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EFFECTS OF 6,6′-DITHIODINICOTINIC ACID,* A THIOL REAGENT ON SEVERAL MITOCHONDRIAL FUNCTIONS: COUPLING MECHANISM, ATPase AND ANION TRANSPORT

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Abstract—CPDS (carboxy pyridine disulfide = 6,6′-dithiodinicotinic acid) was chosen as a thiol reagent to discriminate between -SH implicated in various mitochondrial functions because it is strongly charged and should not penetrate through intact membranes. It does not affect respiratory state 4 in rat liver and pig heart mitochondria, CPDS (4×10^{-4} M) inhibits state 3 in rat liver mitochondria in less than 1 min incubation but Pi prevents this inhibition. With pig heart mitochondria 6×10^{-4} M CPDS is necessary and must react 3·5 min to inhibit state 3 respiration while Pi cannot prevent inhibition. Under the same conditions CPDS binds immediately 3 nmoles -SH/mg protein of rat liver mitochondria, and progressively 16 nmoles -SH in pig heart mitochondria. CPDS inhibits by 50–60% DNP-stimulated ATPase and enhances a DNP-insensitive ATPase in both types of mitochondria. ATP synthesis coupled to oxidative phosphorylation is inhibited by CPDS. Glutamate translocation is not affected. CPDS does not inhibit Pi entry as measured by the swelling of rat liver mitochondria in 120 mM Pi but only delays it slightly: on the contrary Pi exit is inhibited. With pig heart mitochondria CPDS inhibits Pi entry but does not seem to affect Pi exit. Comparisons are made with other thiol agents: CPDS and mersalyl diverge in several ways. CPDS effects can be prevented but not reversed by GSH and DTT. DNP abolishes CPDS inhibition of state 3 respiration.

In previous works the use of 5,5′-dithio-*bis*-(2-nitrobenzoic acid) has proved the implication of very reactive thiols in the process of oxidative phosphorylation in pig heart mitochondria [1, 2]. In recent years numerous papers on the effects of various inhibitors of thiols on several mitochondrial functions have appeared which have been reviewed elsewhere [3]. Since Tyler [4, 5] has shown the participation of thiols in phosphate transport using mersalyl, it was evident that many mitochondrial functions are affected by thiol inhibitors. Our purpose has been to try to discriminate between various populations of -SH linked to the different functions of mitochondria by using 6,6′-dithiodinicotinic acid or carboxypyridine disulphide (CPDS); this very charged molecule should not penetrate into mitochondria and should allow the role of -SH external to inner membrane to be determined. The present work shows that CPDS by blocking only a few nmoles -SH per mg protein affects mitochondrial functions in a way different to that of mersalyl.

* Abbreviation used: CPDS = carboxy pyridine disulfide = 6,6′-dithiodinicotinic acid; GSH = glutathione; DTT = dithiothreitol; DNP = 2,4-Dinitrophenol; Pi = inorganic phosphate.

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MATERIALS AND METHODS

Preparation of mitochondria. Rat liver mitochondria were isolated according to the method of Weinbach [6]. Pig heart mitochondria were prepared as described by Crane *et al.* [7].

Submitochondrial particles were prepared by sonication of rat liver mitochondria according to Kielley and Bronk [8] in a medium containing 30 mM phosphate buffer, 1 mM $MgCl_2$, 6 mM ATP, 4 mg cytochrome *c*/100 mg mitochondrial protein, 1 mg bovine serum albumin/g liver (130 mg mitochondrial protein/5 ml medium), pH 7·4.

Incubation. Mitochondria were incubated at 30° with 0·01 M glutamate in media of the following composition:

(a) For rat liver mitochondria: 24 mM glycyl-glycine, 9·6 mM $MgCl_2$, 60 mM KCl, 87 mM sucrose, pH 7·4.

(b) For pig heart mitochondria: 16 mM Tris–HCl, 112 mM KCl, 6 mM $MgCl_2$, pH 7·4.

Oxygen uptake was determined by oxypolarography.

Protein was determined by the quick Biuret method after solubilizing the particles with 0·2% sodium cholate [9].

ATP was estimated by the firefly luminescence method [10].

ATPase activity was measured in the above media, the mitochondria being incubated in the presence or

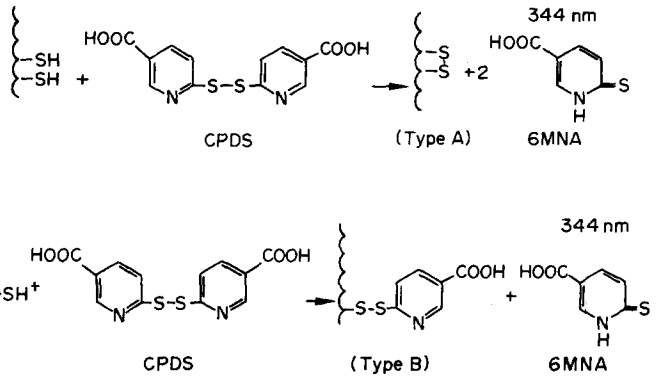

Fig. 1. The two types of reactions possible between CPDS (carboxy pyridine disulfide = 6,6′ dithiodinicotinic acid) and mitochondrial free -SH. Thione released is 6 MNA = 6-mercaptonicotinic acid.

absence of inhibitor. The reaction was started by the addition of 2·5 mM ATP and 2·5 10^{-4} M dinitrophenol and was stopped with 10% trichloracetic acid (final concentration). Inorganic phosphate was determined by the method of Fiske and Subbarow [11].

Mitochondrial swelling was measured according to Chappell [12] by following the decrease of optical density at 520 nm with a Unicam SP 800 spectrophotometer.

Thiol groups were estimated spectrophotometrically by CPDS. This compound can react with thiols in two different ways [13] which both lead to the formation of disulphide bonds with an accompanying release of thione or 6-mercaptonicotinic acid (absorption peak at 344 nm) as shown in Fig. 1. A method was adapted for the estimation of thiol groups of mitochondria, absorbance being measured at 344 nm against a blank consisting of reaction mixture without CPDS. The concentration of the released thione was calculated by the use of a molar extinction coefficient of 1×10^4. The results were expressed as nmoles/mg protein. In some thiol estimations, 0·23% sodium cholate (final concentration) was added to the CPDS reaction mixture to avoid permeability restrictions.

RESULTS

Effects of CPDS on mitochondrial respiration. In rat liver mitochondria (Fig. 2A) CPDS did not affect state 4-respiration but inhibited in 1 min state 3 and suppressed the transition state 4/state 3 when added before Pi at the concentration of 4–5 $\times$ 10^{-4} M. The addition of Pi in sufficient amount before CPDS, prevented completely the CPDS effects; if lower concentrations of Pi were present before CPDS addition, one ADP respiratory stimulation (state 3) was observed and then a further addition of ADP could no longer provoke a stimulation. Addition of Pi after CPDS did not release inhibitions but 2,4-dinitrophenol released CPDS inhibition of respiration.

In pig heart mitochondria also (Fig. 2B) CPDS did not affect state 4 but to obtain the inhibition of state 3 and to abolish the state 4/state 3 transition, a concentration of 6 to 7 $\times$ 10^{-4} M of CPDS and 3·5 min of preincubation were necessary (compare experiments 6 and 9).

Therefore CPDS was incubated 3·5 min. In contrast to rat liver mitochondria, Pi added before or after CPDS did not prevent or release the inhibitions produced in pig heart mitochondria; uncoupling agents released the inhibition.

With both types of mitochondria dithiothreitol and glutathione prevented but could not release the inhibitions.

Kinetics of CPDS binding to mitochondrial SH. CPDS was used to estimate accessible mitochondrial -SH under the same conditions of concentration and incubation described above which produced inhibitions. The kinetics of CPDS binding and thione release was followed as shown in Fig. 3.

In rat liver mitochondria CPDS reacted immediately with about 3 nmoles -SH/mg protein; no further evolution was observed. ADP and Pi concentrations used to provoke state 3 transitions did not seem to affect significantly this level.

In pig heart mitochondria on the contrary, CPDS reacted progressively; a stable value of about 16 nmoles -SH/mg protein was obtained after 10 min reaction; no further evolution was observed. After the 3·5 min incubation necessary to observe the respiratory inhibitions about 12 nmoles -SH/mg protein had reacted with CPDS.

If 0·23% sodium cholate was added to the CPDS mixture, the situation was different. In rat liver mitochondria, after the immediate blocking of the very reactive -SH (about 12 nmoles -SH) CPDS reacted progressively for about 26 min when a final stable value of 59 $\pm$ 1·1, nmoles -SH/mg protein was obtained, as shown in Table 1. With pig heart mitochondria, 0·23% sodium cholate did not affect the kinetics of binding and after 26 min reaction CPDS reacted with about 24·7 $\pm$ 0·7 nmoles -SH/mg protein.

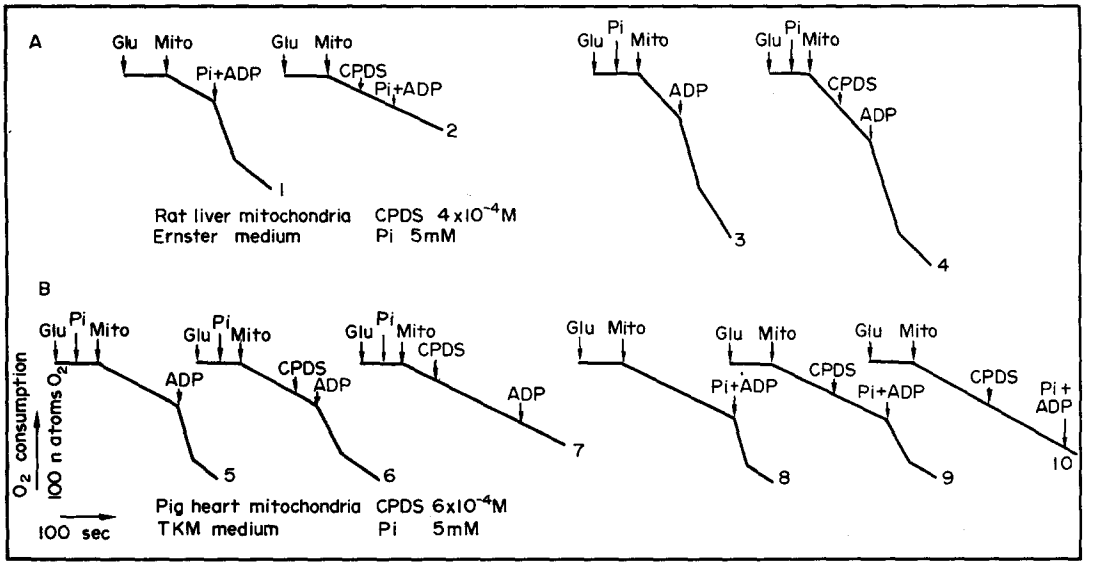

Fig. 2. Effects of CPDS on the transition state 4/state 3 in mitochondria, determined by oxypolarography at 30°. (A) Rat liver mitochondria (2 mg protein/2 ml) were incubated in the medium described in Materials and Methods with 10^{-2} M glutamate and with or without 5 mM Pi; 4×10^{-4} M CPDS was incubated 1 min before the addition of 2×10^{-4} M ADP. (B) Pig heart mitochondria (2 mg protein/2 ml) were incubated in the medium described in Materials and Methods with 10^{-2} M glutamate and with or without 5 mM Pi. 6×10^{-4} M CPDS was incubated 1 or 3·5 min before the addition of 2×10^{-4} M ADP. The plots 1, 3, 5 and 8 are controls.

Effects of CPDS on ATPase activity in intact mitochondria and submitochondrial particles. When CPDS was incubated with mitochondria for times and at concentrations necessary to inhibit state 3 transitions as controlled by oxypolarography, it inhibited only 60 per cent the 2,4-DNP dependent ATPase in a way very similar to mersalyl in both rat liver and pig heart mitochondria (Fig. 4).

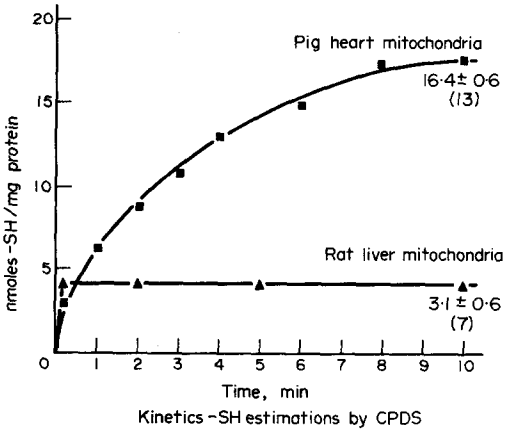

Fig. 3. Kinetics of -SH estimation by CPDS: Mitochondria (rat liver and pig heart) were preincubated for 3 min at 30° in the presence of 10^{-2} M glutamate. Aliquots (2 mg protein) were added to the cuvette containing the titrating mixture with CPDS (4×10^{-4} M in the case of rat liver mitochondria and 6×10^{-4} M in the case of pig heart mitochondria) (final volume 3 ml, pH 7·4). Variations of optical density at 344 nm were followed from 0 to 10 min. ( ) Number of different mitochondrial preparations.

The inhibition was not competitive towards ATP; for rat liver mitochondria K_m-ATP = 0·6 mM, and K_i-CPDS = 1·4 mM; for pig heart mitochondria K_m-ATP = 0·6 mM and K_i-CPDS = 0·15 mM.

On the contrary, CPDS stimulated a 2,4-DNP insensitive ATPase in both type of mitochondria.

Rat liver submitochondrial particles exhibited very high ATPase activities (Fig. 5); the 2,4-DNP dependent ATPase, increased by 8-fold compared with intact mitochondria and the latent ATPase reached 75–80 per cent of the 2,4-DNP dependent activity; CPDS at

Table 1. CPDS estimations of nmoles-SH/mg mitochondrial protein

	nmoles-SH/mg protein			
	In the absence of cholate*		In the presence of cholate*	
Rat liver mitochondria	3·1 ± 6	(7)	59 ± 1·1	(9)
Pig heart mitochondria	16·4 ± 0·6	(13)	24·7 ± 0·7	(7)

* Sodium cholate 0·23% was added to the -SH titrating mixture 26 min before the dosage. Number of experiments in brackets.

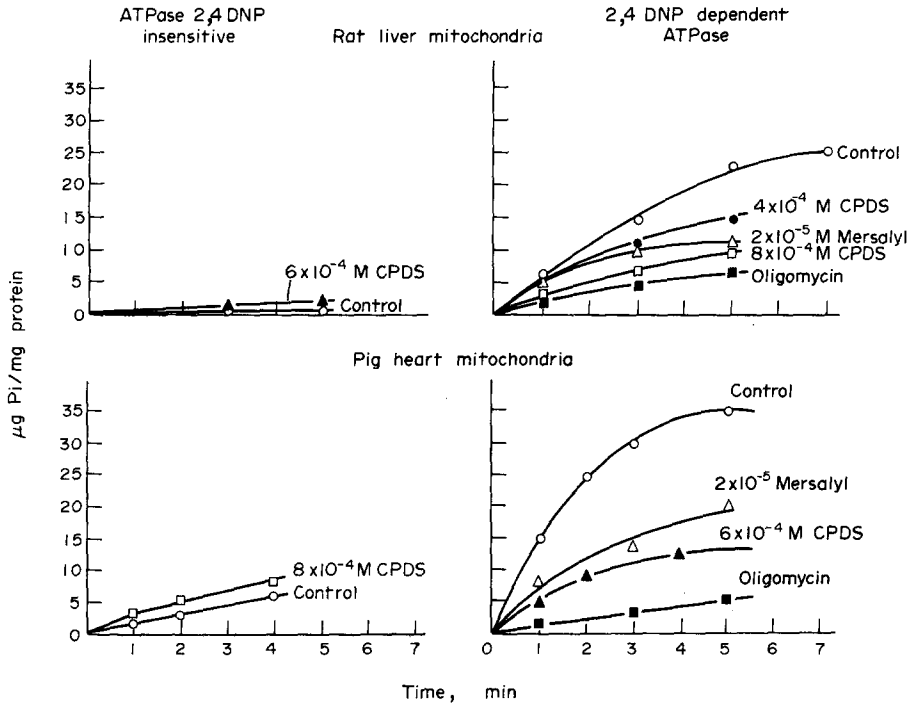

Fig. 4. Effects of CPDS, mersalyl and oligomycin on the 2,4-DNP insensitive and the 2,4-DNP dependent ATPase in rat liver and pig heart mitochondria. Mitochondria (2 mg protein) were incubated in the medium described in Materials and Methods. Different concentration of CPDS were then added and incubated 1 min with rat liver mitochondria and 3·5 min with pig heart mitochondria. The reaction was started by the addition of 2·5 mM ATP with or without $2{\cdot}5 \times 10^{-4}$ M 2,4-DNP; 2×10^{-5} M mersalyl and 2 µg oligomycin were incubated one minute with mitochondria before the addition of ATP.

concentrations (5×10^{-4} M, 10^{-3} M), which inhibited state 3, slightly inhibited 2,4-DNP dependent ATPase and increased slightly the latent ATPase. In the same way mersalyl did not affect both ATPases.

Similar results were obtained with pig heart submitochondrial particles.

Effects of CPDS on ATP synthesis linked to oxidative phosphorylations (oligomycin sensitive) Figs. 6 and 7. Both types of mitochondria behaved differently.

In rat liver mitochondria incubated in the absence of Pi, CPDS completely inhibited ATP synthesis linked to oxidative phosphorylations (Fig. 6). The residual ATP synthesis observed was in fact due to adenylate kinase as shown in Fig. 7, since this residual activity was not inhibited by oligomycin. If mitochondria were preincubated with Pi prior to CPDS addition, no inhibition of ATP synthesis was observed.

On the contrary, with pig heart mitochondria (Fig. 6) CPDS inhibited ATP synthesis linked to oxidative phosphorylations in a way which seems independent of Pi; the residual ATP synthesis was also due to adenylate kinase activity (Fig. 7).

Influence of CPDS on Pi translocation compared with the action of mersalyl. (1) Pi entry as measured by swelling in 0·12 M ammonium phosphate (Chappell's method): rat liver and pig heart mitochondria behaved in a very different way.

If rat liver mitochondria were incubated in the absence of EGTA + Rotenone, mersalyl completely inhibited the swelling, but CPDS at concentrations 2–3-fold higher than those necessary to inhibit state 3 stimulation, only delayed the swelling in a sigmoidal way but did not affect the final amplitude of swelling (Fig. 8A); if 5×10^{-3} M Pi was added before CPDS, the swelling was not delayed and CPDS had almost no effect (Fig. 8C). In the presence of EGTA + Rotenone which blocked electron transfer, no sigmoidal response was observed and only CPDS concentrations 2–4 fold higher than those necessary to inhibit state 3 transition and ATP synthesis could inhibit Pi swelling in a way similar to mersalyl (Fig. 8B); 2×10^{-3} M CPDS abolished completely the swelling as observed with 2×10^{-5} M mersalyl; the preincubation of rat liver mitochondria with 5×10^{-3} M Pi prevented the inhibition of swelling but induced a sigmoidal response (Fig. 8D).

With pig heart mitochondria (Fig. 8) 6×10^{-4} M CPDS never induced a sigmoidal swelling response; when preincubated for 1 min only, it had no effect either on the swelling or on the state 3 transition (Fig. 2); when preincubated for 3·5 min 6×10^{-4} M CPDS

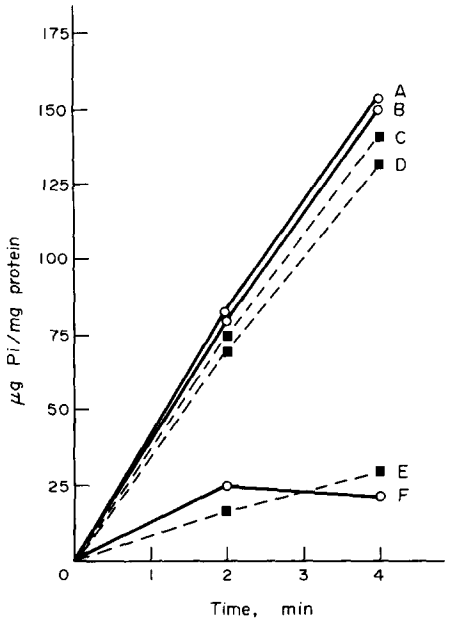

Fig. 5. Effects of CPDS and oligomycin on the 2,4-DNP insensitive and the 2,4-DNP dependent ATPases in rat liver submitochondrial particles (SMP); 0·75 mg protein of SMP are incubated under the same conditions as Fig. 4, but ATP added here was 5 mM. (A) Control of 2,4-DNP dependent ATPase. (B) 2,4-DNP dependent ATPase in the presence of 5×10^{-4} M to 10^{-3} M CPDS. (C) 2,4-DNP insensitive ATPase in the presence of 5×10^{-3} M to 10^{-3} M CPDS. (D) Control of 2,4-DNP insensitive ATPase. (E) and (F) are respectively 2,4-DNP insensitive and 2,4-DNP dependent ATPases in the presence of 2 µg oligomycin.

completely inhibited the Pi swelling of mitochondria as it inhibited the state 3 transition (Fig. 2); 2×10^{-5} M mersalyl had the same effects; preincubation with 2×10^{-3} M Pi or with EGTA + Rotenone had no effect.

(2) Inhibition of Pi exit as measured in the presence of ATP + dinitrophenol. The behaviour of both mitochondria is completely different.

With rat liver mitochondria, according to Tyler [5], the addition of 2,4-dinitrophenol + ATP does not induce swelling as measured at 520 nm. CPDS (6×10^{-4} M–10^{-3} M) preincubated 1 min with rat liver mitochondria prior to addition of 5 mM ATP + $2{\cdot}5 \times 10^{-4}$ M dinitrophenol produced a swelling (Fig. 9A) as observed in the presence of 2×10^{-5} M mersalyl (Fig. 9B); CPDS in the absence of ATP and 2,4-dinitrophenol did not induce any swelling; oligomycin completely inhibited the swelling due to CPDS or mersalyl.

With pig heart mitochondria, under the same conditions, neither CPDS nor mersalyl could induce swelling.

Effects of CPDS on mitochondrial swelling in the presence of isotonic ammonium glutamate. Chappell [12] used the swelling of mitochondria in isotonic ammonium glutamate to measure glutamate entry. Figure 10 shows that neither mM CPDS (±Pi, or ± EGTA + Rotenone) nor 2×10^{-5} M mersalyl, could inhibit the swelling of rat liver mitochondria in 0·12 M ammonium glutamate, while *N*-ethyl maleimide, fuscin and avenaciolide [3, 14] inhibited this swelling by blocking some mitochondrial thiols.

Surprisingly, a series of different pig heart mitochondria preparations were tested in the same conditions but they did not swell in 0·12 M ammonium glutamate.

DISCUSSION

From our results CPDS appears as a specific reagent of mitochondrial SH located on the external face of the inner membrane since with rat liver mitochondria it reacts immediately with very few very reactive thiols, 3 nmoles -SH/mg protein without further evolution while according to the other penetrating reagents 18–150 nmoles -SH can be affected [3] and however it inhibits functions linked specifically to inner membrane. With pig heart mitochondria, the slower reaction of mitochondrial thiols can be relevant of the very tight packing of cristae formed by the inner membrane which delays the accessibility of CPDS; however the thiols affected by CPDS must be also located outside the inner membrane, the greater value estimated, 16 nmoles -SH/mg protein, being due to the very large area of inner membrane in pig heart mitochondria compared with rat liver mitochondria.

Another proof of the non penetration into mitochondria of CPDS is the -SH levels obtained in the presence of cholate.

The correlations (time of action, concentrations, states 4 and 3, effects of uncoupling agents...) between CPDS-bound thiols and inhibition profiles, prove that the inhibition of the ADP respiratory stimulation (state 3) is related to the blockade of very few thiols external to the inner membrane; this is also proved by the fact that thiols could prevent but not release the inhibitions.

Glutamate transport is not affected by CPDS in either type of mitochondria, since uncoupling agents release CPDS inhibition of state 3. This agrees with the suggestion that thiols implicated in glutamate transport are buried inside the inner membrane [3, 14].

We repeatedly observed that pig heart mitochondria do not swell in 0·12 M ammonium glutamate. However in another work [15], we have shown that the K_m of entry of glutamate into pig heart mitochondria as measured by the reduction of intramitochondrial pyridine nucleotides is very low; 76 µM. This seems to indicate that glutamate entry into pig heart mitochondria proceeds by a mechanism different from that of other mitochondria. We cannot yet make any correlation between this observation and HGAP, a proteolipid isolated from pig heart mitochondrial membranes in a precedent work and which exhibited many of the

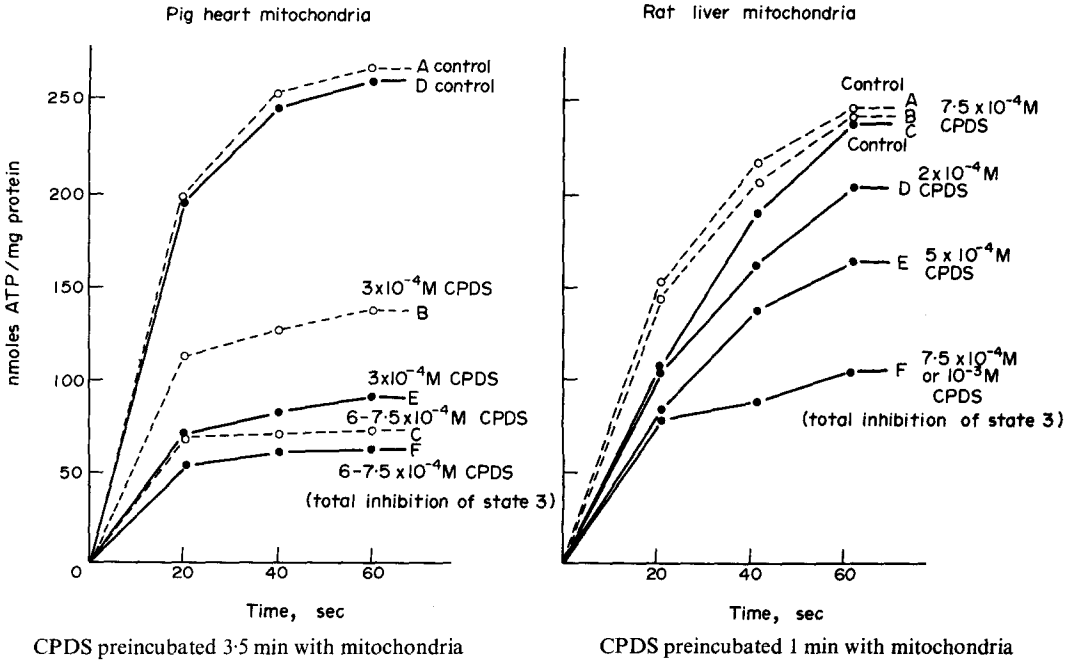

Fig. 6. The influence of CPDS on ATP synthesis. Mitochondria (2 mg protein/2 ml) were preincubated in the medium assay as described in Materials and Methods. ---- Mitochondria were preincubated with 5 mM Pi with or without CPDS (1 min for rat liver mitochondria and $3\frac{1}{2}$ min for pig heart mitochondria). 300 nmoles ADP were added at zero time to start ATP synthesis. —— Mitochondria were preincubated with or without CPDS. 300 nmoles ADP + 5 mM Pi were added at zero time to start ATP synthesis.

properties expected from a glutamate translocator [15].

CPDS exhibits only some of the effects of mersalyl on mitochondrial functions.

CPDS inhibits only 60 per cent of 2,4-DNP dependent ATPase in both types of mitochondria; however in submitochondrial particles, this ATPase is hardly affected by CPDS. In the same way Senior [16] has also observed that beef heart submitochondrial ATPase was very resistant to thiol reagents such as *N*-ethylmaleimide, mercurials and 5,5'-dithiobis-2 nitrobenzoic acid. This could mean that CPDS is less accessible to the submitochondrial ATPase, for example if the submitochondrial particles are inside-out. However this is impossible since the ATPase is on the matrix side of inner membrane and should become more accessible to a reagent like CPDS which does not penetrate through the inner membrane, when the particles are inside-out. Thus CPDS binding on a protein outside the inner membrane may affect the ATPase

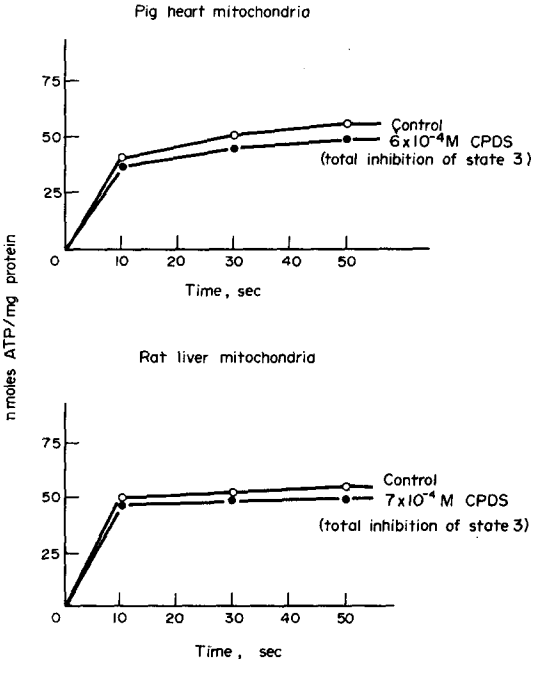

Fig. 7. The ATP synthesis in the absence of added Pi. Mitochondria 2 mg protein/2 ml were incubated with or without CPDS. 300 nmoles ADP were added at zero time. This ATP synthesis was not inhibited by oligomycin (1 μg/mg protein) and due to adenylatekinase.

Fig. 7.

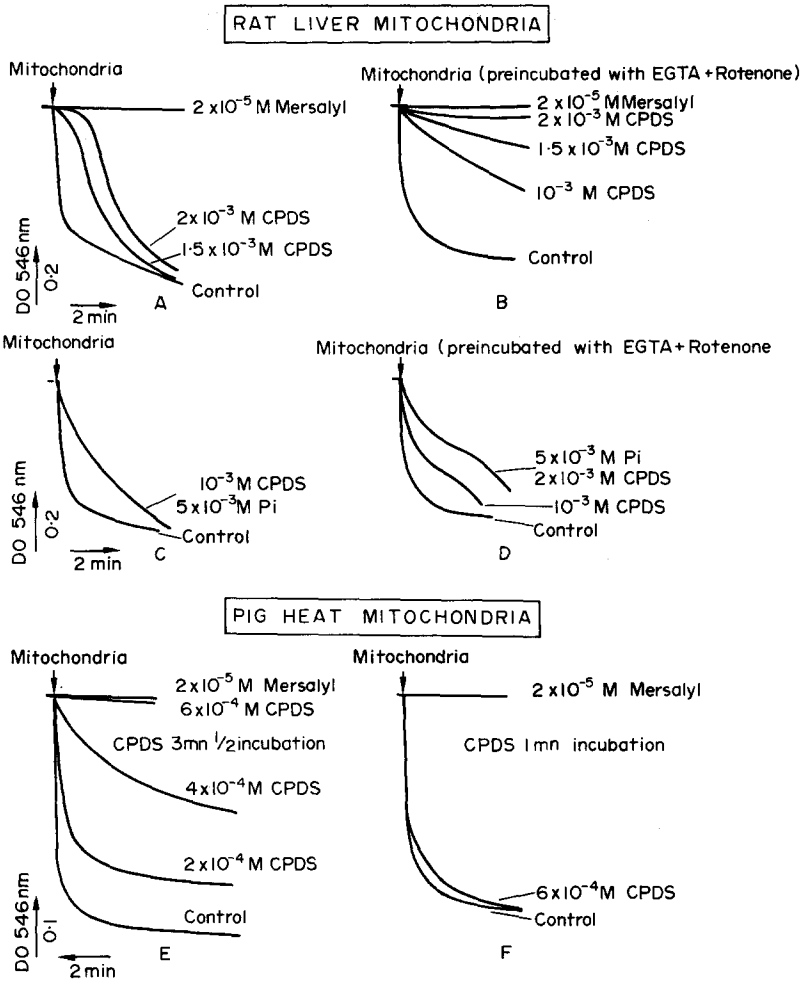

Fig. 8. Effect of CPDS on mitochondrial swelling in ammonium phosphate. The swelling was measured at 546 nm as described under Materials and Methods. Rat liver mitochondria (4·5 mg protein/2·3 ml) were preincubated one minute in the assay medium with different concentrations of CPDS, with or without EGTA $1{\cdot}5 \times 10^{-3}$ M and rotenone 2 μM, and with or without 2×10^{-2} M glutamate (plots A and B). The swelling was initiated by addition of aliquots of 0·45 mg protein to 1 ml 0·12 M ammonium phosphate. In (C) and (D) mitochondria were preincubated with 5 mM Pi. Pig heart mitochondria 4·5 mg protein/2·3 ml were preincubated for 3·5 min (E) or one minute (F) with different concentrations of CPDS, with or without EGTA, Rotenone, Pi and glutamate. The swelling was initiated as above.

either by conformational changes of the inner membrane or by indirect effects. The latent ATPases are stimulated by CPDS. Therefore one could conclude that CPDS acted in the same way as mersalyl and inhibited ATPase by blocking the Pi transporter. However some results do not fit with this conclusion.

In the absence of Rotenone and EGTA, CPDS at the concentrations which inhibit state 3, does not affect the swelling of rat liver mitochondria in ammonium phosphate; therefore the inhibition of state 3 does not seem to be due to an inhibition of Pi entry. Very high concentrations of CPDS, slightly delay the swelling in the absence of EGTA + Rotenone, and in the presence of EGTA + Rotenone provoke inhibitions of the Pi swelling similar to those of mersalyl; however preincubation with Pi protects against inhibitions of swelling as it prevents the inhibitions of state 3. These results suggest that Pi entry is linked to the energized state of mitochondria and that CPDS inhibits this entry only when all energy supply is suppressed. This conclusion is supported also by the fact that CPDS provokes the swelling of mitochondria in the presence of ATP + uncoupling agents; such type of swelling has been related by Tyler [5], in the case of mersalyl, to an inhibition of ATPase due to an accumulation of Pi owing to an inhibition of Pi exit. Therefore it can be concluded that

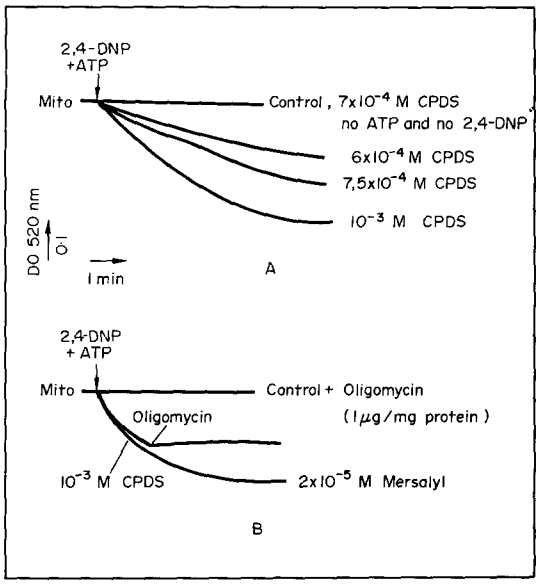

Fig. 9. Inhibition of Pi exit from rat liver mitochondria. Mitochondria (2 mg protein/2 ml) are preincubated one minute in the assay medium with different concentration of CPDS. 5 mM ATP and $2{\cdot}5 \times 10^{-4}$ DNP were added at zero time. The swelling was measured at 520 nm. 1 μg/mg protein oligomycin, and 2×10^{-5} M mersalyl preincubated one minute with mitochondria, were used as control.

CPDS affects Pi transport only when energy recuperation is suppressed (uncoupling agents, Rotenone + EGTA).

In pig heart mitochondria, CPDS inhibits ammonium Pi swelling at the concentrations which inhibit state 3 and ATPase, in the presence or not of Rotenone + EGTA, in a way very similar to that of mersalyl. However, preincubation of mitochondria with Pi cannot prevent the inhibition of state 3 and of the swelling by CPDS. The CPDS inhibitions seem to be due to an inhibition of Pi entry. However no swelling of pig heart mitochondria can be observed by addition of CPDS or mersalyl in the presence of ATP and uncoupling agents.

These results indicate clearly that membrane mechanisms implicated in energy transduction and anion transport are not the same in rat liver and pig heart mitochondria. Pig heart mitochondria in contrast to rat liver mitochondria exhibited the following special features: very reactive -SH were found to be implicated in the coupling mechanism [2]; no swelling was observed in ammonium glutamate or in the presence of ATP + uncoupling agents after addition of CPDS or mersalyl; Pi entry was inhibited by CPDS. A detailed study of pig heart mitochondrial subparticles and of F_1 and other coupling factors will enable us to understand these differences in the membrane mechanisms compared with rat liver mitochondria.

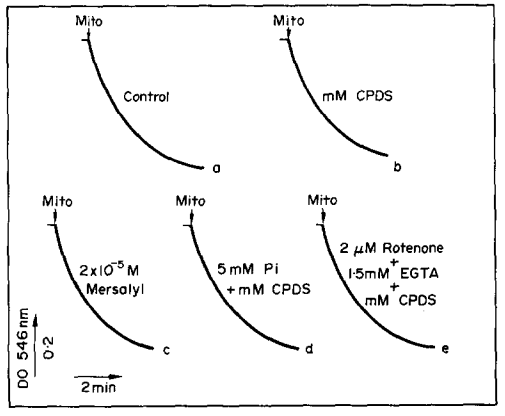

Fig. 10. Swelling of rat liver mitochondria in ammonium glutamate at 546 nm. Mitochondria were preincubated under the same conditions as Fig. 8. The swelling was initiated by the addition of aliquots of 0·45 mg protein to 1 ml 0·12 M ammonium glutamate.

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