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ACTION OF THE FUNGICIDES CAPTAN AND FOLPET ON RAT LIVER MITOCHONDRIA

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Abstract—Captan and folpet action on rat liver mitochondria has been examined. Both fungicides were found to uncouple oxidative phosphorylation. The minimal effective concentration was 20–25 mμmoles fungicide/mg mitochondrial protein. In addition to uncoupling oxidative phosphorylation, captan and folpet inhibited state 3 respiration, DNP-activated respiration, the ATP–P_i exchange reaction, NADH-dehydrogenase, and β-hydroxybutyrate dehydrogenase. Mitochondrial adenosine triphosphatase (ATPase) was activated by both fungicides, but captan was considerably more effective than folpet in this respect. Captan activation of ATPase was observed only upon initial exposure of mitochondria to the fungicides. Preincubation with captan inhibited activation. Preincubation with captan also inhibited DNP-activated ATPase, and the loss of DNP-activated ATPase paralleled the loss of captan-activated ATPase. Mg^{2+} prevented the inhibition of activated ATPase when captan was used, but had little effect on folpet-stimulated ATPase. Cysteine prevented the inhibition of activated ATPase, but did not prevent activation itself. Cysteine also prevented, and reversed, captan and folpet inhibition of state 3 respiration and DNP-activated respiration.

FOLPET [*N*-(trichloromethylthio)phthalimide] and captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2 dicarboximide] are widely used as fungitoxic agents. The toxicity of both compounds appears to be due to their affinity for sulfhydryl groups.[1–6] Since mitochondria contain several sulfhydryl enzymes,[7–11] oxidative phosphorylation could prove vulnerable to the toxic action of captan and folpet. Indeed, it has been previously suggested[12] that captan inhibits both oxidative and phosphorylative processes in *Neurospora* spores, though it was not determined if these reactions were associated with mitochondria. The present study was undertaken to examine the effects of folpet and captan on mammalian mitochondria *in vitro*. The results show that several enzymes of rat liver mitochondria are sensitive to these fungicides and that oxidative phosphorylation is disrupted. Preliminary accounts of this study have been published elsewhere.[13]

METHODS

Liver mitochondria were prepared from Sprague–Dawley rats as described previously.[14] Inner membranes were prepared from rat liver mitochondria by the method of Sottocasa *et al.*[15] Electron transport particles (ETP) were prepared from beef heart by the method of Green and Ziegler[16] and stored at −20° until used.

Oxygen electrode tracings were obtained at 30° with a Clark oxygen electrode. The standard reaction mixture contained 120 mM KCl, 8 mM $MgCl_2$, 5 mM potassium phosphate, 20 mM glycylglycine buffer (pH 7·4), and aliquots of mitochondria (1–4 mg protein) in a total volume of 1·6 ml. Substrates were added in a volume of 20 μl to give

a final concentration of 12·5 mM. Additional compounds (captan, ADP, 2,4-dinitrophenol) were added in volumes of 1–10 μl. ATPase activity was measured in terms of inorganic phosphate released, as described earlier.[14]

The $^{32}P_i$–ATP exchange reaction was carried out in a volume of 1 ml containing 10 mM $MgCl_2$, 10 mM ATP, 20 mM phosphate (pH 7·4), 100,000 counts/min of $^{32}P_i$ and aliquots of mitochondria. The system was incubated for 10 min and the reaction stopped by addition of 0·1 ml of 35% $HClO_4$ acid. $^{32}P_i$-labelled ATP and $^{32}P_i$ were extracted as described by Pullman.[17] The molybdate used in the final step of the extraction produced an intense yellow chromophore in scintillation fluids containing 2,5-diphenylorazole (PPO) or 1,4-bis-2-(4-methyl 5-phenyloxazolyl)benzene (POPOP). Therefore, radioactivity was measured by adding 1 ml of the molybdate extract to 9 ml of distilled water and counting by use of the tritium window in a liquid scintillation counter (Cerenkov counting).[18]

Folpet and captan were generously supplied by Dr. L. Fishbein of this Institute. Folpet (m.p. 181) was recrystallized two times from acetone, and captan (m.p. 173) was recrystallized two times from ethanol prior to use. Solutions were prepared fresh daily as stock 15 mM solutions in dimethylsulfoxide (DMSO). Small aliquots (1–10 μl) of the stock solutions were added directly to the reaction mixture. DMSO controls were routinely run with all experiments. DMSO at concentrations less than 5% (v/v) had no effect upon oxidative phosphorylation.

RESULTS

Both captan and folpet inhibited oxidative phosphorylation in rat liver mitochondria. Results with folpet are shown in Fig. 1. Mitochondria incubated with 75 mμmoles folpet (47 μM) for 1 min did not exhibit state 3 respiration (ADP controlled) or respiratory control (RC) with β-hydroxybutyrate, α-ketoglutarate and glutamate (latter not shown) as the substrates. State 3 respiration was obtained with succinate when mitochondria were incubated under the same conditions. Addition of 12·5 μM 2,4-dinitrophenol (DNP) did not reverse state 3 inhibition, indicating that folpet does not mimic the action of oligomycin.[19] Addition of succinate to captan- or folpet-inhibited mitochondria stimulated respiration. With succinate as substrate (bottom tracing) the ADP:O ratio and respiratory control were only slightly reduced by folpet, and state 3 respiration was inhibited only 19 per cent. Reduction of the RC value is due more to increased state 4 respiration than to decreased state 3, presumably because of uncoupling. Succinate-supported state 3 respiration, however, was inhibited by increasing the incubation time in the presence of the fungicide. This is shown in Table 1 using captan.

The selective inhibition of NADH-linked oxidations was further examined using inner membrane preparations which, unlike intact mitochondria, oxidize exogenous NADH (Table 2). NADH-oxidase was inhibited by 100 μM captan or folpet, and the inhibited site, like the rotenone-inhibited site,[19] could be bypassed with vitamin K_3. Oxidation of succinate and ascorbate plus *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediame (TMPD) was unaffected in experiments where fungicide was added directly to respiring inner membranes and the reaction measured for an additional 1 min. However, if inner membranes were preincubated for 3 min in the presence of fungicides, oxidation of succinate and ascorbate plus TMPD was inhibited 30–50 per cent.

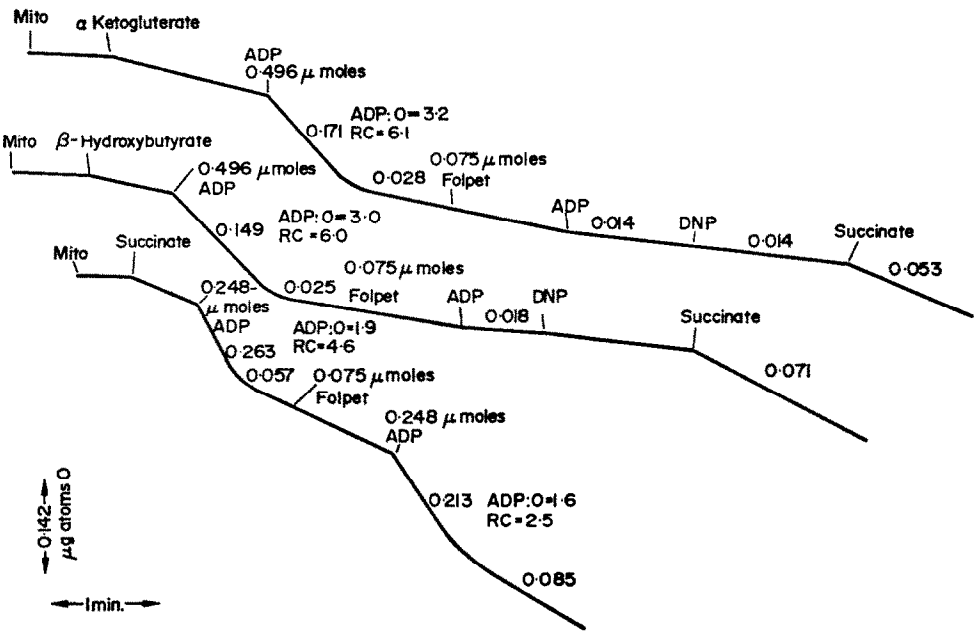

FIG. 1. Folpet inhibition of oxidative phosphorylation. Assay conditions are described in Methods. Each experiment contained 3·5 mg of mitochondrial protein. Final concentration of DNP was 12·5 μM. Total volume of the reaction was 1·6 ml.

TABLE 1. EFFECTS OF CAPTAN ON SUCCINOXIDASE IN RAT LIVER MITOCHONDRIA*

Additions	Preincubation time (sec)	O_2 uptake (μg atoms O/min)			
		State 3	State 4	DNP	RC
None		0·185	0·046	0·306	4·05
Captan	30	0·188	0·064	0·053	2·94
Captan	120	0·125	0·064	0·064	2·00
Captan	180	0·097	0·053	0·053	1·82
Captan + 375 mμmoles cysteine	180	0·142	0·046	0·228	3·09
Captan + 75 mμmoles cysteine	180	0·167	0·053	0·202	3·15

* Polarographic measurements were made as described in Methods. Mitochondria were incubated with 47 μM captan for 0·5 to 3 min prior to initiating state 3 respiration with 0·496 μmole ADP. Cysteine, when added, was present during preincubation. Final concentration of DNP was $2·25 \times 10^{-5}$ M; mitochondrial protein was 1·55 mg. Total volume was 1·6 ml.

The effects of captan and folpet on NADH-cytochrome c reductase, NADH-DCIP* reductase, and NADH-ferricyanide reductase were examined in an attempt to localize the site of fungicidal action in the NADH-dehydrogenase region.[10] For comparison, rotenone and *p*-chloromercuribenzoate (PCMB) were also used (Table 3). Rotenone inhibited only cytochrome c reductase, indicating that DCIP and ferricyanide accept

* DCIP: 2,6-dichlorophenol indophenol.

TABLE 2. EFFECTS OF CAPTAN AND FOLPET ON ELECTRON TRANSPORT IN RAT LIVER MITOCHONDRIAL INNER MEMBRANES*

Substrate	Inhibitor	O_2 uptake (μg atoms O/min/mg protein) Minus inhibitor	Plus inhibitor	Inhibitor + vitamin K_3
NADH	Captan	0·129	0·066	0·233
	Folpet	0·142	0·051	0·240
Succinate	Captan	0·116	0·109	
	Folpet	0·120	0·121	
Ascorbate	Captan	0·154	0·154	
+ 1·5 mM TMPD	Folpet	0·123	0·123	

* Polarographic measurements were made as described in Methods. Respiration was measured in the presence of substrate and then for 1 min after addition of 100 μM fungicide. The assay contained 1 mg NADH, 6·3 mM succinate or ascorbate, $1{\cdot}25 \times 10^{-5}$ M vitamin K_3 and 2·0 mg protein. Total volume was 1·6 ml.

electrons from the substrate, not the oxygen side of the rotenone site, i.e. in the NADH-dehydrogenase region.[19,20] Captan, folpet and PCMB all inhibited reduction of ferricyanide, indicating fungicide inhibition in the NADH-dehydrogenase. PCMB inhibited ferricyanide reduction more strongly than either folpet or captan, however, suggesting some difference in their mechanisms of action. Neither the fungicides nor PCMB inhibited NADH-DCIP reductase. These results might be explained by the observation of Hadler *et al.*[21] that oxidized DCIP itself reacts with sulfhydryl groups. DCIP could thus inhibit the enzyme. Such does not appear to be the case, however,

TABLE 3. EFFECTS OF CAPTAN AND FOLPET ON THE NADH-DEHYDROGENASE REGION OF ELECTRON TRANSPORT PARTICLES (ETP)*

Additions	Specific activity (μmoles acceptor reduced/min/mg protein) NADH-DCIP reductase	NADH-ferricyanide reductase	NADH-cytochrome c reductase
Expt. 1 Control	0·027	0·925	0·036
Captan (75 μM)	0·025	0·805(13)	0·020(45)
Folpet (75 μM)	0·025	0·791(14)	0·020(45)
Rotenone (1 μg/ml)	0·025	0·914(2)	0·013(65)
Expt. 2 Control	0·040	1·040	0·042
PCMB (100 μM)	0·040	0·663(39)	0·000(100)
PCMB (0·2 μM)	0·040	0·667(36)	0·021(50)

* Assay media for measurement of NADH-DCIP reductase contained 0·05 M phosphate buffer (pH 7·4), 3 mM azide, 1 μmole NADH and 0·18 mM DCIP in a total volume of 3 ml. Reduction of DCIP was measured at 600 mμ. NADH-ferricyanide reductase was measured under the same conditions, except that 1 mM ferricyanide replaced DCIP and the reaction was followed at 420 mμ. Assay of NADH-cytochrome c reductase contained 0·05 M phosphate buffer (pH 7·0), 1 μmole NADH, 2·25 mM KCN and 1 mg cytochrome c in a total volume of 1 ml. Cytochrome c reduction was followed at 550 mμ. Measurements were made on a Gilford recording spectrophotometer at 30°, using 133 μg ETP protein. Numbers in parentheses represent per cent inhibition.

when the purified NADH-dehydrogenase is used.[10] All four compounds tested inhibited cytochrome c reductase. Captan, folpet, and PCMB inhibited cytochrome c reductase more than ferricyanide reductase. It is not clear from these results whether the site(s) at which captan and PCMB inhibit cytochrome c reductase is on the substrate or oxygen side of the rotenone site.

Since captan and folpet inhibit state 3 respiration, polarographic detection of uncoupling was not always possible. Thus, manometric methods were also used and the results are given in Table 4. With 25 μM captan (approximately 10–15 mμmoles/mg mitochondrial protein), both respiration and phosphorylation were inhibited with glutamate and β-hydroxybutyrate as the substrate and little, if any, reduction in the P:O ratio was observed. In contrast, succinate-supported oxidative phosphorylation was uncoupled and respiration increased rather than declined. Increasing captan to 75 μM uncoupled oxidative phosphorylation regardless of the substrate used. At this concentration the sensitivity of NADH-linked substrates to captan inhibition was apparent, since respiration supported by glutamate and β-hydroxybutyrate was inhibited 80–85 per cent but succinate-supported respiration was inhibited approximately 10 per cent. Table 5 shows that β-hydroxybutyrate dehydrogenase is very sensitive to inhibition by captan and folpet. Thus, inhibition of β-hydroxybutyrate-supported respiration may be, in part, due to the effects of captan and folpet directly on β-hydroxybutyrate dehydrogenase. In preliminary experiments, glutamate dehydrogenase, assayed only in the direction of NADH formation, was unaffected by the fungicides.

TABLE 4. EFFECTS OF CAPTAN ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	Captan (μM)	P_i esterified (μmoles/flask)	O_2 utilized (μg atoms O/flask)	P:O
β-Hydroxybutyrate	0	12·73	4·59	2·78
	25	8·25	2·92	2·82
	75	0·00	0·79	0·00
Glutamate	0	12·02	4·78	2·56
	25	4·50	2·10	2·14
	75	1·16	0·93	1·25
Succinate	0	5·95	3·61	1·68
	25	3·17	4·34	0·54
	75	1·51	3·18	0·48

* Oxidative phosphorylation was measured manometrically. The reaction mixture contained 30 μmoles glucose, 1 μmole EDTA, 2·5 μmoles ATP, 10 μmoles $MgCl_2$ 50 μmoles substrate, 0·5 mg hexokinase and 4–5 mg mitochondrial protein. Total volume was 2·0 ml. Respiration was measured for 30 min at 30°. Disappearance of P_i from the medium was used as an index of esterification.

Cysteine was found to prevent captan inhibition of succinate oxidation (Figs. 2 and 3). In these experiments, incubation of mitochondria with captan for 3 min prior to adding ADP resulted in inhibition of state 3 respiration and a reduction in respiratory control from approximately 5 in controls to less than 2 in inhibited preparations. State 4 respiration was increased due to uncoupling. Addition of cysteine at concentrations half that of captan partially protected state 3 respiration. However, a second addition of ADP to the same preparation did not stimulate additional respiration (Fig. 2,

TABLE 5. EFFECT OF CAPTAN AND FOLPET ON β-HYDROXYBUTYRATE DEHYDROGENASE ACTIVITY FROM INNER MEMBRANES OF RAT LIVER MITOCHONDRIA*

Fungicide concn (μM)	Specific activity (μmole NAD reduced/min/mg protein)	
	With captan	With folpet
none	0·220	0·220
0·5	0·100	0·143
1	0·060	0·126
10	0·000	0·000
100	0·000	0·000

* Inner membranes were incubated for 1 min at 30° in the presence of captan or folpet. The reaction was then started by addition of NAD. The complete reaction mixture contained 1·5 mM NAD, 0·1 M tris-HCl buffer (pH 8·1), 33 μM KCN, 3·3 μM β-hydroxybutyrate and 1·35 mg protein. Total volume was 3·0 ml. The reaction was run at 30° and NAD reduction was measured at 340 mμ.

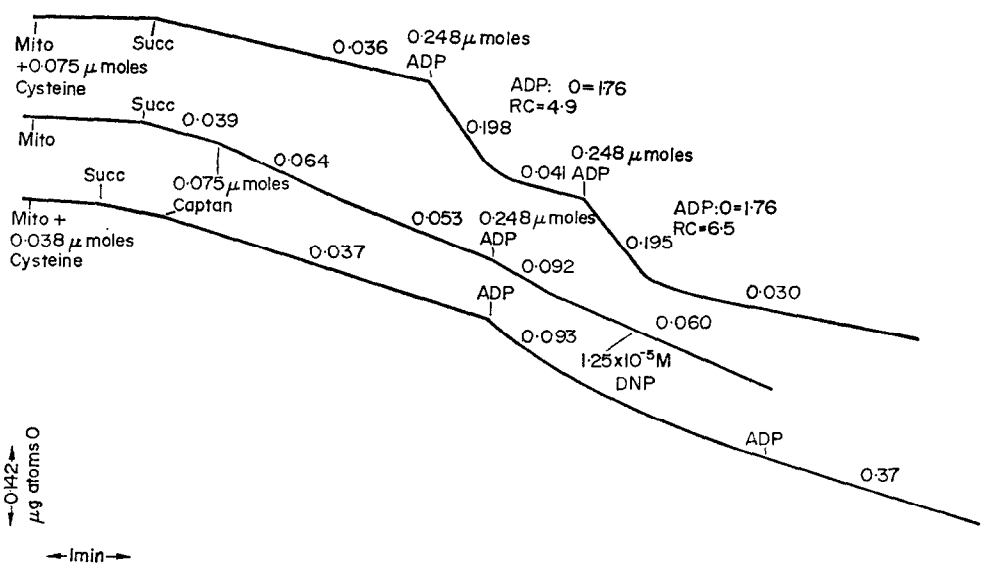

FIG. 2. Effects of cysteine on captan inhibition of succinate-supported oxidative phosphorylation in rat liver mitochondria. Conditions of the assay are described in Methods. Top tracing, control containing 75 mμmoles cysteine; middle tracing, mitochondria treated with 75 mμmoles captan but no cysteine; bottom tracing, 38 mμmoles cysteine added prior to 75 mμmoles captan. Each experiment contained 3·5 mg protein. Total volume was 1·6 ml.

bottom tracing). Addition of equal concentrations of cysteine and captan increased state 3 respiration after a second addition of ADP (Fig. 3, upper tracing). Addition of twice as much cysteine as captan provided excellent protection of respiratory control and state 3 respiration (Fig. 3, middle tracing). Attempts to reverse captan inhibition by adding cysteine to inhibited mitochondria were partially successful (Fig. 3, lower

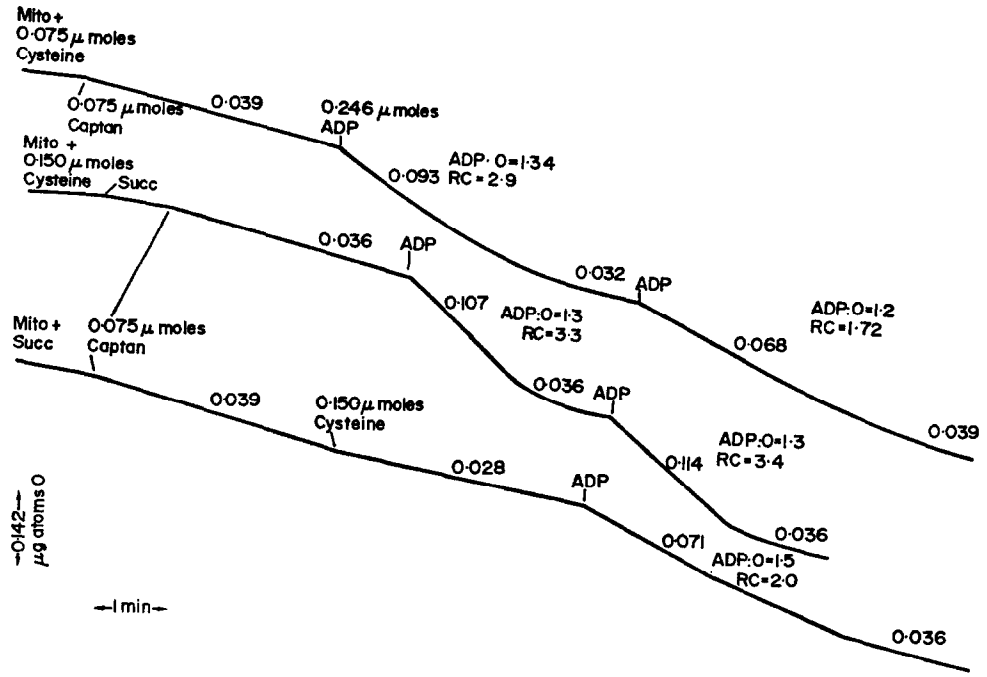

FIG. 3. Continuation of experiments shown in Fig. 2. Top tracing, 75 mμmoles cysteine and 75 mμmoles captan; middle tracing, 150 mμmoles cysteine and 75 mμmoles captan; bottom tracing shows an attempt to reverse captan inhibition. Captan (75 mμmoles) was incubated with mitochondria for 3 min prior to adding 150 mμmoles cysteine. After addition of cysteine, incubation was continued for 3 min at which time state 3 was initiated.

tracing), but state 3 respiration was not increased as much as when cysteine was added before captan. Cysteine had no effect on oxidative phosphorylation (Fig. 2, top tracing). Cysteine additions up to 750 mμmoles were tested. In all experiments in which cysteine "countered" or reversed captan inhibition, oxidative phosphorylation remained partially uncoupled, as indicated by the reduced ADP:O ratios. Cysteine protection of state 3 was observed only with succinate as the substrate, not with glutamate, β-hydroxybutyrate or α-ketoglutarate.

Both folpet and captan activated mitochondrial ATPase (Fig. 4), which is in keeping with their action as uncouplers. ATPase-activation was observed only if captan was added at the time enzyme activity was initiated (Fig. 4), i.e. with no preincubation. ATPase activity became progressively inhibited with time upon preincubation of the enzyme with captan. However, ATPase was not inhibited below endogenous levels, which indicates that captan inhibited only the activation process. DNP-activated ATPase was also inhibited by captan (Fig. 4) and the degree of inhibition was again dependent upon the length of the preincubation period. Preincubation in the absence of captan had no effect on either endogenous or DNP-activated ATPase.

In several experiments, captan inhibited DNP-activated ATPase by 70–90 per cent in the absence of Mg^{2+} but only 10–30 per cent when Mg^{2+} was present. A typical experiment is shown in Table 6. Inhibition resulting from preincubation with captan for 10 min could be partially prevented by adding Mg^{2+} at the end of the preincubation period. In contrast to captan, folpet did not inhibit DNP-activated ATPase extensively

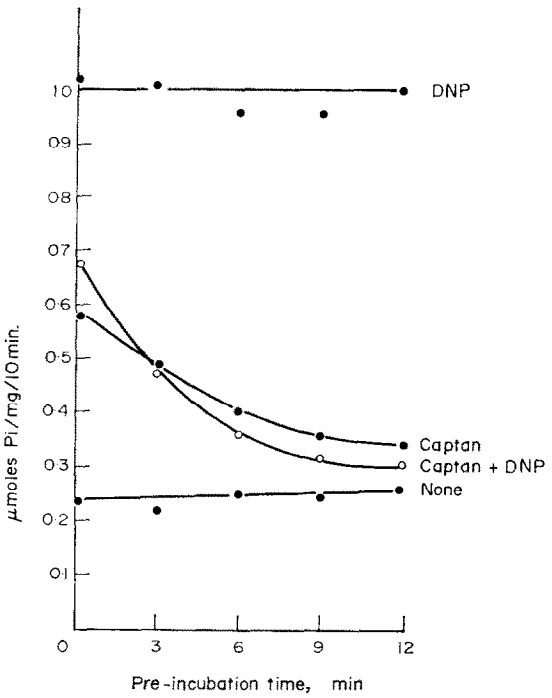

FIG. 4. Captan inhibition of DNP-activated ATPase. Mitochondria (2·6 mg protein) were preincubated for 0–12 min at 30° in 0·25 M sucrose, 0·06 M glycylglycine (pH 7·4), and 75 mμmoles captan. At the times indicated, ATPase activity was initiated by addition of 3 mμmoles ATP and, when added, 10^{-5} M DNP. Incubation was continued for 10 min at 30°. Total volume of the reaction medium was 1·5 ml.

in the absence of Mg^{2+}. Folpet inhibited ATPase activity by approximately 10–30 per cent regardless of the amount of Mg^{2+} present.

Inhibition of both captan- and DNP-activated ATPase could be prevented by 150 mμmoles cysteine (Fig. 5). The progressive inhibition which normally results from

TABLE 6. EFFECT OF CAPTAN AND FOLPET ON DNP-ACTIVATED ATPASE*

Additions	ATPase activity (μmoles P_i/15 min/mg protein)	
	Minus Mg^{2+}	Plus Mg^{2+}
DNP	1·305	1·234
DNP + captan	0·311(76)	0·935(24)
DNP + folpet	0·930(29)	0·856(30)

* Rat liver mitochondria (2·6 mg protein) were pre-incubated at 30° for 10 min in a medium containing 0·25 M sucrose, 0·06 M glycylglycine buffer (pH 7·4), and 50 μM captan or folpet. At the end of preincubation, 6 μmoles $MgCl_2$, 3 μmoles ATP and 10 μM DNP (final concn) were added to initiate enzyme activity. Total volume was 1·5 ml. Incubation was continued an additional 15 min. Numbers in parentheses represent per cent inhibition.

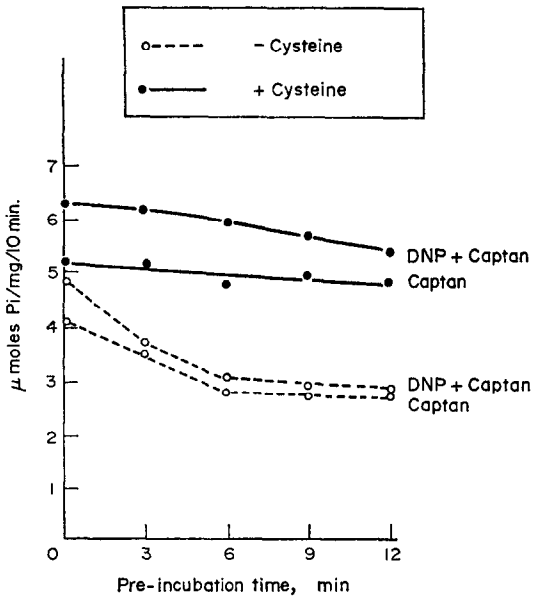

FIG. 5. Effect of cysteine on captan-inhibited ATPase. Conditions are the same as those described in Fig. 4, with the exception that 150 mμmoles cysteine was included in the preincubation with mitochondria (2·5 mg protein) and 75 mμmoles captan. Total volume was 1·5 ml.

prolonged incubation with captan was prevented, and both captan- and DNP-activated ATPase remained activated throughout the incubation period. Cysteine alone had no effect on either the endogenous or the DNP-activated ATPase.

Inhibition of the P_i–ATP exchange by 10 μM and 100 μM captan is shown in Table 7. Preincubation of mitochondria with captan for 10 min prior to initiating the exchange reaction significantly increased inhibition, particularly at lower levels of captan. The exchange was less sensitive to captan than to DNP or oligomycin.

Captan produced a physical alteration to the mitochondrial membrane, as shown by its effect on NADH and cytochrome c oxidation using intact and deoxycholate (DOC)

TABLE 7. CAPTAN INHIBITION OF $^{32}P_i$–ATP EXCHANGE IN RAT LIVER MITOCHONDRIA*

Inhibitor	Inhibitor concn (μM)	Exchange rate (μmoles P_i/10 min/mg protein)	
		No preincubation	Preincubated
None		0·562	0·542
Captan	10	0·486(14)	0·381(30)
Captan	100	0·334(41)	0·232(57)
DNP	4	0·256(55)	0·196(64)
Oligomycin	3 μg	0·208(63)	0·196(64)

* Exchange activity was measured as described in Methods. In some experiments mitochondria were preincubated in the presence of inhibitor for 10 min prior to initiating exchange activity by addition of $^{32}P_i$. The numbers in parentheses represent per cent inhibition.

lysed mitochondria (Table 8). Captan inhibited oxidation of cytochrome c by DOC lysed mitochondria (experiment 1), but stimulated cytochrome c oxidation if cytochrome was added before the mitochondria were lysed (experiment 2). These results suggest that captan alters the membrane and, in doing so, increases the accessibility of the cytochrome c binding site.

TABLE 8. EFFECT OF CAPTAN ON NADH AND CYTOCHROME C OXIDATION BY RAT LIVER MITOCHONDRIA SOLUBILIZED WITH DOC*

Expt	Additions	Respiration rate (μg atoms O/min)	
		Without captan	With captan
1	None	0·021	0·021
	NADH (1 mg)	0·021	0·021
	DOC	0·220	0·156
	Cytochrome c	0·668	0·376
2	None	0·025	0·014
	NADH (1 mg)	0·025	0·014
	Cytochrome c	0·153	0·284
	DOC	0·750	0·326

* Compounds are listed in the order in which they were added. Conditions of the assay are the same as those described in Methods for polarographic measurements of oxidative phosphorylation. Mitochondria were incubated for 3 min in the presence of captan (47 μM) before adding either DOC (0·1% final concn) or 1 mg cytochrome c. In experiment 1, mitochondria were lysed with DOC before adding cytochrome c. In experiment 2, cytochrome c was added before lysing with DOC. The reaction contained 4·4 mg of mitochondrial protein.

DISCUSSION

The present study shows that captan and folpet inhibit mitochondrial function. Inhibition is relatively nonspecific, involving several sites. This is in keeping with the nonspecific action of the fungicides as sulfhydryl group reagents.[1–6] Though no attempts were made to determine the extent of interaction of captan and folpet with mitochondrial sulfhydryl groups, it is assumed that they inhibited most of the enzymes in this manner, particularly since the inhibited enzymes all contain functional sulfhydryl groups. NADH-dehydrogenase, for example, has both sulfhydryl groups and labile sulfides[10] and, as shown in this study and elsewhere,[10,11] is inhibited by sulfhydryl reagents. Captan and folpet inhibition of β-hydroxybutyrate dehydrogenase could also be explained by the interaction of the fungicide with sulfhydryls which participate in its catalytic activity.[9] In addition, captan and folpet inhibit the energy transfer system, again mimicking the effects of other sulfhydryl group reagents.[7,22] Inhibition of the energy transfer reactions was shown in experiments where the fungicides inhibited succinate-supported state 3 and DNP-activated respiration (but not states 2 and 4), as well as the ATP–P_i exchange activity and DNP-activated ATPase.

The possibility that captan acts at more than one site in the energy transfer system is suggested by the response of ATPase to captan. In the absence of cysteine, captan initially activates ATPase, but with continued incubation the activated state becomes progressively inhibited. DNP-activated ATPase is similarly inhibited. This bimodal

effect of captan, i.e. activation followed by inhibition, suggests that at least two sites are involved. That the site responsible for inhibition of ATPase may not involve a sulfhydryl group is suggested by earlier results showing that sulfhydryl reagents which inhibit energy transfer either inhibit ATPase slightly[22] or not at all.[23] These results, however, are in contrast to those obtained by Kielly[24] using PCMB. In addition, they do not appear to agree with the present findings that excess cysteine protects ATPase from captan. An alternate explanation regarding the protective effect of cysteine, however, is that cysteine does not protect sulfhydryl groups, but rather prevents a degradation product from binding to a nonsulfhydryl site. Degradation of captan occurs in the presence of thiols by scission of the N–S bond to form tetrahydrophthalimide and an $-SCCl_3$ moiety. The $-SCCl_3$ forms disulfides with cellular thiols or rearranges to thiophosgene ($Cl_2C{=}S$) and eventually to carbon disulfide and hydrogen sulfide.[1,5,6] The latter products react with amino and hydroxyl groups in the cell.[3,5] Protection by cysteine might be expected if these products had a greater affinity for the sulfhydryl group on cysteine than for a nonsulfhydryl site on the ATPase. Excess cysteine would effectively eliminate binding to the enzyme and, therefore, inhibition.

One possible mechanism of captan action which should be considered is that the fungicides react with membranes, either inducing a passive influx of ions normally separated by the impermeable inner membranes,[8,25] or interfering with the translocation, or exchange, of specific ions.[26] Brierley *et al.*[8,25] have shown that the mercurials, *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulfonate, activate K^+ uptake, which eventually leads to respiratory inhibition.[8,27] The nonmercurial sulfhydryl reagent, *N*-ethylmaleimide, was, however, considerably less effective in this regard.[8] To induce K^+ uptake, the mercurials reacted with only 15–20 mμmoles sulfhydryl per mg of mitochondrial protein,[8,25] a value very close to the minimal amounts of captan needed (20–25 mμmoles/mg of mitochondrial protein) to inhibit state 3 respiration and respiratory control. Tyler[22] and Haugaard *et al.*[28] have suggested that sulfhydryl group reagents inhibit the energy-linked uptake of inorganic phosphate. Interference with P_i uptake could account for captan inhibition of state 3 respiration, the ATP–P_i exchange and uncoupling, but not for inhibition of activated ATPase or DNP-activated respiration. These results, plus swelling experiments presented in the following paper,[29] strongly suggest that captan does not inhibit P_i transport. Neither can inhibition of the ATPase or DNP-activated respiration be explained in terms of fungicide effects on the translocation of either DNP[30] or ATP.[26] Lack of captan inhibition of endogenous ATPase suggests that ATP uptake is not impaired. Similarly, impairment of DNP uptake does not account for the action of captan. Since both DNP- and captan-activated ATPase were inhibited in a parallel manner, the mechanism of inhibition is probably the same in both cases. DNP, however, is not present in both systems.

The inhibitory effect of captan on DNP- and captan-activated ATPase could involve a mechanism similar to that described for cystamine.[31] Cystamine, by reacting with mitochondrial thiols, alters the permeability of the membrane, allowing leakage of Mg^{2+}. Loss of Mg^{2+} was associated with inhibition of Mg^{2+}-activated and DNP-activated ATPase. Such an effect is in keeping with our present observations that captan inhibits activated ATPase only in the absence of added Mg^{2+}. Folpet differs from captan in that it inhibits the activated ATPase less than captan, and Mg^{2+} does not reverse that inhibition. Though an explanation for these differences is not known, it could be related to stereochemical differences[32] between captan and folpet or to a

difference in the rates of hydrolysis or thiol attack on their N–S bonds.[32] Though the effects of captan and folpet on the ATPase differ, both fungicides inhibit state 3 respiration. The inhibitory effect of captan on ATPase would thus seem to be a secondary reaction not necessarily responsible for fungicide-induced loss of ADP-controlled respiration.

The observation that captan increased the rate of cytochrome c oxidation using intact mitochondria suggests that changes in membrane permeability occur and, as shown for other sulfhydryl inhibitors,[8,25,33] may play a significant role in the loss of mitochondrial integrity. Future experiments will be directed toward understanding the response of mitochondrial and other cellular membranes to captan and folpet.

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