The in Vitro Inhibition of Rabbit Muscle Lactate Dehydrogenase by Mirex and Kepone

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Mirex, a polychlorinated pesticide used for fire ant control in the southern United States, accumulates in many species inhabiting treated areas. Because the LD_{50} of Mirex in many species is very high, it was thought that accumulation levels represented no hazard to species which might be naturally exposed, since these accumulated Mirex concentrations were well below typical lethal doses. However, we have found that lactate dehydrogenase (EC 1.1.1.27, crystalline from rabbit muscle) is competitively inhibited by Mirex and by Kepone (a carbonyl analog of Mirex) at levels as low as 0.01 m*M* (10 ppm) during in vitro determinations. This inhibition occurs at test levels well below usual lethal dosages and comparable to accumulation concentrations. Because lactate dehydrogenase occupies a key position in the anaerobic glycolytic pathway of skeletal muscle (one of several tissues which accumulates Mirex), impairment of the function of this enzyme in energy production could result in possible harmful effect on muscle function.

Mirex (dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalene, shown in Figure 1) is a chlorinated hydrocarbon whose large number of chlorine atoms makes it resistant to normal mechanisms of biodegradation (Spenser, 1968; Metcalf et al., 1973). Because of its apparent biological specificity for certain insects, it is used in large amounts in the southern United States for control of the imported fire ant, *Solenopsis saevissima* (Shapely, 1971), and in Hawaii against pineapple and sugarcane pests (Devick, 1974). A number of recent studies, both in the laboratory and in the field, have shown that Mirex is accumulated and stored in several tissues for long periods of time by many species other than its target organisms. Selected data from several of these experiments are summarized in Tables I and II; these data indicate that Mirex is toxic to animals other than the fire ant although at higher lethal dosages. On a cellular level Mirex has been shown to induce hepatic microsomal detoxication enzymes and cause liver enlargement (at levels similar to those in Table II), an effect which is characteristic of many organohalogen compounds (Baker et al., 1972; Mehendale et al., 1973; Kacew and Singhal, 1973; Sell and Davison, 1973). In recent experiments, Abston and Yarbrough (1974) have shown that Mirex administered orally to laboratory rats depresses hepatic lactate dehydrogenase activity. This in vivo observation substantiated our preliminary report on the depression of lactate dehydrogenase activity in vitro by Mirex and other selected pesticides (Hendrickson and Bowden, 1973). This particular enzyme was chosen for our studies because it is found in many species of marine and land animal life that participate in man's food chain (Worblewski, 1958). In addition, the M_4 isozyme occupies a key position in the anaerobic glycolytic pathway of skeletal muscle, one of several tissues which accumulates Mirex (see Table II; Mehendale et al., 1972; Gibson et al., 1972). This report deals with the kinetic determination of the type of inhibition exerted by Mirex and its carbonyl analog Kepone (decachloro-1,3,4-metheno-2*H*-cyclobuta[6*d*]pentalen-2-one) on rabbit muscle lactate dehydrogenase (EC 1.1.1.27).

MATERIALS AND METHODS

Crystalline rabbit muscle lactate dehydrogenase (LDH), sodium pyruvate, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. All other reagent chemicals were of the highest purity available, and water for all solutions was carbon filtered and deionized to at least 1×10^6 ohms. Analytical samples of Mirex and Kepone were supplied by Allied Chemical Co. LDH activity was assayed by following the rate of conversion of pyruvate to lactate in the presence of NADH by the loss of absorbance of NADH at 340 nm in a Beckman DB spectrophotometer at 25°. The assay cuvette (1.0 cm) contained 0.7 m*M* NADH, 0.1–1.0 m*M* pyruvate, and Mirex or Kepone in 95% ethanol solution. The volume was adjusted to a total volume of 3.0 ml with 0.1 *M* potassium phosphate buffer at pH 7.5 after addition of enough ethanol to bring ethanol concentration to 0.3 ml (10%), and the reaction was started by adding 1 IUB unit of LDH. The system incorporated 10% ethanol to solubilize the water-insoluble pesticides and mimic their in vivo solubilization by lipid carriers in the tissue (Freedland and McFarland, 1965). In order to rule out any adverse effects of the addition of ethanol on the catalytic ability of LDH, control experiments with oxamate (a well-characterized inhibitor of LDH (Novoa et al., 1959)) were performed. Experimental data were plotted according to the methods of Lineweaver and Burk (1934), Dixon (1953), and Cornish-Bowden (1974) to determine the apparent inhibition constant (K_i'). Three methods were used in order to clearly determine the type of inhibition.

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RESULTS

The results of the effect of ethanol on LDH activity are presented in Table III. The observation that ethanol did not change the K_i' values or the type of inhibition for oxamate indicates that the catalytic properties of the active site of LDH were not affected by ethanol at this concentration.

Figure 2 shows that the effect of Mirex is competitive with respect to pyruvate with a K_i' of 0.02 m*M* (21.6 ppm). Kepone (Figure 3) is also a competitive inhibitor with respect to pyruvate; its K_i' is also 0.02 m*M*.

DISCUSSION

The K_i' values for Mirex and Kepone indicate that LDH activity is significantly affected by micromolar concentrations of these two pesticides. As a rule, chlorinated pesticides are usually detectable only in small amounts in our surroundings, and are concentrated in the tissues of exposed animals at slightly higher levels (Metcalf et al., 1973; Baker et al., 1972; Mehendale et al., 1973; Kacew and Singhal, 1973; Sell and Davison, 1973; Wiersma et al., 1973). Toxicity studies in higher animals have shown that lethal doses of Mirex are much higher than any reported accumulation levels: 81% mortality was produced in young mallards only after feeding 500 ppm of Mirex for 30 days (Stickel, 1964). Because of this large difference in

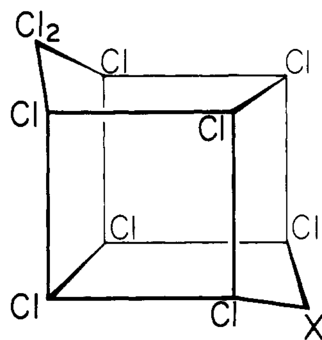

Figure 1. Structure of Mirex and Kepone: Mirex, X is Cl_2; Kepone, X is =O.

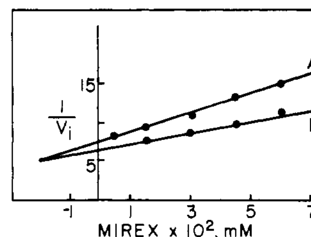

Figure 2. Dixon plot of effect of Mirex on LDH activity. [Mirex] (m*M*) is plotted vs. $1/V_i$, where V_i is initial velocity in millimoles of NADH consumed per minute, and the point of intersection extrapolated to the abscissa is K_i'. Varying concentrations of pesticide were added to cuvettes containing pyruvate (A = 0.66 m*M*, B = 1.3 m*M*), 0.7 mmol of NADH, 10% ethanol, and 0.1 *M* phosphate buffer (pH 7.5) to a volume of 3.0 ml.

availability of Mirex from natural consumption and in the amounts needed for lethal doses in the laboratory, the assumption has been made that there was little potential biochemical hazard to involved species from Mirex accumulation in the environment (Shapely, 1971). However, the data in Table II show that accumulation levels of Mirex in various tissues are of the same order of magnitude as the calculated K_i' values shown in Figures 2 and 3; e.g., goldfish accumulate Mirex in skeletal muscle as high as 232 ppm, and in liver at 1.3 ppm (Van Valin et al., 1968), while 0.02 m*M* (K_i' for Mirex) is approximately 21 ppm.

The inhibition at the active site exhibited by Mirex and Kepone is characteristic of competition by structural analogs of the enzyme's natural substrate. In the case of these inhibitors, no analogous structure to pyruvate is immediately apparent. The question of whether this inhibition occurs in vivo and the reason for the inhibition are currently under investigation by our laboratory. Although the accumulation levels listed in Table I are not lethal, it is possible that partial inhibition of LDH activity (and the production of glycolytic energy by muscle) could cause impairment of muscle function.

If this inhibition holds for the reverse reaction (lactate to pyruvate) catalyzed by the H_4 isozyme in heart muscle, where a constant supply of energy is required, even slight interference with energy production could have a crippling biological effect.

A number of recent studies indicate that these data may represent previously unexplored effects of pesticides

Table I. Toxicity of Mirex to Selected Species

Species	Level of exposure	Effect	Reference
T. pyriformis	0.03 ppb	Reduced growth rate	Cooley et al., 1972
Brown shrimp	0.1–1.0 ppb	40–100% mortality	Lowe et al., 1971
Crayfish	5 ppb for 6 hr	26% mortality at 10 days	Spenser, 1968; Ludke et al., 1971
Goldfish	1 ppm for 224 days	Gill and kidney lesions	Van Valin et al., 1968
Quail	300 ppm for 111 days	12% mortality	Stickel, 1964
Albino rat	10 ppm for 60 days	100% mortality	Ware and Good, 1967
Blue crab	4.4 μg per crab	Paralysis and death	Lowe et al., 1971

Table II. Accumulation of Mirex by Selected Species

Species	Tissue	Accumulated level	Application	Reference
T. pyriformis	Whole cell	50× after 7 days	0.9 μg/l. in seawater	Cooley et al., 1972
Pink shrimp	Hepatopancreas	2.4 ppm (5 weeks)	0.1 ppb in seawater	Lowe et al., 1971
Pink shrimp	Whole body	0.26 ppm (5 weeks)	0.1 ppb in seawater	Lowe et al., 1971
Catfish	Liver	0.023–0.675 ppm	1 year after spray	Collins et al., 1973
Catfish	Adipose	0.105–0.138 ppm	1 year after spray	Collins et al., 1973
Catfish	Skeletal muscle	0.060–0.079 ppm	1 year after spray	Collins et al., 1973
Goldfish	Liver	0–1.350 ppm	1 ppm for 224 days	Van Valin et al., 1968
Goldfish	Muscle	20.8 –232 ppm	1 ppm for 224 days	Van Valin et al., 1968
Quail	Liver	0.013–0.295 ppm	1 year after spray	Baetcke et al., 1973
Quail	Adipose	0.016–3.148 ppm	1 year after spray	Baetcke et al., 1973
Barred owl	Liver	4.072 ppm	1 year after spray	Baetcke et al., 1973
Barred owl	Skeletal muscle	0.934 ppm	1 year after spray	Baetcke et al., 1973
Meadowlark	Heart muscle	1.058–1.931 ppm	1 year after spray	Baetcke et al., 1973
Brown deer	Adipose	0.045–0.305 ppm	1 year after spray	Baetcke et al., 1973
Albino rat	Adipose	27.8% of dose	6 mg/kg orally	Mehendale et al., 1972
Albino rat	Muscle	3.2% of dose	6 mg/kg orally	Mehendale et al., 1973
Blue crab	Whole body	0–6.7 ppm	28 days after exposure to 4.4 μg	Lowe et al., 1971

Table III. Effect of Ethanol on LDH Activity

	K_i'	Type of inhibition
Oxamate	0.04 mM	Competitive
Oxamate + 10% ethanol	0.04 mM	Competitive

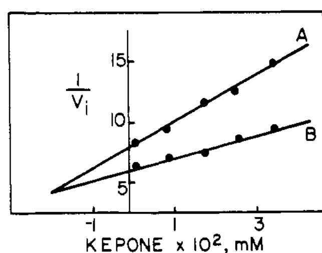

Figure 3. Dixon plot of effect of Kepone on LDH activity. Coordinates and conditions are the same as in Figure 2, except that [pyruvate] for A = 0.33 mM and for B = 0.66 mM.

at the subcellular level. Additional studies by Hendrickson and Bowden (1973) have demonstrated inhibition of lactate dehydrogenase by a number of related compounds, including dieldrin and DDT. Khaikina et al. (1970) have shown that DDT and lindane cause changes in activity of total serum lactate dehydrogenase and disruption in the normal distribution of LDH isozymes. Gertig et al. (1970) demonstrated depression of lactate dehydrogenase activity by aldrin, dieldrin, and lindane. Similarly, glutamate dehydrogenase is competitively inhibited by Kepone (Freedland and McFarland, 1965). It is possible that inhibitory effects of pesticides (Mirex, DDT, dieldrin, etc.) on selected enzymes, if occurring in vivo, could be widespread when one considers the total amounts of biologically stable pesticides used in recent years. The buildup of Mirex and other stable pesticides by passage upward in food chains (Lowe et al., 1971) is a potentially serious problem, and the future effect of these accumulated pesticides on enzymatic function and the resultant harm to susceptible species is a relatively new consideration in both pesticide research and in enzymological studies. However, from the evidence which has been presented, it is likely to become a point of growing interest for workers in both fields.

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