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ENHANCEMENT OF HIGH DOSE CYCLOSPORIN A TOXICITY BY FRUSEMIDE

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Abstract—Adult Sprague–Dawley rats were given cyclosporin A (CyA), frusemide (Fr) or both drugs daily for 14 days. The doses of CyA (50 mg/kg) and Fr (5 mg/kg) were approximately 3–6 times and twice respectively those used in man. Fr on its own produced a diuresis lasting approximately 3 hr. This was characterized by a 10-fold increase in urine flow rate, a 40-fold increase in the rate of sodium excretion, and by 2- and 4-fold increases in urea and creatinine clearance rates, respectively. In addition, there was a doubling in urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity. After 4 days of combination treatment with CyA and Fr, the diuretic-induced increases in urine flow rate, sodium excretion and urinary NAG activity were similar to those following frusemide alone. However, urea and creatinine clearances did not increase during the diuresis. Fr itself did not impair renal function, but rats receiving only CyA did show elevations in serum urea and creatinine, with reductions in clearance rates, which progressed with time. There was also an increase in NAG enzymuria. When the two drugs were exhibited together, renal function was more severely impaired. All animals given CyA showed proximal renal tubular cell vacuolation: in half the damage was confined to the straight segment, while the rest showed additional severe convoluted segment change. Renal function was most abnormal in those rats in which both segments were affected. All animals given both drugs showed both straight and convoluted tubular abnormalities and a 2-fold increase in serum CyA levels. CyA-induced disturbances in hepatic function and lymphoid tissue atrophy were unaffected by the addition of Fr, nor did Fr affect the immunosuppressive action of CyA.

Traditional immunotherapy with azathioprine and steroids produces a non-specific 'blanket' suppression of both cell-mediated and humoral immunity. This form of treatment is associated with many side-effects, including myelotoxicity. Not surprisingly therefore, the advent of the specific immunosuppressant cyclosporin A (CyA), which selectively inhibits T-lymphocyte activation [1, 2] and exhibits relatively low myelotoxicity [3], has evoked considerable interest in transplantation circles. Unfortunately, CyA is not without certain untoward effects, nephrotoxicity being the most serious [4–6].

There is evidence that the toxic properties of CyA may be enhanced by other potentially nephrotoxic drugs, such as the aminoglycoside antibiotic gentamicin, both in human bone marrow transplant recipients [7] and in experimental animals [8]. Since diuretics, and in particular frusemide (Fr), are commonly used following human renal transplantation and because drug-induced nephrotoxicity may be enhanced by diuretic administration [9], we have investigated the effect of frusemide on renal and hepatic function in rats receiving immunotherapeutic doses of CyA. The dose of CyA used was approximately 3- to 6-fold that used clinically.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (mean body wt 300 g) bred in the University Animal Department, Foresterhill, Aberdeen, were used throughout. They were housed in a temperature-controlled environment and received Oxoid pasteurized rat and mouse breeding diet with tap water *ad libitum*, except as specified below.

Cyclosporin A. CyA (OL27-400; Sandoz Ltd., Basel, Switzerland) was dissolved initially at 20° in absolute ethanol. A solution of CyA in 10% ethanol in olive oil B.P. (Boots Company Ltd., U.K.) was then prepared and 0.3 ml was administered to the conscious rat by gastric intubation using a 4-fine gauge intravenous cannula (Portex Ltd., Hythe, Kent, U.K.).

Frusemide. Frusemide (Hoechst U.K. Ltd., Hounslow, Middlesex, U.K.) was administered (5 mg/kg) as a single intraperitoneal (i.p.) injection.

Experimental protocol. Groups of six animals were given CyA alone (50 mg/kg), Fr alone (5 mg/kg) or a combination of both once daily for 14 days. When the agents were given in combination, CyA administration preceded that of Fr by 30 min. The primary humoral immune response to sheep red blood cells (SRBC) was determined in all groups.

Analyses of serum and urine were conducted immediately before the start of treatment and at 7 and 14 days. On day 14 the animals were killed by terminal ether anaesthesia and autopsies performed.

Humoral response to SRBC. SRBC obtained from sheep blood in Alsever's solution (Difco Laboratories) were washed three times in ice-cold

Dulbecco 'A' phosphate-buffered saline (pH 7.2) and 5×10^9 cells (1 ml) administered by i.p. injection on day 0. On day 14, total serum haemagglutinin titres were determined for each individual in U-shaped microtitre plates (Nunc, Denmark) using the microtechnique of Takátsy [10].

Blood and urine sampling. Whole blood samples (1.5 ml) were collected into plain tubes from the tail tips of anaesthetized rats. Serum expressed from the clotted blood (2 hr at ambient room temperature) was stored at −20° until assayed. Urine free of faecal contamination was collected overnight (16 hr) from animals placed in metabolic cages and starved throughout the collection period.

Radioimmunoassay of CyA. Estimations of CyA levels in serum samples obtained 16 hr after drug administration were performed using radioimmunoassay kits provided by Sandoz Ltd. (Basel, Switzerland) as described by Donatsch *et al.* [11].

Biochemical investigations. Estimations of serum urea, creatinine, total protein, albumin, total bilirubin, aspartate aminotransferase (AAT) and alkaline phosphatase were conducted as previously described, as were determinations of urine levels of urea, creatinine and NAG, and glomerular filtration rate (GFR) [12]. NAG activity was expressed as IU per mg creatinine in the urine.

Preparation of tissues for microscopy. For light microscopy, tissue (kidney, liver, bone marrow, thymus, spleen and lumbar lymph nodes) was fixed in 10% neutral buffered formalin and processed to acrylic resin: 2 μm sections were stained with haematoxylin and eosin. For electron microscopy, 1 mm^3 blocks were fixed in 4% formaldehyde and 1% glutaraldehyde, then processed to epoxy resin. Sections were stained with uranyl acetate and lead citrate prior to examination in a Jeol 100S electron microscope.

Statistics. The significance of differences between means was determined using Student's *t*-test for paired or independent samples as appropriate. P values <0.05 were considered significant.

RESULTS

Renal function

Following administration of Fr alone, a diuresis was observed which lasted approximately 3 hr. This was characterized (Table 1) by a 10-fold increase in urine flow rate, a 40-fold increase in the rate of sodium excretion, and by 2- and 4-fold increases in urea and creatinine clearance rates, respectively. In addition, there was a doubling of urinary NAG activity. This diuresis also resulted in an average weight loss of 20 g (7% body wt), which was replaced during the subsequent 16 hr. The parameters named above also returned to pretreatment levels during this period. Following 4 days of both CyA and Fr treatment (Table 2), the Fr-induced increases in urine flow rate, sodium excretion and NAG activity were similar to those observed in the absence of CyA. However, both urea and creatinine clearance rates were reduced in this group in comparison to animals given Fr alone. During the diuretic period this reduction in clearance rates was particularly marked and was maintained for the subsequent 16 hr post-diuresis, as were the increased NAG levels.

CyA treatment alone produced significant increases in serum urea levels and NAG enzymuria by day 7, with a consequent reduction in urea clearance rates (Table 3). By day 14, further increases were observed in both serum urea and creatinine levels with reduced clearance rates of both: NAG enzymuria was maintained at around its day 7 value. Frusemide treatment alone for 14 days did not affect any of the indices of renal function measured. Combined CyA and Fr administration produced a more severe degree of renal impairment on both days 7 and 14 than could be attributed to summation of the effects of both drugs given independently ($P < 0.001$). By day 14 glomerular filtration was reduced to *ca* 20% of the pretreatment value and NAG levels had risen steeply.

Hepatic function

The effect of CyA alone and in combination with Fr on serum indices of hepatic function is given in Table 4. CyA treatment alone produced a decrease in total protein and albumin levels observed on days 7 and 14, a rise in total bilirubin on day 7 and maintained on day 14, and a progressive rise in alkaline phosphatase activity, which was significant on day 14. As we have shown previously at various CsA doses in the rat [8, 12], serum AAT levels fell significantly by day 7, but returned to pretreatment levels by day 14. Fr administration alone over 14 days did not have any significant effect on any of these parameters.

Combined CyA and Fr treatment produced similar serum biochemical changes to those observed with CyA alone, except that alkaline phosphatase activity was significantly increased on day 7. However, by day 14 serum protein and albumin levels were not significantly different from pretreatment results and alkaline phosphatase activity was significantly reduced when compared to its day 7 level. Serum

Table 1. Characteristics of Fr-induced diuresis

	Urine flow rate (ml hr^{-1})	Sodium excretion (μmol hr^{-1})	Urea clearance (ml hr^{-1})	Creatinine clearance (ml hr^{-1})	Urine NAG (IU/mg)
Pretreatment (0–3 hr)	0.5 ± 0.4	9 ± 2	56 ± 17	127 ± 14	540 ± 65
Treatment (4–7 hr)	5.0 ± 1.9*	402 ± 230*	201 ± 55*	253 ± 52*	953 ± 361†
Post treatment (8–24 hr)	0.6 ± 3.0	11 ± 8	63 ± 22	125 ± 34	651 ± 178

Results are means ± S.D. for six animals. Values compared to pretreatment levels: *, $P < 0.001$; †, $P < 0.05$.

Table 2. Effect of CyA on Fr-induced diuresis 4 days after start of treatment

	Urine flow rate (ml hr^{-1})	Sodium excretion (μmol hr^{-1})	Urea clearance (ml hr^{-1})	Creatinine clearance (ml hr^{-1})	Urea NAG (IU/mg)
Pretreatment (0–3 hr)	1.1 ± 0.3	10 ± 3	27 ± 10	75 ± 28	1333 ± 298
Treatment (4–7 hr)	4.5 ± 0.5*	407 ± 69*	14 ± 6*§	62 ± 27§	1817 ± 828†
Post treatment (8–24 hr)	0.7 ± 0.1	8 ± 2	17 ± 3*§	48 ± 13‡§	1701 ± 148†§

Results are means ± S.D. for six animals. Results compared to pretreatment values: *, $P < 0.001$; †, $P < 0.01$; ‡, $P < 0.05$. Results compared to values in Fr-treated group (Table 1): §, $P < 0.001$.

AAT levels were significantly increased by day 14, and the same trend was observed with total bilirubin levels, although in this case it was not significant.

Humoral immunity

The influence of CyA, Fr or a combination of both drugs on the humoral response to SRBC is shown in Table 5. Fr had no effect on the CyA-induced reduction in haemagglutinin titre.

Morphological observations

There were no structural abnormalities in any of the tissues (kidney, liver, marrow, thymus, spleen and lymph nodes) examined from the group of six animals given Fr alone for 2 weeks.

Kidney

All six rats dosed only with CyA showed renal tubular abnormalities. In three of the animals the damage was similar to that which we have previously ascribed to CyA, viz. varying degrees of vacuolation of a proportion of epithelial cells lining the proximal straight tubules. In the remaining three animals, the straight tubular cell damage was accompanied by pronounced focal proximal convoluted tubular cell vacuolation (Fig. 1). These vacuoles were larger than those observed in the straight tubules and although fewer in number, they caused gross distension of the affected cells. Electron microscopy showed that the vacuolation in both convoluted and straight segments was due to gross distension of the endoplasmic reticulum. An additional feature was an increase in the number of large lysosomes within proximal tubular cells whether these showed vacuolation or not. Notably, serum urea and creatinine levels were highest in those animals with both proximal straight and convoluted tubular cell damage.

In all six animals receiving both CyA and Fr, the

Table 3. Effect of CyA alone or in combination with Fr on renal function 7 and 14 days after start of treatment

		Serum urea (mmol/l)	Serum creatinine (μmol/l)	Urea clearance (ml hr^{-1})	Creatinine clearance (ml hr^{-1})	Urine NAG (IU/mg)
Pretreatment		7.7 ± 1.1	49 ± 9	55 ± 17	127 ± 14	649 ± 67
Day 7	Fr	6.8 ± 1.9	44 ± 6	48 ± 6	109 ± 16	746 ± 146
	Cy A	10.3 ± 1.0*	56 ± 9	35 ± 15†	97 ± 18	1066 ± 296*
	Cy A/Fr	36.2 ± 11.7*	106 ± 24*	16 ± 7*	50 ± 21*	1843 ± 383*
Day 14	Fr	7.9 ± 1.4	52 ± 7	44 ± 9	98 ± 19	946 ± 220‡
	Cy A	18.4 ± 10.8*	86 ± 34*	18 ± 11*	50 ± 20*	986 ± 289‡
	Cy A/Fr	44.6 ± 19.1*	177 ± 98*	8 ± 6*	25 ± 12*	2565 ± 918*

Results are means ± S.D. for six animals and compared to pretreatment values: *, $P < 0.001$; †, $P < 0.01$; ‡, $P < 0.05$. Urine values and clearances based on 16 hr urine collections; serum samples were taken at the end of this period.

Table 4. Effect of CyA alone or in combination with Fr on hepatic function 7 and 14 days after start of treatment

		Total protein (g/l)	Albumin (g/l)	Total bilirubin (μmol/l)	AAT (IU/L)	Alk.phos. (IU/L)
Pretreatment		63 ± 1	35 ± 1	1 ± 1	137 ± 13	276 ± 36
Day 7	Fr	64 ± 2	36 ± 2	1 ± 1	146 ± 16	280 ± 48
	Cy A	60 ± 2‡	33 ± 2	8 ± 3*	83 ± 9†	300 ± 83
	Cy A/Fr	59 ± 3‡	33 ± 2	10 ± 2*	83 ± 12†	360 ± 48‡
Day 14	Fr	63 ± 3	35 ± 2	1 ± 1	140 ± 22	255 ± 36
	Cy A	57 ± 1*	32 ± 1†	8 ± 3*	131 ± 37	350 ± 95‡
	Cy A/Fr	62 ± 8	35 ± 5	12 ± 7*	225 ± 108†§	206 ± 55‖

Results are means ± S.D. for six animals. Comparisons with pretreatment values: *, $P < 0.001$; †, $P < 0.01$; ‡, $P < 0.05$; and between Cy and Cy/Fr: §, $P < 0.05$; ‖, $P < 0.01$ at days 7 and 14.

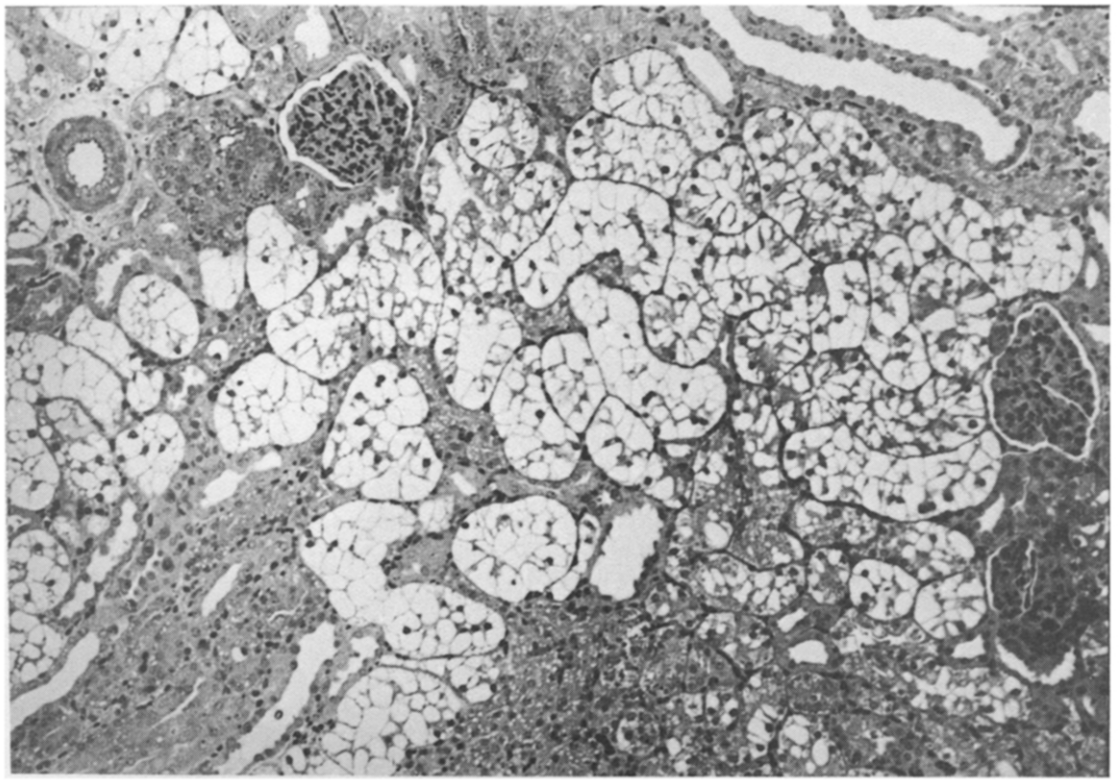

Fig. 1. Renal cortex after CyA treatment for 14 days. There is focal gross proximal convoluted tubular cell vacuolation and ballooning. Proximal straight tubules showing minor basal vacuolation are also included in the field (bottom left and centre). Haematoxylin and eosin, × 180.

kidneys showed the dual convoluted and straight tubular abnormalities described after CyA alone; in three, the changes were similar to those of the CyA group, while in the remainder they were more severe in terms of vacuolation and cellular distension.

Liver

In four of the six rats given CyA alone, and in all animals given CyA and Fr, either a centrilobular or periportal hepatic fatty change was observed.

Bone marrow and lymphoid tissue

In the six animals given CyA alone there was a reduction in marrow cellularity, lymph node cortical atrophy and a loss of thymic parenchyma: five of the six rats also exhibited diminution of the splenic white pulp. All rats treated with both CyA and Fr showed the same degree of marrow and lymphoid changes noted after CyA alone.

Serum levels of CyA

Trough serum concentrations of CyA in animals treated either with CyA alone or CyA in combination with Fr are shown in Table 6. Whereas at day 7 there was no significant difference between the groups, a 2-fold increase in CyA level was observed in the combination group on day 14. Moreover, a linear correlation existed between the serum levels of CyA and both urea ($r = 0.89$; $P < 0.01$) and creatinine ($r = 0.80$; $P < 0.01$).

Table 5. Haemagglutinin titres in rats treated with CyA and Fr

Treatment	Titre ($-\log_2$) Day 14
Untreated	5.1 ± 0.5
Fr	4.6 ± 0.4
Cy A	<1.0*
CyA/Fr	<1.0*

Results are means ± S.D. for six animals and compared to those of the untreated group: *, $P < 0.001$.

Table 6. Serum CsA levels in rats treated with CyA and Fr

	CsA (μg/ml)	
Treatment	Day 7	Day 14
Cy A (4)	4.26 ± 1.23	6.91 ± 1.96
Cy A/Fr (4)	5.23 ± 0.95	13.80 ± 4.19*

Number of animals in parentheses. Results are means ± S.D. Groups compared at each time point by Student's *t*-test for independent samples. *, $P < 0.001$.

DISCUSSION

Evidence is accumulating that when other therapeutic agents are given as part of combination therapy the toxic properties of CyA may be enhanced. Thus gentamicin, cimetidine and the antifungal agent ketoconazole have all been shown to augment the nephrotoxicity of CyA [7, 8, 13, 14]. The aminoglycoside antibiotic gentamicin is nephrotoxic in its own right, whereas ketoconazole is known to depress hepatic cytochrome P-450-dependent drug metabolism [14]. Clearly, therefore, it is important to recognize such potentially hazardous drug combinations, especially as CyA also depresses hepatic cytochrome P-450 levels [15].

In previous studies, we have demonstrated that the nephrotoxicity associated with CyA is centred on the straight segment of the proximal renal tubule. However, in this study, we have shown that the proximal convoluted tubule may also be affected by CyA in the rat, an observation which has also been made in human transplant recipients treated with CyA [16]. In addition, we have shown that the administration of a non-toxic dose of frusemide (approximately 2-fold that recommended in man) together with CyA causes enhancement of the nephro- and hepatotoxicity observed with the immunosuppressant alone, as manifested either biochemically or histologically and without affecting the immunosuppressive property of the drug. Whether this enhancement is related to sodium depletion caused by frusemide, as postulated for the enhancement of gentamicin nephrotoxicity by the diuretic in the dog [9], awaits further investigation of the renal tubular handling and metabolism of CyA.

An alternative explanation for the enhancement of CyA toxicity by frusemide may be related to hepatic drug metabolism. High doses of frusemide (400 mg/kg) cause a 300% reduction in hepatic cytochrome P-450 levels and a 50% reduction in *N*-demethylation activity in the mouse [17]. This is manifested histologically as a structural change in the hepatic endoplasmic reticulum, an observation similar to that which we have previously reported with CyA [6]. Although toxic doses of frusemide were not used in this study, the combination of a potentially toxic dose (50 mg/kg) of CyA with a subtoxic dose of the diuretic may have resulted in a synergistic interaction, further reducing cytochrome P-450-dependent drug metabolism in the liver. This would in turn lead to higher serum levels of the parent CyA molecule, which we have previously suggested is responsible for the nephrotoxic effect of the drug [15]. Indeed, as we have clearly demonstrated in this study, Fr caused a doubling in trough CyA levels. Moreover, there was a good correlation between those and indices of renal function.

Extensive clinical use of frusemide has not resulted in any reports of liver damage, and the drug is presumably not hepatotoxic at normal therapeutic doses (40–600 mg/day) in man. However, frusemide is converted to a toxic arylating metabolite by microsomes in both mouse and man [18], and the safety of the drug at therapeutic dosage may be related to a dose-threshold phenomenon similar to that found in the mouse. Frusemide is eliminated from the body primarily by renal excretion of the unchanged drug [19], and impaired renal function as a result of CyA therapy would only serve to increase hepatic and other tissue levels, out of step with dosage regimes, resulting in further reduction in cytochrome P-450.

There is accumulating evidence that CyA-induced nephrotoxicity may be exacerbated by decreases in the hepatic metabolism of the drug. It is therefore clinically important that concomitant administration of CyA with other drugs which might also affect hepatic drug metabolism should be avoided. The likely outcome is enhancement of the toxic properties of the immunosuppressant due to a further reduction in cytochrome P-450-dependent drug metabolism.

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REFERENCES

1. J. F. Borel, C. Feurer, C. Magnée and H. Stähelin, *Immunology* **32**, 1017 (1977).
2. A. W. Thomson, *Aust. J. exp. Biol. med. Sci.* **61**, 147 (1983).
3. M. Y. Gordon and J. W. Singer, *Nature, Lond.* **279**, 422 (1979).
4. R. Y. Calne, D. J. G. White, S. Thiru, D. B. Evans, P. McMaster, D. C. Dunn, G. N. Craddock, B. D. Pentlow and K. Rolles, *Lancet* **ii**, 1323 (1978).
5. R. L. Powles, A. J. Barrett, H. M. Clink, H. E. M. Kay, J. Sloane and T. J. McElwain, *Lancet* **ii**, 1327 (1978).
6. A. W. Thomson, P. H. Whiting and J. G. Simpson, in *Cyclosporin A* (Ed. D. J. G. White), p. 177. Elsevier/North Holland, Amsterdam (1982).
7. J. Hows, S. Palmer, S. Want, C. Dearden and E. C. Gordon-Smith, *Lancet* **ii**, 145 (1981).
8. P. H. Whiting, J. G. Simpson, R. J. L. Davidson and A. W. Thomson, *Br. J. exp. Path.* **63**, 554 (1982).
9. R. D. Adelman, W. L. Spangler, F. Beasom, G. Ishizaki and G. M. Conzelman, *J. infect. Dis.* **140**, 342 (1979).
10. G. Takátsy, *Acta microbiol. Acad. Sci. Hung.* **4**, 365 (1955).
11. P. Donatsch, E. Abisch, M. Homberger, R. Traber, M. Trapp and R. Voges, *J. Immunoassay* **2**, 19 (1981).
12. A. W. Thomson, P. H. Whiting, J. T. Blair, R. J. L. Davidson and J. G. Simpson, *Transplantation* **32**, 271 (1981).
13. A. J. Wood, G. Maurer, W. Niederberger and T. Beveridge, *Transplantation Proc.* Suppl. (in press).
14. R. M. Ferguson, D. E. R. Sutherland, R. L. Simmons and J. S. Najarian, *Lancet* **ii**, 882 (1982).
15. C. Cunningham, P. H. Whiting, M. D. Burke, D. N. Wheatley and J. G. Simpson, *Transplantation Proc.* Suppl. (in press).
16. M. J. Mihatsch, *Transplantation Proc.* Suppl. (in press).
17. S. S. Thorgeirsson, H. A. Sasame, J. R. Mitchell, D. J. Jollow and W. Z. Potter, *Pharmacology* **14**, 205 (1976).
18. J. R. Mitchell, S. D. Nelson, S. S. Thorgeirsson, R. J. McMurty and E. Dybing, in *Progress in Liver Diseases* (Eds. H. Popper and F. Schaffner), p. 259. Grune & Stratton, New York (1976).
19. J. R. Mitchell, W. Z. Potter, J. A. Hinson, W. R. Snodgrass, J. A. Timbrell and J. R. Gillette, in *Concepts in Biochemical Pharmacology* (Eds. J. R. Gillette and J. R. Mitchell), p. 383. Springer, Berlin (1975).