Effect of the Herbicide Prometryne [2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine] on Mitochondria

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The effects of the herbicide prometryne (2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine) on rat liver and corn shoot (*Zea mays*) mitochondria were investigated. Prometryne, at 2.7 – 6.7 × $10^{-4}M$, inhibited state 4 respiration to a certain extent but state 3 respiration was inhibited to a much greater degree. The P-to-O ratio of corn mitochondria was greatly reduced. The inhibition by prometryne of the state 3 respiration of rat-liver mitochondria was partially relieved by the uncoupling agents DNP, *m*-Cl-CCP and Ca^{2+}. Prometryne had no effect on the endogenous or DNP-induced ATPase of rat-liver mitochondria. The possible site of action of prometryne inhibition is discussed in relation to the suggested sites of action of other inhibitors.

Prometryne [2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine] is one of the *s*-triazine group of herbicides produced by the Geigy Chemical Corp. Of this family of compounds, the 2-chloro triazine class, which includes simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine], atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], and propazine [2-chloro-4,6-bis(isopropylamino)-*s*-triazine] is the most important commercially. Prometryne shows a herbicide selectivity different from the 2-chloro triazines. It shows promise for use with small grain crops such as wheat and barley and is recommended for the control of broad leaved weeds and grasses in cotton.

The *s*-triazine herbicides are known to inhibit the Hill reaction (Good, 1961; Gysin and Knusli, 1960; Moreland and Hill, 1962; Sikka and Davis, 1968). Simazine at a concentration >1 p.p.m. almost completely blocks $^{14}CO_2$ fixation in bean (Ashton, 1960), and similar results have been reported for atrazine (Couch and Davis, 1966; Zweig and Ashton, 1962). Similarly, prometryne blocks the synthesis of sucrose from $^{14}CO_2$ in soybean and, to a lesser extent, in cotton (Sikka and Davis, 1969).

Prometryne shows some contact injury to sensitive species whereas the 2-chloro triazines usually require several days for toxic symptoms to develop. This suggests that some basic physiological process in addition to photosynthesis may be involved in prometryne toxicity.

A number of herbicides have been shown to affect oxygen uptake and occasionally phosphorylation by plant mitochondria (Ashton, 1963; Davis, 1968; Foy and Penner, 1965; Lotlikar *et al.*, 1968; Stenlid and Saddik, 1962; Wedding and Black, 1962). The *s*-triazine herbicides have not yet been explored fully in this respect. Lotlikar *et al.* (1968) found that simazine at a concentration of 6.0 × 10^{-5} *M* inhibited cabbage mitochondrial phosphorylation by 12% and oxygen uptake by 6%. Foy and Penner (1965) reported that atrazine at a concentration of 3.25 × 10^{-6} *M* inhibited the oxidation of succinate and α-ketoglutarate by cucumber mitochondria. However, Davis (1968) could detect no effect of atrazine on oxygen uptake or P-to-O ratio of corn shoot mitochondria at a concentration of 4.0 × 10^{-4} *M*. Results presented in this paper show that prometryne inhibits oxygen uptake to a certain extent and will completely prevent ADP stimulation of oxygen uptake by mitochondria isolated from both plants and animals.

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PROCEDURE

Mitochondrial Isolation. Mitochondria were isolated from three-day-old corn shoots (*Zea mays*) as described by Kenefick and Hanson (1966b). Final suspension was in a volume of 0.4*M* sucrose such that a 0.1-ml. suspension was equivalent to approximately 0.1 mg. of nitrogen as determined by digestion and nesslerization. Rat liver mitochondria were isolated according to the method of Johnson and Lardy (1967) from Charles River CDA weanling rats, final suspension was in 0.25*M* sucrose (0.1-ml. suspension equivalent to approximately 0.4 mg. of nitrogen).

Measurement of P to O. The P-to-O ratios of corn preparations were determined at 30° C. using conventional manometric techniques in a medium of the following final millimolar concentrations: 25 potassium phosphate buffer (pH 7.5), 20 pyruvate, 20 malate, 50 glucose, 50 sucrose, 1 $MgSO_4$, 1 ATP, 0.3 NAD, 0.17 TPP, 0.06 CoA + 25 k*M* units hexokinase, and 1 mg. per ml. of bovine serum albumin (total volume = 2.5 ml.).

Oxygen Uptake. Rates of oxygen uptake were calculated from polarographic traces obtained with the Yellow Springs Instrument Co., Model 53 Biological Oxygen Monitor working in conjunction with a Bausch and Lomb VOM 5 potentiometric recorder. Experiments were conducted at 30° C. The final composition of the media used was as follows:

Corn: 0.4*M* sucrose, 33 m*M* malate, 10m*M* KCl, 5 m*M* $MgSO_4$, 10 m*M* KH_2PO_4, and 1 mg. per ml. of bovine serum albumin, pH 7.2.

Table I. Effect of Prometryne on P-to-O Ratio of Mitochondria from Three-Day-Old Corn Shoots[a]

	O_2 Uptake[b]	Pi Esterified[c]	P-to-O Ratio
Control	53	122	2.3
+ $3.2 \times 10^{-4}M$ prometryne	35	58	1.7
+ $6.4 \times 10^{-4}M$ prometryne	31	15	0.5

[a] The prometryne was added in a small volume of ethanol, an equal amount of ethanol being added to the control. Each treatment was duplicated and the experiment was repeated five times. The results presented are those of one typical experiment.
[b] μatoms per mg. mitochondrial nitrogen per hour.
[c] μmoles per mg. mitochondrial nitrogen per hour.

Rat-liver: 0.25*M* sucrose, 3.3 m*M* succinate, 20 m*M* KCl, 5 m*M* $MgSO_4$, 10 m*M* KH_2PO_4, 20 m*M* triethanolamine, and 1 mg. per ml. of bovine serum albumin, pH 7.4.

ATPase Activity. The ATPase activity of liver mitochondria was determined at 30° C. over a period of 10 minutes, using the method of Weinbach (1956). The medium contained: 40 m*M* glycylglycine, 5 m*M* $MgSO_4$, 6 m*M* ATP, 0.05 ml. of mitochondrial suspension in sucrose + other additives as indicated in Table III, in a total volume of 1 ml., pH 7.4.

Chemicals. Prometryne (99.0% pure) is only slightly soluble in water (48 μg. per ml. at 20° C.). Stock solutions were prepared in absolute ethanol so that volumes of 20 to 50 μl., when added to the reaction system, gave the stated final concentrations. An equivalent amount of alcohol was added to all controls. The addition of even these small amounts of alcohol to 3.0 ml. of medium in the Model 53 polarographic system, which uses a membrane covered oxygen electrode, causes a temporary deflection of the polarographic trace. Corrections have been made to the traces to allow for such alcohol-caused deflections.

RESULTS AND DISCUSSION

The effect of two concentrations of prometryne on the P-to-O ratio of corn mitochrondria is shown in Table I. The esterification of phosphate was depressed more than oxygen uptake, and there was increased inhibition with increasing prometryne concentration.

Prometryne prevented the increased rate of oxygen uptake associated with the addition of ADP to a coupled, but acceptorless, system. The degree of inhibition of state 3 oxidation with born corn and liver mitochondria was dependent upon

Table II. Prometryne Inhibition of State 3 Oxidation by Corn and Liver Mitochondria[a]

	Prometryne Concn.	Stimulation of Oxygen Uptake with Addition of ADP ($10^{-4}M$)
Corn Shoot	Control	100
	$+2.7 \times 10^{-4}M$	29
	$+4.0 \times 10^{-4}M$	22
	$+5.3 \times 10^{-4}M$	9
	$+6.7 \times 10^{-4}M$	1
Rat Liver	Control	100
	$+2.4 \times 10^{-4}M$	24
	$+3.6 \times 10^{-4}M$	11
	$+4.8 \times 10^{-4}M$	7
	$+6.0 \times 10^{-4}M$	1

[a] Prometryne was added in small volumes of ethanol. An equal amount of ethanol was added to the controls.

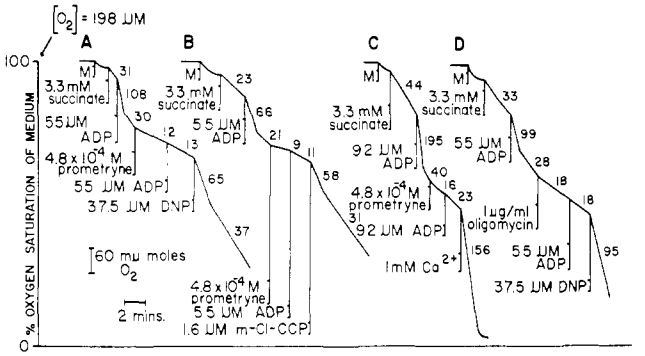

Figure 1. Polarographic traces of oxygen uptake by liver mitochondria showing inhibition by prometryne (traces *A*, *B*, and *C*) and oligomycin (trace *D*) of state 3 respiration and its relief by uncoupling agents

Measurements made in 3 ml. of medium as described in methods Section. Figures on traces represent rate of oxygen uptake in mμ-moles per minute

prometryne concentration (Table II). The inhibition was relieved to a certain extent in liver mitochondria by the addition of DNP, *m*-Cl-CCP (carbonyl cyanide *m*-chlorophenyl hydrazone) or Ca^{2-} (Figure 1, traces *A*, *B*, and *C*). The results obtained using corn shoot mitochondria were similar to those obtained and illustrated for rat liver mitochondria. There was one difference, however. With the corn preparations, inhibition was relieved with DNP, but the authors were not able to relieve the inhibition with Ca^{2+}. Note that the rate of oxygen uptake following the addition of DNP or *m*-Cl-CCP to prometryne-inhibited liver mitochondria did not represent a total release of inhibition, and the relief was relatively short lived. The results of an experiment carried out in a similar manner substituting oligomycin, a classical inhibitor of phosphorylating oxidation, for prometryne are shown in Figure 1, trace *D*. Oligomycin, at the concentration of 1 μg. per ml. used, inhibited the state 3 rate to a similar extent, but DNP completely released the inhibition, and this high rate of oxygen uptake was sustained until virtually all of the oxygen of the medium had been exhausted.

The data presented in Figure 1 suggest that prometryne acts in a manner similar to that of oligomycin. Table III shows the effects of prometryne and oligomycin on the endogenous and DNP-induced ATPase of liver mitochondria. The complete data were analyzed according to the procedure described by Snedecor (1956), and the means were compared for significance by Duncan's (1955) multiple range test. That analysis showed that prometryne, unlike oligomycin, had no effect on the ATPase activity.

The data presented show that prometryne inhibited mitochondrial oxygen uptake and oxidative phosphorylation. The fact that the inhibition was partially relieved by uncoupling agents such as DNP and *m*-Cl-CCP indicates that the block due to prometryne is at some point, in the sequence of reactions leading to ATP formation, beyond where DNP reacts. DNP uncouples in the absence of Pi and is generally considered to be interacting with a nonphosphorylated intermediate of the oxidative phosphorylation sequence of reactions (Slater, 1963; Vignais, 1963). The site of action of oligomycin is in dispute. Racker (1965) and Bruni *et al.*, (1964) believe it prevents the formation of $X \sim P$ from $X \sim I$, but Lardy *et al.*, (1964), from their observations on rat liver mitochondria, and Kenefick and Hanson (1966a), working with corn shoot mitochondria, believe it prevents the transfer of $\sim P$ from $X \sim P$ to ADP. Prometryne may be acting at a

Table III. Effect of Prometryne and Oligomycin on Liver Mitochondrial ATPase[a]

	μmoles Pi Liberated per Mg. N per Hr.	
	Expt. 1, Mean of 4 replicates ± S.D.	Expt. 2, Mean of 4 replicates ± S.D.
Control	22.8 ± 2.2	39.0 ± 3.1
$+2.4 \times 10^{-4}M$ prometryne	23.3 ± 1.0	31.4 ± 16.0
$+6.0 \times 10^{-4}M$ prometryne	25.9 ± 3.5	35.5 ± 12.6
$+1.0 \times 10^{-4}M$ DNP	103.0 ± 2.7	131.6 ± 3.5
$+1.0 \times 10^{-4}M$ DNP + $2.4 \times 10^{-4}M$ prometryne	105.6 ± 4.2	137.4 ± 3.7
$+1.0 \times 10^{-4}M$ DNP + $6.0 \times 10^{-4}M$ prometryne	100.5 ± 3.1	126.6 ± 6.2
	Expt. 3, Mean of 2 replicates ± S.D.	**Expt. 4, Mean of 2 replicates ± S.D.**
Control	50.1 ± 5.9	31.1 ± 7.1
+2.3 μg./ml. oligomycin	31.4 ± 3.6	8.1 ± 1.0
$+1.0 \times 10^{-4}M$ DNP	128.7 ± 0.0	90.4 ± 2.2
$+1.0 \times 10^{-4}M$ DNP + 2.3 μg./ml. oligomycin	41.6 ± 8.2	24.1 ± 0.0

[a] The reaction was started by the addition of mitochondrial suspension (0.15–0.25 mg. N) and terminated after 10 minutes with 50 μl. of 60% perchloric acid. The different experiments represent different mitochondrial preparations. Prometryne and oligomycin were added in a small volume of ethanol, and an equal amount of ethanol was added to the controls.

point near to that of oligomycin; however, its action cannot be identical to that of oligomycin because prometryne inhibition was not relieved completely by uncoupling agents such as DNP, (Figure 1). More strikingly, it did not inhibit ATPase activity (Table III). The decrease with time in the rate of oxygen uptake following the addition of DNP or *m*-Cl-CCP to prometryne treated mitochondria (Figure 1), may represent some secondary effect and probably does not conflict with the basic assumption that such uncoupling agents do relieve prometryne inhibition. The relief by Ca^{2+} did not slow with time. This is interpreted as indicating that the uncoupling due to Ca^{2+} is different in nature to that of the relief due to DNP and *m*-Cl-CCP. Hanson *et al.* (1965) attribute Ca^{2+} uncoupling of corn mitochondria to a diversion of ~P into Pi + Ca uptake rather than to its utilization in the synthesis of ATP. Such a situation may be applicable here also and could account for the observed difference in response. The failure to obtain relief of prometryne-inhibited corn mitochondria with Ca^{2+} probably reflects the extreme sensitivity of corn mitochondria to relatively low concentrations of Ca^{2+}, since concentrations of less than 1 m*M* tend to inhibit respiration (Hanson *et al.*, 1965).

The ability of prometryne to inhibit ATP formation, and yet its failure to affect ATPase activity, is presently not explicable. A similar situation occurs with aurovertin which prevents ATP formation but has no marked effect on ATP hydrolysis (Lardy *et al.*, 1964; Slater, 1967; Truelove and Hanson, 1966). Aurovertin does not prevent the accumulation of phosphate by corn mitochondria, which is an indication that it does not prevent the formation of the phosphorylated intermediate X ~ P from the nonphosphorylated X ~ I (Truelove and Hanson, 1966). Experiments are in progress to determine whether prometryne-treated mitochondria can accumulate phosphate; the results should enable us to circumscribe better its site of action.

In view of its effect on mitochondria, it is somewhat surprising that prometryne is not a particularly poisonous compound. The technical literature supplied by the Geigy Co. indicates an acute oral LD_{50} for rats and mice of 3750 mg. per kg. This suggests that ingested prometryne is not absorbed or that rats and mice possess a very effective detoxification system. In plants, there is evidence that certain species are capable of converting prometryne into the nontoxic hydroxypropazine to a limited extent (Montgomery and Freed, 1964; Sikka and Davis, 1969; Whitenberg, 1965). In cotton, prometryne is only slightly metabolized and tolerance in this case has been partially attributed to poor translocation from roots to shoots and to accumulation in the lysigenous glands (Sikka and Davis, 1969; Whitenberg, 1965).

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Received for review January 9, 1969. Accepted April 21, 1969. This research was supported in part by Public Health Service Grant No. UI 00113-08 from the National Center for Urban and Industrial Health, by Geigy Chemical Corporation, and by a National and Aeronautics Space Administration Traineeship.