Biochemical Pharmacology, Vol. 33, No. 19, pp. 2995–3000, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

MECHANISM OF ACTION OF PARACETAMOL PROTECTIVE AGENTS IN MICE *IN VIVO*

JOHN O. MINERS,* ROGER DREW and DONALD J. BIRKETT
Department of Clinical Pharmacology, Flinders University Medical Centre, Bedford Park, Adelaide, South Australia 5042

(*Received* 18 *January* 1984; *accepted* 16 *April* 1984)

Abstract—The mechanism of action of cysteine, methionine, *N*-acetylcysteine (NAC) and cysteamine in protecting against paracetamol (APAP) induced hepatotoxicity in male C3H mice *in vivo* has been investigated by, (i) characterising the effect of the individual protective agents on the metabolism of an hepatotoxic dose of APAP, and (ii) determining the efficacy of the protective agents in animals treated with buthionine sulphoximine (BSO), a specific inhibitor of glutathione (GSH) synthesis. Co-administration of cysteine, methionine or NAC increased, while co-administration of cysteamine decreased, the proportion of GSH-derived conjugates of APAP excreted in the urine of mice administered APAP, 300 mg/kg. Pretreatment of animals with BSO abolished the protective effect of cysteine, methionine and NAC, whereas cysteamine still afforded protection against APAP after BSO treatment. In conjunction with other data, these results suggest the most likely mechanism for the protective effect of cysteine, methionine and NAC is by facilitating GSH synthesis, while the most likely mechanism for the protective effect of cysteamine is inhibition of cytochrome P-450 mediated formation of the reactive metabolite of APAP.

Although paracetamol (*N*-acetyl-*p*-aminophenol; APAP) is non-toxic in therapeutic doses, hepatotoxicity develops following overdosage with the drug. Animal studies have shown that APAP is metabolically activated by cytochrome P-450 to an electrophilic, arylating intermediate [1]. This reactive intermediate is usually inactivated by conjugation with glutathione (GSH) but, with higher doses of APAP, hepatic GSH levels are progressively depleted and subsequent covalent binding of the toxic metabolite to liver cell macromolecules initiates cell damage [2]. In addition, the dose threshold that exists for APAP toxicity following the depletion of hepatic GSH is also dependent upon the amount of drug which can be conjugated by the saturable sulphate pathway [3].

Elucidation of the role of GSH in detoxication of the APAP reactive metabolite provided the rationale for the use of other thiol-containing nucleophiles as antidotes to prevent hepatic necrosis in APAP overdose patients. Initially it was shown that cysteine was effective in protecting against APAP-induced hepatotoxicity in mice [4] and subsequently cysteamine [5], methionine [5, 6] and *N*-acetylcysteine (NAC) [7] have all been successfully used to treat severe APAP poisoning in humans. Of these compounds NAC has emerged as the preferred agent for the treatment of APAP poisoning [8]. Although NAC has found widespread clinical acceptance, the hepato-protective mechanism of this compound remains controversial. Indeed, at least 5 mechanisms have been proposed on the basis of *in vivo* and *in vitro* data. These are: (i) direct conjugation of NAC with the APAP reactive metabolite [9, 10], (ii) conversion of NAC to cysteine leading to enhanced synthesis of GSH for conjugation with the reactive metabolite [11, 12, 13], (iii) conversion of NAC to cysteine leading to enhanced synthesis of inorganic sulphate for conjugation with APAP [14, 15], (iv) chemical reduction of the reactive metabolite back to APAP [16], and (v) slowed APAP absorption due to inhibition of gastric motility by NAC [17].

The present study was initiated to determine the mechanism of the protective effect of NAC, cysteine, methionine and cysteamine in mice *in vivo*. In particular, the relative importance of these protective agents in facilitating GSH synthesis was investigated by characterising the effect of the individual protective agents on the metabolism of an hepatotoxic dose of paracetamol, and by determining the efficacy of the protective agents in animals treated with buthionine sulphoximine (BSO), a specific inhibitor of glutathione synthesis [18, 19, 20].

MATERIALS AND METHODS

Chemicals. Paracetamol, L-cysteine, cysteamine, L-methionine and *N*-acetylcysteine were purchased from the Sigma Chemical Co. (St. Louis, MO). Buthionine sulphoximine (BSO) was supplied by the Chemical Dynamics Corp. (South Plainfield, NJ).

Experimental. All experiments were carried out using male C3H mice (23–27 g). Animals were allowed food and water *ad libitum*. In all experiments solutions of APAP, 30 ml/kg in 0.9% saline, were administered by gavage. The protective agents in saline (cysteine, 500 mg/kg; NAC, 500 mg/kg; methionine, 1000 mg/kg; cysteamine, 150 mg/kg) were injected intraperitoneally at the same time as APAP administration. Mice were placed in specially constructed glass metabolic cages and urine collected for 24 hr. All parts of the cage exposed to urine were washed with 10 ml methanol–water (1:1) and the washings added to the urine. In those studies investigating the effect of BSO on the efficacy of the

Table 1. Effect of protective agents on plasma ALT activity and hepatic GSH concentration after an hepatotoxic dose of paracetamol

Treatment	Plasma ALT (U/l)	Hepatic GSH concentration (μmole/g)
Saline	33 ± 5	6.46 ± 0.30†
APAP	16,273 ± 2032*	1.37 ± 0.22*
APAP + NAC	36 ± 6	4.04 ± 0.16*†
APAP + cysteine	36 ± 5	4.07 ± 0.23*†
APAP + methionine	38 ± 6	4.81 ± 0.39*†
APAP + cysteamine	42 ± 7	4.15 ± 0.50*†

Plasma samples for measurement of ALT collected 7 hr after paracetamol dose. Livers removed 2 hr after paracetamol dose for determination of GSH concentrations.

The APAP dose was 300 mg/kg (p.o.); protective agents were administered as described in Materials and Methods.

Data represent mean ± S.E. for 6 mice.

Compared to saline only, *$P < 0.001$; compared to APAP only, †$P < 0.001$.

protective agents, BSO (1600 mg/kg, i.p.) was administered 1 hr prior to and 6 hr post APAP and protective agent as previously described [18].

Assays. Livers were excised 2 hr after the APAP dose and GSH content determined by a modification [18] of the *o*-phthalaldehyde derivatisation procedure of Cohn and Lyle [21]. Blood was collected from animals 7 hr after APAP administration for the measurement of alanine transaminase (ALT) [22]; mice were lightly anaesthetised with ether and mixed venous/arterial blood collected from a pouch under the right fore leg after severing the axial blood vessels. Unchanged APAP and its glucuronide, sulphate, cysteine and mercapturic acid conjugates in urine were determined by high performance liquid chromatography [23]. The cysteine and mercapturic acid conjugates were summed and are reported as total GSH derived conjugates.

Analysis of results. Results are expressed as mean ±S.E. The significance of differences between treatment groups was evaluated using Students *t*-test for unpaired samples. LD_{50} values and 95% confidence intervals were determined according to the method of Litchfield and Wilcoxon [24].

RESULTS

Effects of protective agents on plasma ALT and hepatic GSH content. Blood samples were collected 7 hr after administration of APAP, 300 mg/kg (± protective agents), for the determination of plasma ALT activity. The results are summarised in Table 1 and show that ALT was massively increased after APAP administration. However, co-administration of NAC (500 mg/kg), cysteine (500 mg/kg), methionine (1000 mg/kg) or cysteamine (150 mg/kg) with APAP maintained ALT at control levels, demonstrating that the doses of these agents were optimal

Table 2. Effect of protective agents on the pattern of urinary paracetamol metabolites†

Treatment	Mean fractional excretion‡				Mean %dose recovered§
	G‖	S	GSH	APAP	
APAP (N = 10)	50.1 ±2.1	6.8 ±0.3	16.4 ±0.6	13.3 ±1.7	86.6 ±3.9
APAP + NAC (N = 5)	47.8 ±2.9	8.2* ±0.3	21.4* ±0.9	11.1* ±1.9	88.5 ±2.4
APAP + cysteine (N = 5)	50.1 ±3.9	8.3* ±0.3	21.7** ±0.9	10.7* ±1.9	90.8 ±3.5
APAP + methionine (N = 5)	46.8 ±3.1	8.1* ±0.5	22.4** ±1.4	12.9 ±2.0	90.2 ±3.2
APAP + cysteamine (N = 5)	59.5* ±2.5	7.9 ±0.5	10.4** ±0.6	16.1* ±1.5	93.9* ±3.6

Compared with controls (APAP alone); *$P < 0.05$, **$P < 0.01$.

† APAP dose administered to all animals was 300 mg/kg; dose of protective agent as described in Materials and Methods.

‡ Expressed as percentage of dose administered.

§ Total paracetamol-derived products recovered expressed as percentage of the dose administered.

‖ G = glucuronide, S = sulphate, GSH = glutathione-derived conjugates (cysteine + mercapturate), APAP = unchanged paracetamol.

Table 3. Effect of BSO on the efficacy of paracetamol protective agents†

Treatment	LD_{50} (mg/kg)	95% Confidence interval
APAP	295	268–325
APAP + BSO	110	100–121
APAP + NAC	750	683–824
APAP + NAC + BSO	120	109–132
APAP + cysteine	860	775–955
APAP + cysteine + BSO	120	110–130
APAP + methionine	910	856–967
APAP + methionine + BSO	120	113–127
APAP + cysteamine	800	740–865
APAP + cysteamine + BSO	185	174–197

† APAP, BSO and protective agents administered as described in Materials and Methods.

and equally effective for protection against APAP-induced hepatotoxicity. Data in Table 1 further show that 2 hr after administration of APAP hepatic GSH content was reduced to 21% of the control level. Although co-administration of NAC, cysteine, methionine or cysteamine prevented this marked depletion of hepatic GSH, the levels of GSH in the animals treated with the protective agents were still 26–33% lower than in the control group.

Effects of protective agents on the pattern of urinary paracetamol metabolites. Table 2 summarises the effects of the individual protective agents on the metabolism of a 300 mg/kg APAP dose. Co-administration of NAC, cysteine and methionine increased the proportion of the dose excreted as the GSH-derived conjugates by 30, 32 and 36% respectively ($P < 0.01$). These agents caused smaller (19–22%), but nevertheless statistically significant, increases in the fractional excretion of the sulphate conjugate. Cysteine and NAC decreased the proportion of the dose excreted as unchanged APAP. Neither NAC, cysteine nor methionine altered the fractional excretion of APAP-glucuronide. In contrast, cysteamine treatment reduced (by 37%) the amount of GSH-derived conjugates excreted, this effect being compensated for by an increase in the proportion of the dose eliminated as the glucuronide and as unchanged APAP.

Effects of BSO on the efficacy of the protective agents. At least four different doses of APAP known to cause between 0 and 100% mortality were administered to mice (12 animals per dose) in order to construct dose–lethality curves for the following treatments; APAP alone, APAP with co-administration of protective agent, APAP 1 hr after BSO treatment, and APAP plus protective agent 1 hr after BSO treatment. Data from these experiments are summarised in Table 3. The LD_{50} for APAP in the male C3H mice was 295 mg/kg and, as expected, pretreatment with BSO, an inhibitor of GSH synthesis, decreased the LD_{50} for APAP to 110 mg/kg. Co-administration of NAC, cysteine, methionine or cysteamine resulted in a 2.5 to 3.1-fold increase in the APAP LD_{50}. However, when either NAC, cysteine or methionine were co-administered with APAP to animals pretreated with BSO no protective effect was observed and the LD_{50} values were not significantly different to those when APAP alone was given to BSO pretreated animals. In contrast, the LD_{50} for BSO pretreated animals administered both cysteamine and APAP (185 mg/kg) was higher than BSO pretreated animals given APAP alone (110 mg/kg). Dose–lethality curves for the studies with NAC (which gave similar results to cysteine and methionine) and cysteamine are shown in Fig. 1 and 2 respectively.

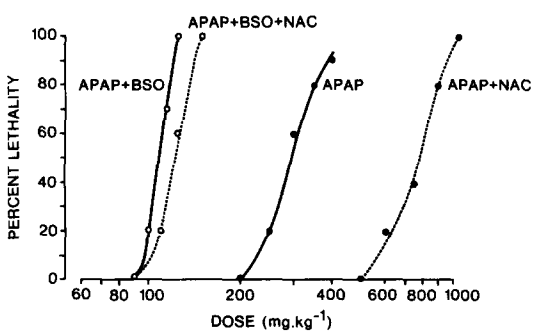

Fig. 1. Effects of NAC, BSO and NAC + BSO on the cumulative dose–lethality curves for APAP in male C3H mice. NAC and BSO doses as described in Materials and Methods. Twelve animals received each APAP dose in each treatment phase.

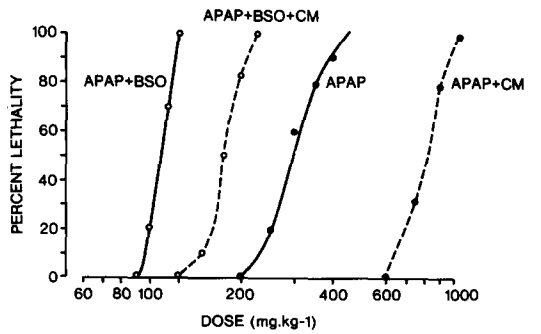

Fig. 2. Effects of cysteamine (CM), BSO and CM + BSO on the cumulative dose–lethality curves for APAP in male C3H mice. CM and BSO doses as described in Materials and Methods. Twelve animals received each APAP dose in each treatment phase.

DISCUSSION

Although cysteamine, methionine and NAC have all found widespread clinical use for the treatment of APAP overdose, the mechanism of the hepatoprotective effect of these compounds remains controversial. This study has investigated the mechanism of action of cysteine, cysteamine, methionine and NAC *in vivo* in the C3H mouse, which we have previously shown [23] to be a good model for APAP metabolism studies.

Concomitant treatment of animals with APAP and either cysteine, methionine or NAC caused an increase in the proportion of the APAP dose excreted as the GSH-derived and sulphate conjugates. These results are consistent with conversion of methionine and NAC to cysteine, which in turn leads to enhanced synthesis of GSH and inorganic sulphate. Alternatively, cysteine (or NAC) once formed may directly conjugate with the APAP reactive metabolite. The ratio of metabolite(s) to unchanged APAP in urine (called the metabolic ratio) reflects the clearance to that metabolite assuming that all products of the pathway are measured in urine and that renal clearance of unchanged APAP remains constant [23]. For the oxidative pathway at hepatotoxic APAP doses the first of these assumptions will not be valid as it is known that a proportion of the reactive metabolite binds covalently to cellular constituents rather than being excreted as GSH conjugates. However, the metabolic ratio will be a valid measure of oxidative clearance in the presence of adequate GSH reserves. The apparent increase in metabolic ratio (Table 4) for this pathway in NAC, cysteine and methionine treated animals is, therefore, more likely to be due to decreased covalent binding (a consequence of increased GSH availability) and increased recovery of GSH conjugates rather than an increase in oxidative metabolism. Both the urinary metabolite excretion and metabolic ratio data suggest that in unprotected animals administered 300 mg/kg APAP, 30–40% of the reactive intermediate formed covalently binds to cellular macromolecules. The metabolic ratio data (Table 4) further show that clearance through the sulphation pathway is increased by 22–47% after treatment with cysteine, methionine or NAC, presumably due to increased availability of sulphate for conjugation with APAP. Although the metabolic ratio for the glucuronide was significantly higher (by 18%) in animals treated with cysteine, the data show that these protective agents generally have no effect on the activity of the glucuronidation pathway.

Table 4. Effect of protective agents on metabolic ratios

Treatment	Metabolic ratio for G†	S	GSH
APAP	3.96 ±0.21	0.55 ±0.02	1.29 ±0.16
APAP + NAC	4.35 ±0.28	0.74** ±0.04	2.01** ±0.09
APAP + cysteine	4.70* ±0.36	0.81** ±0.05	2.07** ±0.20
APAP + methionine	3.76 ±0.22	0.67** ±0.04	1.79** ±0.18
APAP + cysteamine	3.79 ±0.19	0.52 ±0.04	0.66** ±0.09

Compared with control (APAP alone); *$P < 0.05$, **$P < 0.01$.

†Abbreviations as described in Table 2.

By contrast to the above effects, co-administration of cysteamine caused a decrease in the fractional excretion of the GSH-derived conjugates of APAP. When the results for cysteamine are reconsidered in terms of metabolic ratios (Table 4), it is apparent that cysteamine has no effect on the activity of the glucuronidation and sulphation pathways. If it is assumed that the metabolic ratio for the GSH-derived products in animals pretreated with NAC or similar agents reflects the true clearance to the APAP reactive metabolite (i.e. the situation where clearance is not, or only minimally, affected by GSH availability), then the data indicate cysteamine reduced the clearance to the reactive intermediate by approximately two-thirds. These results could be due to a number of possible processes; decreased synthesis of the reactive metabolite, reduction of the reactive metabolite back to APAP, and/or direct conjugation of cysteamine with the reactive metabolite. It should be noted that while conjugation of cysteine or NAC with the reactive intermediate would produce the same urinary metabolites of APAP as from GSH conjugation, direct conjugation with cysteamine or methionine would produce different compounds. Although authentic samples of the cysteamine and methionine conjugates of APAP were not available, no additional peaks were observed in the chromatograms of urines from animals treated with these protective agents compared with urines from mice administered cysteine or NAC and total recoveries of the measurable metabolites tended to increase rather than decrease.

To characterise further the mechanism of the protective agents, the efficacy of cysteine, cysteamine, methionine and NAC in protecting against APAP-induced toxicity was investigated in mice pretreated with BSO. BSO is a specific inhibitor of γ-glutamylcysteine synthetase [18, 19, 20] which subsequently results in lowered tissue GSH levels. We have shown [18] in the C3H mouse that the dose of BSO used in this study causes maximal inhibition of γ-glutamylcysteine synthetase in liver and kidney within 1–2 hr of administration. Furthermore, we demonstrated [18] that BSO has no effect on the activity of a range of enzymes of the hepatic mixed function oxidase system or on the activity of sulphotransferase, glucuronyl transferase or GSH-transferase. Any effect of BSO on APAP toxicity would therefore appear to be solely due to the inhibitory effect of BSO on GSH synthesis.

Thus, pretreatment of animals with BSO markedly increased the toxicity of APAP, causing a 63% reduction in APAP LD_{50}. Consistent with the protective effect demonstrated by the ALT measurements, co-administration of cysteine, methionine or NAC markedly increased the APAP LD_{50}. However, the protective effect of these agents was abolished in animals pretreated with BSO indicating

the mechanism of their hepato-protective action was facilitation of GSH synthesis, presumably through provision of precursor cysteine. As would be expected from such a mechanism, treatment of animals with cysteine, methionine or NAC reduced the extent of depletion of hepatic GSH normally occurring after an hepatotoxic dose of APAP. Further support for our conclusion is provided by the observation that cysteine, methionine and NAC are all effective in maintaining GSH levels in isolated hepatocytes, whereas exclusion of glutamate and/or glycine does not markedly affect the rate of GSH resynthesis [25]. It is known that the rate of GSH synthesis is significantly influenced by the intracellular concentration of cysteine since this amino acid is present in cells at a limiting concentration [26]. Although the urinary metabolite studies indicated cysteine, methionine or NAC treatment increased the amount of sulphate available for conjugation with APAP, presumably through cysteine as a common intermediate [28], the lack of a protective effect of these agents in the presence of BSO indicates enhanced sulphate synthesis is quantitatively unimportant in preventing APAP toxicity. Lauterberg *et al.* [13] have also recently shown that while APAP sulphate formation was increased after NAC administration to rats, the increased sulphate conjugation did not significantly affect the extent of APAP toxic metabolite formation.

Whereas pretreatment of animals with BSO abolished the protective effective of cysteine, methionine and NAC, cysteamine still afforded some protection after BSO treatment. Indeed, this result was not surprising since cysteamine cannot be converted to cysteine and hence enhance GSH synthesis. The results from the BSO experiments are consistent with the urinary metabolite data which indicated cysteamine exerted its protective effect by a different mechanism to the other agents studied here. In the urinary metabolite studies the lack of an additional peak corresponding to the APAP-cysteamine conjugate, which should have separated under the chromatography conditions, suggests direct conjugation with the reactive metabolite is unlikely to occur to any significant extent. Harvey and Levitt [28], using ^{35}S labelled cysteamine, have also reported that the APAP-cysteamine conjugate does not appear to be formed in mice *in vivo*. Cysteamine has been shown to inhibit acetanilide metabolism [28] and steroid hydroxylation [29] *in vitro*. Inhibition of cytochrome P-450 mediated reactive metabolite formation would be consistent with data from our metabolic and BSO experiments. Like other thiol-containing molecules, however, cysteamine has a low redox potential and could conceivably reduce the reactive intermediate back to APAP. This mechanism is unlikely since it has previously been shown not to be involved in the protective effect of NAC [13] and is also not consistent with our results for NAC, cysteine and methionine.

In summary, data presented here suggest that in the C3H mouse cysteine, methionine and NAC share a common mechanism of action in protecting against APAP toxicity, namely facilitation of GSH synthesis. Other suggested effects such as enhanced sulphate synthesis, chemical reduction of the APAP reactive metabolite and slowed gastric absorption would appear to be quantitatively unimportant in the protective mechanism of these agents. By contrast, the protective effect of cysteamine against APAP toxicity is not dependant on GSH synthesis, the most likely mechanism being inhibition of cytochrome P-450 mediated reactive metabolite formation. These proposed mechanisms are consistent with the clinical observation [7] that cysteamine, methionine or NAC are only effective if administered within 12 hr of APAP overdosage.

Acknowledgements—This work was supported by a grant from the National Health and Medical Research Council of Australia. The authors wish to thank Mrs. J. Attwood for her excellent technical assistance.

REFERENCES

1. W. Z. Potter, S. S. Thorgiersson, D. J. Jollow and J. R. Mitchell, *Pharmacology* **12**, 129 (1974).
2. W. Z. Potter, D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. ther.* **187**, 203 (1973)
3. J. T. Slattery and G. Levy, *Clin. pharmac. Ther.* **25**, 184 (1979).
4. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. ther.* **187**, 211 (1973).
5. L. F. Prescott, R. W. Newton, C. P. Swainson, N. Wright, A. R. W. Forrest and H. Matthew, *Lancet* **i**, 588 (1974)
6. L. F. Prescott, J. Park, G. R. Sutherland, I. J. Smith and A. T. Proudfoot, *Lancet* **ii**, 109 (1976).
7. L. F. Prescott, J. Park, A. Ballantyne, P. Adriaenssens and A. T. Proudfoot, *Lancet* **ii**, 432 (1977).
8. L. F. Prescott, R. N. Illingworth, J. A. J. H. Critchley, M. J. Stewart, R. D. Adam and A. T. Proudfoot, *Br. med. J.* **2**, 1097 (1979).
9. A. R. Buckpitt, D. E. Rollins and J. R. Mitchell, *Biochem. Pharmac.* **28**, 2941 (1979).
10. J. M. Tredger, H. M. Smith, M. Davis and R. Williams, *Toxicol. Lett.* **5**, 339 (1980).
11. E. Piperno, A. H. Mosher, D. A. Berssenbruegge, J. D. Winkler and R. B. Smith, *Pediatrics* **62**, 880 (1978).
12. T. E. Massey and W. J. Racz, *Toxicol. appl. Pharmac.* **60**, 220 (1981).
13. B. H. Lauterburg, G. B. Corcoran and J. R. Mitchell, *J. clin. Invest.* **71**, 980 (1983).
14. R. E. Galinsky and G. Levy, *Life Sci.* **25**, 693 (1979).
15. R. E. Galinsky and G. Levy, *J. Pharmac. exp. ther.* **219**, 14 (1981).
16. G. B. Corcoran, J. R. Mitchell, Y. N. Vaishnav and E. C. Horning. *Molec. Pharmac.* **18**, 536 (1980).
17. L. W. Whitehouse, L. T. Wong, G. Solomonraj, C. J. Paul and B. H. Thomas, *Toxicology* **19**, 113 (1981).
18. R. Drew and J. O. Miners, *Biochem. Pharmac.* **33**, 2989 (1984).
19. O. W. Griffith and A. Meister, *J. biol. Chem.* **254**, 7558 (1979).
20. O. W. Griffith and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5606 (1979).
21. H. Cohn and J. Lyle, *Analyt. Biochem.* **14**, 434 (1966).
22. R. J. Henry, N. Chiamori, O. J. Golub and S. Berkman, *Am. J. clin. Path.* **34**, 381 (1960).
23. J. O. Miners, J. Adams and D. J. Birkett, *Clin. exp. Pharmac. Physiol.* **11**, 209 (1984).
24. J. Litchfield and F. Wilcoxon, *J. Pharmac.* **96**, 99 (1949).

25. H. Thor, P. Moldeus and S. Orrenius, *Archs. Biochem. Biophys.* **192**, 405 (1979).
26. A. Meister and S. Tate, *Ann. Rev. Biochem.* **45**, 559 (1976).
27. B. Sorbo, *Scand. J. clin. Lab. Invest.* **17**, 21 (1976).
28. F. D. Harvey and T. E. Levitt, *J. int. Med. Res.* **4**, (suppl. 4), 130 (1976).
29. K. Flemming, B. Geierhass and V. Seydewitz, *Biochem. Pharmac.* **22**, 1241 (1973).