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STUDIES *IN VITRO* AND *IN VIVO* OF THE EFFECTS OF CHLORPROMAZINE ON RAT LIVER LYSOSOMES

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Abstract—Evidence is presented demonstrating a stabilization of the rat liver lysosomal membrane by chlorpromazine both *in vivo* and *in vitro*. The lysosomal membrane is labilized *in vitro* by various incubation manipulations and *in vivo* by injection of vitamin A or *Escherichia coli* endotoxin. These data bear implications for some previously advanced hypotheses concerning the phenothiazine mode of action and may explain the protective effect of chlorpromazine in certain shock states.

THE RELEASE of hydrolytic enzymes from lysosomes has been implicated in many pathophysiological states such as traumatic shock,[1] endotoxin shock, and irradiation syndromes.[2, 3] In these same conditions and others wherein lysosomal enzyme release may be an important response to pathologic stimuli, chlorpromazine (CPZ), and other phenothiazines have a protective effect. For example, animals pretreated with CPZ have been protected against X-irradiation,[4] shock from endotoxins of Brucella,[5] *Escherichia coli*, *Salmonella typhosa*, and *S. marcescens*,[6] Noble-Collip shock,[7] hemorrhagic shock,[7] and heat and tourniquet shock.[8]

Preliminary investigations[9] have demonstrated the stabilizing effect of CPZ on isolated rat liver lysosomes *in vitro*. It was felt that an extension of these investigations to the *in vivo* situation might provide explanations for the protective effects of CPZ in the above-mentioned pathophysiological states.

METHODS

The *in vitro* investigations employed pH- and temperature-induced release of lysosomal enzymes. The *in vivo* investigations employed the lysosomal labilizers, *E. coli* endotoxin* and vitamin A.† Female Holtzman albino rats, 300–400 g weight, were used throughout these experiments.

In vitro

The light mitochondrial fraction of rat liver containing typical lysosomal enzyme activity was isolated by the technique of Sellinger and Verster[10] with the modification that the sucrose concentration was changed to 0·6 M. All subsequent incubations were in 0·6 M sucrose as well. Variations in pH, tonicity, and duration of incubation were made to determine the most suitable conditions for the study of lysosomal enzyme release. Experiments employing pH 5 and 0·6 M sucrose yielded greatest

* Difco Labs., Bacto lipopolysaccharide W, *E. coli* 0111: B_4.

† Mann Research Labs., vitamin A (dry), water dispersible.

total phosphatase activities and most sensitivity to the presence of CPZ. After incubation, the lysosomal suspension was centrifuged at 525,000 *g*-min, and supernatants were examined for acid phosphatase activity by the methods of Sellinger and Verster[10] and Fiske and Subbarrow,[11] and protein by the method of Folin and Ciocalteu.[12]

In vivo

Each experiment involved four rats deprived of food overnight. One rat served as control. One was injected with lysosomal labilizer (either endotoxin, 40 mg/kg, or vitamin A, 300,000 units/kg) i.p. One was injected with CPZ at various dose levels i.p. One rat was injected with both CPZ and a lysosomal labilizer. After 2 to 4 hr, the rats were sacrificed. When the postinjection interval was greater than 2 hr, it was found necessary to reinject the CPZ at the 2-hr period. After sacrifice, the livers were removed and the light mitochondrial fraction containing lysosomal enzyme activities harvested. Total acid phosphatase activity and total protein remaining in the light mitochondrial fractions were determined as previously.[11, 12]

Neither the endotoxin nor vitamin A proved to be a completely reliable lysosomal labilizer. With both substances there seemed to be a loss of labilizing activity on storage. Of the two, vitamin A was more reliable, and the bulk of the study was done with it. Four experiments employing trans-retinol in ethanol also demonstrated the

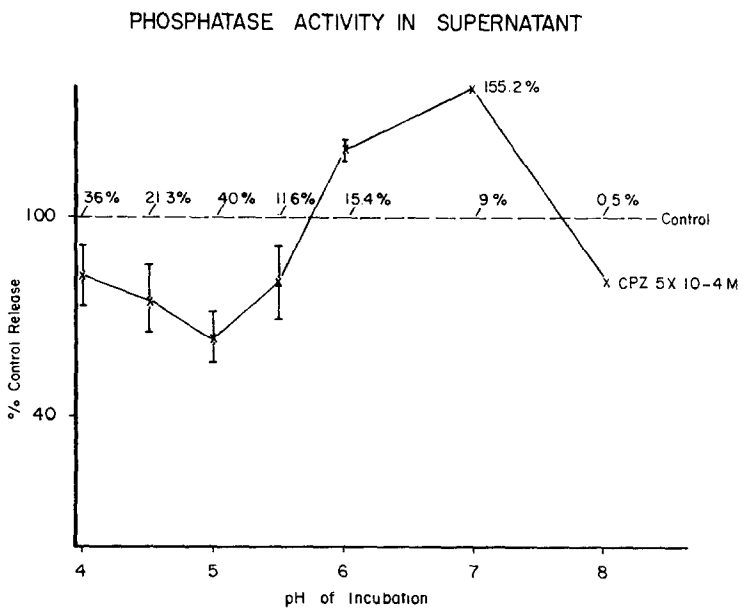

FIG. 1. Control releases of acid phosphatase activity into the supernatant upon incubation at various pH values for 1 hr at 37° in 0·6 M sucrose have been normalized at 100 per cent. The numbers along the horizontal 100 per cent line are the actual percentages of total (pellet plus supernatant) enzyme activity found in supernatant. The solid wandering line indicates the percentage of control supernatant enzyme activities found at various pH's in the presence of CPZ, 5×10^{-4} M. The bars are the standard errors of the means. Each point, except pH 7 and 8, is the mean of at least six determinations.

lysosomal labilizing action of vitamin A. These experiments also served as controls for the palmitate form used in the majority of experiments.

Appropriate control experiments were performed to determine whether CPZ affects enzyme activity or interacts with any of the reagents used in the determination of protein or acid phosphatase activity. No such interactions or inhibitions were found to occur.

RESULTS

In vitro

Figure 1 depicts the release of acid phosphatase activity into supernatant fluid upon incubation at various pH levels for 1 hr at 37° in 0·6 M sucrose. Note that pH 5 incubation yielded the greatest control release into the supernatant and was most susceptible to inhibition of release by drugs. The enhanced release occurring at pH 6 and 7 with CPZ might be due to greater drug binding at these pH's producing, in effect, a higher local concentration. However, there is no direct evidence on this point at the moment. Experiments with CPZ interactions with other phenomena have demonstrated a biphasic effect, generally inhibitory at low concentration and facilitatory at high.[13]

Figure 2 depicts both acid phosphatase activity release and total protein release into the supernatant fluid upon incubation for various times at pH 5, 37°, in 0·6 M

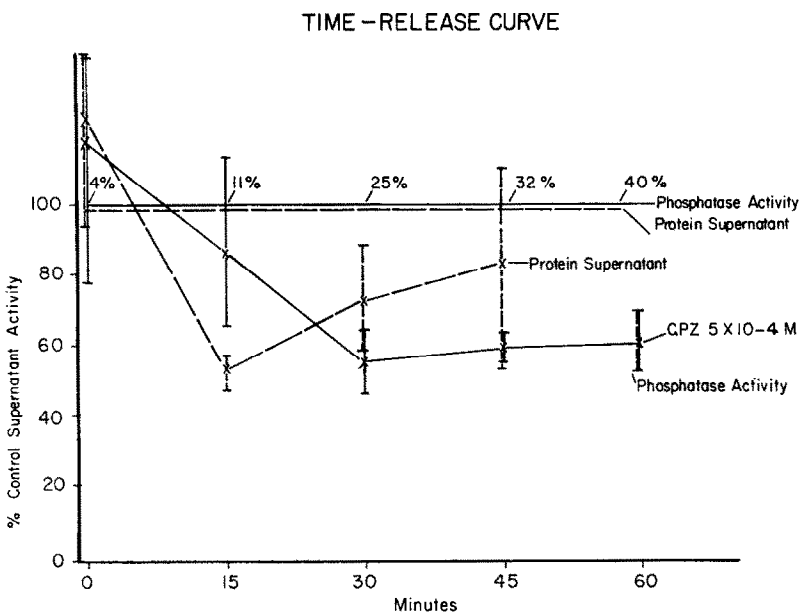

FIG. 2. Control releases both of acid phosphatase activity and total protein into the supernatant upon incubation at pH 5 in 0·6 M sucrose at 37° for various time periods have been normalized at 100 per cent. The numbers along the horizontal 100 per cent line are the actual percentages of total (pellet plus supernatant) enzyme activity found in the supernatant. The solid wandering line indicates the percentage on control supernatant enzyme activity found at various times in the presence of CPZ, 5×10^{-4} M. The dashed wandering line indicates the percentage of control supernatant protein found at various times, up to 45 min, in the presence of CPZ, 5×10^{-4} M. The bars are the standard errors of the means. Each point represents the mean of at least six determinations.

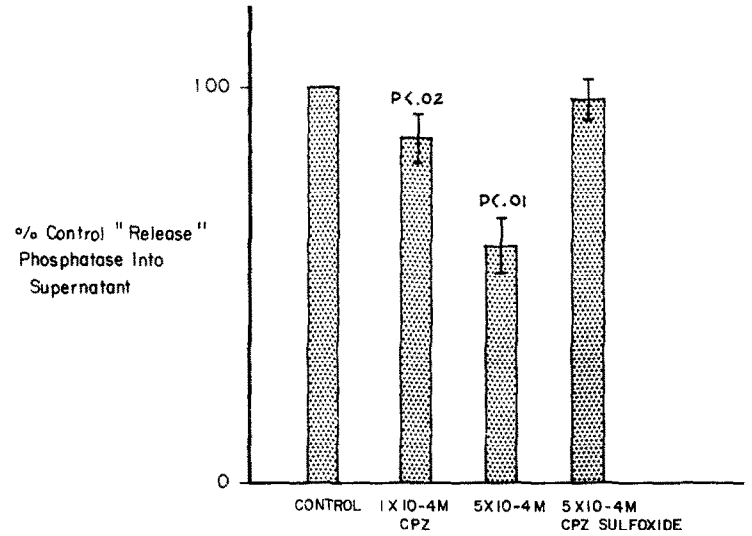

FIG. 3. Control releases of acid phosphatase activity into the supernatant upon incubation at pH 5, 37°, for 1 hr in 0·6 M sucrose have been normalized at 100 per cent. The additions of 1×10^{-4} M CPZ and 5×10^{-4} M CPZ diminish the release into the supernatant; bars indicate the standard errors of the means. Three experiments were performed with CPZ sulfoxide and six with each concentration of CPZ.

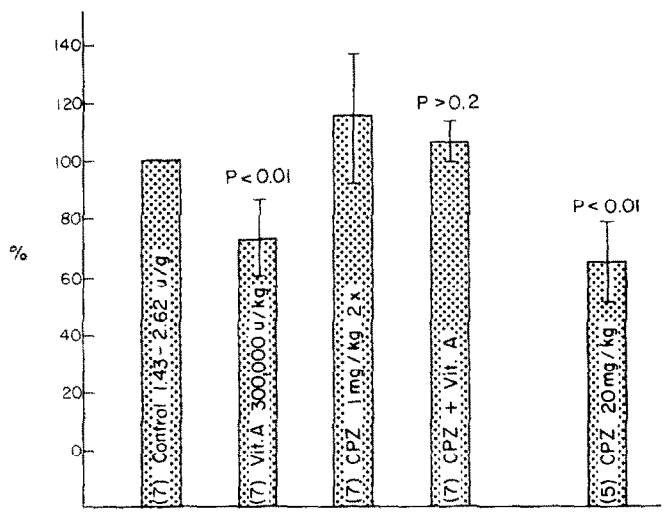

FIG. 4. *In vivo* antagonism of a lysosomal labilizer (A) by CPZ. Residual light mitochondrial fraction acid phosphatase activities of control rat liver have been normalized at 100 per cent. The actual range of values in enzyme activity units per gram of liver are given alongside the *control* column. The effects of vitamin A and CPZ alone and combined are expressed as percentages of the control figure; bars indicate the standard errors of the means. The numbers of experiments are given in parentheses at the bottoms of the columns. The animals were sacrificed 4 hr after injection. The 1 mg/kg dose of CPZ was administered twice at 2-hr intervals.

sucrose with and without CPZ at 5×10^{-4} M. Under these conditions, CPZ at 5×10^{-4} M inhibits the release of both protein and enzyme activity into the supernatant fluid. The apparent increased release at zero time is not significant.

Figure 3 compares the effects of different drug concentrations on release of acid phosphatase activity into supernatant under previously mentioned incubation conditions; *P* values, as determined by a Students "t" test, indicated that 5×10^{-4} and 10^{-4} M CPZ both produced significant inhibition of enzyme liberation. CPZ sulfoxide at 5×10^{-4} M, or CPZ at 1×10^{-3} M (not depicted), were unable to inhibit enzyme "leakage" or release. All these data were obtained under the previously mentioned incubation conditions.

In vivo

Figure 4 summarizes the most important *in vivo* data. Again, control values have been normalized to 100 per cent, but the actual range of control values in phosphatase units is given alongside the "control" bar. Injection of vitamin A, 300,000 units i.p. 4 hr before sacrifice, produces a significant reduction in residual total hepatic light mitochondrial acid phosphatase activity. CPZ at 1 mg/kg i.p. every 2 hr caused no significant change from control levels, although the trend seemed to be toward an increase. CPZ and vitamin A administered simultaneously, as described above, caused no significant change in acid phosphatase activity. Thus it may be concluded that CPZ administration completely antagonized lysosomal labilization by vitamin A. Also shown in Fig. 4 is the reduction in residual acid phosphatase activity caused by 20 mg CPZ/kg. Here again a biphasic effect may be noted; that is that high doses of CPZ caused a reduction in enzyme activity, possibly by an increase in lysosomal membrane permeability, whereas low doses tended to cause an increase in residual levels, indicating perhaps a decrease in lysosomal membrane permeability.

Several experiments using whole homogenate acid phosphatase activity rather than the activity of the light mitochondrial fraction were performed. These experiments also showed a CPZ inhibition of the labilizing action of vitamin A. The data of the

TABLE 1. *In vivo* ANTAGONISM OF A LYSOSOMAL LABILIZER (ENDOTOXIN) BY CPZ

On two occasions out of five attempts, *E. coli* endotoxin produced a significant decrease in light mitochondrial fraction acid phosphatase activity of rat liver. The data from these two experiments are given below. Drugs were administered i.p. 4 hr before sacrifice.

	Acid phosphatase (units/g liver)	Average
Control	1·75, 1·67	1·71
E. coli endotoxin (40 mg/kg)	1·04, 0·44	0·76
CPZ (20 mg/kg)	1·37, 1·19	1·28
CPZ + endotoxin	1·18, 1·32	1·25

whole homogenate experiments are more variable, however. Nevertheless, the whole homogenate data are consistent with the interpretation that the CPZ effect occurs *in vivo*, and not during the centrifugal separation of the light mitochondrial fraction.

Finally, Table 1 shows the results of the two experiments out of five in which *E. coli* endotoxin caused a pronounced fall in residual acid phosphatase activity of

the liver light mitochondrial fraction. CPZ itself at 20 mg/kg caused a reduction, as noted previously. However, in spite of the reduction caused by the drug alone, the drug-treated animals were partially protected against the lysosomal labilization induced by *E. coli* endotoxin.

DISCUSSION

Now yet another membrane, that of the lysosome, may be added to the list of those upon which CPZ appears to exert some stabilizing effect. This list includes the erythrocyte, nerve-ending particle, mitochondrion, adrenomedullary particle, capillary wall, blood-brain barrier and muscle fascicle.[13] Such catholic membrane effects probably explain the wide variety of systems known to be influenced by the phenothiazines. Certainly some researches with these drugs have been performed in membraneless systems such as isolated enzymes.[14] Nevertheless, the great majority of such research has employed membrane-endowed systems. Thus, in the present research, had the "leakage" or release of acid phosphatase activity into the supernatant not been accounted for, one might have concluded that CPZ inhibits acid phosphatase activity because the CPZ-influenced soluble activity was always less than control. In fact, no enzyme inhibition was found. Therefore, the large number of enzyme inhibitions reportedly caused by CPZ in membrane-endowed systems, such as in mitochondrial fractions, slices, or homogenates,[13] should be viewed in a new light.

The main significance of the reported research resides in the fact that an effect of CPZ originally shown to occur *in vitro* appears to occur *in vivo* as well. Thus, further weight may now be given to the hypothesis that the phenothiazines exert an effect on membranes where these drugs are sufficiently concentrated in the body.[13] Previous criticism of this hypothesis was aimed at those organs, such as the liver,[13] where CPZ had been shown to concentrate, yet no membrane-stabilization effect had been demonstrated. Aside from the possibility that phenothiazine-induced jaundice may be membrane-based, the present *in vivo* findings may stand as an example of an hepatic membrane effect.

Beyond this, an explanation may now be at hand for such diverse and abstruse phenothiazine effects as tissue preservation,[15] prolongation of life in various shock states such as endotoxin, hemorrhagic,[16] traumatic, and heat; and protection against irradiation, in all of which lysosomal enzyme release has been implicated. Alternative explanations, such as the protective effects of hypothermia on these shock states, must also be kept in mind.

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