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SHORT COMMUNICATIONS

Studies of *in vitro* treatment of rat liver plasma membranes with carbon tetrachloride

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PLASMA membranes isolated from liver of rats treated with carbon tetrachloride (CCl_4) show reduced activities of the three enzymes ($Na^+ - K^+$)-activated Mg^{2+}-dependent ATPase, Mg^{2+}-dependent ATPase (EC 3.6.1.3), and 5′-nucleotidase (EC 3.1.3.5).[1] The two ATPases of plasma membranes are dependent on associated lipids for maximal activity whereas 5′-nucleotidase appears not to have this requirement.[2] In view of the solubility of lipid in CCl_4, studies were made *in vitro* of the effects of this toxin on the activities of rat liver plasma membrane enzymes.

Plasma membranes were isolated from liver homogenates of male Sprague–Dawley rats (250–280 g) (Animal House, Department of Pathology, University of Melbourne), by the method of Neville[3], essentially, as modified by Emmelot *et al.*[4] The final membrane pellet was resuspended in 7 mM Tris-buffer pH 7·4 to a protein concentration of approximately 2 mg/ml and 0·1 ml samples were used in all assays. This isolation procedure gives a relative specific activity for 5′-nucleotidase of 20–25. Membrane preparations were further checked for purity by phase contrast microscopy, electron microscopy and the testing of enzyme activities characteristic of possible contaminating organelles. Enzyme assays were made on freshly prepared membranes according to the methods described by Emmelot *et al.*[5] except for the following; (a) assays were carried out in Warburg flasks without sidearms, (b) the plasma membrane samples were treated *in vitro* in the incubation medium at 37° without substrate and with 0, 5, 10, 20 or 100 μl respectively of CCl_4(AR) added to the centre well (using a Hamilton microsyringe) immediately prior to the stoppering of the flasks. Preincubation was carried out in this condition for 0, 5, 10 or 20 min after which time the assays were started by addition of substrate. The flasks were unstoppered during the actual incubation, and the assays were terminated by the addition of trichloroacetic acid. It should be noted that the stated μl quantities of CCl_4 do not represent concentrations as such, but the amounts of CCl_4 allowed to diffuse at 37° from the centre well of the Warburg flask into 1·6 ml of suspension containing plasma membranes. Enzyme activities are expressed as μmoles, inorganic phosphorus (*Pi*)[1] released/hr/mg membrane protein $\pm$S.D.

($Na^+ - K^+$)-ATPase showed no activity when plasma membranes were exposed to CCl_4 at the lowest concentration (5 μl) and for the shortest preincubation time of 5 min (Table 1). Activity of Mg^{2+} ATPase decreased progressively with increase in concentration of CCl_4 and was completely inactive when the incubation medium was saturated with CCl_4, 100 μl (Table 1). At the concentrations of CCl_4 tested the specific activity of Mg^{2+} ATPase was similar when the time of preincubation with CCl_4 was 5, 10 or 20 min. Activity of 5′-nucleotidase was unchanged in plasma membranes exposed to CCl_4 in the test system used in these experiments (Table 1).

TABLE 1.

CCl_4 (μ)	0	5	10	20	100
($Na^+ - K^+$)-ATPase	7·5 ± 3·8 n = 16	0	0	0	0
Mg^{2+} ATPase	132·3 ± 2·5 n = 4	101·0 ± 1·5 n = 4	68·1 ± 2·7 n = 4	16·1 ± 2·1 n = 4	0
5′-nucleotidase	81·4 ± 1·3 n = 4	82·1 ± 1·8 n = 4	86·0 ± 1·6 n = 4	84·3 ± 1·6 n = 4	80·0 ± 1·1 n = 4

Specific activities (±S.D.), expressed as μmoles inorganic phosphorus produced/hr/mg membrane protein, of plasma membrane ($Na^+ - K^+$)-ATPase, Mg^{2+} ATPase, and 5′-nucleotidase after preincubation for 10 min with different dose levels of CCl_4 *in vitro*.

Since the period of preincubation was found to be of little further consequence in depressing the activity of Mg^{2+} ATPase, perhaps CCl_4 does not chemically attack the enzyme *per se* in the *in vitro* system, but rather in some way affects its affinity for substrate. The observed changes may have been a manifestation of the action of CCl_4 on the associated lipid. This suggestion is based on the fact that $(Na^+ - K^+)$-ATPase and Mg^{2+} ATPase activities were both shown to be dependent on associated lipid for activity and were affected by CCl_4 *in vitro*, while 5′-nucleotidase was not shown to be lipid-dependent for activity and was not affected by CCl_4 *in vitro*.[2] It has been shown that 5′-nucleotidase is a lipoprotein with only one phospholipid, sphingomyelin associated with it, but this enzyme's dependence on the associated lipid was not necessarily indicated.[6]

The above proposal was tested by the removal of as much CCl_4 as possible from the incubation media after preincubation, but prior to the starting of the assay by the addition of substrate. CCl_4 was removed by subjecting the flasks containing the incubation medium plus membranes to a low air pressure (about 12 mm Hg) for 15 min at 2° following the period of preincubation. Plasma membranes were treated as above, but were tested with 10 or 100 μl of CCl_4 with a 10 min preincubation. As shown in Table 1 these conditions (i.e. when the CCl_4 was not removed prior to adding substrate) were suitable to completely inactivate $(Na^+ - K^+)$-ATPase and Mg^{2+} ATPase respectively. Flasks containing both control and test material were evacuated or left at atmospheric pressure then substrate added. Data from estimations of activities of $(Na^+ - K^+)$-ATPase and Mg^{2+} ATPase with and without removal of CCl_4 is given in Table 2. The data shows that there was a partial recovery of activity of both enzymes when CCl_4 was removed from the incubation medium by this means.

TABLE 2.

CCl_4 (μl)	0	10		100	
	Evacuated and atmospheric pressure	Atmospheric pressure	Evacuated	Atmospheric pressure	Evacuated
$(Na^+ - K^+)$-ATPase	7·5 ± 3·8 n = 16	0 n = 16	3·2 ± 2·1 n = 16	—	—
Mg^{2+} ATPase	132·3 ± 2·5 n = 4	66·3 ± 0·6 n = 4	71·2 ± 2·0 n = 4	0 n = 4	59·1 ± 2·2 n = 4

Specific activities (±S.D.), expressed as μmoles inorganic phosphorus produced/hr/mg membrane protein, of plasma membrane $(Na^+ - K^+)$-ATPase and Mg^{2+} ATPase after preincubation for 10 min with 0, 10, or 100 μl CCl_4 followed by 15 min evacuation (12 mmHg) at 2°.

In the present study the plasma membrane associated enzymes $(Na^+ - K^+)$-ATPase, and Mg^{2+} ATPase were both found to decrease in activity following *in vitro* treatment with CCl_4, while 5′-nucleotidase remained unchanged. These ATPases were found to recover activity, at least partially, with the removal of CCl_4 from the incubation media. It is considered possible that CCl_4 passes into or in some way associates with the membrane lipids, altering the tertiary or quaternary structure of the enzyme protein thus lowering its affinity for substrate, and with the removal of CCl_4 a partial return to active configuration is achieved. Lipid dependent enzymes have been located as components of mitochondrial and microsomal membranes.[7–11] The precise role of this lipid in the activity of these enzymes is not yet fully understood, though certain possibilities have been discussed.[2] It would seem most likely that the lipids hold the enzyme protein in a state of required activity by imposing certain tertiary or quaternary conformations possibly by electrostatic and/or hydrophobic interactions. In the case of $(Na^+ - K^+)$-ATPase, lipid may be involved in the coupling of the component reactions on opposite sides of the membrane, and in other instances, holding enzyme components in pathway sequence.

In the present experiments conditions of incubation did not favour the metabolism of CCl_4 and therefore the formation of active radicals, such as are considered responsible for the *in vivo* toxicity of CCl_4 on the liver.[12] Nevertheless we have demonstrated an effect, possibly a direct effect of CCl_4 on

liver cell plasma membrane ATPases. This kind of effect is reversible and it may be considered as having a possible marginal role in the pathogenesis of the liver lesion initiated by CCl_4.

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REFERENCES

1. R. N. LE PAGE and P. R. DORLING, *Aust. J. exp. biol. med. Sci.* **49,** 345 (1971).
2. P. EMMELOT and C. J. BOS, *Biochim. biophys. Acta* **150,** 341 (1968).
3. D. M. NEVILLE, *J. biophys. biochem. Cytol.* **8,** 413 (1960).
4. P. EMMELOT, C. J. BOS, E. L. BENEDETTI and PH. RÜMKE, *Biochim. biophys. Acta* **90,** 126 (1964).
5. P. EMMELOT and C. J. BOS, *Biochim. biophys. Acta* **120,** 369 (1966).
6. C. C. WIDNELL and J. C. UNKELESS, *Proc. natn. Acad. Sci. U.S.A.* **61,** 1050 (1968).
7. I. SEKUZU, P. JURTSHUK and D. E. GREEN, *J. biol. Chem.* **238,** 975 (1963).
8. S. FLEISCHER, G. BRIERLEY, H. KLOUWEN and D. B. SLAUTTERBACK, *J. biol. Chem.* **237,** 3264 (1962).
9. A. MARTONOSI, *Biochem. biophys. Res. Commun.* **13,** 273 (1963).
10. R. TANAKA and K. P. STRICKLAND, *Archs Biochem. Biophys.* **111,** 583 (1965).
11. R. COLEMAN and G. HUBSCHER, *Biochim. biophys. Acta* **56,** 479 (1962).
12. T. C. BUTLER, *J. Pharmac. exp. Ther.* **134,** 311 (1961).

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Effects of 5-diazoimidazole-4-carboxamide and 3-diazopyrazole-4-carboxamide and related thioazo compounds on xanthine oxidase, uricase, and hypoxanthine–guanine phosphoribosyltransferase

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RECENTLY, Iwata *et al.*[1] showed that 5-diazoimidazole-4-carboxamide (Diazo-ICA) and its thioazo compounds, but not triazeno compounds, were potent inhibitors of rat liver xanthine oxidase and milk xanthine oxidase, both *in vivo* and *in vitro*. This finding stimulated further investigations of the effects of these compounds on purine metabolism.

This communication describes the actions of Diazo-ICA and its ring analog, 3-diazopyrazole-4-carboxamide (Diazo-PCA), and related thioazo derivatives on xanthine oxidase, uricase and hypoxanthine–guanine phosphoribosyltransferase (HG–PRTase) *in vitro*.

Bovine milk xanthine oxidase was prepared by the method of Klenow and Emberland.[2] Xanthine oxidase activity was determined by a modification of the methods described by Plesner and Kalckar,[3] and by Della Corte and Stirpe.[4] Uricase activity was determined by the method of Fridovich.[5] A soluble powder of bovine kidney uricase (Type II) was used as the uricase preparation. The HG–PRTase activity of rat erythrocyte hemolysates was assayed isotopically by the method of Kelley *et al.*[6]

Table 1 shows the relative potencies of Diazo-ICA and Diazo-PCA and their thioazo derivatives as inhibitors of xanthine oxidase, uricase and HG–PRTase; for comparison the inhibition of xanthine oxidase by allopurinol, of uricase by oxonate, and of HG–PRTase by 6-mercaptopurine (6-MP) are included. In our previous paper,[1] Diazo-ICA and two related thioazo compounds, 5-(2-aminoethylthioazo)imidazole-4-carboxamide (Aminoethylthioazo-ICA) and 5-(2-amino-2-carboxyethylthioazo)-