albeit marginal, against paracetamol toxicity by reducing the oxidative formation of a potentially toxic reactive intermediate. Such an effect upon paracetamol metabolism would also reinforce the present recommendation that children be given paracetamol rather than aspirin during febrile illness.

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