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INDUCTION OF MITOCHONDRIAL SWELLING BY THE FUNGICIDE CAPTAN

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Abstract—The fungicide captan induces large-amplitude swelling in rat liver mitochondria. In the absence of an energy source, initial swelling, but not later swelling, can be inhibited by 2,4-dinitrophenol and KCN, suggesting two phases in the process—the first energy-linked, the second passive. Sucrose inhibits both phases of swelling. Though sucrose prevents swelling, it does not prevent captan inhibition of state 3 respiration. Captan does not prevent P_i-induced swelling, indicating that captan inhibition of oxidative phosphorylation is not due to interference with the transport of P_i into the mitochondria. Two moieties of the captan molecule (trichloromethylthiol moiety and tetrahydrophthalimide) were tested for their abilities to induce swelling; only the former compound proved active. The results suggest that captan interacts with mitochondrial membranes, presumably with sulfhydryl groups, altering their permeability to endogenous and exogenous ions.

IN A PREVIOUS paper,[1] we reported that the fungicide captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2 dicarboximide] inhibited oxidative phosphorylation in rat liver mitochondria, presumably due to reactions with mitochondrial sulfhydryl groups.[2–4] Included in the list of inhibited enzymes were NADH-dehydrogenase, β-hydroxybutyrate dehydrogenase and one or more enzymes of the energy transfer system. Inhibition of the dehydrogenases undoubtedly resulted from direct action of the fungicide on the enzymes.[5,6] Inhibition of the energy transfer reactions, however, could be due to an indirect action of the fungicide, i.e. on membrane permeability[7,8] rather than on specific enzymes. Sulfhydryl reagents have been shown to react with mitochondrial membranes, activating energy-dependent K^+ uptake and inducing swelling.[7,8] Inhibition of inorganic phosphate (P_i) transport by sulfhydryl reagents has also been reported.[9–11] In both cases the alteration in ion uptake subsequently led to respiratory inhibition[9–11] and uncoupling of oxidative phosphorylation.[8]

The present study was undertaken to determine if captan, because of its high affinity for sulfhydryl groups,[2–4] interfered with mitochondrial function by altering membrane permeability. This possibility was suggested by an earlier finding that captan stimulated oxidation of exogenous cytochrome c in intact mitochondria.[1] The results show that captan does indeed induce large-amplitude swelling associated with loss of state 3 respiration. Captan does not, however, appear to inhibit P_i transport.

METHODS AND MATERIALS

Liver mitochondria were prepared from male, Sprague–Dawley rats. Liver tissue was placed in 0·25 M sucrose to give a final concentration of 33 per cent (w/v) and then

homogenized for 30 sec in a Potter–Elvehjem homogenizer. The homogenate was diluted to 10 per cent (w/v) with 0·25 M sucrose and the cell debris was removed by centrifugation at 600 *g* for 10 min. Mitochondria were removed by centrifugation at 8500 *g* for 10 min and then washed twice in 30 ml of 0·25 M sucrose.

Mitochondrial swelling was measured as a decrease in absorbance at 520 mμ. Studies were carried out in a water-jacketed Gilford recording spectrophotometer at 30°. The standard swelling medium contained 125 mM KCl, 20 mM Tris-chloride (pH 7·4), and mitochondria, to give an absorbance at 520 mμ of 0·8–0·9. Total volume was 3 ml. Unless otherwise indicated, no energy source was used.

Captan was recrystallized twice from ethanol before use. Fresh stock solutions (15 mM) were prepared daily in dimethylsulfoxide (DMSO). Small aliquots of stock solution (1–10 μl) were added directly to the reaction vessels. DMSO at 20 μl/3 ml of reaction mixture had no effect upon mitochondrial swelling.

Oxidative phosphorylation was measured as previously described.[1] Mitochondria were prepared for electron microscopy as described by Hackenbrock.[12]

RESULTS

Figure 1 shows the effects of several concentrations of captan on mitochondrial swelling in the absence of an energy source. The time of onset and the degree of swelling are both dependent upon the concentration of captan, though neither is a linear

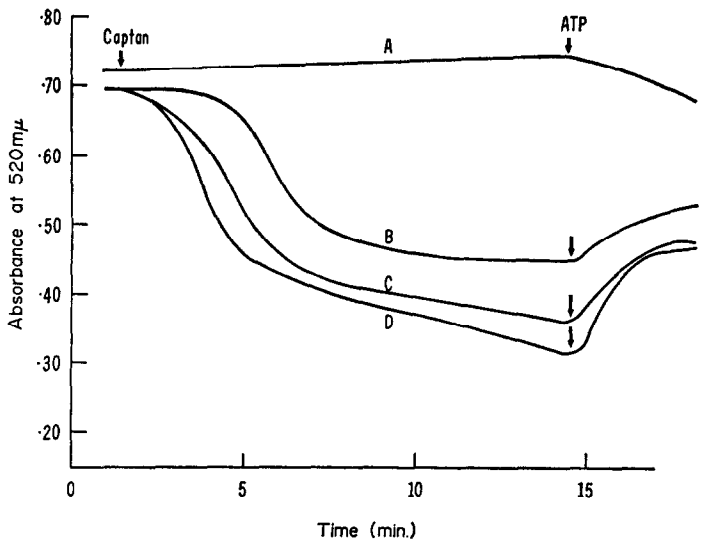

FIG. 1. Mitochondrial swelling in response to varying concentrations of captan. Curve (A), no captan; (B), 15 mμmoles; (C), 45 mμmoles; (D), 75 mμmoles. Swelling was carried out in 125 mM KCl, 20 mM tris-chloride buffer (pH 7·4) and 0·76 mg mitochondrial protein at 30° in a 3-ml volume. Final concentration of ATP was 3 mM.

function of the captan concentration. The lowest concentration of captan that induced swelling was 15–20 mμmoles/mg mitochondrial protein. Captan-swollen mitochondria could be partially recontracted with ATP (Fig. 1). ATP-induced contraction varied in several experiments from 5 to 35 per cent of the O.D. change which occurred in the

swelling phase. The extent of contraction was independent of captan concentrations. Captan also produced rapid large-amplitude swelling when energy was supplied through the oxidation of succinate, glutamate, β-hydroxybutyrate and α-ketoglutarate. The degree of swelling was as great in the absence of an energy source as in its presence.

Degradation of captan occurs by scission of the N–S bond, releasing tetrahydrophthalimide and an $-SCCl_3$ moiety.[2] The latter either forms mixed disulfides with cellular components or rearranges to thiophosgene ($Cl_2C{=}S$),[2–4] which in turn reacts with cellular components. In an attempt to determine what part of the captan molecule is responsible for induction of mitochondrial swelling, the tetrahydrophthalimide moiety and sulfur-containing trichloromethylsulfenylchloride ($ClSCCl_3$) moiety were tested (Table 1). The trichlormethylsulfenylchloride induced large-amplitude swelling at a concentration similar to that at which captan acts. Tetrahydrophthalimide was ineffective. The response to $ClSCCl_3$ was not as consistent as the response to captan, probably because of the instability of the former. To demonstrate $ClSCCl_3$-induced swelling, solutions of the chemical had to be used within 10–15 min of preparation.

TABLE 1. EFFECTS OF CAPTAN, TETRAHYDROPHTHALIMIDE AND TRICHLOROMETHYLSULFENYLCHLORIDE ($ClSCCl_3$) ON MITOCHONDRIAL SWELLING*

Additions	Concn (mμmoles)	Mitochondrial swelling ($-\Delta$ in absorbance at 520 mμ after 10 min) Expt 1	Expt 2
None	—	0·050	0·040
Captan	15	0·390	0·410
$ClSCCl_3$	15	0·250	0·500
Tetrahydrophthalimide	15	0·050	0·050

* Swelling medium contained 125 mM KCl, 20 mM tris-chloride (pH 7·4), and mitochondria to give an O.D. of 0·80–0·90 at 520 mμ. Total volume was 3 ml. The temperature was 30°.

Uncouplers of oxidative phosphorylation and inhibitors of electron transport prevent energy-dependent mitochondrial swelling, but not passive swelling.[13–16] To determine if captan-induced swelling is passive or energy-dependent, mitochondria were incubated for 3 min in the presence of the uncoupling agent, 2,4-dinitrophenol (DNP), before adding captan (Table 2). DNP at 10^{-6} M had no effect on captan-induced swelling. At 10^{-4} M, however, DNP retarded the onset of swelling, but did not completely abolish it. The ability of DNP to retard captan-induced swelling is dependent upon the concentration of captan (Table 3). Addition of 60 mμmoles captan to swelling medium containing $2{\cdot}5 \times 10^{-5}$ M DNP produced little swelling in the first 10 min. Addition of 150 mμmoles captan produced large-amplitude swelling immediately.

KCN, like DNP, retarded but did not abolish swelling (Table 4). KCN at concentrations of 0·05 and 0·1 mM had little or no observable effects, but 0·5–5 mM KCN retarded swelling. Increasing KCN to 10 mM increased the rate of swelling; this observation remains unexplained. Though neither DNP nor KCN completely inhibited

TABLE 2. EFFECTS OF DNP CONCENTRATION ON CAPTAN-INDUCED MITOCHONDRIAL SWELLING*

DNP concn (M)	Captan	Mitochondrial swelling (−Δ in absorbance at 520 mμ) Swelling time (min) 5	10	15
0	0	0·000	0·000	0·000
0	+	0·350	0·410	0·455
10^{-4}	+	0·030	0·150	0·350
5×10^{-5}	+	0·030	0·140	0·350
10^{-5}	+	0·030	0·140	0·350
5×10^{-6}	+	0·075	0·260	0·400
4×10^{-6}	+	0·100	0·270	0·430
3×10^{-6}	+	0·210	0·320	0·440
10^{-6}	+	0·370	0·420	0·470

* Mitochondria were incubated 3 min with DNP before adding 75 mμmoles captan. Conditions of assay were the same as in Table 1.

TABLE 3. EFFECTS OF DNP ON CAPTAN-INDUCED MITOCHONDRIAL SWELLING*

Additions	Captan concn (mμmoles)	Mitochondrial swelling (−Δ in absorbance at 520 mμ) Swelling time (min) 5	10	15
Captan	60	0·030	0·240	0·400
Captan + DNP	60	0·030	0·090	0·220
Captan	150	0·330	0·370	0·400
Captan + DNP	150	0·200	0·340	0·400

* Final concentration of DNP was $2{\cdot}5 \times 10^{-5}$ M. Other conditions of the system are described in Table 1.

captan-induced swelling, both prevented P_i-induced swelling (data not shown), indicating that the concentrations of inhibitors used were sufficient to prevent energy-linked swelling.

Passive transport of ions across the mitochondrial membrane, and thus swelling, can be prevented by establishing an opposing osmotic force with sucrose in the medium.[13–16] Swelling due to energy-linked ion translocation is not affected by sucrose.[13–15] Table 5 shows that 0·15 M sucrose retarded captan-induced swelling and that 0·3 M sucrose completely prevented swelling over a 15-min incubation period. In spite of its protective effect on swelling, sucrose did not prevent captan from inhibiting

TABLE 4. EFFECTS OF KCN ON CAPTAN-INDUCED MITOCHONDRIAL SWELLING*

KCN concn (mM)	Mitochondrial swelling (−Δ in absorbance at 520 mμ) Swelling time (min) 5	10	15
0	0·390	0·420	0·460
0·05	0·390	0·430	0·470
0·1	0·310	0·370	0·410
0·5	0·080	0·190	0·270
1·0	0·055	0·135	0·240
5·0	0·065	0·140	0·250
10·0	0·075	0·245	0·310

* Mitochondria were incubated for 3 min in the presence of KCN before initiating swelling by addition of 75 mμmoles captan. Conditions of assay were the same as in Table 1.

TABLE 5. SUCROSE INHIBITION OF CAPTAN-INDUCED MITOCHONDRIAL SWELLING*

Sucrose concn (M)	Captan	Mitochondrial swelling (−Δ in absorbance at 520 mμ) Swelling time (min) 5	10	15
0	−	0·000	0·000	0·000
0	+	0·460	0·600	0·640
0·15	+	0·040	0·140	0·240
0·20	+	0·040	0·140	0·240
0·25	+	0·040	0·100	†
0·30	+	0·010	0·040	0·080

* The absorbance at 520 mμ was 1·98 at the start of the experiment. Swelling was initiated by adding 75 mμmoles captan. Other conditions of the system are given in Table 1.

† Sample removed for assay in O_2 electrode.

state 3 respiration (Table 6). Thus swelling *per se* is not necessary for captan to induce loss of respiratory control.

Certain antibiotics stimulate mitochondrial swelling by inducing the translocation of selected monovalent cations.[17] To determine if captan selectively activates ion uptake, swelling was measured using media in which K^+ was replaced by Li^+, Na^+ or NH_4^+ (Table 7). In the presence of captan, neither the rate nor the extent of swelling

differed significantly in the presence of the various cations. These findings indicated either that captan does not induce selective ion translocation or that ion selectivity is very broad.

TABLE 6. CAPTAN-INDUCED SWELLING AND IMPAIRMENT OF OXIDATIVE PHOSPHORYLATION*

Additions	Swelling (−Δ in absorbance at 520 mμ)	Respiration (μg atoms O/min)			
		Succinate	Succinate + ADP	RC†	ADP:O
None	0·000	0·021	0·103	4·9	1·67
Captan	0·420	0·014	0·021	1·5	0·00
Captan + sucrose	0·006	0·021	0·021	1·0	0·00

* Swelling was carried out at 30° in the oxygen electrode mixture containing 0·12 M KCl, 8 mM $MgCl_2$, 5 mM K_2HPO_4 and 20 mM glycylglycine (pH 7·4) in a total volume of 3 ml. The final concentration of sucrose was 0·25 M. Mitochondrial protein was adjusted to give an absorbance at 520 mμ of 1·90. Swelling was initiated by addition of captan. After 5 min of swelling, 1·6 ml of the sample was transferred to the oxygen electrode vessel for determination of oxidative phosphorylation.

† Respiratory control (RC) is defined as the ratio of respiration in the ADP-controlled state to respiration in the uncontrolled state.

TABLE 7. CAPTAN-INDUCED SWELLING IN THE PRESENCE OF DIFFERENT MONOVALENT CATIONS*

Additions	Captan	Mitochondrial swelling (−Δ in absorbance at 520 mμ)		
		Swelling time (min)		
		5	10	15
KCl	−	0·000	0·000	0·020
KCl	+	0·390	0·430	0·450
NaCl	−	0·000	0·070	0·110
NaCl	+	0·300	0·330	0·360
NH_4Cl	−	0·180	0·220	0·240
NH_4Cl	+	0·340	0·400	0·430
LiCl	−	0·000	0·060	0·070
LiCl	+	0·350	0·400	0·410

* Cation concentration was 150 mM. Mitochondria were incubated for 3 min before initiating swelling by addition of 75 mμmoles captan. Other conditions are described in Table 1.

Earlier[1] we suggested that captan could interfere with oxidative phosphorylation by competing with, or preventing completely, P_i transport into the mitochondria. Results of experiments designed to test this are shown in Fig. 2. With 0·25 M sucrose in the swelling media, captan failed to induce swelling even after 10–15 min of incubation. Addition of P_i, however, produced an immediate, large-amplitude swelling in both control and captan-treated mitochondria, indicating that captan-treated mitochondria

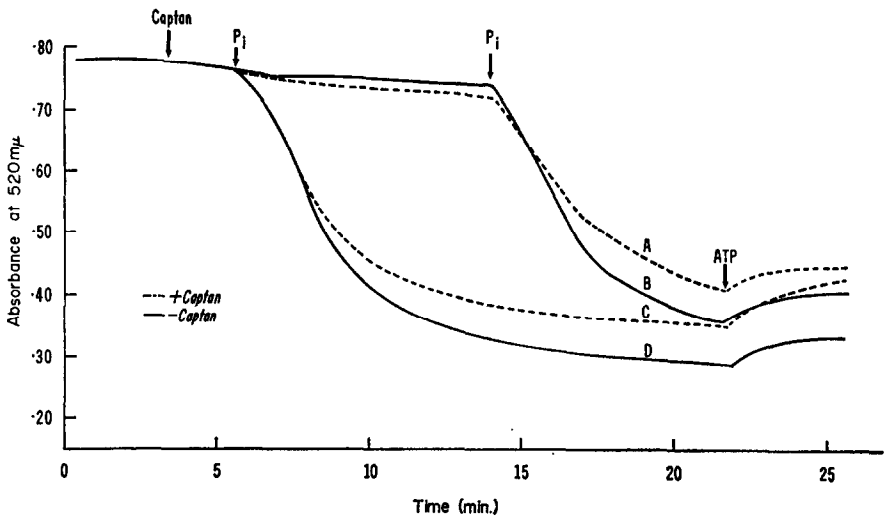

FIG. 2. Effects of captan on P_i-induced swelling. The reaction was carried out in KCl swelling medium containing 0·25 M sucrose to prevent captan-induced swelling. Captan (75 mμmoles) was added to curves A and C as indicated. P_i was adddd to curves C and D after 1 min of incubation with captan, and to curves A and B after 10 min of incubation. The final concentration of P_i was 3·3 mM, and the final concentration of ATP was 3 mM.

are still capable of transporting P_i. Since captan inhibits state 3 respiration in 3–5 min (reference 1 and Table 6), but has no effect on P_i-induced swelling after 15 min of incubation, it is apparent that captan does not inhibit respiration and phosphorylation by preventing P_i transport.

Ultrastructural changes occurring during captan-induced swelling are shown in Fig. 3. Figure 3A shows control mitochondria incubated for 3 min in KCl swelling media. Nearly all of these mitochondria were highly contracted, with expanded intracristal spaces and condensed, electron-dense matrices. They resemble control mitochondria incubated in 0·25 M sucrose at 4°. Incubation in the presence of captan (Fig. 3B) for 3 min resulted in nearly complete ultrastructural transformation into a swollen type which, in many cases, contained no visible cristae. In these mitochondria, the matrix was diluted and appeared as small clumps of electron-dense material. Increasing the incubation time in the presence of captan produced progressively greater numbers of mitochondria with no visible cristae. In view of these ultrastructural changes, the small amount of ATP-induced contraction (Fig. 1) observed with captan-treated mitochondria probably reflects the numbers of mitochondria still containing intact inner membranes rather than a limited amount of contraction by the entire population.

DISCUSSION

Mitochondrial swelling results from either energy-linked or passive transport of ions across the inner membrane.[13–16] During the energy-linked swelling, the uptake of ions and water[18,19] is supported by substrate oxidation or hydrolysis of ATP, and utilizes energy derived from oxidative phosphorylation.[13–16] Energy-linked swelling is characterized by its sensitivity to electron transport inhibitors or uncouplers of oxidative phosphorylation.[14,15] Passive swelling, on the other hand, occurs when the permeability barriers of the inner membrane are removed and passive ion exchange takes

place.[14,15] Large-amplitude passive swelling is not affected by metabolic inhibitors, but is inhibited by establishment of an opposing osmotic force using sucrose or high molecular weight compounds.[14,15]

The present experiments show that captan induces passive, large-amplitude swelling in rat liver mitochondria, since swelling was not entirely inhibited by KCN or DNP, but was inhibited by sucrose. Higher concentrations of DNP (0·10 mM) and KCN (5 mM) did, however, retard the onset of swelling, suggesting that the earlier phase is at least partially energy dependent. Thus captan-induced swelling apparently occurs in two phases and resembles that caused by *p*-chloromercurisulfonate (*p*CMS).[8]

The level of captan needed to induce swelling (15–20 mμmoles/mg protein) compares closely with that necessary to inhibit state 3 and DNP-activated respiration.[1] The time course of swelling also compares well with the rate of captan-induced loss of respiratory control; maximal swelling occurs in 3–5 min and respiratory control is lost within 3 min after addition of captan.[1] These data suggest a temporal correlation between inhibition of mitochondrial function and swelling. The amount of captan needed to induce early DNP- and KCN-sensitive swelling is also similar to the number of mitochondrial sulfhydryl groups which must react with sulfhydryl group reagents (15–20 mμmoles/mg protein) before energy-dependent K^+ accumulation and swelling occur.[20] In their experiments, Knight *et al.*[20] also found that passive swelling did not occur until 40 mμmoles sulfhydryl per mg protein had reacted, suggesting that late-phase swelling induced by captan is due to something other than additional binding to sulfhydryl groups. Our finding that DNP on retardation of early-phase swelling could be overcome by increasing the concentration of captan is, however, in keeping with the conclusion of Knight *et al.*[20] that passive swelling can be induced with higher concentrations of sulfhydryl reagents.

Several reports have shown that sulfhydryl inhibitors prevent oxidative phosphorylation by preventing P_i transport.[9–11] Though captan reacts strongly with sulfhydryls,[2,4] the present results indicate that it does not interfere with P_i transport. We have shown that mitochondria incubated in swelling media containing captan and sucrose lose the ability to carry out state 3 respiration, while P_i-induced swelling is unaffected. If P_i is transported into the mitochondria via a specific exchange reaction, as suggested by Chappell and Crofts,[21] it is obvious that captan does not compete for that reaction and, therefore, is not the mechanism through which captan inhibits state 3 respiration. This result is in agreement with the conclusion of Brierley[8] that sulfhydryl group reagents can induce swelling by reacting with sites independent of the P_i exchange reaction.

It seems unlikely that captan-induced swelling is caused by inducing ion specific transport, as described for certain antibiotics[17] and mercurials.[7,8] Captan induces swelling in the presence of K^+, Na^+, Li^+ and NH_4^+. These findings add support to the belief that captan induces a breakdown in the permeability barriers of the inner membrane. Ultrastructural alterations also support this interpretation. Inner membranes are almost entirely absent in captan-treated mitochondria and the matrix is completely disorganized. Captan-treated mitochondria were considerably more damaged than those reversibly uncoupled by pentacholorophenol.[22] Such drastic ultrastructural alterations undoubtedly account for the activation of cytochrome c oxidation previously observed,[1] using captan-treated mitochondria.

The reaction of captan with cellular thiol groups results in its degradation to thio-

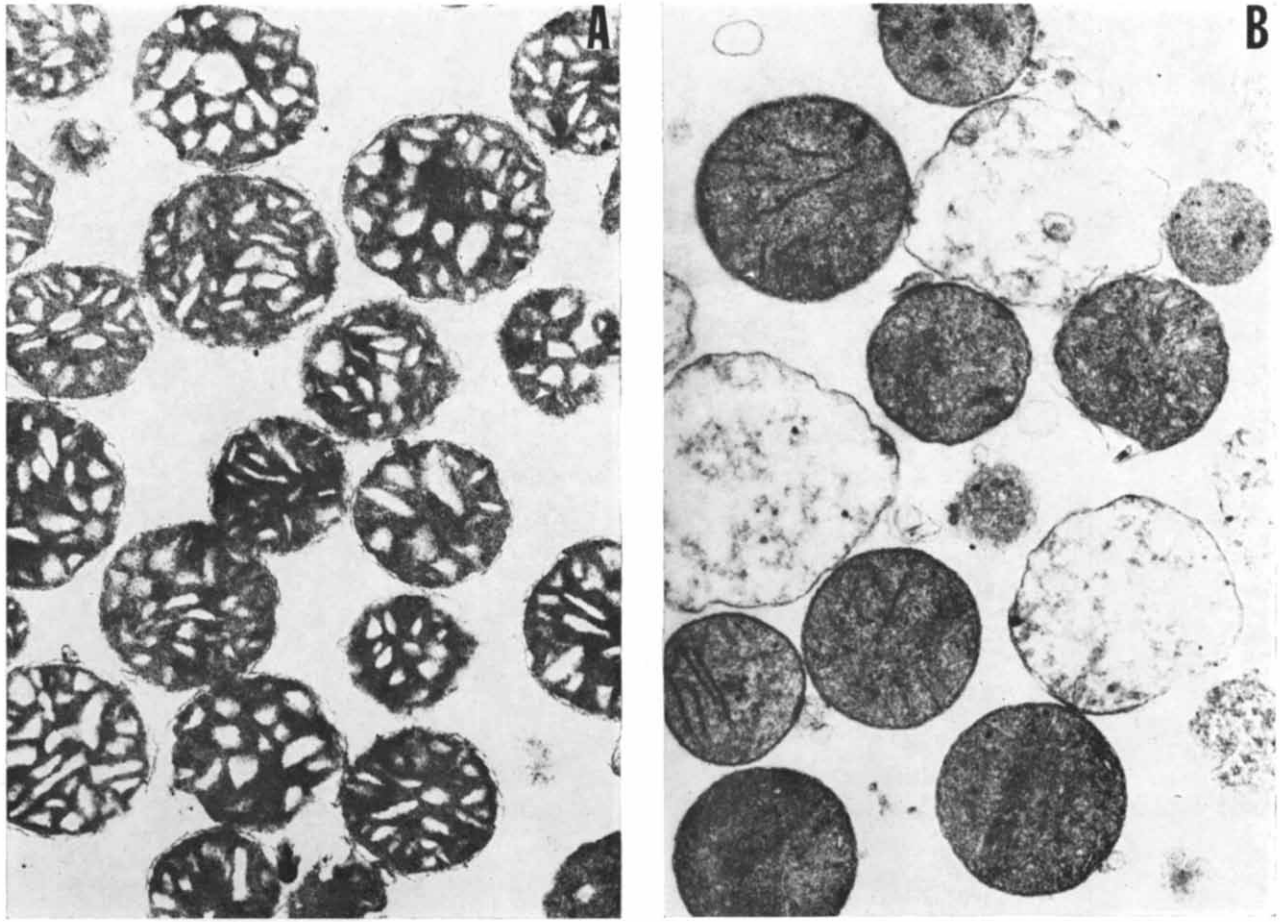

FIG. 3. Ultrastructural changes in mitochondria swollen by captan. (A), control; (B), treated with 75 mμmoles captan for 3 min at 30°.

phosgene ($Cl_2C{=}S$) and tetrahydrophthalimide.[2] The sulfur-containing moiety is believed to be the toxic portion of the captan molecule, since it (and not tetrahydrophthalimide) is toxic to yeast[2] and binds to yeast and purified proteins.[23,24] These observations are extended by the present finding that $ClSCCl_3$ and not tetrahydrophthalimide induces extensive swelling in mitochondria. This is also in keeping with unpublished results from this laboratory that $ClSCCl_3$ inhibits state 3 respiration and uncouples oxidative phosphorylation, whereas tetrahydrophthalimide has no effect. In many experiments, however, no effects of $ClSCCl_3$ were observed. This variation is probably due to the instability of $ClSCCl_3$, since respiratory inhibition was obtained only within the first 10–15 min after preparing a fresh solution of $ClSCCl_3$.

This report established that, in addition to inhibiting individual enzymes or enzyme systems,[1] captan reacts with mitochondrial membranes inducing permeability changes. These results confirm our earlier suggestion that some of the observed effects of captan could be due to alterations in the mitochondrial membrane.[1] In view of this additional action of captan, however, assignment of a primary site of action becomes more difficult. Indeed, whether the primary site is on specific enzymes, enzyme systems or membranes will remain an open question, until more is known about the mechanisms of oxidative phosphorylation and the role of the mitochondrial membrane in the generation of a high energy intermediate[25] or "state".[26] In spite of these difficulties, the present results do open the possibility that captan toxicity could result from the interaction with cell membranes rather than specific enzymes or enzyme systems.

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