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EFFECTS OF CHLORPROMAZINE ON BIOLOGICAL MEMBRANES—III.

SWELLING AND SHRINKAGE OF RAT LIVER MITOCHONDRIA SUSPENDED IN ISO- OR HYPOTONIC SUCROSE MEDIA IN THE PRESENCE OF CYANIDE OR CYANIDE PLUS CHLORPROMAZINE

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Abstract—Sucrose entry, but not the entry of water, into rat liver mitochondria suspended in 0·15 M sucrose–0·02 M Tris buffer, pH 7·4, could be prevented by 3×10^{-4} M cyanide. Water entry under such conditions consists of two phases. The first is a rapid phase which cannot be affected by chlorpromazine (CPZ). It is evidenced by a rise in the 0-min value of mitochondrial water to 72% from the 67–68% determined for mitochondria suspended in 0·30 M sucrose–Tris solution. The second phase is the swelling occurring in the second 10-min period after the onset of the experiment as evidenced by a drop in optical density and a further increase in per cent water content of the mitochondria to 78%. The second phase can be inhibited by 10^{-5} M CPZ The mitrochondrial swelling at the end of the 20-min experimental period could be reversed by centrifuging the mitochondria at 20,000 *g* for 20 min, removing the supernatant fluid and replacing it by the same volume of 0·30 M sucrose–0·02 M Tris (pH 7·4)–3×10^{-4} M cyanide. This shrinkage could *not* be prevented by the addition of 10^{-5} M CPZ to the replacement medium.

RECENTLY[1] it was reported that CPZ simultaneously inhibited the spectrophotometrically measured optical density (O.D.) drop, the change in water content, and the entrance of sucrose into rat liver mitochondria suspended in 0·30 M sucrose–0·02 M Tris buffer, pH 7.4, at 25° over a period of 10–20 min. In a similar series, chlorpromazine sulfoxide was found to be ineffective in bringing about any inhibition of the parameters measured.

Since the reactions were carried out only under aerobic conditions, and did not result in an answer to the question whether CPZ primarily inhibited sucrose or water entry or both, they have been repeated in the presence of 3×10^{-4} M cyanide, enough to effectively stop endogenous O_2 consumption. Such experiments soon confirmed the fact that this cyanide concentration completely blocked the previously noted changes in O.D., as reported by others.[2, 3] Simultaneously, water content and sucrose entry changes were also found to be blocked.

In order again to initiate water entry in the presence of cyanide, mitochondria originally made up in 0·30 M sucrose were exposed to hypotonic (0·15 M) sucrose–Tris solution at pH 7·4 containing 3×10^{-4} M cyanide. Over the same 20-min period, water was now found to enter the mitochondria, and this event was accompanied by a large O.D. drop. *However, little or no sucrose entered.* Furthermore, even in the hypotonic sucrose, the mitochondria in the presence of cyanide resisted swelling for

more than 10 min. The prior addition of CPZ now partially prevented entrance of the water at concentrations as low as 10^{-5} M and often at 5×10^{-6} M. It has therefore been possible to separate the effect of CPZ on sucrose entry from that on water entry and to show that CPZ can effectively prevent the entry of water alone into the mitochondria. However, the same drug did not prevent the exit of water from mitochondria, first swollen and then shrunken, both in the presence of cyanide.

METHODS

Chemicals and the methods for preparing rat liver mitochondria, for measuring mitochondrial swelling spectrophotometrically, for analysis of per cent water gain, and for measuring mitochondrial pellet protein, total nitrogen, and sucrose were those previously published.[1] Sodium cyanide, when used, was added to the Beckman cuvet with the water and 0·15 M sucrose–0·02 M Tris buffer at pH 7·4 to make a volume of 2·94 ml, after which the final addition of mitochondria was made, the mixture inverted three times, and the experiment begun. To prevent cyanide evaporation, the cuvet cover was kept in place during the entire period of the reaction. Cyanide analysis at the end of the reaction performed by the picric acid method[4] indicated no cyanide had been lost.

Mitochondrial shrinkage

Such experiments followed the measurement of rat liver mitochondrial swelling that occurred after suspension in 0·15 M sucrose–0·02 M Tris buffer, pH 7·4, in the presence of 3×10^{-4} M Na cyanide for 20 min (Table 3). Two portions of 33 ml each of such mixtures were prepared at the start of the incubation; 3 ml of the first was removed and its O.D. followed for 20 min as described. The rest of the first portion and the whole of the second were allowed to stand for 20 min at room temperature (Ref. 1, under Estimation of Mitochondrial Pellet H_2O), after which both portions were spun down as indicated and the first mixture of 30 ml immediately tested for mitochondrial pellet water and sucrose. When the supernatant was removed from the second portion (33 ml), it was replaced with enough 0·30 M sucrose–0·02 M Tris buffer (pH 7·4) and 3×10^{-4} M Na cyanide to make up to a volume of about 25 ml. This was mixed briefly in the centrifuge tube, transferred to a glass homogenizer, and homogenized (2 or 3 strokes) to a smooth mixture. The mixture was now made up to the original starting volume of 33 ml. Three ml was immediately removed and the O.D. determined and followed for 20 min. The remaining 30 ml was allowed to stand at 25° for 20 min. An estimation of mitochondrial pellet water and sucrose was then carried out on this second portion.

EXPERIMENTAL

The overall changes in mitochondrial pellet water and sucrose content and in the O.D. of the organelle suspensions over a period of 20 min are recorded in Tables 1–3. Table 1 indicates that the 20 min *gain* in sucrose content for mitochondria suspended in 0·30 M sucrose–0·02 M Tris buffer (pH 7·4) was $3{\cdot}17 \pm 1{\cdot}2$ mg, the 0-min value (not recorded here) being 0·3–0·5 mg/pellet. In the presence of 3×10^{-4} M Na cyanide, practically no gain occurs, as is shown by the 20-min gain of 0·58 mg/pellet. The mitochondrial water increases by 6·9% in the absence of cyanide and by only 2·4%

in the presence of the latter. The per cent water content of both groups was the same (68%) at 0 min. The drop in O.D. which accompanies mitochondrial water content changes amounted to 0·207 unit in the absence of cyanide and only 0·017 unit in its presence.

TABLE 1. SUMMARY OF THREE PARAMETERS AFFECTED BY CYANIDE IN A RAT LIVER MITOCHONDRIAL SUSPENSION IN ISOTONIC SUCROSE*

	Mitochondrial suspension	Mitochondrial suspension + 3×10^{-4} M NaCN	P
Gain in mg mitochondrial pellet sucrose in 20 min	3·17 ± 1·2	0·58 ± 0·85†	< 0·001
Gain in % mitochondrial pellet water in 20 min	6·9 ± 2·8	2·4 ± 2·4‡	< 0·001
O.D. Change at 520 mμ in 20 min	− 0·207 ± 0·050	− 0·017 ± 0·016	< 0·001

* Mitochondrial suspension in 0·30 M sucrose–0·02 M Tris buffer, pH 7·4, at 25°; final volume 3 ml containing 0·06 ml of mitochondrial suspension. Tenfold multiples were used for the sucrose and water entry studies. The 0-min water content of the mitochondrial was 67–68%. All values are expressed as averages ± standard deviations from the mean of 12 different mitochondrial suspensions.

† The gain of 0·58 mg sucrose per pellet was barely significant ($P < 0·02$).

‡ The gain of 2·4% water was not significant ($0·05 < P < 0·10$).

TABLE 2. SUMMARY OF THREE PARAMETERS AFFECTED BY CHLORPROMAZINE (CPZ) IN A SUSPENSION OF RAT LIVER MITOCHONDRIA IN HYPOTONIC SUCROSE AND CYANIDE*

	Mitochondrial suspension + cyanide	Mitochondrial suspension + cyanide + 10^{-5} M CPZ	P
Gain in mg of mitochondrial pellet sucrose in 20 min	1·38 ± 1·15	0·04 ± 0·16	< 0·001
Gain in % mitochondrial pellet H_2O in 20 min	7·5 ± 2·9	1·7 ± 1·0†	< 0·001
O.D. Change at 520 mμ in 20 min	− 0·245 ± 0·081	− 0·036 ± 0·045	< 0·001

* Mitochondrial suspension, 0·06 ml of a mitochondrial preparation in an amount of 0·15 M sucrose–0·02 M Tris buffer, pH 7·4, + 3×10^{-4} M NaCN to make a final volume of 3·0 ml at 25°. Tenfold multiples of the control were used for the sucrose and water studies. All values are expressed as averages ± standard deviations from the mean of 12 different mitochondrial suspensions.

† The gain of 1·7% for mitochondrial water in the presence of CPZ was *not* significant ($0·1 < P < 0·2$).

Table 2 records the values of the same parameters as above when the liver mitochondria were suspended in 0·15 M sucrose–0·02 M Tris buffer (pH 7·4) + 3×10^{-4} M NaCN in the presence and absence of 10^{-5} M CPZ. The gain in pellet sucrose is now only 1·38 mg over the 20-min period. This figure would have been under 1·0 mg and close to that of the pellets from mitochondria suspended in isotonic sucrose + cyanide (see Table 1), were it not for two experiments in which considerably higher figures of about 3 mg were noted (see Table 3). When 10^{-5} M CPZ was present, the

sucrose gain disappeared entirely. Likewise, the gain in per cent mitochondrial water of 7·5 fell significantly in the presence of CPZ to only 1·7. The drop in O.D. accompanying the increase in water content was 0·245 in the absence of CPZ and only 0·036 in its presence.

TABLE 3. EFFECT OF CPZ ON RAT LIVER MITOCHONDRIAL SWELLING OR SHRINKAGE IN THE PRESENCE OF CYANIDE AT 25°

Parameter measured	Mitochondria in 0·15 M sucrose + NaCN			Same mitochondria resuspended in 0·3 M sucrose + NaCN	Same mitochondria resuspended in 0·3 M sucrose + NaCN + 10^{-5} M CPZ
	0 min	20 min	20–0 min	0 min	0 min
Mitochondrial water (%)	72·7 ± 1·1	78·0 ± 1·1	+ 5·3	73·6 ± 1·5	72·3 ± 1·2
Sucrose in pellet (mg)	0·68 ± 0·1	0·88 ± 0·1	+ 0·20	3·10 ± 0·29	3·0 ± 0·21
O.D. of the mitochondrial suspensions	0·480 ± 0·001	0·268 ± 0·017	− 0·212	0·393 ± 0·03	0·374 ± 0·02
Protein-N per mitochondrial pellet (mg)	0·446 ± 0·04	0·402 ± 0·02	− 0·044	0·392 ± 0·01	0·402 ± 0·04

* NaCN at 3×10^{-4} M; CPZ at 10^{-5} M. Rat liver mitochondria from 1 g liver made up to 1·0 ml with 0·30 M sucrose, pH 7·4; 0·6 ml made up to 30·0 ml with suspending fluid and spun down at 20,000 *g* after 20 min then resuspended at same concentration as before with and without CPZ. All values are expressed as averages ± standard deviations from the mean of 12 different mitochondrial suspensions.

In the third set of experiments, the liver mitochondria, in addition to incubation in 0·15 M sucrose–0·02 M Tris buffer + 3×10^{-4} M NaCN for 20 min, were spun down at the end of that period and the pellet resuspended in 0·30 M sucrose–Tris buffer–cyanide as described under Methods, in order to measure mitochondrial shrinkage. Water and sucrose content as well as O.D. changes were measured at 0 min, after 20 min of swelling and after resuspension of the mitochondria in the 0·30 M sucrose–Tris–cyanide mixture. The results in Table 3 were essentially the same as in Table 2 for the initial portion of the experiment, except that the sucrose gain was exceedingly small (0·2 mg) in spite of the large gain in water content (5·3%). When the mitochondria were resuspended in 0·30 M sucrose–Tris–cyanide, the per cent water fell back from 78 almost to the 0-min value (73·6%), in spite of the fact that considerable sucrose *entered* the pellet. Concomitant with the exit of water from the particles, an increase in O.D. occurred from 0·268 O.D. unit to 0·393. It could also be seen in Table 3 that a 10% loss in the mitochondrial pellet nitrogen occurred during the swelling phase of the experiment, but no further losses were recorded upon resuspension of the organelles in 0·30 M sucrose–Tris–cyanide. Furthermore, the addition of 10^{-5} M CPZ to the resuspension fluid altered none of the changes noted in Table 3. Possible reasons for the sucrose entry and the failure of the O.D. to return to the 0-min value upon resuspension of the organelles in 0·30 M sucrose–Tris–cyanide will be discussed.

A closer analysis of the changes in O.D. resulting from the suspension of liver mitochondria in 0·30 M sucrose–0·02M Tris buffer (pH 7·4) and in 0·15 M sucrose–0·02 Tris buffer (pH 7·4) in the presence and absence of cyanide and CPZ, indicates

first that mitochondria are stabilized by the addition of 3×10^{-4} M cyanide alone (Fig. 1). Suspended in 0·30 M sucrose–Tris, the O.D. begins to fall after 6 min and is significantly lower after 9 min ($P < 0{\cdot}05$); in 0·15 M sucrose–Tris, the O.D. begins to drop immediately, and the change is significant after less than 1 min. The swelling rate is also faster, as indicated by the slope of the lines representing these changes. When 3×10^{-4} M CN is added to the 0·15 M sucrose–Tris, the O.D. of the mitochondrial suspension is practically unchanged for 10 min and drops significantly only after 13 min. The swelling rate has decreased until it is about equal to that for mitochondria suspended in 0·30 M sucrose–Tris alone. There is a tendency in the last 10 min for the rate to increase when the organelles are in the hypotonic sucrose–Tris–cyanide and to decrease in the isotonic sucrose–Tris.

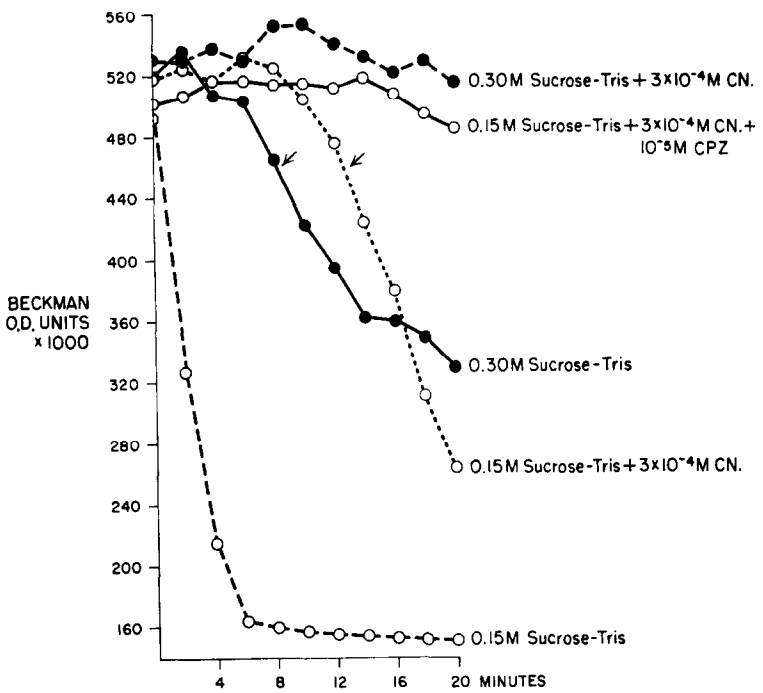

FIG. 1. Effects of cyanide or cyanide + CPZ on the swelling rates of rat liver mitochondria suspended in iso- and hypotonic sucrose–Tris media. Arrows indicate the O.D. values at which the O.D. fall has become statistically significant.

Not noted in Fig. 1 is the fact that after the initial rise in O.D. following isotonic resuspension of the mitochondria swollen in 0·15 M sucrose–Tris–cyanide (see Table 3), there was a little further drop in the O.D. followed for the next 10–20 min.

DISCUSSION

Chappell and Greville,[5] Hunter,[3] Lehninger,[6] and others explained the lack of swelling of mitochondria suspended in an isotonic medium containing cyanide by the supposition that electron flux over the cytochrome electron-transport system was blocked and that this flux in some manner facilitated the swelling of the organelles

brought about by various means such as different ions, thyroxine, etc. Such water movement across mitochondrial membranes would, therefore, presumably also fail to occur in mitochondria suspended in hypotonic 0·15 M sucrose + cyanide. Yet water entered (Table 2) at a rate and amount at least equal to that for the same particles suspended in isotonic sucrose alone. A portion of this water entry in the presence of cyanide was therefore, in all probability, "passive" or "osmotic" in nature; i.e. it required little energy and was mainly due to the hypotonicity of the sucrose in which the mitochondria were suspended. The fact that little or no concomitant sucrose entry occurred under these conditions also indicates that some of the water entry was due to the osmotic effects of the hypotonic sucrose medium in which the mitochondria were suspended. However, it must be taken into consideration that the entry of water could be the result of a secondary osmotic event brought about by an intramitochondrial increase in the number of chemical entities resulting from anaerobic processes such as hydrolyses and related phenomena or reductive splitting. Whatever the cause, water entry transpired in two phases. Phase 1 was rapid and unaffected by 10^{-5} M CPZ. Thus, the 0-min water content increased to 72% from the 67–68% found at 0 min for mitochondria suspended in 0·30 M sucrose–Tris with or without cyanide present. The second phase for the organelles in 0·15 M sucrose–Tris–cyanide, transpiring during the second 10 min of suspension, was delineated by a rapid but measurable drop in O.D. (Fig. 1) and a further increase in water content measured directly (78%, see Table 3). This phase could be inhibited by 10^{-5} M CPZ (Table 2). A comparison of the effective inhibitory CPZ concentrations between water entry under *hypotonic* conditions in the presence of cyanide (Table 2) and entry in the presence of isotonic sucrose without cyanide[1] shows that they are approximately the same (between 5×10^{-6} and 10^{-5} M).

The direct measurement of O_2 consumption of the very dilute mitochondrial suspension used in the swelling experiments in the absence of exogenous substrates is technically very difficult and was not attempted. Therefore it is possible that the concentration of cyanide in our experiments (3×10^{-4} M) does not cause complete cessation of O_2 consumption, although it *is* sufficient to stop mitochondrial swelling in 0·30 M sucrose for at least 20 min (Fig. 1). From this fact it is reasonable to assume that the extent of the inhibition of O_2 consumption by such a cyanide concentration would be the same when the mitochondria are suspended in 0·15 M sucrose, in which state delayed swelling did occur and could be prevented by 10^{-5} M CPZ. Nevertheless, because of the possible occurrence of some O_2 consumption, conditions corresponding to *total* anaerobiasis were sought to see whether similar results could be obtained. The substitution of antimycin A for cyanide is found to satisfy such conditions, when 10 μg antimycin A is added to the mitochondrial suspension in 0·15 M sucrose solution. A preliminary determination of O_2 consumption, with 2 mg succinate as substrate plus 10 times the amount of organelles usually added to follow swelling, was carried out by the Warburg technique at 25°. The oxygen uptake was completely inhibited by the addition of 10 μg antimycin A for 3 ml of suspension. The assumption was then made that the same concentration of antimycin A was certainly sufficient to prevent O_2 consumption of suspensions such as are used in the swelling experiments, i.e. containing 1/10 the amount of mitochondria and only those endogenous substrates as are already present. The results of such experiments, analogous to those obtained in the presence of cyanide, show that mitochondrial swelling is completely stopped for

at least 20 min in 0·30 M sucrose but not in 0·15 M sucrose. As with cyanide, the swelling in hypotonic sucrose could be inhibited by 10^{-5} M CPZ.

It is also possible, in the absence of electron flux, that mitochondrial swelling takes place with the aid of the small amount of high-energy phosphate compounds known to be present in the mitochondria under any conditions. Neither cyanide nor antimycin A is known to block the utilization of such entities. If this were true, then their presence, upon the addition of electron flux blocking agents, should still allow swelling to take place. Figure 1 indicates, however, that such swelling does *not* occur when the mitochondria are suspended in 0·30 M sucrose + cyanide. Similar results in the presence of 0·30 M sucrose + antimycin A have also been previously mentioned. The amount of such high-energy compounds in the mitochondria under our conditions may therefore be inadequate, or they may have little to do with swelling. A third postulate still remains a possibility, i.e. that such high energy compounds are not effective in 0·30 M sucrose because they are too tightly bound in the organelles and therefore unavailable for use; in 0·15 M sucrose such compounds may become more available. This postulate has not yet been ruled out experimentally. However, since oligomycin is thought by some observers to prevent some types of utilization of ATP,[7, 8] a combination of 10 μg antimycin A and 10 μg oligomycin was added to mitochondrial suspension in 0·15 M and 0·30 M sucrose–Tris solutions. The results were similar to those of the experiments in which antimycin A alone was added. Chlorpromazine was therefore effective in preventing swelling of mitochondria in 0·15 M sucrose–Tris in the presence of inhibition of the electron transport chain and when utilization of at least some of the ATP present was probably blocked. Details concerning these inhibitor experiments, which considerably strengthen the hypothesis that CPZ is blocking passive mitochondrial swelling under our conditions, will subsequently be published.

From the spectrophotometric evidence of Fig. 1 of this article it is obvious that water entry, at least in part, is a relatively slow process, measurable over a period of 10–20 min. The exit of water from mitochondria which have undergone swelling in 0·15 M sucrose, achieved by spinning them down and resuspending in a 0·30 M sucrose medium (see Methods under *Mitochondrial shrinkage*), is a somewhat quicker process, since the 0-min per cent water value (spun down again for 8 min) is already back to the pre-swelling value of 73·6% (Table 3). This water exit could *never* be influenced by the presence of 10^{-5} M CPZ in the 0·30 M sucrose resuspending fluid.

Other interesting facts emerge from the swelling–shrinkage experiments. First, a 10% loss in mitochondrial nitrogen occurs during the original swelling when the organelles are in 0·15 M sucrose + cyanide (Table 3). If this is due to mitochondrial destruction, it could partly have accounted for the fact that the O.D. of the shrunken suspensions did *not* return to the original O.D. of the organelles suspended in 0·15 M sucrose + cyanide at 0 min. Subtracting this 10% from the 0-min O.D. reading of 0·480, the O.D. upon shrinkage could have reached at best about 0·430.

The percentage of water in the shrunken mitochondria decreased from 78% to 73%. Mitochondria originally suspended in 0·30 M sucrose + cyanide averaged about 67–68% water content. The reason the contracted mitochondria returned only to 73% is seen in Table 3 which indicates that sucrose entered the mitochondria at this stage even in the presence of cyanide, probably owing to damage to the organelles. This caused the retention of some water and accounts for the partial exit of H_2O to

73% instead of to the 67–68% level (Table 1). Such water retention could have been another reason the O.D. of the mitochondria, upon shrinking in 0·30 M sucrose, reached only 0·374–0·393 instead of a possible 0·430. Neither the exit of water nor the sucrose entry occurring with mitochondrial shrinkage was ever inhibited by 10^{-5} M CPZ.

In comparing the initial O.D. of the same mitochondrial preparations while following the swelling in 0·30 M sucrose in the presence or absence of cyanide, it is interesting to note that the two values are identical (Fig. 1). Likewise the 0-min water content values are the same (these values are not listed in Table 1). This is because changes in both samples began to occur only 8–14 min after their preparation, if at all. The 0-min samples of mitochondrial suspensions obtained for per cent water determinations are centrifuged for only 8 min before the supernatants are removed, the tube-sides dried, and the water determinations begun (see Methods). Since, after 8 min, no significant drops have occurred (Fig. 1), the per cent water values for the 0-min samples (although actually measured 8 min later) would still be expected to reflect the actual water content at 0 min. The same considerations hold for the 0-min per cent water values obtained for mitochondrial suspensions in 0·15 M sucrose–Tris + 3×10^{-4} M cyanide and in the same suspensions plus 10^{-5} M CPZ and cyanide, although both values are somewhat higher (72–73% in Table 3) because of the rapid, probably osmotic, phase than is the case in 0·30 M sucrose (67–68% in Table 1). However, they do not hold for mitochondria suspended in 0·15 M sucrose–Tris solution alone, since in this case the O.D. fall begins immediately, is very rapid, and is finished after only 8 min (Fig. 1), so that meaningful mitochondrial water content studies could not be performed in this case by the method used.

The "early mitochondrial swelling", in extremely hypotonic sucrose media recently reported by Smith *et al.*,[9] is measured by the resulting very rapid (1–2 min) and extensive fall in O.D. and could not be influenced by CPZ. Duplicating their experimental conditions, we were able to confirm this finding. However, the O.D. change occurring in this very short time is as great or greater than that resulting in the present experiments after 20 min. It is possible that the very rapid and extensive fall also took place in two stages, similar to those here reported, but that, in the absence of cyanide and in the presence of extremely hypotonic sucrose, Smith *et al.* were unable to separate them. Furthermore, even under our milder conditions for swelling, CPZ occasionally failed to influence the O.D. drop appearing 10–20 min after the onset of the experiment, if this fall was greater than 0·2 unit. These infrequent failures of the mitochondria suspended in 0·15 M sucrose + cyanide to respond to CPZ with an *inhibition* of swelling were thought to be due to the isolation procedure, which sometimes resulted in more extensively damaged mitochondria. Likewise, it is believed that exposure of the mitochondria to extremely hypotonic sucrose damages mitochondria too extensively to allow CPZ to exert any effect. This could explain why Smith *et al.* failed in their attempts to inhibit mitochondrial swelling with this drug.

Since there was some evidence presented by Bernheim[10] that CPZ might act as an antioxidant in preventing mitochondrial swelling concomitant with the appearance of lipoperoxide in the suspension, we thought it advisable, using the same thiobarbiturate determination, to investigate the possibility that such peroxides might be formed during the 20-min course of the present experiments. This proved *not* to be the case.

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