tal food fermentation is considered significant for those people whose diets are often nutritionally inadequate. Conceivably ingestion of this material may confer disease resistance.

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## Cytotoxicity of Phenothiazines on Chang Liver Cells as Measured by Enzyme Leakage (33931)

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Previous studies in this laboratory have utilized *in vitro* systems to demonstrate cytotoxicity of known and suspected hepatotoxic agents (1–3). Exposure to $CCl_4$ of suspensions of cells grown in tissue cultures led to rapid loss of intracellular enzymes (1). Chlorpromazine (CPZ), 10-(dimethylaminopropyl)-2-(chlorphenothiazine hydrochloride), an agent known to produce jaundice or hepatic dysfunction in humans (4), also led to leakage of intracellular enzymes from rabbit liver slices (3), while promazine (PZ) 10-(dimethylaminopropyl)-(phenothiazine hydrochloride) an agent which rarely produces hepatic injury in man (4) did not lead to this effect (3). In a further effort to develop a suitable *in vitro* model for the testing of hepatotoxicity, human liver cells (Chang) from tissue culture lines were exposed to PZ and CPZ at different concentrations. Leakage of lactate dehydrogenase, malate dehydrogenase, and asparatate aminotransferase into the medium was utilized as an index of injury to the liver cells.

*Material and Methods.* Chang human liver cells from tissue culture were obtained from Flow Laboratories, Rockville, Maryland. The cells were processed within 24 hr of arrival during which time they were kept at 4° refrigeration. Immediately before the experiment, the cells were centrifuged for 10 min at 1000 rpm, the original medium was discarded, and the cells were suspended in Hanks' base media 119 (IX), free of antibiotics and tested free of enzyme activity. For each experiment 1 ml of suspended cells containing approximately $10^6$ cells/ml was mixed with 1 ml of the same medium used for suspension (controls) or 1 ml of the medium containing CPZ[3] or PZ[3] in different concentrations. The

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[3] Chlorpromazine hydrochloride (CPZ) and promazine hydrochloride (PZ), as the pure powders were each made available through the courtesy of the respective manufacturers: Smith, Kline and French, Philadelphia; and Wyeth Laboratories, Radnor, Pennsylvania.

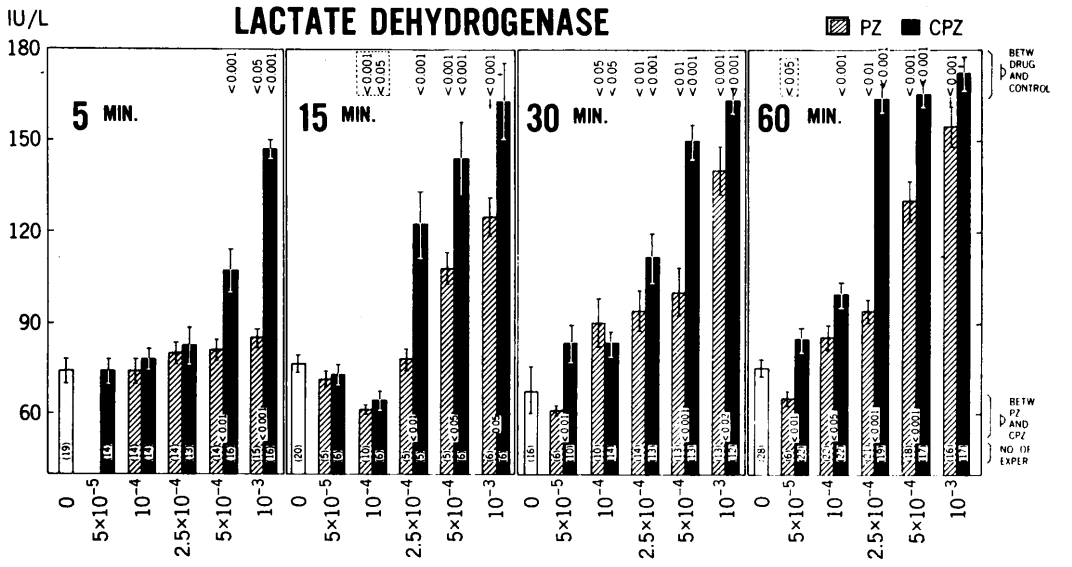


FIG. 1. Levels of activity of LDH in medium after exposure for periods ranging from 5 to 60 min at 37°, to promazine (PZ) or chlorpromazine (CPZ) at concentrations shown along abscissa: values for the mean and standard error (IU/liter) are shown by height of bars; $p$ values are stated if 0.05 or less (obtained by Student's $t$ test).

final concentration of each drug in the systems ranged from $5 \times 10^{-5}$ to $10^{-3}$ $M$ (Fig. 1–3). The pH of the media was adjusted to 6.7 for all solutions of drugs and controls to fulfill the pH requirement for optimal solubility of the phenothiazines. Samples were incubated for 5, 15, 30, and 60 min after which time they were centrifuged for 5 min

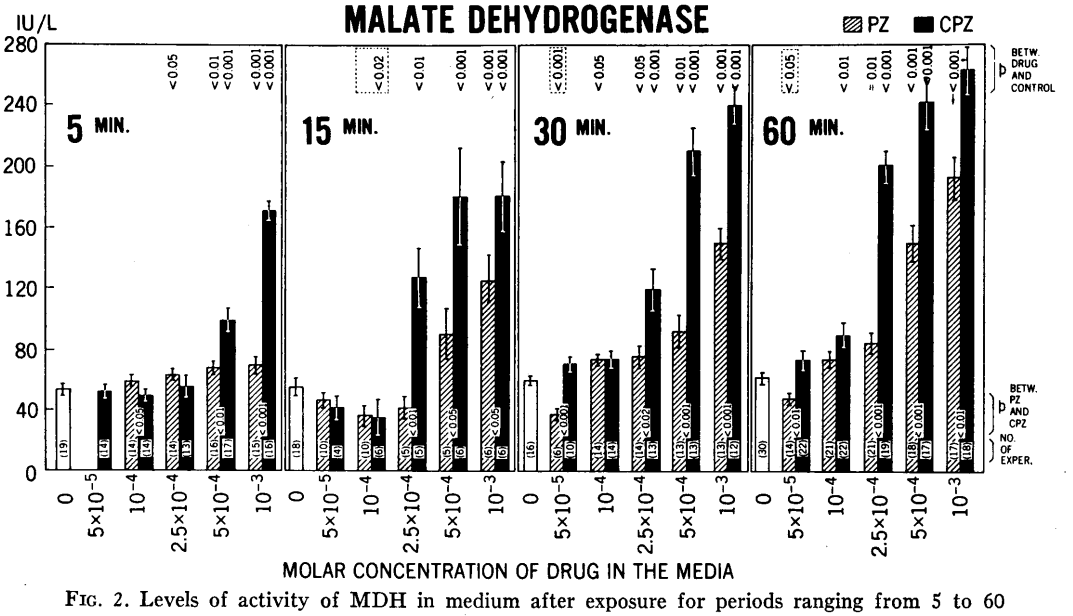


FIG. 2. Levels of activity of MDH in medium after exposure for periods ranging from 5 to 60 min at 37°, to promazine or chlorpromazine at concentrations shown along abscissa: Values for the mean and SE (IU/liter) are shown by height of bars; $p$ values are stated if 0.05 or less (obtained by Student's $t$ test).

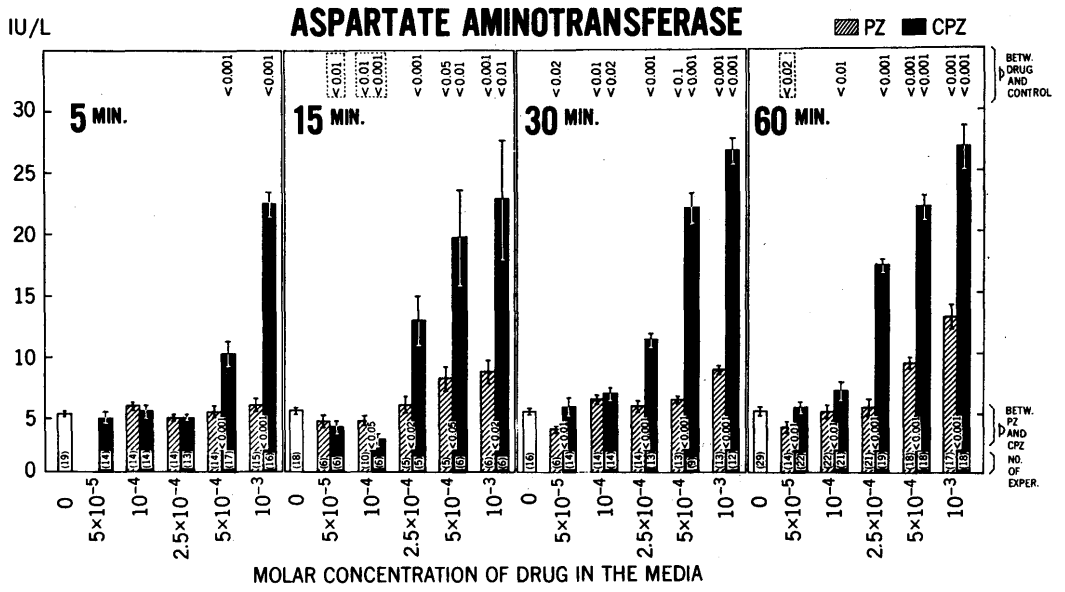


FIG. 3. Levels of activity of GOT in medium after exposure for periods ranging from 5 to 60 min at 37°, at concentrations shown along abscissa: values for the mean and SE (IU/liter) are shown by height of bars; $p$ values are stated if 0.05 or less (obtained by Student's $t$ test).

at 1000 rpm. The supernatant was decanted for enzyme assay. The aspartate aminotransferase [glutamic-oxalacetic transaminase (GOT)],[4] lactate dehydrogenase (LDH)[4] and malate dehydrogenase (MDH)[4] were measured according to previously reported methods (1). The activity of these enzymes tested as "pure preparations"[5] was not significantly affected by either of the phenothiazines (CPZ and PZ) when tested at the concentrations and conditions utilized in the study. All assays were conducted in duplicate.

*Results.* Values for enzyme activity in the medium after exposure of cells to each phenothiazine in varying concentrations, for periods of 5–60 min are shown in Fig. 1–3. Variable degrees and rates of leakage occurred after exposure to each drug depending on the concentration. Chlorpromazine appeared more potent in producing this effect as demonstrated by the "leakage" induced at lower concentrations, the greater rapidity of release at high concentrations, and the greater apparent amount of enzyme leaked when equal concentrations of the two phenothiazines were compared (Fig. 1–3).

Concentrations of CPZ of $5 \times 10^{-4}$ $M$ by 5 min and of $2.5 \times 10^{-4}$ $M$ by 15 min led to leakage of the three enzymes from the cells in amounts significantly greater than those of the controls. For PZ to produce leakage of all three enzymes at a concentration of $5 \times 10^{-4}$, required exposure for 30 min. In the exposures for 60 min, concentrations of CPZ, but not of PZ, as low as $10^{-4}$ $M$ were able to induce leakage of all three enzymes.

At all the "effective" concentrations and durations of exposure, the amount of enzyme that apparently leaked into the medium was greater after exposure to CPZ than to PZ. In general, LDH appeared to leak from the cells at lower concentrations of either drug and after shorter periods than did the two other enzymes. Demonstrable leakage of aspartate aminotransferase appeared to require higher concentrations of the drugs or longer exposure periods. Malate dehydrogenase occupied an intermediate position in this regard.

The medium used contained no enzyme activity before the cells were suspended in it.

[4] International Union of Biochemistry (IUB) classification of these enzymes are: GOT = 2.6.1.1; LDH = 1.1.1.27; MDH = 1.1.1.37.

[5] Obtained from Sigma Chemical Company, St. Louis, Missouri.

The activity that was detected in the medium of the control preparations after incubation of cells derived presumably from spontaneous leakage from the cells. Low concentrations ($5 \times 10^{-5}$ *M*) of both phenothiazines at some periods appeared to inhibit this spontaneous leakage. This was more clearly evident for PZ than CPZ (see *p* values inside broken line rectangles in Fig. 1–3).

*Discussion.* The demonstration in the present study of enzyme leakage from tissue culture cells of hepatic origin exposed to drugs *in vitro*, is presumptive evidence that those drugs produce damage to the cells or alter the permeability of their membranes. The effect of both phenothiazines on the enzyme leakage was dependent on the concentration of the drug and the duration of exposure. Chlorpromazine appeared to have exerted a greater effect than PZ, since lower concentrations and shorter incubation periods were necessary for CPZ to produce enzyme leakage from the cells into the medium. Furthermore, under the same condtions of concentration and time, CPZ led to higher levels of the enzymes in the medium.

Spirtes and Guth (5) demonstrated that low concentrations of phenothiazines protect mitochondria against osmotic swelling while high concentrations ($3–5 \times 10^{-4}$ *M*) appear to enhance this adverse effect. The present study also demonstrated that low concentrations ($5 \times 10^{-5}$ *M*) of the phenothiazines prevented spontaneous enzyme leakage from cells, while higher concentrations ($5 \times 10^{-4}$ *M*) induced or accelerated leakage. High concentrations also inhibited respiration and uncoupled oxidative phosphorylation in mitochondria (6), and led to lysis of erythrocytes (7). Seeman and Bialy (8), showed that several phenothiazines lower the surface tension activity of Ringer solution. They found that PZ, (which differs from CPZ only by absence of a chlorine atom) required a concentration six times as great as that of CPZ to produce an equal effect, and drew attention to the correlation between these observations and the different therapeutic potency of the two compounds. They inferred that different effects of the two drugs on the surface tension activity might result not only in substantial differences of action on enzymes residing in the cell membranes, but also on damage to cell structures. Guth *et al.* (9) demonstrated that the CPZ effect on lysosomal enzyme released *in vitro* also appeared *in vivo* with a similar and proportional dose–effect relationship.

The studies cited above and the results of the present study indicate that both phenothiazines can exert an effect on the membranes of the cells and their organelles at a concentration approximately equal to those reached in the blood and liver parenchyma in humans who receive these drugs (10, 11). Each drug in physiologically obtainable concentrations, produced enzyme "leakage" from the cells into the surrounding media as early as 5 min after exposure. This rapidity of effect may constitute evidence of noxious effect on the cell membranes by the unchanged drug. No attempt was made in the present study, however, to establish the presence of metabolic products of the drugs. Studies with these compounds indicated that metabolites may be as active pharmacologically as the unchanged drug (12). Whether this applies to potential toxic activity is unclear.

Promazine apparently had less damaging effect than CPZ on the membranes in the present study. Moreover, when at lower concentrations, both drugs appeared to prevent "spontaneous leakage" of enzymes (as deduced by comparison with the controls), PZ seemed more effective than CPZ. This phenomenon appears to parallel the previous observations from this laboratory of phenothiazine inhibition of $CCl_4$ cytotoxicity *in vitro* (2). Phenothiazines have prevented apparent injury to cells and organelles exposed to noxious agents and prevented hepatic necrosis *in vivo* (13).

The results of the present study support the view that phenothiazines may exert a toxic effect on cellular membranes of organs in which, as in the liver, these drugs are sufficiently concentrated. Phenothiazine-induced hepatic injury may, in part, be a result of this toxic effect on membranes. The hepatotoxic potency of the individual com-

pound, would depend on the chemical composition and the quantitative tissue levels and presumably would be modified by individual idiosyncracy of the host. Chlorpromazine produced impaired liver function in approximately 50% of patients who received this drug and jaundice in 1–5% of recipients (14–17). Promazine apparently did not produce liver dysfunction (18) and rarely was reported to produce jaundice (4). This lower incidence of promazine-induced hepatotoxic effects in man appears to be paralleled by the different cytotoxic potency *in vitro* demonstrated in the present study.

The experimental model and the results described suggest that this system of cultured human liver cells might serve as a suitable experimental method for routine testing of drug hepatotoxicity. The applicability of data derived from *in vitro* conditions to the *in vivo* hepatotoxicity remains to be established. Particularly uncertain is the relevance of adverse effects of CPZ on isolated cells to the intrahepatic cholestasis induced by the drug in humans.

*Summary.* Suspensions of cultured human fetal liver cells were exposed to promazine (PZ) or chlorpromazine (CPZ) at concentrations ranging from $10^{-3}$ to $10^{-5}$ *M* for periods of 5–60 min. Identical preparations without drugs served as controls. The levels of three enzymes: lactate dehydrogenase, malate dehydrogenase and asparatate aminotransferase were measured in the surrounding media. The differences between the controls and drug preparations were considered to be the result of cytotoxic effect of the drugs. The CPZ appeared more potent in its cytotoxic activity than did PZ, as demonstrated by the effectiveness at lower concentrations and the greater rapidity and amount of enzymes released from the cells at equal concentrations. The results obtained may have a bearing on the known differences in the development of apparent hepatotoxicity in humans who receive the two phenothiazines.

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