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THE EFFECT OF CHLORPROMAZINE, PROMETHAZINE, AND DIPHENHYDRAMINE ON SWELLING OF ISOLATED LIVER MITOCHONDRIA*

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Abstract—Diphenhydramine, chlorpromazine, and promethazine do not inhibit the initial adjustment of isolated liver mitochondria to hypotonic medium. Chlorpromazine and promethazine in optimum concentrations do inhibit glutathione- or ascorbate-induced swelling-lysis, late isotonic swelling, and a late or second phase of hypotonic swelling. In isotonic medium they do not inhibit electron transport-dependent swelling except in very much greater concentrations, and then only partially. Diphenhydramine does not inhibit glutathione- or ascorbate-induced swelling-lysis, late isotonic swelling, or electron transport-dependent swelling in isotonic medium. In most cases it does inhibit late swelling of mitochondria in hypotonic media.

Late swelling of fresh mitochondria in very hypotonic medium may have some relation to electron transport, since it is inhibited by 2,4-dinitrophenol and most electron transport inhibitors, but late swelling under other circumstances is not. The late swelling of fresh mitochondria in very hypotonic medium, like the late swelling in isotonic medium and most other circumstances, is inhibited by the antioxidant butylated hydroxyanisole, so that lipid peroxidation may be responsible for the changes.

With DPHA there is clear indication of a membrane-stabilizing effect independent of electron transport inhibition, uncoupling, or antioxidant activity. With the phenothiazines, the powerful antioxidant action makes it more difficult to determine whether the stabilizing action is direct like that of DPHA or is the result of preventing peroxidation, but they do inhibit swelling in some circumstances when BHA is inactive.

The aging of isolated mitochondria results in some membrane change which greatly slows their initial adjustment to hypotonic medium.

SPIRTES and Guth[1] reported that 10^{-5} M chlorpromazine inhibits uptake of water and sucrose by rat liver mitochondria and suggested that chlorpromazine affects the passive transport of water and ions across biological membranes. Earlier, Halpern and Reuse[2] and Eckhardt and Govier[3] reported the ability of certain phenothiazines to inhibit water uptake of frog muscle suspended in distilled water. Several groups have reported the ability of phenothiazines to protect mammalian erythrocyte membranes

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Abbreviations used in this paper are as follows: PMZ, promethazine; CPZ, chlorpromazine; DPHA, diphenhydramine; NHQNO, 2-nonyl-4-hydroxyquinoline-N-oxide; DNP, 2,4-dinitrophenol; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; SN 5949, 2-hydroxy-3-(2-methyl octyl)-1,4-naphthoquinone; GSH, reduced glutathione; GSSG, oxidized glutathione; BOHB, β-hydroxybutyrate.

from lysis.[4–6] Judah[7] reviewed the protective effects of antihistamines against various forms of swelling and discussed several mechanisms by which this protection might be produced. He concluded that these drugs inhibit a widely distributed mechanism which results in membrane degradation, the process being initiated by any one of several stimuli. He observed that this degradation allows water and ions both to leave and to enter the mitochondria, lysozomes, or cell membranes involved. Antihistamines always acted to preserve the *status quo*. Bernheim[8] reported the antioxidant effect of phenothiazines, and Judah[7] mentions antioxidant effects of chlorpromazine as a possible explanation for part of its protective action in liver.

Earlier workers[7] grouped promethazine (PMZ), chlorpromazine (CPZ), and diphenhydramine (DPHA) together as antihistamines, although there may be less reason for doing so now in light of current knowledge about them. This report deals with the effects of these three drugs on swelling of rat liver mitochondria in isotonic and hypotonic media, or induced by phosphate + β-hydroxybutyrate, ascorbate or glutathione. In each case a comparison is made with other substances known to inhibit specific types of mitochondrial swelling. As will be seen, an understanding of differences in the causes of swelling permits conclusions of greater significance concerning the action of these drugs.

METHODS

Rat liver mitochondria were prepared as described by Hunter *et al.*[9] and finally suspended in 0·33 M sucrose so that 1 ml of stock mitochondrial suspension was equivalent to 1 g original liver. Experimental incubations were carried out at room temperature in three different media: 0·33 M sucrose–0·025 M Tris buffer (pH 7·4) as the isotonic medium; and two hypotonic media, 0·075 M sucrose–0·025 M Tris (pH 7·4) and 0·025 M sucrose–0·025 M Tris (pH 7·4). Except where otherwise indicated, enough mitochondrial suspension was added in a total volume of 3·5 ml to produce optical density readings of approximately 0·650 in isotonic medium (150–160 μg mitochondrial protein/ml) in selected 13 × 100 mm Pyrex test tubes. The tubes were read in a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 520 mμ. The initial reading was made immediately after mixing, which required 4–5 sec. Throughout the experiments the tubes were checked for possible aggregation of mitochondria by swirling the tube under a strong light. All solutions were prepared in water redistilled in a two-step all-quartz still, except for BHT, BHA, SN 5949, antimycin A, NHQNO, and rotenone. For these substances concentrated stock solutions were prepared in 95% ethanol. The concentration of ethanol present after dilution in the test medium had no effect. Stock solutions of CPZ, PMZ, and DPHA were usually 5 or 10 mM. All chemicals were of analytical reagent grade whenever available. Otherwise the highest purity obtainable was used. CPZ was from the Smith, Kline and French Laboratories, PMZ from the Wyeth Institute for Medical Research, and DPHA from Parke, Davis and Co. Special biochemicals were from the Sigma Chemical Co., the Nutritional Biochemicals Corp., the Wisconsin Alumni Research Foundation, and the California Biochemical Corp.

EXPERIMENTAL RESULTS

Effects of CPZ, PMZ, and DPHA on isotonic swelling

Under our experimental conditions fresh rat liver mitochondria in 0·33 M sucrose–0·025 M Tris at room temperature commonly remain unswollen for as long as 1·5 to

2·5 hr, but then start to swell. As shown in Fig. 1, 25 and 100 μM PMZ and 25 μM CPZ protected well against this late isotonic swelling; 5 μM PMZ and 5 μM CPZ did not protect for so long, while 100 μM CPZ in some cases protected and in other experiments promoted a rapid and dramatic drop in turbidity or optical density (D_{520}). Ten to 200 μM concentrations of DPHA produced a D_{520} decrease a little sooner than that of the control, but paralleling the control curve; 1 mM DPHA, after a moderate lag, produced a rapid D_{520} decrease for some time, but then the curve leveled off instead of continuing downward.

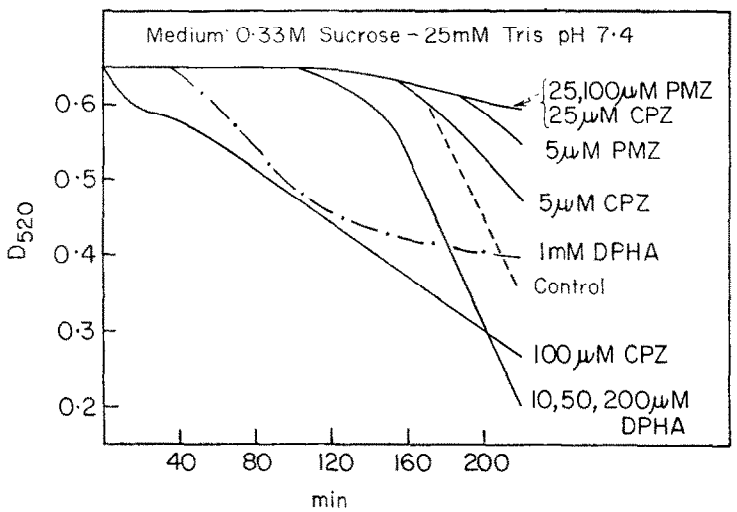

FIG. 1. Optical density changes of fresh mitochondria caused by spontaneous swelling in 0·33 M sucrose–0·025 M Tris with and without CPZ, PMZ, or DPHA.

Ten μM EDTA, or the commercial antioxidant BHT at 5 μM, protected against this late swelling as effectively as the optimum concentrations of the phenothiazines. Five μM BHA, another antioxidant, and 5 μM antimycin A protected moderately well, whereas none of the other electron transport-blocking agents (0·1 and 5 μM SN 5949, 0·1 μM antimycin A, and 0·1 and 5 μM NHQNO), nor 20 and 100 μM DNP (an uncoupler of phosphorylation) afforded any protection at all. Indeed, in some cases they induced earlier swelling. For example, 5 μM SN 5949, after a short lag period, caused a considerable drop in D_{520} which was more rapid than that produced by 100 μM CPZ, although not so extensive.

Effects of the three drugs on hypotonic swelling in 0·025 *M sucrose–*0·025 *M Tris*

A. Fresh mitochondria. When fresh mitochondria were diluted in this medium there was an immediate large drop in the D_{520} as compared with that of the isotonic controls (Fig. 2). This drop occurred so rapidly that it was impossible to read the tubes beforehand. It was followed by a plateau which lasted for varying lengths of time, after which there was a slower D_{520} decrease. The initial fall was not affected by the presence of any of the drugs, but in optimum concentrations they did prevent the secondary or late swelling. As is shown in Fig. 2, somewhat lower concentrations of the phenothiazines were optimum for the prevention of late swelling in this medium than were

required in isotonic medium. PMZ at 100 μM, which protected very well against late isotonic swelling, promoted rapid second-phase swelling in this very hypotonic medium, whereas 25 μM CPZ, which gave good protection in isotonic medium, sometimes protected in very hypotonic medium, but at other times caused swelling during the early part of the experiments. DPHA at concentrations of 10, 50, and 200 μM, which promoted slightly earlier swelling in isotonic medium, protected very well in 0·025 M sucrose–0·025 M Tris. EDTA at 10 μM gave excellent protection, just as it did in isotonic medium. DNP and the electron transport inhibitors antimycin A, SN 5949, and rotenone did not affect the rapid initial D_{520} drop, but low (0·1 μM) concentrations did inhibit late hypotonic swelling (Fig. 2), whereas they did not inhibit late isotonic swelling. Ten μM BHA, an antioxidant, also inhibited late hypotonic swelling very well.

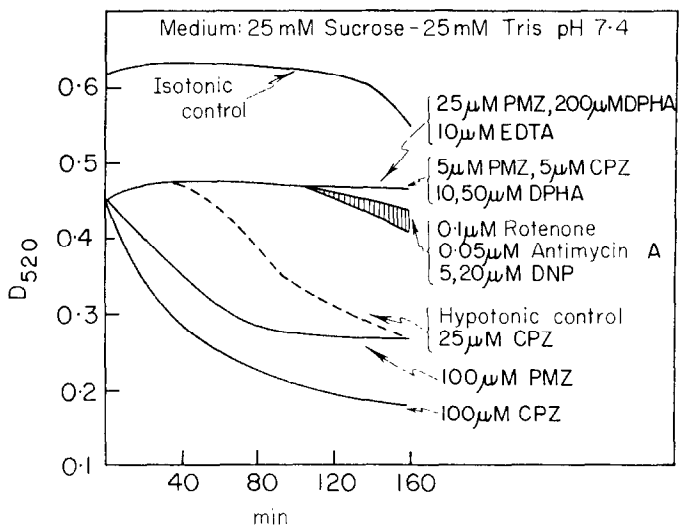

FIG. 2. Optical density changes of fresh mitochondria caused by spontaneous swelling in a very hypotonic medium, with and without CPZ, PMZ, DPHA, or other inhibitors.

We also studied the effects of 3, 20, and 100 μM CPZ and PMZ and of 10, 50, and 200 μM DPHA on suspensions of fresh mitochondria in this hypotonic medium with 10 μM EDTA + 0·05 μM antimycin A present to prevent electron transport-dependent swelling[9] The initial very rapid D_{520} decrease was not influenced by any of these drug–inhibitor combinations. As expected on the basis of experiments described above, the antimycin A + EDTA combination prevented late hypotonic swelling, and this inhibition was unaffected by low concentrations of PMZ and CPZ. However, the EDTA–antimycin A combination was unable to prevent the large D_{520} decrease caused by high (100 μM) PMZ or CPZ. When 20 μM DNP was substituted for EDTA–antimycin A to prevent the late hypotonic swelling, the results were similar to those just described. The uncoupler DNP, like electron transport inhibitors, was unable to prevent the D_{520} fall seen with 100 μM CPZ and 100 μM PMZ. Ten, 50, or 200 μM DPHA appeared not to interfere with, or be additive with, the action of EDTA + antimycin A or with that of DNP.

Experiments were also carried out in which 1 mM β-hydroxybutyrate was added to a portion of the fresh mitochondrial stock suspension to ensure a supply of substrate. The presence of added substrate had little or no influence on the effect of most concentrations of antihistamines with the exception of 25 μM CPZ, which protected much better (resembling its effect with fresh mitochondria in isotonic medium).

B. Aged mitochondria. With mitochondria aged for 24 hr at 0° and then for 20 min at 25°, the initial adjustment to hypotonicity was much slower, and the fall of the D_{520} readings could be recorded. Since this was not always the case with mitochondria aged for 24 hr at 0° alone or for 20 min at 25° alone, we adopted the double-aging procedure for study of this difference from fresh mitochondria. Our results are shown in Fig. 3. EDTA and most of the electron transport inhibitors used, with the exception

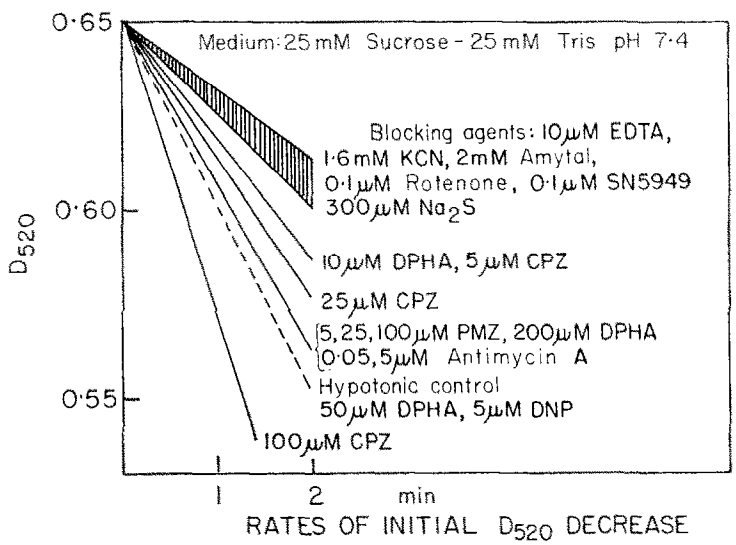

FIG. 3. Initial optical density changes of aged mitochondria in a very hypotonic medium, with and without CPZ, PMZ, DPHA, or other inhibitors. See text for aging procedure.

of antimycin A, slowed this initial swelling. 5–20 μM DNP had no effect. Among the three drugs tested only 10 μM DPHA, and 5 and 25 μM CPZ, appeared to slow this swelling, and that to a rather slight extent.

Like fresh mitochondria, aged mitochondria in 0·025 M sucrose–0·025 M Tris, after the initial D_{520} drop, showed a lag period followed by a second phase of swelling (Fig. 4). All three drugs could prevent this late swelling, but slightly higher concentrations appeared to be optimal with aged rather than with fresh mitochondria. Of the electron transport inhibitors tested, only 0·1 μM rotenone gave any protection, and this was only partial. The others, if anything, hastened swelling. Twenty μM DNP had little if any effect, while 10 μM BHA was fairly protective.

Adding 1 mM β-hydroxybutyrate to the aged mitochondrial stock suspension gave experiments in which the initial D_{520} fall was somewhat accelerated, but not so fast as with fresh mitochondria. The three drugs studied had no effect on the lower zero time reading or on the total initial drop. In the second or late phase of hypotonic

swelling, the only difference noted when aged mitochondria were pretreated with substrate was that they were somewhat less protected by 25 μM CPZ.

C. Mitochondrial concentrations. Higher concentrations of CPZ result in aggregation of mitochondria visible to the naked eye, as reported by Spirtes and Guth[1] and also observed in this laboratory. We have not encountered this complication with

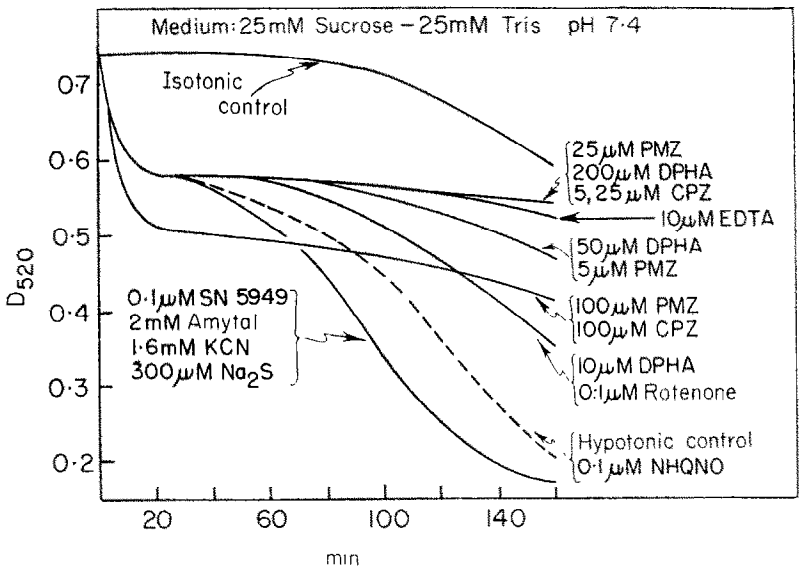

FIG. 4. Optical density changes of aged mitochondria caused by spontaneous swelling in a very hypotonic medium, with and without CPZ, PMZ, DPHA, or other inhibitors. See text for aging procedure.

PMZ in concentrations up to 200 μM, although 500 μM PMZ produced aggregation in both isotonic and very hypotonic media. Some experiments using 10, 50, 100, and 200 μM PMZ and fresh mitochondria were carried out to test for interrelationships of the amount of mitochondrial material with the concentrations of PMZ. Figure 5 shows the results with 80 and 160 μg protein/ml in terms of percentage of optical density, with the isotonic control plateau as 100%. Ten μM PMZ had the same protective effect in both cases. After a small amount of swelling, 50 μM also protected; 100 μM PMZ caused more extensive swelling during the first part of the experiments with the more dilute mitochondria, but in both cases a plateau was reached and maintained for very long periods. It appears, therefore, that the amount of fresh mitochondrial material does influence to some degree the early swelling caused by high concentrations of PMZ.

The results with aged mitochondria, representing 80 μg mitochondrial protein/ml, closely resembled those with fresh mitochondria. With 160 μg mitochondrial protein/ml, all the concentrations of PMZ except 200 μM protected during the first 20–30 min. After 30 min, even 200 μM protected against further swelling.

D. Pre-exposure of mitochondria to drugs. To determine the effect of exposing mitochondria to the drugs before exposure to hypotonic medium, we treated 56·2 μl of fresh mitochondrial stock suspension (8–10 mg mitochondrial protein/ml) to

varying concentrations of the three drugs at 0° in a total volume of 210 μl of 0·33M sucrose–0·025 M Tris (pH 7·4) for 7 min. Then sufficient 0·025 M Tris (pH 7·4) at room temperature was added to make a total volume of 3·5 ml of 0·025 M sucrose–0·025 M Tris, and the tubes were read immediately. When necessary, more drug was added with the 0·025 M Tris to produce the desired final concentration. With all

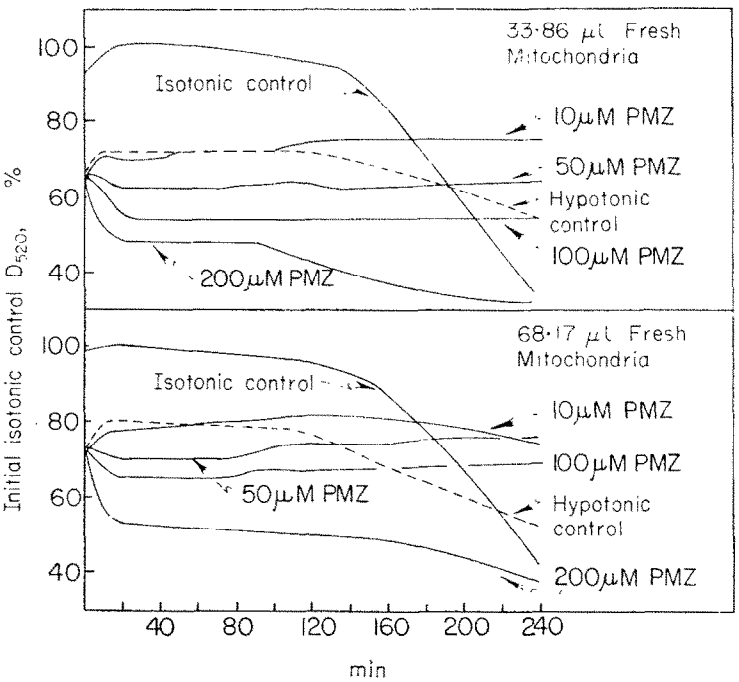

FIG. 5. Effect of four concentrations of PMZ upon the optical density changes of two concentrations of fresh mitochondria in 0·025 M sucrose–0·025 M Tris.

three drugs it was found that the final drug concentration was the important factor, with the pre-exposure concentration exerting influence only when it was unreasonably high (325 μM CPZ or PMZ, or 648 μM DPHA), in which case it promoted some D_{520} decrease during the early part of the experiments.

Effects of CPZ, PMZ, and DPHA on hypotonic swelling in 0·075 M sucrose–0·025 M Tris

A. Fresh mitochondria. When fresh mitochondria were placed in this moderately hypotonic medium the initial D_{520} drop was much less than in 0·025 M sucrose–0·025 M Tris, but was similar in that it was too rapid to read and was unaffected by these three drugs. As is shown in Fig. 6, the concentrations of phenothiazines affording very good protection against late swelling in this medium were intermediate between the higher concentrations optimum for protection in isotonic medium and the lower concentrations optimum in the very hypotonic medium. The concentrations of the phenothiazines promoting a D_{520} decrease were also intermediate between those seen in the other two media. This was particularly noticeable with PMZ, as 100 μM

afforded excellent protection in 0·33 M sucrose–0·025 M Tris, moderate protection in 0·075 M sucrose–0·025 M Tris, and promoted swelling in 0·025 M sucrose–0·025 M Tris. DPHA followed a similar pattern; no concentration that was tested protected in isotonic medium, 200 μM gave the best protection in 0·075 M sucrose, and all concentrations down to even 10 μM protected completely in the very hypotonic

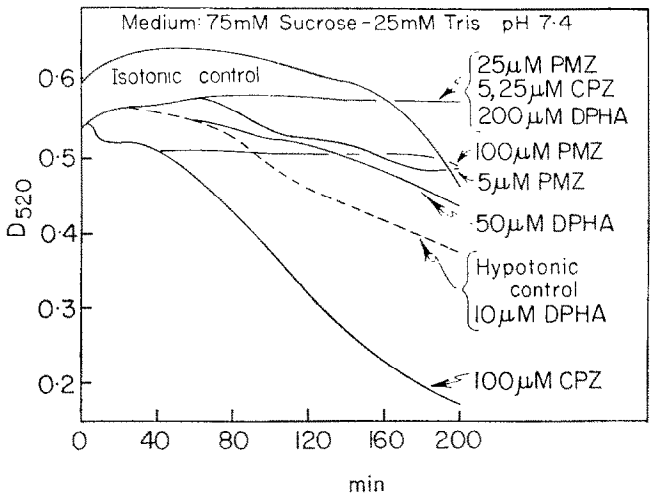

FIG. 6. Optical density changes of fresh mitochondria caused by spontaneous swelling in a moderately hypotonic medium, with and without CPZ, PMZ, or DPHA.

medium. With the 0·075 M sucrose medium, 10 μM EDTA and 0·1 μM rotenone gave nearly complete inhibition of late swelling, 2 mM amobarbital (Amytal) briefly delayed it, and all other electron transport inhibitors tested (300 μM NaCN, 300 μM Na_2S, and 0·1 μM NHQNO) hastened the D_{520} fall. Twenty μM DNP had little or no effect.

B. Aged mitochondria. After aging, the mitochondria showed no initial D_{520} decrease in 0·075 M sucrose–0·025 M Tris (Fig. 7). After a lag period there occurred a second-phase swelling which was more rapid and extensive than that seen with fresh mitochondria in this medium. The effects of the various concentrations of the phenothiazines were similar to their effects with fresh mitochondria in this medium, but none of the DPHA concentrations protected aged mitochondria at all. Ten μM EDTA protected quite well, while 20 μM DNP had no effect. The electron transport chain inhibitors showed some inhibitory effects which varied from experiment to experiment. Only 0·1 μM rotenone at any time protected as well as did EDTA, and this was not consistent. It would seem likely that there are variables in aged mitochondria which, while not affecting the consistent results obtained with the CPZ, PMZ, DPHA, or EDTA, have considerable influence on the effect of metabolic inhibitors on swelling. Special note should be taken of the fact that the antioxidant BHA afforded no protection, since this appears to be the main difference between fresh and aged mitochondria in this moderately hypotonic medium.

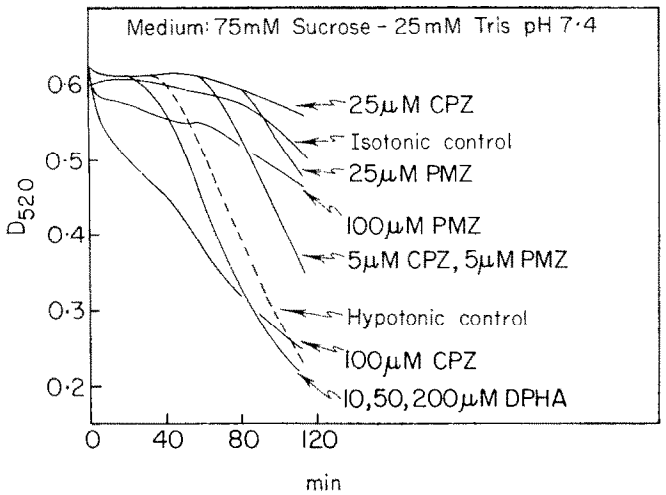

FIG. 7. Optical density changes of aged mitochondria caused by spontaneous swelling in a moderately hypotonic medium, with and without CPZ, PMZ, or DPHA.

Effects of CPZ, PMZ, and DPHA on electron transport-dependent swelling

With 0·33 M sucrose–0·025 M Tris as the medium, the effects of 25, 100, and 500 μM PMZ on swelling of fresh mitochondria supported by 2 mM β-hydroxybutyrate + 5 mM orthophosphate were studied (Fig. 8). The results showed that 25 and 100 μM had extremely slight if any influence on this electron transport-supported swelling, while 500 μM decreased the rate of swelling but not the final degree of change. Twenty μM CPZ gave results similar to those with 25 and 100 μM PMZ, and 100 μM CPZ

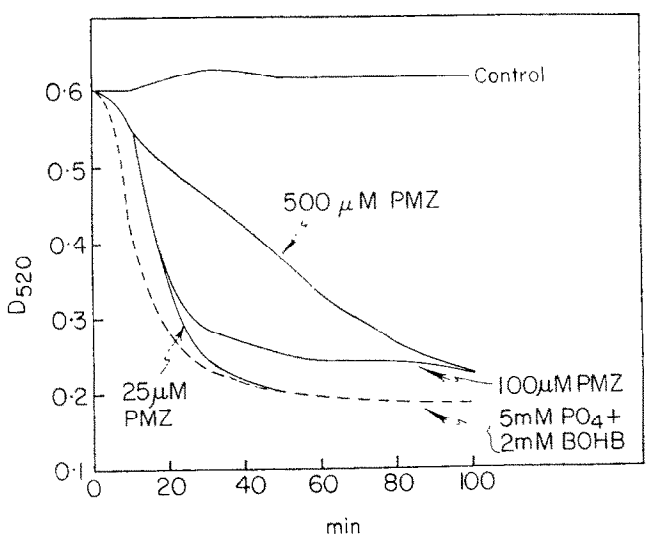

FIG. 8. Optical density changes of fresh mitochondria induced by electron transport-dependent swelling, and the effects of three concentrations of PMZ upon this type of swelling in 0·33 M sucrose–0·025 M Tris. PO_4 and BOHB were present in all tubes except the control.

inhibited about as much as 500 μM PMZ. No concentration of DPHA afforded any protection.

When the same experiment was repeated with mitochondria aged for 20 min at 25°, the results with 25 and 100 μM PMZ were the same as with fresh mitochondria, but 500 μM inhibited less than with fresh mitochondria. CPZ gave results similar to PMZ; 10, 50, and 200 μM DPHA gave virtually no protection. Except for very high concentrations of the phenothiazines with fresh mitochondria, it appears that all three drugs have little protective ability against electron transport-supported swelling.

Effects of CPZ, PMZ, and DPHA on ascorbate-induced swelling-lysis of mitochondria

Previous reports[10] have shown that low concentrations of ascorbate induce swelling and lysis in mitochondria. Further work[11, 12] linked ascorbate-induced lysis with the formation of lipid peroxide and showed that this lysis could be prevented by antioxidants. Bernheim[8] reported that CPZ inhibits lipid peroxide formation in rat liver homogenates, and that this inhibition is proportional to the concentration of CPZ/amount homogenate present. She also reported that as little as 50 μM CPZ could inhibit lipid peroxidation induced by 1 mM Fe^{2+}, so that chelation of the metal by CPZ is not likely to be the explanation for the antioxidant effect of CPZ. She suggested it might be connected with the tendency of phenothiazine and its derivatives to form resonance-stabilized free radicals.

Figure 9 shows the effects of various concentrations of PMZ, CPZ, and DPHA on

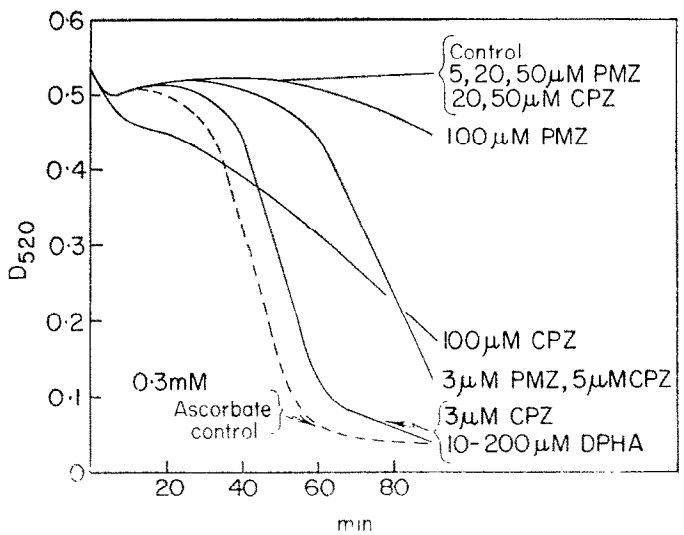

FIG. 9. Effect of CPZ, PMZ, or DPHA on ascorbate-induced swelling-lysis of fresh mitochondria in 0·33 M sucrose–0·025 M Tris. Ascorbate was present in all tubes to which the drugs were added.

swelling-lysis of fresh mitochondria induced by 0·3 mM ascorbate in 0·33 M sucrose–0·025 M Tris medium. DPHA at 10 to 200 μM showed virtually no protection; 3 μM PMZ and 5 μM CPZ produced significant delays; 5, 20, and 50 μM PMZ, and 20 and 50 μM CPZ, gave excellent protection. PMZ (100 μM) protected well at first, but later the D_{520} started to decrease; with 100 μM CPZ the D_{520} decrease started from the

beginning of the experiment. The D_{520} decrease observed with 100 μM CPZ can be ascribed to the direct effect of high concentrations of CPZ in this medium. PMZ appears to be a somewhat better antioxidant than CPZ insofar as lipid peroxidation in mitochondria is concerned.

Effects of the three drugs on glutathione-induced swelling-lysis

GSH induces swelling of isolated liver mitochondria,[13] and GSSG markedly accelerates this swelling.[14–16] Hunter *et al.*[9] reported that glutathione-induced swelling appeared to be more closely related to ascorbate-induced swelling and lysis than to swelling caused by phosphate and substrate because of the extremely low optical densities reached. Further work[11, 17] demonstrated that there is an excellent correlation between lipid peroxide formation and GSSG + GSH-induced swelling and lysis.

As is shown in Fig. 10, we found that from 5 to 100 μM PMZ afforded excellent

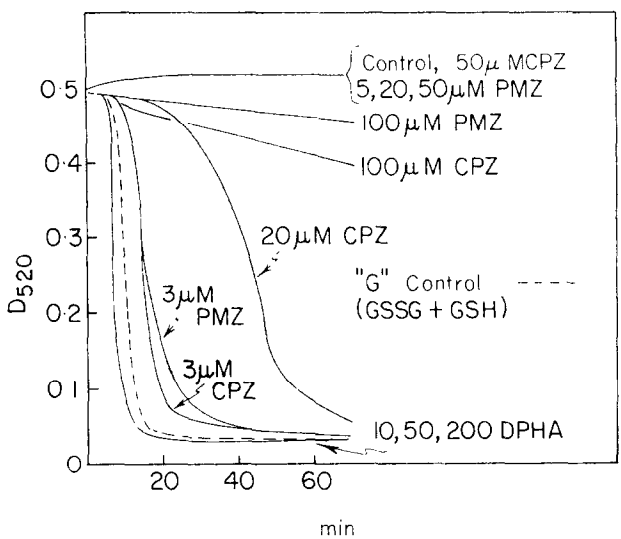

FIG. 10. Effect of CPZ, PMZ, or DPHA on swelling-lysis of fresh mitochondria induced by 5 mM GSSG + 1 mM GSH in 0·33 M sucrose–0·025 M Tris. GSSG + GSH was present in all tubes to which the drugs were added.

protection against swelling and lysis produced by 5 mM GSSG + 1 mM GSH, presumably by antioxidant action. More CPZ was required for protection, with 50 and 100 μM protecting very well, but 20 μM protected for only a short period of time. Repeated additions of 20 μM CPZ at 0, 5, 15, and 25 min gave good protection throughout the length of the experiments. Twenty μM CPZ at 0, 10, 20, and 30 min gave moderate protection. However, if GSSG + GSH-swelling is allowed to start and 20 μM CPZ is then added, it exerts no protective effect at all. DPHA at 10–200 μM did not inhibit glutathione-induced changes.

DISCUSSION

Of the five different mitochondrial swelling patterns studied in this work, three (phosphate + substrate, ascorbate, and GSSG + GSH-initiated) are much better

understood than are those patterns produced by mitochondria standing at room temperature in isotonic or in hypotonic sucrose-Tris media. Phosphate + substrate-induced swelling is known to be prevented by a great variety of electron transport inhibitors and also by numerous agents that uncouple oxidative phosphorylation from the electron transport chain,[9, 18] but not by the antioxidants BHT and BHA. Hunter and co-workers,[11, 12, 17] have shown the close relationship between the swelling-lysis induced by both ascorbate and GSSG + GSH and the production of lipid peroxide. This swelling-lysis is prevented by BHT and BHA, only slightly delayed by many of the electron transport inhibitors, and unaffected by DNP and other uncoupling agents.

None of the drugs tested inhibited electron transport-dependent swelling except for a partial inhibition of the rate with very high concentrations of phenothiazines. These results are consistent with some older reports that CPZ acted as an electron transport inhibitor and uncoupling agent, since concentrations as high as 1 mM CPZ were used in those studies. Such concentrations are entirely out of the range expected to be present in living cells.

Relatively low concentrations of the phenothiazines prevented GSSG + GSH- or ascorbate-induced swelling-lysis, while DPHA had no effect. These results are consistent with our knowledge that this type of swelling is intimately associated with lipid peroxidation and prevented by antioxidants. Phenothiazines can act as antioxidants whereas DPHA is not an antioxidant.

Swelling of liver mitochondria in hypotonic media has been reported to be inhibited by antihistamines. However, our data suggests that the initial rapid adjustment to hypotonicity is not affected to any great degree by either the phenothiazines or by DPHA (Table 1). There is a second or late phase of swelling in hypotonic media, but

TABLE 1. SUMMARY OF THE EFFECTS OF VARIOUS AGENTS ON THE INITIAL OSMOTIC ADJUSTMENT OF RAT LIVER MITOCHONDRIA

Type of swelling	Mitochondria	Phenothiazines	DPHA	Electron transport inhibition	DNP (uncoupler)
Very hypotonic	Fresh	No	No	No	No
	Aged	No	No	Slowed	No

No: Little or no protection was afforded by the agents against D_{520} decrease.

it is very doubtful that this can be considered a part of hypotonic swelling due to simple adjustment to osmotic differences. In many investigations some of this second-phase change may have been measured together with the initial rapid adjustment, since with some preparations the time interval between these two phases is very short. This second swelling phase is inhibited by CPZ, PMZ, and DPHA under most circumstances. This may have led to the mistaken conclusion that these drugs inhibited hypotonic adjustment *per se*. The nature of this late, secondary phase of hypotonic swelling appears to be quite complex, since known electron transport chain inhibitors and uncoupling agents can inhibit this swelling under some conditions and are ineffective under others. This is also true of the antioxidant BHA.

In a very hypotonic medium, the late-phase swelling of fresh mitochondria was inhibited by low concentrations of electron transport inhibitors, the uncoupler DNP, and the antioxidant BHA, as well as by low concentrations of the phenothiazines and all concentrations of DPHA (Table 2). This type of swelling therefore cannot be clearly

TABLE 2. SUMMARY OF THE EFFECTS OF VARIOUS AGENTS UPON SECOND-PHASE SWELLING OF RAT LIVER MITOCHONDRIA

Type of swelling	Mito-chondria	Pheno-thiazines	DPHA	Electron transport inhibition*	DNP (un-coupler)	Anti-oxi-dants
Late isotonic	Fresh	Yes	No	No	No	Yes
Moderately hypotonic	Fresh	Yes	Yes	No	No	Yes
	Aged	Yes	No	No	No	No
Very hypotonic	Fresh	Yes	Yes	Yes	Yes	Slowed
	Aged	Yes	Yes	No	No	Yes
Pi + BOHB-induced swelling	Fresh	Slowed	No	Yes	Yes	No
	Aged	No	No	Yes	Yes	No
Ascorbate-induced swelling-lysis	Fresh	Yes	No	No	No	Yes
GSH + GSSG-induced swelling-lysis	Fresh	Yes	No	No	No	Yes

* Under a number of conditions rotenone afforded protection against D_{520} decrease, although no other electron transport inhibitor tested did so. Rotenone appears to have some more direct action in addition to its properties as an electron transport inhibitor. The same is true for very high concentrations of antimycin A, SN 5949, and NHQNO, which show antioxidant properties and can inhibit or delay ascorbate- or GSSG + GSH-induced changes.[12, 17]

Yes: Optimum concentration of the agents protected against D_{520} decrease.

No: Little or no protection was afforded by the agents against D_{520} decrease.

classified as either electron transport-dependent or lipid peroxidation-induced. The very low concentrations of phenothiazines that are inhibitory are probably not acting as uncouplers or electron transport inhibitors, even though hypotonically swollen mitochondria may allow greater penetration of drug to inhibitory sites, for known electron transport-dependent swelling in isotonic medium is only partially inhibited by 100-fold greater concentrations. The phenothiazines and BHA could inhibit late-phase swelling of mitochondria in a very hypotonic medium by acting as antioxidants if lipid peroxidation plays a part in this swelling. However, DPHA, which is not an antioxidant, an electron transport inhibitor, or an uncoupler, also can inhibit. Therefore we can conclude that DPHA must be inhibiting by some other means, possibly by a direct effect on membrane permeability. At the same time we must recognize that some electron transport inhibitors, phenothiazines, and BHA may also have direct stabilizing actions not related to their antioxidant properties. Stabilization and labilization of membranes by various organic compounds has been studied by Shanes.[19]

With fresh mitochondria in moderately hypotonic medium and aged mitochondria in very hypotonic medium it is clear that the phenothiazines do not act by electron transport inhibition, but they might act as antioxidants (Table 2). However, with aged mitochondria in moderately hypotonic medium, electron transport inhibitors, uncouplers, or antioxidants cannot produce the inhibition seen with CPZ and PMZ.

It must therefore be concluded that the phenothiazines also have some direct membrane action. This action is not identical with that of DPHA, since there is at least one situation in which the phenothiazines prevent swelling but DPHA and antioxidants do not (Table 2). These experiments also revealed that rotenone probably has membrane-stabilizing effects not related to electron transport inhibition.

Fresh mitochondria in isotonic medium were protected from late spontaneous swelling by the phenothiazines and by the antioxidants BHT and BHA, but not by DPHA, DNP, or electron transport inhibitors. It can be concluded that this swelling is not electron transport-dependent but apparently is associated with lipid peroxidation. Since the general membrane-stabilizing action of DPHA is ineffective here, the phenothiazines are presumably acting either by their antioxidant action or by a direct membrane stabilization with a mechanism different from that with DPHA.

Under hypotonic, but not isotonic, conditions 100 μM PMZ caused a characteristic fall in D_{520} from zero time. This appears to be swelling induced by high concentrations of PMZ, since careful examination failed to detect aggregation of mitochondria in these tubes. The nature of this action is not known, but electron transport blockers and DNP do not affect it. CPZ presented a complicated picture because, under either isotonic or hypotonic conditions, 100 μM or more CPZ caused an even more rapid and marked fall in D_{520}. This decrease cannot be interpreted exclusively as swelling, since mitochondrial aggregation is easily demonstrated. It was noted that under some conditions higher concentrations of both CPZ and PMZ first caused a D_{520} fall and later in the experiments protected against further change.

The optimum concentration of CPZ for protection against second-phase swelling in hypotonic media appears to be lower than that of PMZ. The more hypotonic the medium, the lower the optimum concentrations of CPZ, PMZ, and DPHA tend to be for both fresh and aged mitochondria. This may result from better penetration of the drugs into the more swollen mitochondria. With aged mitochondria slightly higher concentrations of the drugs are optimum.

Aging of mitochondria markedly slows the initial adjustment to hypotonic conditions. Thus the membrane must have become appreciably less permeable to water. Only further work can determine the possible mechanisms and implications of this effect.

These studies with phenothiazines and with DPHA emphasize that under certain conditions quite low concentrations of these drugs can have marked effects on tissues and membranes, but these effects probably have little or no relationship to antihistamine action. This must be kept in mind when making studies with isolated tissues, cell components, or membranes.

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