Weisgerber et al. (1975) also identified photoaldrindiol, one of the products from the soil treated with [^{14}C]photodieldrin. Khan et al. (1970) showed that the antioxident sesamex affects the toxicity and metabolism of photodieldrin in insects. The formation of ketone and photodieldrindiol in the present study shows the involvement of oxidative and hydroxylating detoxication mechanisms. That both of these types of enzyme systems are involved in the metabolism of dieldrin in insects was also shown by other authors (Oonithan and Miskus, 1964; Tomlin, 1968; Sellers and Guthrie, 1972; Nelson and Matsumura, 1973). It was also observed that about 1% of the applied photodieldrin was excreted unchanged in feces of house flies. Similar excretion of unchanged dieldrin through feces has been reported in flies (Sellers and Guthrie, 1972) and locusts (Cohen and Smith, 1961). However, no dieldrin was detected in the feces of cockroaches treated with [^{14}C]dieldrin (Nelson and Matsumura, 1973).

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LITERATURE CITED

Cohen, A. J., Smith, J. N., *Nature (London)* **189**, 600 (1961).
Dorough, H. W., Cardona, R. A., Lolmaugh, S. E., *Drug Metab. Disp.* **2**, 129 (1974).
Edwards, C. A., "Persistent Pesticides in the Environment", CRC Press, Cleveland, Ohio, 1970, 170 pp.
Feil, V. J., Hedde, R. D., Zaylskie, R. G., Zachrison, C. H., *J. Agric. Food Chem.* **18**, 120 (1970).
Jones, L. R., Riddick, J. A., *Anal. Chem.* **24**, 569 (1952).
Khan, M. A. Q., Khan, H. M., Sutherland, D. J., "Survival in Toxic Environments", Khan, M. A. Q., Bederka, J. P., Ed., Academic Press, New York, N.Y., 1974, p 333.
Khan, M. A. Q., Rosen, D. J., Sutherland, D. J., *Science* **164**, 318 (1969).
Khan, M. A. Q., Sutherland, D. J., Rosen, D. J., Carey, W. F., *J. Econ. Entomol.* **63**, 470 (1970).
Klein, W., Kaul, R., Parlor, Z., Zimmer, M., Korte, F., *Tetrahedron Lett.*, 3197 (1969).
Matsumura, F., "Survival in Toxic Environments", Khan, M. A. Q., Bederka, J. P., Ed., Academic Press, New York, N.Y., 1974, p 129.
Menzie, C. M., "Metabolism of Pesticides: An Update", 1974, 486 pp.
Nelson, J. O., Matsumura, F., *Arch. Environ. Contam. Toxicol.* **1**, 224 (1973).
Oonithan, E. S., Miskus, R., *J. Econ. Entomol.* **57**, 425 (1964).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **22**, 910 (1974).
Reddy, G., Khan, M. A. Q., *J. Agric. Food Chem.* **23**, 861 (1975).
Roburn, J., *Chem. Ind. (London)* **38**, 1555 (1963).
Rosen, J. D., Sutherland, D. J., Lipton, G. R., *Bull. Environ. Contam. Toxicol.* **1**, 133 (1966).
Sellers, L. G., Guthrie, F. E., *J. Econ. Entomol.* **65**, 378 (1972).
Tomlin, A. D., *J. Econ. Entomol.* **61**, 855 (1968).
Weisgerber, I., Bieniek, D., Kohli, J., Klein, W., *J. Agric. Food Chem.* **23**, 873 (1975).

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In Vitro Inhibition of Lactate Dehydrogenases by Kepone

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Lactate dehydrogenase catalyzed reactions, both the oxidation of lactate and the reduction of pyruvate, are effectively inhibited by low concentrations of the polychlorinated hydrocarbon pesticide, Kepone. The previous report [Hendrickson, C. M., and Bowden, J. A., *J. Agric. Food Chem.* **23**, 407 (1975)] of Kepone inhibition of rabbit muscle lactate dehydrogenase catalyzed reduction of pyruvate was confirmed; however, a lower K_i value and a different type of inhibition were observed in the present study. The extension of these studies to the investigation of homologous isozymes of lactate dehydrogenase revealed Kepone to be an effective inhibitor of several M_4 isozymes while showing no inhibition (within limits of solubility) of several H_4 isozymes. When detailed studies of the Kepone inhibition were made, mixed inhibition with respect to both substrates and noncompetitive inhibition with respect to the pyridine nucleotide coenzymes were observed. Changes in pH, solvents for Kepone solubilization, and types of buffer had little effect on the Kepone inhibition. The inhibition was observed through dilution experiments to be a fully reversible type of inhibition and no time-dependent inactivation of lactate dehydrogenases was observed in these studies. Studies were carried out with homologous isozymes from rabbit, beef, pig, and chicken.

The polychlorinated hydrocarbon pesticide, Kepone (decachloro-1,3,4-metheno-2*H*-cyclobuta[6*d*]pentalen-2-one), has been observed in in vitro studies to inhibit at least two pyridine nucleotide dependent dehydrogenases, rabbit muscle lactate dehydrogenase (EC 1.1.1.27) (Hendrickson and Bowden, 1975) and beef liver glutamate dehydrogenase (EC 1.4.1.3) (Freedland and McFarland, 1965). In the latter study, some specificity of the glutamate dehydrogenase inhibition by Kepone was suggested by the lack of effective inhibition by other chlorinated hydrocarbons. The inhibition of rabbit muscle lactate dehydrogenase by Kepone was reported to be competitive with respect to pyruvate although no structural analogy to this substrate was apparent. In these studies (Hendrickson and Bowden, 1975), the closely related, fully

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chlorinated pesticide, Mirex, was also demonstrated to be a substrate-competitive inhibitor of the lactate dehydrogenase. Hendrickson and Bowden (1975) suggested a possible physiological importance for the lactate dehydrogenase inhibition by Mirex and Kepone since the very low K_i values (20 μM) for these inhibitory processes can be related to concentrations of these compounds readily attainable through tissue accumulation.

Many questions remain concerning the inhibition of lactate dehydrogenase by polychlorinated hydrocarbons. The present study is an attempt to provide further information about the inhibition by these pesticides of the lactate dehydrogenase catalyzed conversion of lactate to pyruvate. The present studies suggest that lactate dehydrogenase M_4 isozymes may be more sensitive to Kepone inhibition than previously reported and that lactate dehydrogenase H_4 isozymes are relatively unaffected by low concentrations of this compound.

MATERIALS AND METHODS

Crystalline rabbit muscle lactate dehydrogenase (LDH), NAD^+, NADH, L(+)-lactic acid, lithium salt, human erythrocyte LDH, and beef LDH, H_4 isozyme, were obtained from Sigma Chemical Company. Pig LDH, M_4 isozyme, pig LDH, H_4 isozyme, rabbit LDH, M_4 isozyme, rabbit LDH, H_4 isozyme, and beef LDH, M_4 isozyme, were purchased from the Boehringer Mannheim Corp. Samples of chicken LDH, M_4 and H_4 isozymes, were kindly supplied by Dr. William S. Allison of the University of California. Analytical samples of Kepone and Mirex were supplied by the Environmental Protection Agency, Research Triangle, N.C.

The reactions catalyzed by the variety of LDH preparations employed were studied under various conditions in 3-ml reaction mixtures at 25 °C. In the conversion of lactate to pyruvate, the formation of NADH was followed spectrophotometrically at 340 nm. The disappearance of NADH was followed for studies of the conversion of pyruvate to lactate. Either 10% ethanol or 10% dimethylformamide was used in these reaction mixtures to solubilize the polychlorinated hydrocarbons studied as inhibitors. Spectrophotometric measurements were made on a Beckman Acta M VI recording spectrophotometer. pH measurements were made with a Radiometer PHM 52 pH meter and a type 202 C glass electrode.

RESULTS

The lactate dehydrogenase catalyzed oxidation of lactate to pyruvate was studied at 25 °C in 3-ml reaction mixtures containing 50 mM Tris-HCl buffer (pH 8.1), 10% ethanol, 2 mM NAD^+, and concentrations of lithium lactate varying from 3.67 to 10 mM. Reactions were initiated by the addition of 0.39 IUB unit of rabbit lactate dehydrogenase, M_4 isozyme. Inhibition of this reaction by 4.5 and 8.5 μM Kepone was observed and the data obtained, plotted according to Lineweaver and Burk (1934), are shown in Figure 1. The inhibition was indicated to be mixed with respect to lactate and a K_i of 9.86 μM was calculated from these data. Inhibition of this reaction by Kepone was also studied at two constant concentrations of lactate (2.93 and 5.86 mM) and varying the Kepone concentration from 0 to 7.67 μM. Data obtained in this experiment were plotted according to Dixon (1953), and are shown in Figure 2. The relationship obtained is consistent with mixed inhibition and a K_i value of 6.7 μM was calculated from these data.

Kepone inhibition of the LDH-catalyzed oxidation of lactate was also studied as a function of varying coenzyme concentration. These reactions were carried out in 3-ml reaction mixtures containing 50 mM Tris-HCl buffer (pH 8.1), 10% ethanol, 75 mM lactate, and NAD^+ varying from

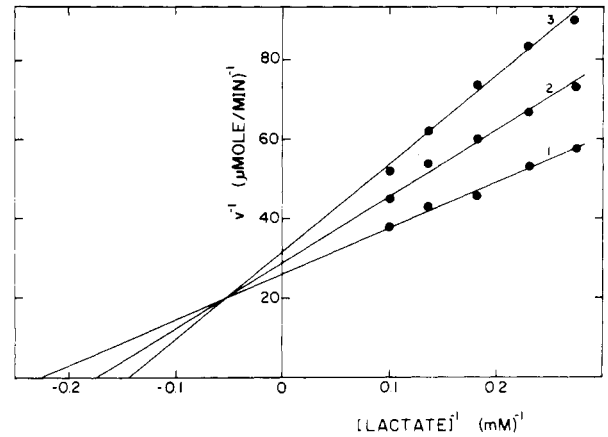

Figure 1. Inhibition of rabbit muscle lactate dehydrogenase (M_4 isozyme) by Kepone. Lactate concentration was varied from 3.67 to 10.0 mM. Reaction mixtures contained 0.05 M Tris-HCl buffer (pH 8.1), 2.0 mM NAD^+, 0.38 unit of rabbit muscle lactate dehydrogenase (M_4 isozyme), lactate as indicated, and inhibitor at concentrations of 0 (line 1), 4.5 μM (line 2), and 9.0 μM (line 3), in a total volume of 3.0 ml.

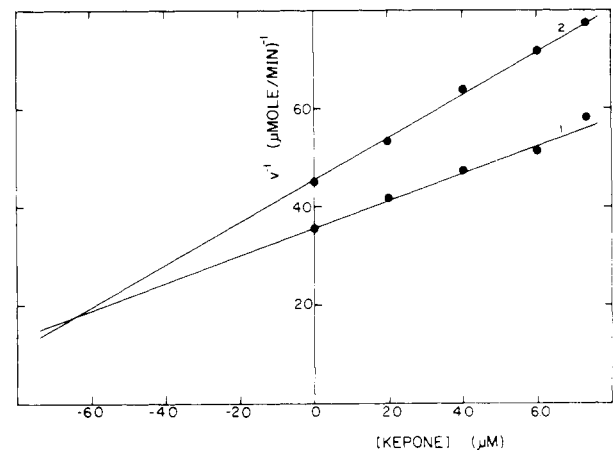

Figure 2. Inhibition of rabbit lactate dehydrogenase (M_4 isozyme) as a function of the concentration of Kepone. Reaction mixtures contained 0.05 M Tris-HCl buffer (pH 8.1), 2.0 mM NAD^+, 0.38 unit of rabbit muscle lactate dehydrogenase (M_4 isozyme), two lactate concentrations, 2.4 and 4.8 mM, and inhibitor varying from 0 to 7.67 μM in a total volume of 3.0 ml.

90.7 to 408 μM. The data, plotted according to Lineweaver and Burk (1934) (Figure 3), show a noncompetitive inhibition by Kepone with respect to NAD^+ with a K_i value of 9.28 μM. The K_i value obtained for Kepone in these studies was in good agreement with those obtained when lactate was used as the variable substrate. It should also be noted that when the experiments described in Figures 1–3 were performed using rabbit muscle LDH in place of the homologous rabbit M_4 isozyme, the properties of the Kepone inhibition were essentially the same, showing mixed inhibition with respect to lactate, noncompetitive inhibition with respect to NAD^+, and K_i values from 6 to 9 μM.

Experiments identical with those described in Figures 1–3 were carried out using rabbit LDH homologous H_4 isozyme. Regardless of whether the concentration of NAD^+ or lactate was varied, no inhibition of the H_4 isozyme-catalyzed oxidation of lactate was observed with concentrations of Kepone up to 33 μM. The low solubility of Kepone in the LDH reaction mixtures precluded the study of higher concentrations of this pesticide in the 10%

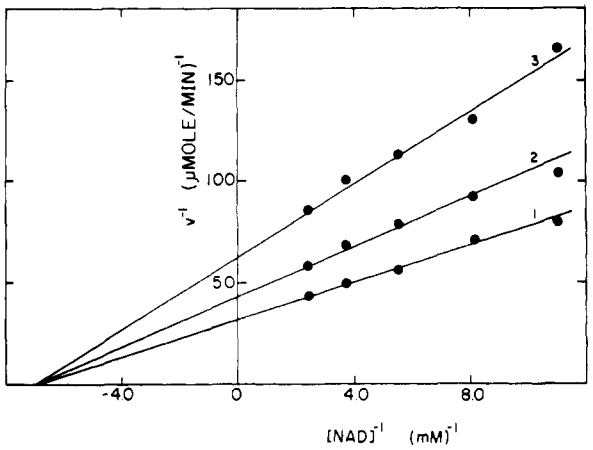

Figure 3. Noncompetitive inhibition of rabbit muscle lactate dehydrogenase (M_4 isozyme) by Kepone. NAD^+ concentration was varied from 90.7 to 408 μM. Reaction mixtures contained 0.05 M Tris-HCl buffer (pH 8.1), 0.075 M lactate, 0.38 unit of rabbit muscle lactate dehydrogenase (M_4 isozyme), NAD^+ as indicated, and inhibitor at concentrations of 0 (line 1), 6.9 μM (line 2), and 13.8 μM (line 3) in a total volume of 3.0 ml.

Table I. Kepone Inhibition of LDH Isozymes at pH 8.1

Enzyme	Inhibition	K_i, μM
Rabbit muscle (isozyme mixture)	Mixed (lactate)	6.0
	Noncompetitive (NAD^+)	9.0
Rabbit LDH M_4	Mixed (lactate)	8.43
	Noncompetitive (NAD^+)	9.28
Rabbit LDH H_4	None up to 80 μM Kepone	
Beef LDH M_4	Mixed (lactate)	35
	Noncompetitive (NAD^+)	30
Beef LDH H_4	None up to 80 μM Kepone	

ethanol reaction mixtures. However, if 10% dimethylformamide is used in place of the 10% ethanol, higher concentrations of Kepone were obtained and no inhibition of the H_4 isozyme was observed at 80 μM Kepone. When 10% dimethylformamide was used in place of 10% ethanol in reactions catalyzed by rabbit M_4 isozyme, values for the K_m for lactate and K_i for Kepone inhibition comparable to those obtained in the ethanol-containing reaction mixtures were observed. The absence of inhibition of the H_4 isozyme in the Kepone concentration range studied was not restricted to rabbit lactate dehydrogenases. In studies of beef LDH homologous isozymes, the H_4 isozyme was also not inhibited by concentrations of Kepone up to 80 μM. As in the earlier experiments, these reactions were studied by varying both substrate and coenzyme. The beef LDH M_4 isozyme, however, behaved the same as the rabbit LDH M_4 isozyme in that the beef LDH M_4 isozyme-catalyzed oxidation of lactate was inhibited by Kepone, the inhibition was mixed with respect to lactate and noncompetitive with respect to NAD^+, and the average K_i value was calculated to be 32.5 μM. For comparison, the properties of Kepone inhibition of various lactate dehydrogenases are listed in Table I. Kepone inhibition was also studied with the homologous LDH isozymes of chicken and pig. Again, the pig H_4 and chicken H_4 isozymes were not inhibited by Kepone at concentrations up to 80 μM. The pig M_4 and chicken M_4 isozymes were inhibited by Kepone under conditions of saturating coenzyme concentrations and varying lactate concentrations. Fifty percent inhibition of pig M_4 isozyme occurred at approximately 10 μM and approximately 40 μM in the case of chicken M_4 isozyme. A precise measurement of K_i values and type of inhibition were complicated in these cases by the fact that the Kepone inhibition showed a nonlinear response to Kepone concentration. Human erythrocyte LDH, which has an isozymic distribution of 5:1 H_4–H_3M, was likewise not inhibited by Kepone at concentrations up to 80 μM. As in the case of the other H_4 isozymes studied, this LDH preparation was investigated for Kepone inhibition under conditions of saturating coenzyme, varying lactate and saturating lactate, varying coenzyme with no inhibition at 80 μM Kepone observed in either case.

Table II. Substrate Effects on Kepone Inhibition of Rabbit LDH M_4 Isozyme at pH 7.5

Reaction	Inhibition	K_i, μM	Comments[a]
Lactate oxidation	Mixed (lactate)	10.1	50 mM Tris-HCl buffer
Lactate oxidation	Mixed (lactate)	10.2	33 mM potassium phosphate buffer
Pyruvate reduction	Mixed (pyruvate)	3.9	50 mM Tris-HCl buffer
Pyruvate reduction	Mixed (pyruvate)	7.25	33 mM potassium phosphate buffer

[a] Reaction mixtures contained 10% ethanol.

In order to compare results with previously reported polychlorinated hydrocarbon inhibition of lactate dehydrogenase catalyzed reactions, both lactate oxidation and pyruvate reduction as catalyzed by rabbit LDH M_4 isozyme were studied at pH 7.5 in either 50 mM Tris-HCl buffer or 33 mM potassium phosphate buffer. Kepone inhibition was investigated in the Kepone concentration range of 10–15 μM. In studying lactate oxidation, the 3-ml reaction mixtures contained 2 mM NAD^+, 10% ethanol, and lactate varying from 3.67 to 10 mM. In studying pyruvate reduction, the 3-ml reaction mixtures contained 0.22 mM NADH, 10% ethanol, and pyruvate varying from 0.15 to 0.77 mM. The data obtained in these studies were plotted according to Lineweaver and Burk (1934) and, as shown in Table II, the inhibition by Kepone was mixed inhibition with respect to both pyruvate and lactate and K_i values for this inhibition were somewhat lower when measured as a function of pyruvate reduction.

Since most reported studies of Kepone inhibition of dehydrogenases interpret the Kepone-induced loss of catalytic activity as a reversible inhibitory process, it was felt necessary to investigate the reversibility of the Kepone inhibition of rabbit LDH M_4 isozyme. Rabbit M_4 isozyme (1.3 IUB units) was incubated at 25 °C in 3-ml reaction mixtures containing 50 mM Tris-HCl buffer (pH 8.1), 30 μM Kepone, and 10% ethanol. At timed intervals, 0.6-ml aliquots of this incubation mixture were transferred to a LDH assay mixture containing 50 mM Tris-HCl buffer (pH 8.1), 2 mM NAD^+, 4 mM lithium lactate, and 10% ethanol, and initial velocities were determined spectrophotometrically. The initial velocity obtained in this fashion at zero time of incubation indicated 40% inhibition when compared to initial velocities obtained for aliquots taken from incubation mixtures containing no Kepone. No further inhibition was observed as a function of time of incubation of the enzyme with the Kepone up to 2 h of incubation. The 40% inhibition observed at zero time of incubation could also be obtained by adding 6 μM Kepone directly to the assay mixture. Therefore, any inhibition caused by mixing the enzyme with 30 μM Kepone was reversed by dilution into the assay mixture.

DISCUSSION

The lactate dehydrogenase catalyzed oxidation of lactate to pyruvate was observed to be effectively inhibited at pH

8.1 by low concentrations of Kepone (Table I) when either rabbit muscle LDH isozyme mixture or the specific rabbit LDH M_4 isozyme was used as the enzyme source. Properties of the Kepone inhibition were essentially the same for these two LDH preparations, in that K_i values were very similar and the inhibition was mixed with respect to lactate (Figure 1) and noncompetitive with respect to the coenzyme, NAD^+ (Figure 3). This similarity in properties is consistent with the predominant M-subunit composition of the rabbit muscle LDH preparation. The observed mixed inhibition with respect to substrate can be interpreted to mean that the binding of the inhibitor interferes with both the catalytic process and the specific binding of substrate (Webb, 1963). The apparent lack of structural analogy of Kepone to the substrates for LDH would suggest that the binding of Kepone is not specifically site directed but rather provides an enzyme–inhibitor complex that can prevent the proper functioning of catalytic groups and concomitantly reduce the effectiveness of substrate binding. This inhibition was observed to be fully reversible and not to involve any time-dependent inactivation process. Incubation of rabbit LDH M_4 isozyme with 30 μM Kepone followed by dilution into an assay mixture where the Kepone concentration would be 6 μM gave inhibition corresponding to that expected for 6 μM Kepone. Any effect of the higher concentration of Kepone was reversed by the dilution process.

Although Kepone was observed to be an effective reversible inhibitor of the lactate oxidation catalyzed by rabbit LDH M_4 isozyme, no inhibition by Kepone of the identical reaction catalyzed by rabbit LDH H_4 isozyme was observed up to concentrations of 33 μM Kepone. Actually, by substituting 10% dimethylformamide for 10% ethanol in the reaction mixtures, a change that had no effect on the properties of the enzyme-catalyzed lactate oxidation, concentrations as high as 80 μM Kepone were shown not to inhibit the H_4 isozyme. This is an important observation since the oxidation of lactate catalyzed by the H_4 isozyme of heart muscle is an important factor in energy production for this tissue. Inhibition of the H_4 isozyme could have drastic physiological consequences. Whether or not higher concentrations of Kepone can affect the H_4 isozyme is not known since such studies have been limited by the solubility of Kepone in the solvent mixtures used.

The difference between homologous isozymes in the sensitivity to Kepone inhibition is not limited to the rabbit enzymes. Beef LDH M_4 isozyme was observed to be inhibited by Kepone, showing the same type of inhibition with respect to lactate and NAD^+ and a slightly higher K_i value (Table I). Beef LDH H_4 isozyme was again insensitive to Kepone inhibition at a concentration of 80 μM (Table I). Furthermore, in studies of the homologous LDH isozymes of chicken and pig, the M_4 isozymes were inhibited by Kepone while the H_4 isozymes were not. Considering the nonpolar nature of Kepone and the effectiveness of the reversible inhibition of LDH M_4 isozymes by this compound, it can be suggested that the enzyme–inhibitor complexes involved are stabilized by nonpolar interactions. The lower sensitivity of LDH H_4 isozymes to Kepone inhibition may simply reflect the inability of these isozymes to provide as effective stabilizing nonpolar interactions nearby catalytic sites. Evidence for such a difference has been presented in studies of the *N*-alkylmaleimide inactivation of LDH isozymes (Anderson et al., 1974), in which chain-length effects on the rates of inactivation were much more pronounced with the M_4 isozyme.

It was of interest that the properties of the Kepone inhibition of rabbit LDH M_4-catalyzed reactions did not change when dimethylformamide was substituted for ethanol in stock solutions and assay mixtures. This would indicate that hemiketal formation with ethanol is not a factor in the enzyme inhibition by Kepone. Hemiketal formation was indicated in earlier chemical characterization studies of Kepone (McBee et al., 1956).

Inhibition of rabbit LDH M_4 isozyme by Kepone was also studied at pH 7.5 to provide data at a more physiological pH and for comparison to data reported previously concerning Kepone inhibition of rabbit LDH-catalyzed reduction of pyruvate (Hendrickson and Bowden, 1975). As shown in Table II, Kepone inhibition of lactate oxidation at pH 7.5 is essentially identical with that observed at pH 8.1. Kepone inhibition at pH 7.5 appears to be more effective when studied as a function of pyruvate reduction and the K_i values obtained were somewhat lower than those reported previously (Hendrickson and Bowden, 1975). As pointed out by these workers, the Kepone inhibition occurs at concentrations comparable to or, as indicated in the present study, below those observed to accumulate in various tissues.

The present study indicates that Kepone effectively inhibits the M_4 isozymes of lactate dehydrogenases by the reversible formation of enzyme–inhibitor complexes stabilized by nonpolar interactions. The binding of Kepone to the rabbit LDH M_4 isozyme does not exclude the binding of the coenzyme as witnessed by the lack of a competitive relationship in the simultaneous binding of NAD^+ and the pesticide. The binding of Kepone to rabbit LDH M_4 isozyme as indicated by the mixed type of inhibition does, however, decrease the substrate binding while interfering with the catalytic process. Preliminary studies indicate that the pesticide Mirex behaves essentially in the same fashion, inhibiting LDH M_4 isozymes but not H_4 isozymes, and, as in the case of Kepone, the inhibition observed was mixed with respect to substrates.

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LITERATURE CITED

Anderson, B. M., Vercellotti, S. V., Fisher, T. L., *Biochim. Biophys. Acta* **350**, 135 (1974).
Dixon, M., *Biochem. J.* **55**, 170 (1953).
Freedland, R. A., McFarland, L. Z., *Life Sci.* **4**, 1735 (1965).
Hendrickson, C. M., Bowden, J. A., *J. Agric. Food Chem.* **23**, 407 (1975).
Lineweaver, H., Burk, D., *J. Am. Chem. Soc.* **56**, 658 (1934).
McBee, E. T., Roberts, C. W., Idol, J. D., Jr., Earle, R. H., Jr., *J. Am. Chem. Soc.* **78**, 1511 (1956).
Webb, J. L., "Enzymes and Metabolic Inhibitors", Academic Press, New York, N.Y., 1963, p 160.

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