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TOXICITY OF PENTACHLOROPHENOL ON ISOLATED PLANT MITOCHONDRIA

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Phaseolus aureus*; Leguminosae; mung bean; pentachlorophenol; plant mitochondria; uncoupler; inhibitor; succinate dehydrogenase; BSA.

Abstract—Pentachlorophenol (PCP) is a powerful uncoupler of the oxidative phosphorylations on isolated potato or Mung bean mitochondria; a 50% uncoupling is obtained at less than 1 μM in a medium devoid of Bovine serum albumin (BSA). Furthermore, at higher concentrations PCP inhibits the electron transfer at the level of Complex II and also at a level located near the quinone pool. The high uncoupling activity is dependent on the presence of five chlorines on the phenyl ring, which increase the lipophily of the phenol and decrease its pK_a. The 2,6-dichloro substitution is favorable to the Complex II inhibition. The presence of BSA 0.1% in the reaction medium causes the disappearance of the uncoupling effect of 3 μM PCP and the restoration of the normal increase in the oxidation rate which depends on ADP. Comparison of the uncoupling effect of PCP in plant and rat liver mitochondria suggests that some proteic components of the animal mitochondria play the same role as exogenous BSA in the case of plant mitochondria.

INTRODUCTION

Chlorinated phenols and their derivatives are frequently used in industry and agriculture [1, 2]. Of these, pentachlorophenol (PCP) is the most widely used compound (worldwide yearly production: 50000 tonnes [3]), especially for wood preservation. Because of its lipophily and its resistance to environmental degradation, PCP is responsible for severe contamination of the environment. The toxicity of PCP and its mode of action have been extensively studied in animals and humans in recent years [4–6]. However, very little information is available regarding the effects of PCP on plants. The purpose of this work is to reach a better understanding of the toxic action of PCP on plant mitochondria.

RESULTS

Two types of effect of PCP on plant mitochondria have been studied: (a) coupling between oxidative phosphorylation and electron transfer; (b) respiratory electron transfer from several substrates to O_2.

(a) *Uncoupling activity of PCP*

In the conditions described in the Experimental (see uncoupling test), in the presence of 0.1% BSA in the reaction medium, 20 μM PCP induced a 400% increase in the oxidation rate which remained unaffected after the addition of the classical and potent uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (Fig. 1a). These results indicated a full uncoupling activity of PCP at a concentration twenty times greater than the FCCP concentration needed for the same effect. This uncoupling activity was also confirmed by showing that, after the addition of 20 μM PCP, in the absence of carboxyatractyloside, no increase in the oxidation rate was obtained after the addition of ADP. Figure 1b represents the titration curve of the uncoupling activity in the presence of BSA. A partial uncoupling effect appeared for 0.5 μM PCP and the 50% effect was obtained at 8 μM. The same uncoupling effect was obtained using NADH, pyruvate or α-ketoglutarate as substrate, the lowest concentration of PCP inducing a full uncoupling activity varying between 20 and 50 μM (Table 1).

Previous results have shown an important binding between PCP and BSA [7, 8]. In these conditions, it was interesting to find out if BSA, commonly used in the reaction medium to prevent alterations of the plant mitochondrial membrane [9], was able to change the uncoupling activity of PCP. As shown in Table 1, it could be seen that the concentration of PCP giving a 50% uncoupling effect was ten times lower when the reaction medium was devoid of BSA. Figure 2 shows that after a full uncoupling effect induced by 3 μM PCP in a medium devoid of BSA, this effect could be reversed by addition of BSA 0.1%. In fact, after BSA addition, the oxidation rate decreased to values corresponding to state IV and the ADP-dependent increase of this rate reappeared. Figure 3 indicates that there was a direct relationship between the PCP concentration for half-maximum uncoupling and the mitochondrial concentration in the reaction cuvette when BSA was not present. From Fig. 3, the amount of PCP inducing half-maximum uncoupling was calculated to be about 1 nmol/mg mitochondrial protein. This stoichiometric relationship between PCP and mitochondria was not observed when BSA was added. In fact, the uncoupling activity (where D_{50} is the concentration giving a half-maximum uncoupling effect; D_{100}: concentration

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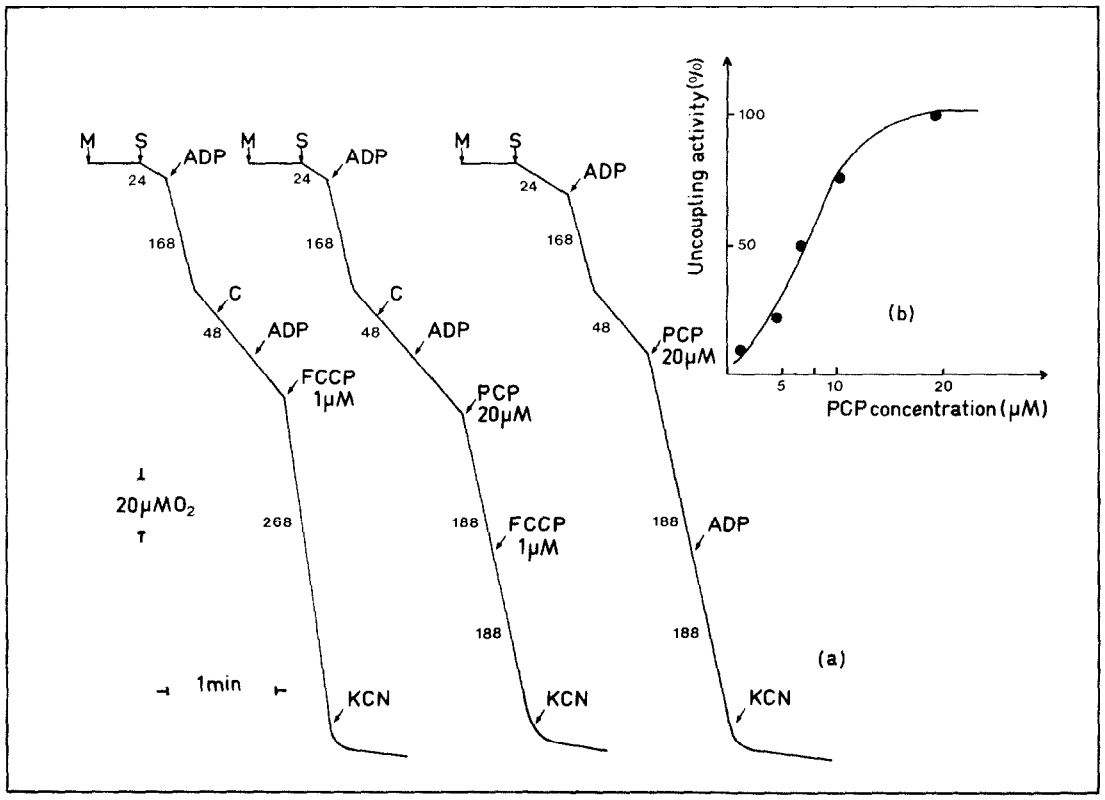

Fig. 1. Uncoupling activity of pentachlorophenol in potato tuber mitochondria in the presence of BSA: (a) polarographic traces; (b) titration curve. M, Purified mitochondria; S, 6 mM succinate + 0.3 mM ATP; ADP, 200 μM; C, 10 μM carboxyatractyloside; KCN, 30 μM. Numbers on traces refer to nmol O_2 consumed/mg mitochondrial protein/min.

Table 1. Effect of 0.1 % BSA in the reaction medium on the uncoupling activity of PCP (μM), with different substrates

Substrate	− BSA		+ BSA	
	D_{50}	D_{100}	D_{50}	D_{100}
Succinate	0.7	3	8	20
NADH	1	3	10	30
Pyruvate	2	4	15	30
α-Ketoglutarate	3	8	15	50

Succinate 6 mM + ATP 0.3 mM; NADH 1 mM; pyruvate 5 mM + NAD 1 mM; α-ketoglutarate 5 mM + thiamine pyrophosphate 0.3 mM + NAD 1 mM.

giving a maximum uncoupling effect) was practically the same ($D_{50} \simeq 10\ \mu$M and $D_{100} \simeq 30\ \mu$M with NADH as substrate) when using 0.11, 0.22, 0.44, 0.88 and 1.32 mg of mitochondrial protein in the presence of 0.1 % BSA.

Furthermore, the protonophoric activity of PCP was studied spectrophotometrically by measuring the swelling rate of potato mitochondria suspended in an iso-osmotic medium. Figure 4 shows the mitochondrial swelling obtained in an NH_4Cl iso-osmotic medium corresponding to a rapid H^+ transmembrane transfer induced by PCP. These results and those presented in Table 2 indicate that the same concentrations of PCP induced the uncoupling activity seen by polarography and the mitochondrial swelling. In the two cases the same preservative activity of BSA was shown. Furthermore, as indicated in Table 2, in the same experimental conditions, PCP was able to induce a K^+ transmembrane transfer (measurements of the swelling rate in KCl iso-osmotic medium). This last effect was obtained for higher concentrations than for the uncoupling effect.

The experiments described above have been carried out using potato mitochondria. They were repeated with mitochondria isolated from etiolated mung bean hypocotyls. The same results were obtained but PCP concentrations needed for a full uncoupling effect were generally slightly higher in this case (Table 3).

(b) *Inhibitory activity of PCP on the respiratory electron transfer*

The inhibitory activity of PCP was always measured with mitochondria in an uncoupled state (uncoupling was obtained with 1 μM FCCP). NADH, succinate, citrate, α-ketoglutarate, malate (at pH 6.5 and 7.5), duroquinol and ascorbate-*N*,*N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine (TMPD) were successively used as electron donors, each one being oxidized in electrode medium with and without BSA. Results are presented in Table 4 as I_{50} and I_{100}

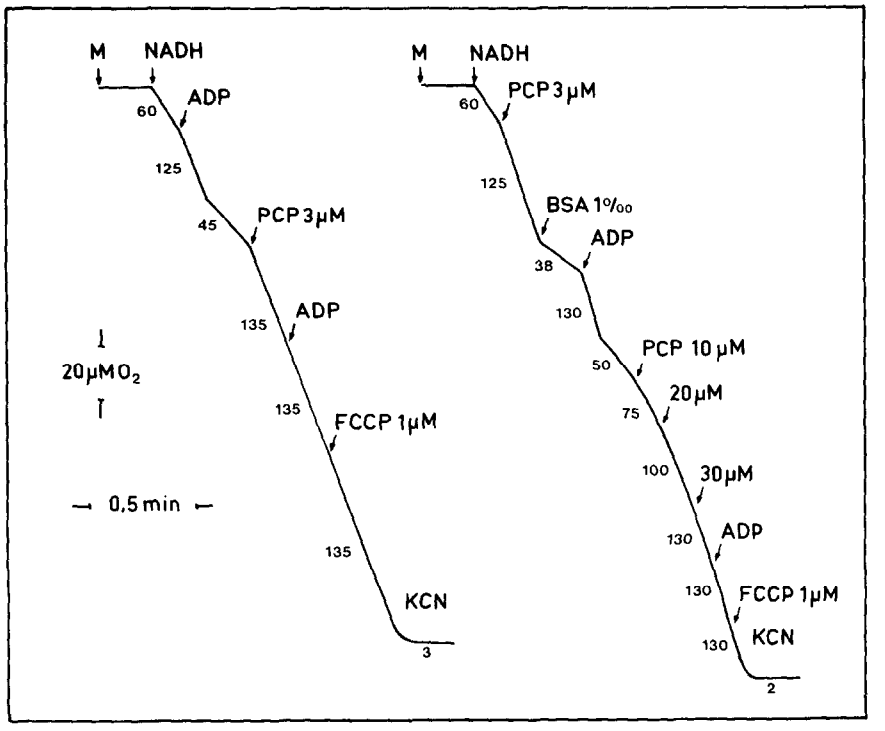

Fig. 2. Changes in PCP uncoupling activity after addition of BSA to potato tuber mitochondria. M, Purified mitochondria; NADH, 1 mM; ADP, 100 μM; FCCP, 1 μM; KCN, 30 μM. Numbers on traces refer to nmoles O_2 consumed/mg mitochondrial protein/min. The amount of mitochondrial protein was 1 mg for each trace.

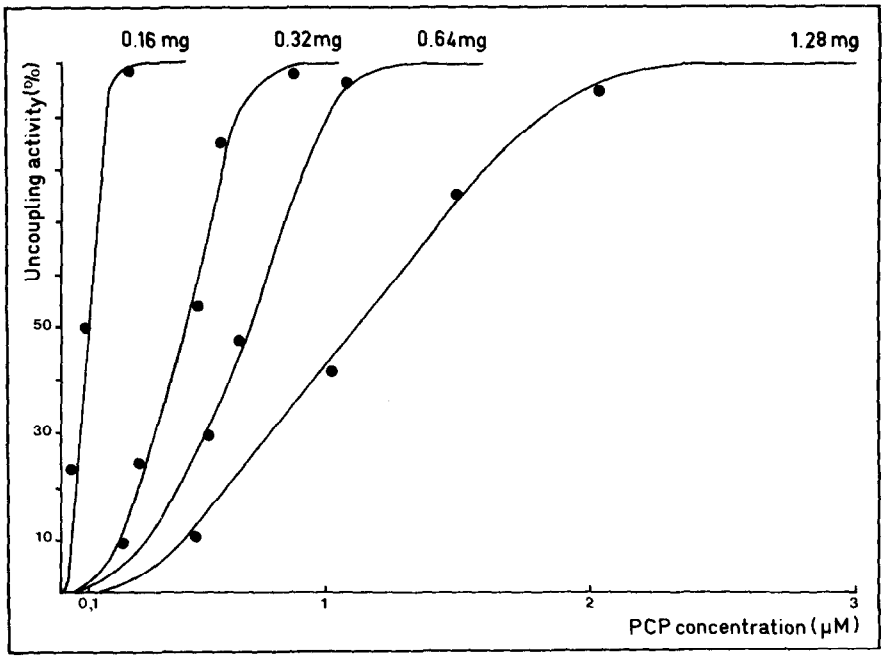

Fig. 3. Relationship between uncoupling effect of PCP and mitochondrial concentration. These results were obtained using potato tuber mitochondria oxidizing NADH (1 mM) as substrate in a reaction medium devoid of BSA.

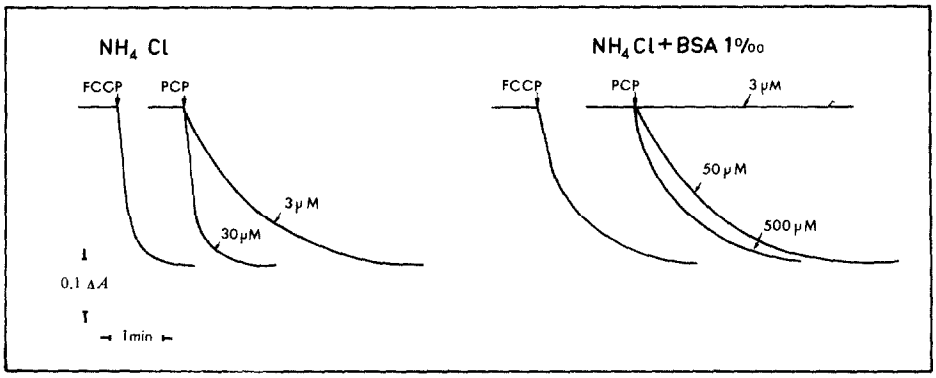

Fig. 4. Uncoupling activity of pentachlorophenol shown by mitochondrial swelling in NH_4Cl iso-osmotic solution in the absence or in the presence of BSA. The decrease of the optical density (ΔA) induced by PCP for mitochondrial suspensions (0.45 mg protein for 2 ml medium) was measured at 540 nm (see Experimental).

Table 2. Ionophoric effect of PCP in the presence or absence of 0.1% BSA

	NH_4Cl				NH_4NO_3				KCl			
	−BSA		+BSA		−BSA		+BSA		−BSA		+BSA	
	ΔA	T	ΔA	T	ΔA	T	ΔA	T	ΔA	T	ΔA	T
FCCP 1	0.30	1	0.30	3	0.35	1	0.35	2				
PCP 3	0.30	4	0		0.35	2	0		0			
PCP 10									0.30	5		
PCP 30	0.30	1			0.35	1			0.30	3		
PCP 50			0.27	4			0.30	2	0.30	2	0	
PCP 300											0.30	4
PCP 500			0.30	3					0.30	0.5	0.30	1

These results were obtained spectrophotometrically (540 nm) using 0.25 mg mitochondrial protein per ml in different iso-osmotic medium (NH_4Cl, NH_4NO_3, KCl). ΔA represents the full decrease of the optical density induced by PCP. Concentrations are given in μM, FCCP is used as reference. The time needed to obtain a full decrease (T) is expressed in min.

Table 3. PCP concentrations (μM) giving a half-maximum (D_{50}) and a maximum (D_{100}) uncoupling effect in mitochondria isolated from etiolated mung bean hypocotyls in the presence or absence of 0.1% BSA.

	−BSA		+BSA	
Substrate	D_{50}	D_{100}	D_{50}	D_{100}
NADH	2	6	20	50
Succinate	0.7	2	15	50

NADH 1 mM; succinate 6 mM + ATP 0.3 mM.

(concentration giving respectively a 50% and a 100% inhibition of the electron transfer). Clear changes of I_{50} or I_{100} were obtained following the respiratory substrate. With reduced TMPD as donor, no PCP-induced inhibition of the electron transfer could be observed. The electron transfer chain between cytochrome *c* and cytochrome oxidase was therefore unaffected by PCP. When reduced duroquinone was used as electron donor, PCP induced visible inhibition ($I_{50} = 40\ \mu$M without BSA). PCP was therefore able to inhibit the electron flow between the quinone pool and the cytochrome *c*. When succinate, NADH, citrate, α-ketoglutarate and malate were used as substrates, the inhibitory efficiency of PCP underwent important changes. Only the oxidation of succinate was inhibited at PCP concentrations smaller than those inhibiting duroquinol oxidation. With NADH as substrate, the PCP-dependent inhibition of the electron flow was exactly the same as when duroquinol was the substrate. These results suggest a site of inhibition located between succinate and the quinone pool. The very small inhibitory effect of PCP when α-ketoglutarate, malate or citrate were the substrates shows that there was no important site of inhibition located at the level of complex I or before. The fact that the inhibitions obtained with these substrates were smaller than with duroquinol can be explained by the changes of the electron flow intensity following the substrate. With citrate, α-ketoglutarate, or malate, the intensity of the

Table 4. PCP concentrations (μM) giving a 50% (I_{50}) and a 100% (I_{100}) inhibition of the electron transfer in uncoupled potato mitochondria supplemented with different substrates, with or without BSA

Substrate	BSA	I_{50}	I_{100}	Substrate	BSA	I_{50}	I_{100}
NADH	−	40	1000	Malate	−	80	500
	+	100	1000	pH 6.5	+	150	500
Succinate	−	10	500	Malate	−	100	750
	+	50	500	pH 7.5	+	250	1000
Citrate	−	100	1000	Duroquinol	−	40	1000
	+	500	1000		+	200	1000
α-Keto-	−	100	500	Ascorbate	−	> 2000	—
glutarate	+	200	2000	+TMPD	+	> 2000	—

NADH 1 mM; succinate 6 mM + ATP 0.3 mM; citrate 5 mM + NAD 1 mM; α-ketoglutarate 5 mM + NAD 1 mM; malate 15 mM + NAD 1 mM; duroquinol 0.5 mM; ascorbate 2 mM + TMPD 15 mM.

electron flow was three to six times smaller than with duroquinol, probably because of a relatively small activity of the corresponding Krebs cycle enzymes. This could explain why a partial inhibition between the quinone pool and cytochrome *c* was not apparent at such small electron flow intensities. With NADH as substrate, the electron transfer rate was as large as for duroquinol and the inhibition was the same in the two cases, showing that there was no selective and powerful inhibition located at the level of the external NADH dehydrogenase of the inner membrane. Figure 5 shows the difference between the inhibitory effects of PCP in the presence or absence of BSA in the reaction medium when NADH was the substrate. BSA appears as a preserving agent, as seen in the uncoupling activity study, but in this case the protective effect against electron transfer inhibition was much smaller.

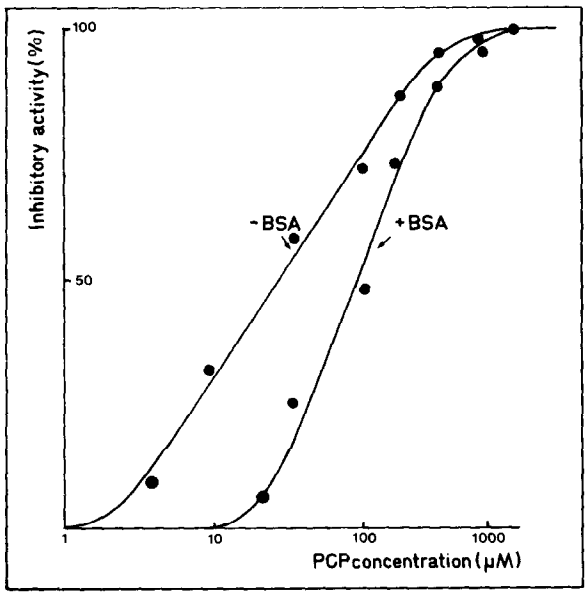

Fig. 5. Inhibitory activity of PCP on the oxidation of NADH (1 mM) by uncoupled potato tuber mitochondria in the absence (−BSA) and in the presence of BSA (+BSA).

The activity of PCP on the electron transfer through the KCN-insensitive pathway was previously studied using mung bean mitochondria [10]. It is interesting to note that the PCP-dependent inhibitions observed with the different substrates when the cytochrome oxidase pathway was operating were also observed when the KCN-insensitive pathway was operating alone. This suggests that the PCP inhibitory site located between the quinone pool and cytochrome *c* was actually located in the part of the electron transfer chain which is common to the KCN-insensitive pathway and to the quinone–cytochrome *c* chain, i.e. the quinone pool itself.

DISCUSSION

The present results show that PCP is a potent uncoupler of the oxidative phosphorylations in plant mitochondria as demonstrated by stimulation of state IV respiration, although the uncoupling activity was masked at higher concentrations by progressive inhibition of the electron transport. When considering the uncoupling concentrations, it can be seen that, among the substituted phenols, PCP is one of the best uncouplers [10], better than the well-known 2,4-dinitrophenol. Nevertheless, PCP remains twenty times less active when BSA is present, rather than FCCP or platanetin (3,5,7,8-tetrahydroxy-6-isoprenyl flavone) which is a very potent natural uncoupler isolated from plane-tree buds [manuscript in preparation]. The uncoupling activity of PCP is dependent on the presence of an acid dissociable group ($pK_a = 4.74$ [11]), the presence of the chlorine substituents inducing the decrease of the pK_a from alkaline value (phenol: $pK_a = 9.1$ [11]) to a slightly acidic value, and also inducing a large increase in lipophily [log P = 5.19 for PCP, and 1.49 for phenol itself [12] where log$P$ is the lipophilic parameter (partition between octanol and water)] which allows good mobility in the lipidic phase of the inner membrane. The uncoupling properties of PCP appear to be in accordance with a mechanism previously described [13]: PCP is able to shuttle protons across the membrane in a neutral form, which is membrane permeable, donating protons to the

matrix side. The anionic conjugate base thus produced, moves back electrophoretically to the positively charged outside, from the negatively charged inside of the mitochondrial membrane. As shown by swelling experiments, it could be shown that this mechanism could probably be extended to cation transport, especially K^+ transport which was demonstrated for slightly higher concentrations of PCP.

The presence or the absence of BSA in the reaction medium greatly influences the uncoupling efficiency of PCP and it can be seen that the effectiveness of this molecule is lowered approximately ten times by the addition of albumin. Moreover, the H^+ gradient disruption induced by 3 μM PCP can be completely reversed by the addition of BSA, restoring a good respiratory control. This indicates that BSA is able to pick out the molecules of PCP engaged in the inner membrane for the transmembranar H^+ transport. It is interesting to note that the fully uncoupling concentration in rat liver mitochondria was 25 μM [14, 15] in a medium devoid of BSA. This concentration is almost the same as for uncoupling potato mitochondria in the presence of BSA. This suggests that a proteic part of the rat mitochondrial membrane was able to bind itself to a large amount of PCP, in contrast with plant mitochondria. Such a binding of PCP by animal mitochondria was also shown by Bakker *et al.* [8].

Our results have also shown that PCP was able to inhibit the electron transfer through the plant mitochondrial inner membrane, at concentrations clearly greater than the uncoupling concentration. Two sites of inhibition were shown: one of them being near the quinone pool, the other concerning specifically complex II. A similar situation was described for PCB series using rat liver mitochondria [16]. Such a selective inhibition of complex II has been described before for compounds like malonate or, more recently, for the experimental herbicide UKJ72J [17]. In the case of chlorinated phenols, the inhibition at the complex II level seems clearly to depend on 2,6-substitution, the other derivatives being devoid of such a selective activity [10]. In the case of PCP, inhibition at the level of complex I or of external NADH dehydrogenase is doubtful: no inhibitory activity stronger than in the duroquinol test appears with NADH or malate, citrate or α-ketoglutarate as substrates. Furthermore, the selective test showing inhibition at the level of complex I is negative: this test which has been explained in detail elsewhere [18, 19] is based upon the appearance of an inhibition of the electron transfer at pH 7.5 with malate as substrate, no inhibition affecting the oxidation of malate at pH 6.5 when the malic enzyme is operating. PCP has a comparable effect on malate oxidation at pH 7.5 and 6.5. A binding of PCP to complex I is therefore doubtful. The effect of BSA on the inhibitory activity of PCP has been studied and illustrated (Fig. 5). The effect of BSA on the uncoupling action of PCP is much more powerful than on the PCP inhibitory effect on the electron transfer, probably for two reasons: (1) the inhibitory concentrations of PCP needed for inhibition are greater than those needed for uncoupling. The amount of PCP bound to 0.1% BSA is therefore insufficient to give important changes of the I_{100}; (2) the uncoupling activity needs free mobility of PCP in the membrane; inhibition is associated with binding to a membrane protein. The binding to BSA is probably no better than on complex II or on the first site of inhibition. An equilibrium between the binding to BSA and to membrane proteins like complex II could therefore occur.

As a whole, it appears that PCP is firstly an uncoupler for plant mitochondria and secondly an inhibitor of electron transfer at higher concentrations. An acute toxicity has been described in animals [6, 20] and on isolated plant cell cultures [21, 22] for concentrations which are near the uncoupling concentrations (20–50 μM); it seems evident that the uncoupling activity of PCP is a major, perhaps the only cause explaining the acute toxic effect in plants as well as in animals or in bacteria. Furthermore, in plants, the uncoupling effect of PCP can also be seen on photophosphorylations in thylakoids inducing the inhibition of photosynthesis (publication in preparation).

It can therefore be suspected that plant cells are as sensitive as animal cells to PCP. A possible resistance to PCP could therefore only come from detoxification intensity, binding of the toxic to non-living parts of the organism or to low access to the essential parts of the plant (meristems in the case of angiosperms).

EXPERIMENTAL

Preparation of mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L.) and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown 5 days in the dark at 26° and 60% relative humidity were prepared and purified by methods previously described [23]. All operations were carried out at 0–4°. Following purification, the mitochondria appeared to be virtually free of extramitochondrial contamination and their membranes were found to be almost fully intact, as judged by electron microscopy and by low activities of the inner membrane and matrix marker enzymes (antimycin A-sensitive NADH cytochrome *c* oxidoreductase and malate dehydrogenase). In addition, the mitochondria were tightly coupled: average ADP/O ratio for succinate was 1.8 and respiratory control ratio for the same substrate was about 3. Protein was determined using BSA (Sigma, fraction V) as the standard.

O_2 uptake was followed polarographically at 25° using a Clark-type electrode system purchased from Hansatech Ltd (Hardwick Industrial Estate, King's Lynn, U.K.). The reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM NaPi buffer, known amounts of mitochondrial proteins and in some cases 0.1% defatted BSA. Unless otherwise stated, all incubations were carried out at pH 7.2.

Uncoupling test. Intact mitochondria were suspended in the electrode medium containing a substrate. After a state III-state IV transition, 10 μM carboxyatractyloside was added in order to inhibit the nucleotide carrier. The uncoupling effect of substance added at this stage corresponded to an increase in the oxidation rate: 100% uncoupling effect was obtained when the rate of O_2 consumption was not further stimulated by the addition of FCCP (1 μM).

Mitochondrial swelling measurements. Intact mitochondria were suspended in a reaction medium (NH_4Cl, NH_4NO_3 or KCl 150 mM, Tris–HCl 10 mM, pH 7.2). Passive swelling reactions were measured as optical density decreases at 540 nm in a Kontron spectrophotometer (model UVIKON 810) as previously described [24]. A rapid passive swelling was induced by uncouplers (in NH_4^+ salts) and by valinomycin-like ionophores (in K^+ salts).

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