The cells used in the present study were washed in saline. Since washing is known to affect the coagulant properties of platelets(5), this may have affected the venom sensitivity in the present experiments. If so, it may indicate that the washing process exposes phospholipid molecules in the platelet but not in the red cell. It may be that the intact (unwashed) platelet is also resistant to venom action but that washing in saline removes cell components that interfere with the action of the enzyme. The red cell is clearly not affected in the same way, perhaps because the non-lipid membrane components are more firmly bound or qualitatively different. These considerations, admittedly speculative, may also be pertinent with regard to the availability of phospholipids for the coagulation process.

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Phenothiazine Inhibition of Carbon Tetrachloride Cytotoxicity *in vitro** (31633)

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The hepatotoxicity of carbon tetrachloride has been demonstrated by administering the agent to the intact animal(1-4), by perfusing the isolated liver with this agent(5,6), and by exposing isolated liver cell mitochondria to it(7). A number of studies have demonstrated that the hepatotoxic effects of CCl_4 are reflected in the elevation of a number of enzymes in the plasma(8-12). Studies in this laboratory have shown that the cytotoxicity of CCl_4 *in vitro* also can be demonstrated by the leakage of intracellular enzymes from non-hepatic cells (grown in tissue culture) after brief exposure to the agent(13).

The present study was designed to evaluate the suitability of the *in vitro* system employing non-hepatic cells as a model for the study of CCl_4 hepatotoxicity. It demonstrated that agents known(14-18) to protect against CCl_4 hepatotoxicity *in vivo* could prevent manifestations of cytotoxicity *in vitro*.

Methods and materials. The cells studied were the strain of laryngeal carcinoma cells maintained in tissue cultures (H. Ep. #2) originally isolated by Moore, Sabuheurh, and

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Toolan(19) that were employed in our previous studies(13). For each experiment, 1.5 ml of cell suspension containing approximately 250,000 cells/ml, were transferred to each of a number of Leighton tubes containing fresh medium. After 72 hours of growth, the cells were harvested and the spent medium discarded. The cells were then suspended in fresh medium (controls), or in fresh medium to which graded amounts of CCl_4 alone or with various amounts of chlorpromazine or promazine had been added. In all instances there were approximately 10^6 cells suspended in 1.5 ml of medium. At the end of 60 min-

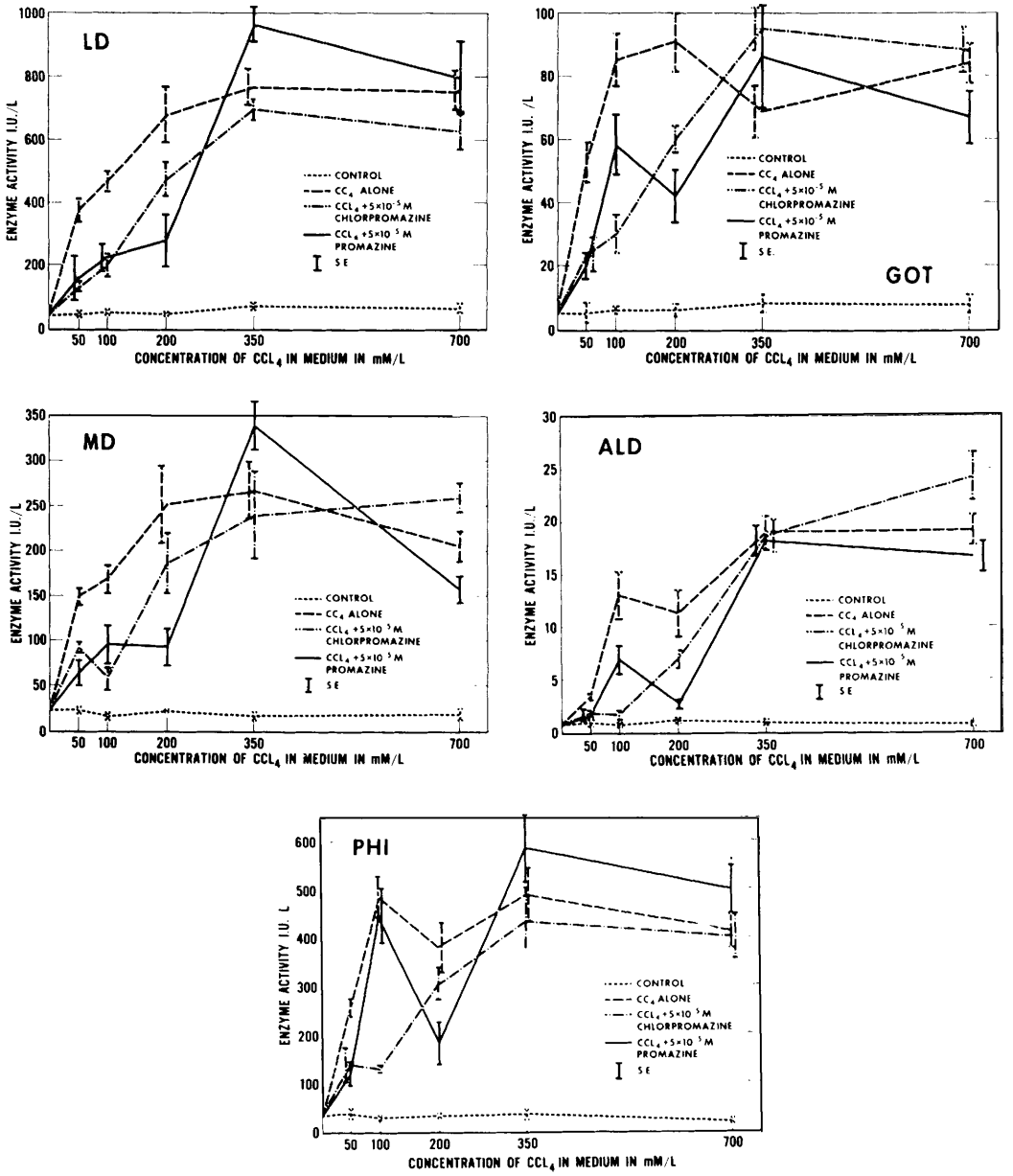

FIG. 1. Enzyme levels in levels in medium in international units per l, after 60-min exposure of cells to various concentrations of CCl_4 alone or in presence of 5×10^{-5} M chlorpromazine or promazine. "Control" refers to enzyme level in medium of untreated cells. See text for abbreviations for respective enzymes.

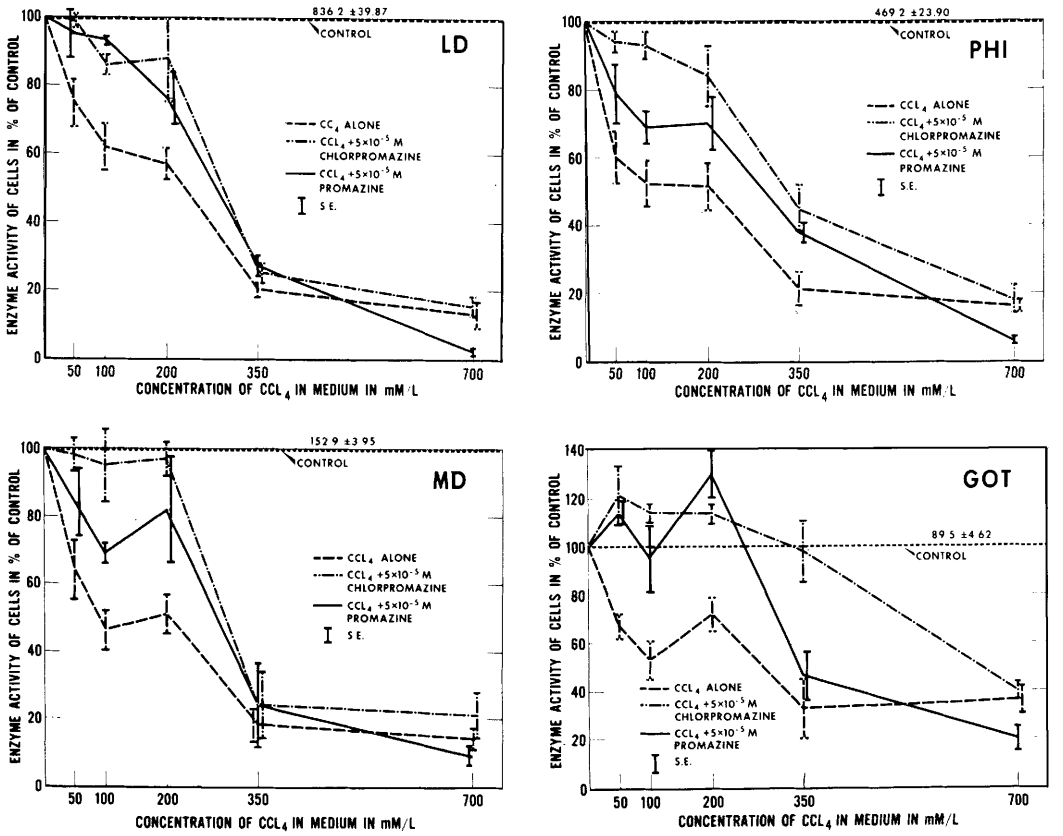

FIG. 2. Enzyme levels of cells in per cent of control after 60-min exposure of cells to various concentrations of CCl_4 alone or in presence of 5×10^{-5} M chlorpromazine or promazine. "Control" value, shown in international units per l, at top of each graph, represents enzyme levels of untreated cells. See text for abbreviations for respective enzymes.

utes, the cells were separated from the medium by centrifugation and the enzyme activity of cells and supernatant were each measured. Previously described methods were used to determine the activity of glutamic oxalacetic transaminase (GOT), lactic dehydrogenase (LD), aldolase (Ald), phosphohexose isomerase (PHI), and malic dehydrogenase (MD)(13).

Results. Effects of increasing concentrations of CCl_4 and modification by a fixed concentration of phenothiazine. Exposure of cells to CCl_4 in concentration of 5×10^{-2} to 70×10^{-2} M led to the appearance of all enzymes measured in the medium (Fig. 1) and decrease in the content of the cells (Fig. 2). The addition of chlorpromazine or promazine in a concentration of 5×10^{-5} M significantly inhibited the leakage of enzymes induced by CCl_4 concentrations between 5 and 20×10^{-2} M. The phenothiazine failed to prevent the effects of CCl_4 at a concentration of 70×10^{-2} M and yielded equivocal modification of the effects of a concentration of 35×10^{-2} M of CCl_4. The effects of the two phenothiazines were similar (Fig. 1 and 2).

Effects of graded concentration of phenothiazine on the enzyme leakage induced by a fixed concentration of carbon tetrachloride. The effect of various concentrations of each of the 2 phenothiazines on the increase in enzyme content of the medium and the decrease in enzyme content of the cells, induced by exposure to 10×10^{-2} M concentration of CCl_4, is shown in Fig. 3 and 4 respectively. Decreases in the enzyme leakage were observed at concentrations ranging from 1×10^{-5} to 5×10^{-5} M for both chlorpromazine and promazine. For promazine, this inhibi-

tion was most apparent at a concentration of 2×10^{-5} M and for chlorpromazine at 5×10^{-5} M. At higher concentrations of phenothiazine of 10×10^{-10} M, or above, the inhibition of the CCl_4 effect was decreased or became negligible. Indeed, at concentrations of 20×10^{-5} M, the phenothiazines seemed to enhance the CCl_4 induced leakage of LD

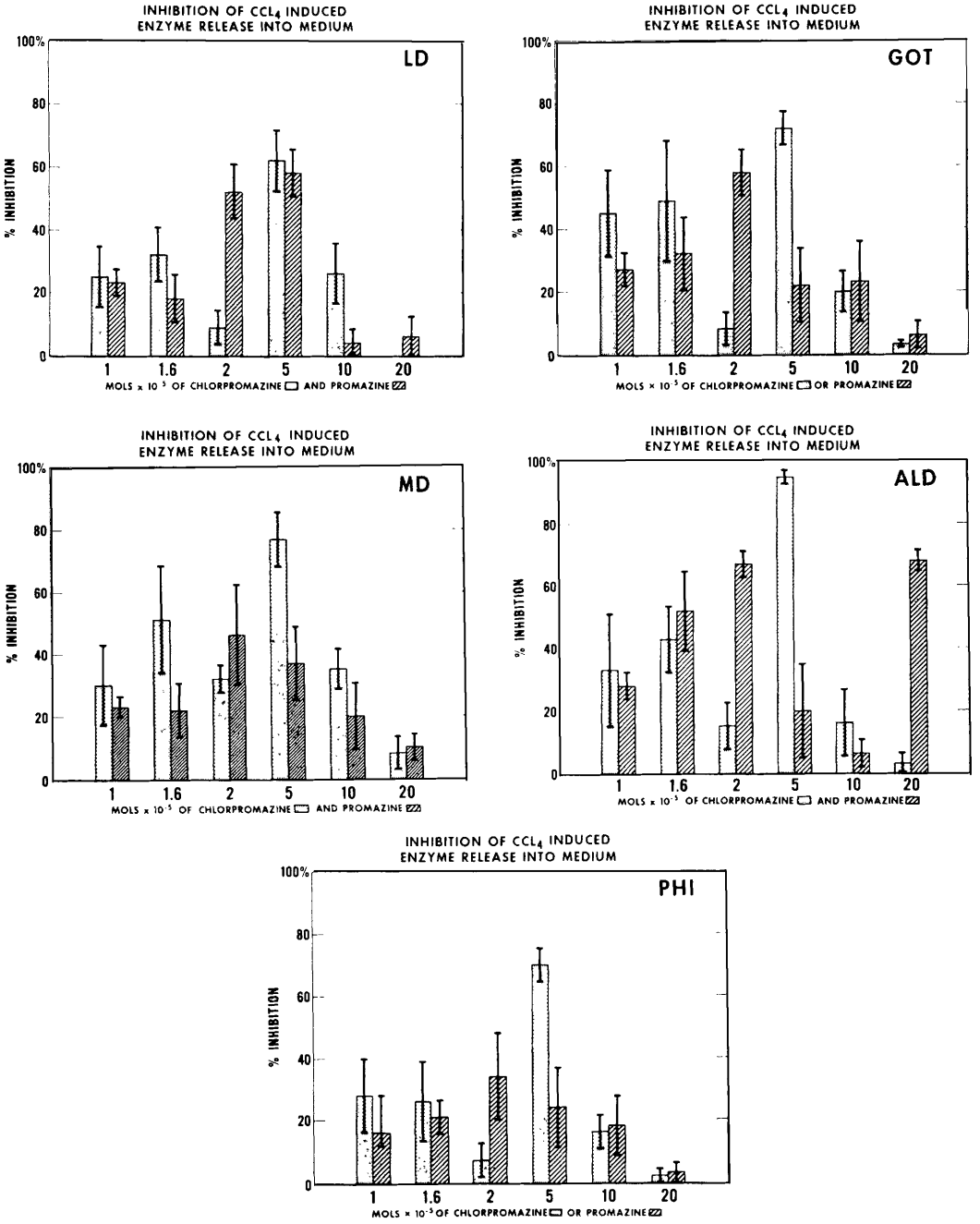

FIG. 3. Effect of various concentrations of chlorpromazine or promazine on enzyme leakage into medium induced by 200 mM concentration of CCl_4. Effect is expressed as per cent inhibition of leakage induced by this dose of CCl_4 alone. See text for abbreviations for respective enzymes.

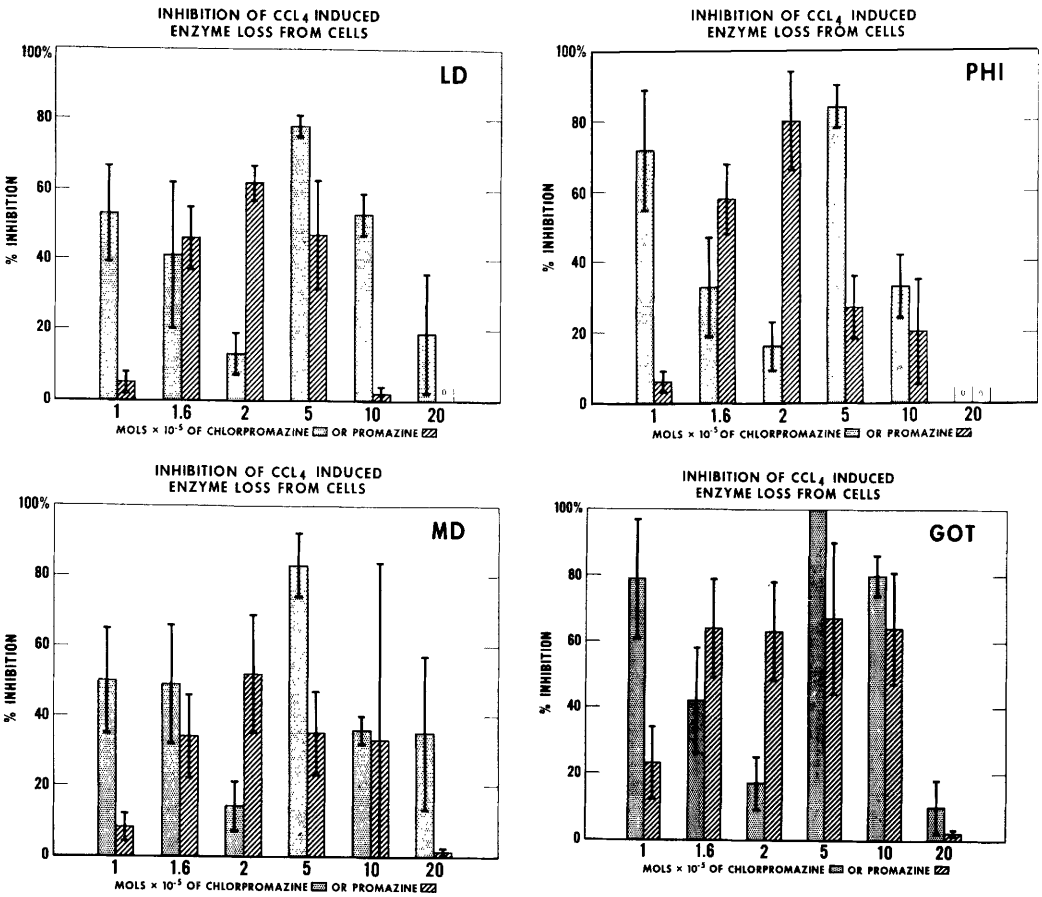

FIG. 4. Effect of various concentrations of chlorpromazine or promazine in enzyme loss from cells induced by 200 mM concentration of CCl_4. Effect is expressed as per cent inhibition of loss induced by this dose of CCl_4 alone. See text for abbreviations for respective enzymes.

and PHI by approximately 20%.

Discussion. The leakage of enzymes from non-hepatic cells induced by carbon tetrachloride *in vitro,* demonstrated in this and in a previous study(13) supports the view that carbon tetrachloride is a protoplasmic poison which can exert its effects on a variety of tissues. The validity of this *in vitro* demonstration of CCl_4 cytotoxicity, as a model for the study of direct hepatotoxins, is supported by the "protective" effects of phenothiazines demonstrated in the present study.

Several phenothiazines (chlorpromazine, promazine, promethazine) have been shown to prevent hepatic necrosis induced by hepatotoxins(14-18). This protective effect appears to be a manifestation of the membrane-stabilizing effects of this series of compounds(18, 20,21). The observation that the protective effect of these agents against CCl_4-induced necrosis *in vivo* can be demonstrated by the inhibition of serum enzyme rise is paralleled by the *in vitro* observations of the present study.

The lowest concentration of CCl_4 used in this study is higher than that found in blood or in liver of intact animals given hepatotoxic doses of CCl_4(22). However, lower concentrations of CCl_4, in a range similar to that of the hepatic concentrations after systemic hepatotoxic doses, also have been shown to produce enzyme leakage from non-hepatic cells *in vitro.*

The concentrations of phenothiazines, shown to inhibit CCl_4-induced enzyme leakage are similar to those used in other *in vitro*

studies(18) with "membrane stabilizers." The effective concentration of promazine and chlorpromazine appeared to be quite specific. Concentrations as high as 10^{-4} M and as low as 1.6×10^{-5} M were ineffective; or less effective than those intermediate. Furthermore, the optimal "protective" concentration of phenothiazine ($2\text{-}5 \times 10^{-5}$ M) inhibited the effects of concentrations of CCl_4 of 20×10^{-2} M or below, but not of higher concentrations. These relatively precise concentration relationships suggest that the *in vitro* CCl_4 effect and its inhibition by phenothiazines are specific biologic phenomena. The evidence that enzyme leakage from nonhepatic cells can be induced by exposure to specific concentrations of CCl_4 *in vitro*, which can be inhibited by "membrane stabilizers," is consistent with the view that CCl_4 is a protoplasmic poison which exerts its hepatotoxic effects by a general cytotoxicity mediated through adverse effects on cellular membranes. The *in vitro* model described here would appear to be useful for the study of the mechanism for these effects and for that of the "membrane-protective" effects of various compounds.

Summary. Enzyme leakage from nonhepatic cells, induced by *in vitro* exposure to CCl_4 was inhibited by simultaneous exposure to promazine or chlorpromazine. Concentrations of either phenothiazine of $2\text{-}5 \times 10^{-5}$ were more effective than higher or lower ones. It seems likely that this phenomenon is an *in vitro* equivalent of the "protective effects" of the phenothiazines against hepatotoxicity *in vivo*.

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