THE EFFECT OF PENTACHLOROPHENOL ON OXIDATIVE PHOSPHORYLATION

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Since pentachlorophenol is used widely, not only as a powerful molluscacide, but also in a variety of industries to suppress the growth of undesirable microorganisms, the mechanism of its toxicity to vertebrates merits investigation.

Loomis (1) found that the di- and trihalophenols inhibit the coupling between phosphorylation and oxidation in rat tissues. Other studies (2) suggested that these compounds block cleavage of fertilized eggs of *Arbacia* by interfering with the formation and transfer of high energy phosphate. By analogy to the trihalophenols it might be anticipated that pentachlorophenol would affect adversely oxidative phosphorylation. However, trinitrophenol, while analogous in structure to the potent uncoupling agent, dinitrophenol, had no effect upon oxidative phosphorylation in rat tissues.[1] Thus, it seemed necessary to study experimentally the effect of PCP[2] on phosphorylation coupled to respiration. This report summarizes the data concerning such studies and shows that PCP prevents the uptake of inorganic phosphate during the oxidation of α-ketoglutarate by rat liver mitochondria.

EXPERIMENTAL

The mitochondria were prepared from rat liver in isotonic (0.25 M) sucrose, essentially by the procedure described by Schneider (4); resuspension and recentrifugation of the nuclear fraction were omitted. The mitochondria were washed once in a volume of cold isotonic sucrose equal to twice the original tissue weight, and the final suspension was made in an equal volume of the same medium. All operations (except centrifugation in the International refrigerated centrifuge) were conducted as quickly as possible in a cold room at 5°, with all tubes chilled in ice. The nitrogen content of the mitochondria was determined by a micro-Kjeldahl procedure.

[1] Unpublished data of E. C. Weinbach (*cf.* (2, 3)).

[2] The following abbreviations are used: PCP = pentachlorophenol, DNP = 2,4-dinitrophenol, ATP and ADP = adenosinetri- and adenosinediphosphate, respectively, KG = α-ketoglutaric acid.

The majority of the experiments were performed with α-ketoglutarate as substrate with the use essentially of the phosphorylation system of Copenhaver and Lardy (5). There were also a few experiments with β-hydroxybutyrate as substrate (6).

ATPase activity was measured (7, 8) in small test-tubes that were shaken manually several times during the incubation period.

Hexokinase was prepared by the procedure of Berger, Slein, Colowick, and Cori (9) through Step 3*a*. Stock solutions in 1 per cent glucose were kept frozen in small vials until needed.

The barium salts of ATP and ADP, as well as cytochrome *c*, α-ketoglutaric acid, *dl*-β-hydroxybutyric and malonic acids, glycylglycine, and pentachlorophenol, were high grade commercial products. Their identity was determined by appropriate tests. Solutions of the potassium salts of ATP and ADP were prepared by treating solutions of the barium salts in 0.1 M HCl with a slight excess of Na_2SO_4 and removing the $BaSO_4$ which was washed. The supernatant solution was adjusted to pH 7.4 with KOH and kept frozen in small vials until needed. Solutions of the other reagents, when required, were adjusted to pH 7.4 before addition to the incubation mixture.

Inorganic phosphate was determined (10) on aliquots of the reaction mixture after deproteinization with cold trichloroacetic acid. The net uptake of phosphate was calculated from the differences between the inorganic phosphate content of the experimental reaction mixtures and the corresponding zero time values.

The net consumption of α-ketoglutaric acid as determined by analysis (11) was calculated from the differences in content found between the various experimental mixtures and corresponding zero time assays.

Oxygen consumption was measured in the Warburg apparatus with air as gas phase. The readings were started 8 minutes after the chilled flasks were placed in the bath, at which time the zero time flasks were removed.

Results

Table I summarizes the data reflecting the effect of various concentrations of PCP upon the uptake of inorganic phosphate associated with the one-step oxidation of α-ketoglutarate to succinate. In these experiments no attempt was made to obtain maximal P:O ratios, nor were corrections introduced for phosphate "leaks," as these refinements were not considered necessary for the present study. The data show clearly that at low concentrations of PCP there was marked suppression of the phosphate uptake while oxidation was relatively unaffected. At concentrations of 1×10^{-4} M, PCP usually caused a slight inhibition of the oxygen uptake, while at the lower concentrations a definite stimulation occurred. These varia-

tions of oxygen consumption under the influence of PCP were paralleled by changes in the utilization of ketoglutarate, thus maintaining a uniform O:KG ratio. This ratio approached unity, indicative of a successful block of succinate oxidation by malonate. Usually there was a slight uptake of inorganic phosphate in PCP concentration ranges of 1×10^{-4} to 1×10^{-5} M; however, in several experiments there was no net uptake whatsoever, and an increase in inorganic phosphate above the zero time level has been observed. At PCP concentrations of 10^{-3} M and higher, oxidation as well as phosphorylation was abolished completely.[3] The uncoupling action of PCP is not to be attributed to inhibition of hexokinase, for PCP in concentrations up to 10^{-4} M had no effect upon the hexokinase reaction when studied independently.

TABLE I

Effect of PCP on Phosphorylation Associated with Oxidation of α-Ketoglutarate to Succinate

Each flask contained in the main compartment 1.3×10^{-2} M potassium phosphate, pH 7.4, 2×10^{-3} M ATP, 1.2×10^{-5} M cytochrome *c*, 7.5×10^{-3} M $MgSO_4$, 6.7×10^{-3} M α-ketoglutarate, 1×10^{-2} M malonate, 1×10^{-2} M fluoride, and 0.5 ml. of mitochondria. Each side arm contained 1.6×10^{-2} M glucose and 0.1 ml. of hexokinase. Final volume made up to 3.0 ml. with 0.15 M KCl. Average N per flask, 1.3 mg. Incubation period, 20 minutes; temperature, 30°. The figures are averages of at least six experiments for each case, with ranges indicated in parentheses.

Incubation system	Δ P	Δ O	Δ KG	P:O	O:KG
M	μM	*micro-atoms*	μM		
Control	−11.3	4.0	4.7	2.8 (2.0–3.3)	0.8 (0.7–1.1)
PCP, 1×10^{-4}	−1.2	3.5	4.3	0.3 (0–0.5)	0.8 (0.7–1.0)
" 1×10^{-5}	−1.4	4.6	6.0	0.3 (0–0.6)	0.8 (0.7–1.0)
" 1×10^{-6}	−9.1	6.1	6.7	1.5 (1.3–1.8)	0.9 (0.8–1.2)

A few experiments with β-hydroxybutyrate as substrate confirmed the uncoupling action of PCP. When Lehninger and Smith's incubation mixture (6), supplemented with the addition of hexokinase and glucose, was used to follow phosphorylation coupled to β-hydroxybutyrate oxidation, there was a rapid drop in the phosphate level in the control flasks, owing to lower initial levels of inorganic phosphate and more efficient phosphorylation of the ADP added as phosphate acceptor in these experiments. Such a mixture quickly became phosphate-deficient accompanied by a decline of oxygen consumption in the control flasks. In the flasks containing PCP the oxygen consumption as well as inorganic phosphate remained at a

[3] PCP in high concentrations (1×10^{-2} M) completely inhibited the succinoxidase and cytochrome *c* oxidase activity of rat liver mitochondria.

higher level; the net effect was a marked "stimulation" of the oxidation by PCP.

In view of the known action of DNP to evoke the ATPase[4] activity of fresh mitochondria (8, 12), it was of interest to determine whether PCP had a similar action. These data are summarized in Table II. Confirming the findings of Kielley and Kielley (13), we found carefully prepared fresh mitochondria to have little ATPase activity. In the absence of activating cations such as Ca^{++} and Mg^{++}, there was no significant ATPase activity. In such preparations the presence of PCP in low concentrations caused a marked release of phosphate from ATP. The effect was maximal at PCP concentrations of 5×10^{-5} M. At either extreme of high (5×10^{-3} M) or

TABLE II

Effect of PCP on Phosphate Release from ATP

Each tube contained 4×10^{-2} M glycylglycine buffer, pH 7.4, 4×10^{-3} M ATP, 0.2 ml. of mitochondria plus other components as indicated, and sufficient isotonic sucrose to make a final volume of 1.0 ml. Average N per flask, 0.6 mg. Incubation period, 15 minutes; temperature, 30°. The figures are averages of at least five experiments for each case, with ranges indicated in parentheses.

Incubation system	Δ P, μM	
	No addition	1.5×10^{-2} M NaF added
M		
Control	0	
PCP, 5×10^{-4}	1.7 (1.3–1.9)	1.3 (1.2–1.4)
" 5×10^{-5}	3.2 (2.4–3.8)	2.3 (2.0–2.5)
" 5×10^{-6}	2.9 (2.5–3.6)	1.4 (1.2–1.6)

low (5×10^{-7} M) concentrations, the effect of PCP was greatly diminished.[5] Under strictly anaerobic conditions (experiments conducted in 5 ml. Warburg flasks in an atmosphere of pure N_2) essentially the same stimulating effect of PCP upon ATPase was observed.

As detailed in Table II, fluoride, 1.5×10^{-2} M, diminished somewhat the ATPase stimulation by PCP. This suppressing effect of fluoride was quite marked at the lower concentration (5×10^{-6} M) of PCP. The addition of $MgCl_2$, 5×10^{-3} M, to the control incubation mixture caused a slight, but definite, release of phosphate from ATP (0.45 μM per mg. of N per 15 minutes). This liberation of phosphate was not additive to that evoked by PCP; usually the stimulating effect of PCP was diminished slightly in the presence of Mg^{++}. Similar effects had been observed with DNP (8).

[4] ATPase is used here to designate the enzyme (or enzymes) in the mitochondrial preparations which split inorganic phosphate from added ATP.

[5] 5×10^{-3} M PCP rendered the mitochondria soluble.

Pentachlorophenol had no effect on the release of phosphate from AT in the absence of mitochondria and caused no release of phosphate from mitochondria in the absence of added ATP.

DISCUSSION

The results presented in this paper on the effects of PCP upon oxidative phosphorylation in rat liver mitochondria support the hypothesis that this compound is a potent uncoupling agent. Although PCP resembles DNP in its stimulation of respiration while dissociating phosphate uptake, the increase in oxygen consumption is not as marked as usually observed with DNP. It is pertinent to this discussion that PCP increases the respiration of living snails (14), and a similar effect upon laboratory animals has been reported (15). A striking similarity of PCP to DNP is its acceleration of ATP breakdown in fresh mitochondrial preparations. As discussed by Hunter (12), it is not known whether this action is owing to an activation of ATPase, *per se*, or to an alteration in the permeability of mitochondria. The results of a few experiments with the specific ATPase prepared from disintegrated mitochondria (16) suggested that, in the case of PCP, permeability may be the more important factor. Not only did PCP fail to enhance the activity of such a system, but, with concentrations of 5×10^{-4} M and higher, appreciable inhibition was observed.

The uncoupling effects of PCP upon oxidative phosphorylation provide at least a partial explanation of the toxic characteristics of this compound. It would be anticipated that interference with the generation and transfer of energy-rich phosphate would result in eventual damage to the cell, especially if alternative energetic processes were not available. This does not imply that all uncoupling agents are necessarily toxic; indeed, Aureomycin, which has been shown to uncouple oxidative phosphorylation in rat tissues (17), usually is not considered to be toxic to the mammalian host. Undoubtedly, many unknown factors such as permeability effects and selective absorption have an important rôle in this respect.

SUMMARY

Pentachlorophenol in low concentrations (1×10^{-5} M) prevented the uptake of inorganic phosphate associated with the oxidation of α-ketoglutarate by rat liver mitochondria. The oxidation of this substrate was relatively unaffected except in phosphate-deficient systems in which a stimulation was observed.

PCP greatly enhanced the liberation of inorganic phosphate from ATP by fresh mitochondrial preparations but had no such effect upon ATPase prepared from disintegrated mitochondria. The enhancement of ATPase

activity of fresh mitochondria by PCP was depressed by fluoride and by magnesium ions.

It is postulated that the uncoupling of phosphorylation from oxidation by PCP may account, at least in part, for the toxic manifestations of this compound.

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