Suzuki, K., Goto, S., Kashiwa, T., *Bunseki Kagaku* 17(10), 1279 (1968).
Thompson, J. A., Markey, S. P., *Anal. Chem.* 47, 1313 (1975).
Thompson, J. F., Ed., "Analysis of Pesticide Residues in Human and Environmental Samples", Section 6, Revised Dec 1974.
Van Wazer, J. R., Ed., "Phosphorus and Its Compounds", Interscience, New York, N.Y., 1961, p 1930.
Walker, W. W., Stojanovic, B. J., *J. Environ. Qual.* 2, 474 (1973).
Wolfenden, R., Spence, G., *Biochim. Biophys. Acta* 132, 296 (1967).
Yu, C. C., Sanborn, J. R., *Bull. Environ. Contam. Toxicol.* 13, 543 (1975).
Zech, R., Wigand, K. D., *Experientia* 31, 157 (1975).
Zuckerman, B. M., Deubert, K., Mackiewicz, M., Gunner, H., *Plant Soil* 33, 273 (1970).

Received for review August 29, 1975. Accepted December 8, 1975. This research was supported in part by National Institutes of Health Trainee Grant No. ES00125, NIEHS.

In Vitro Inhibition of Lactate Dehydrogenase by Insecticidal Polychlorinated Hydrocarbons. I. Mechanism of Inhibition: Possible Association of Reduced Nicotinamide Adenine Dinucleotide with Mirex

Constance M. Hendrickson and Joe A. Bowden*

In order to aid in the clarification of the inhibitory mechanism operating in vitro on NADH-dependent dehydrogenases, we determined the effect of dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalene (Mirex) on the activity of crystalline rabbit muscle lactate dehydrogenase (EC 1.1.1.27, M_4 isozyme) at 340 nm. Mirex competitively inhibited with respect to both pyruvate and NADH (K_i values are 0.02 and 0.03 mM, respectively). A time-dependent association of Mirex with NADH was also observed as were changes in both uv and CD spectra. Based on the mechanism of lactate dehydrogenase (Holbrook, J. J., Gutfreund, H., *FEBS Lett.* 31, 157 (1973)), we have postulated a mechanism for in vitro inhibition. This mechanism involves formation of a weak association complex between Mirex and NADH, and possibly some binding of Mirex with the enzyme molecule itself subsequent to formation of the Mirex–NADH–enzyme complex. The probable nature of the complex and implications of these findings are discussed.

Inhibition of lactate dehydrogenase and other NADH- or FADH-dependent enzymes by high molecular weight organochlorines possessing insecticidal activity has been observed by a number of groups (Abston and Yarbrough, 1974; Byczkowski, 1973; Gertig et al., 1970; Hendrickson and Bowden, 1973, 1975; McCorkle and Yarbrough, 1974; Freedland and McFarland, 1965). These inhibitions are of two types: in vitro inhibition of the enzyme itself (in cell-free systems) and in vivo inhibition, where enzyme activity is lowered after ingestion or injection of the compound. For example, the in vitro inhibition of lactate dehydrogenase by a number of chlorinated compounds appears to be the result of some direct (but unknown) effect on the enzyme molecule itself (Hendrickson and Bowden, 1973; Gertig et al., 1970). It is not known whether both types of inhibition are the result of the in vitro direct enzymatic effect, or whether separate reactions cause each type of inhibition. Even if enzymatic inhibition is not wholly responsible for the metabolic alterations observed in vivo (Byczkowski, 1973), it is of interest to study the mechanism by which this effect occurs, in order to delineate and clarify the various effects of these compounds on living systems at the cellular and subcellular levels, as well as on a gross physiological level. In a previous paper (Hendrickson and Bowden, 1975), we discussed the implications of inhibition of enzymatic activity by pesticides present at accumulation levels in affected species.

The compound under consideration, dodecachlorooctahydro-1,3,5-metheno-2*H*-cyclobuta[*cd*]pentalene (Mirex, shown in Figure 1) has been implicated in several instances of both in vitro and in vivo inhibition; among these are direct enzymatic inhibition of succinate dehydrogenase (McCorkle and Yarbrough, 1974) and of lactate dehydrogenase (Hendrickson and Bowden, 1973). In this paper, we have attempted to clarify the manner in which crystalline rabbit muscle lactate dehydrogenase (EC 1.1.1.27; M_4 isozyme) is inhibited by Mirex and have postulated a mechanism for this inhibition.

Department of Biochemistry, Louisiana State University, Baton Rouge, Louisiana 70803.

EXPERIMENTAL PROCEDURE

Materials. Crystalline rabbit muscle lactate dehydrogenase, sodium pyruvate, and NADH were obtained from Sigma Chemical Co. (St. Louis, Mo.). Analytical grade samples of Mirex (crystalline, 99+% purity) were supplied as a gift by Allied Chemical Corporation (Agricultural Division, Jackson, Miss.). All other compounds, solvents, and inhibitors were reagent grade, and water was carbon filtered and deionized.

Methods. *Determination of Initial Velocity.* Lactate dehydrogenase activity was assayed by following the rate of conversion of pyruvate to lactate in the presence of NADH by observing a change in absorbance of NADH at 340 nm with a recording Beckman DB spectrophotometer at 25 °C. Pyruvate and NADH solutions were prepared in 0.1 M potassium phosphate buffer at pH 7.5. NADH solutions were freshly prepared every 4 h. Mirex solutions were made up in 95% ethanol. Lactate dehydrogenase was diluted in 2.1 M ammonium sulfate (pH 6.0–6.1) to a final concentration of 10 IUB units per ml. Pyruvate was held constant at 1.3 mM and NADH varied from 0.005 to 0.36 mM, or NADH was held constant at 0.18 mM and pyruvate varied from 0.1 to 1.0 mM. NADH, pyruvate,

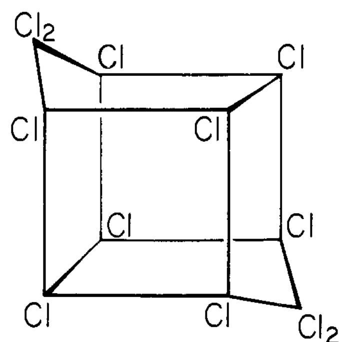

Figure 1. Structure of Mirex.

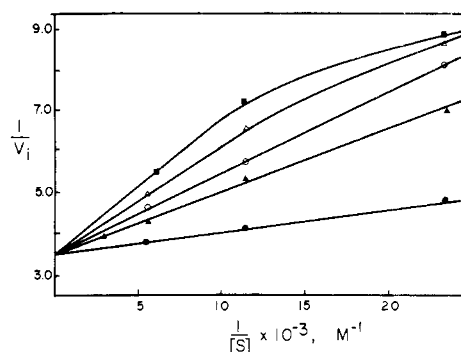

Figure 2. Lineweaver-Burk plot of the effect of Mirex on lactate dehydrogenase with respect to NADH. [S] is the concentration of NADH; initial velocity (V_i) is expressed in millimoles of NADH consumed per minute. Experimental conditions: [pyruvate] = 1.3 mM; [Mirex] = 0 (●); 0.015 mM (▲); 0.023 mM (○); 0.031 mM (△); 0.047 mM (■). Reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH 7.5).

Mirex, ethanol to 10% (0.3 ml total), and buffer to a total volume of 2.9 ml were mixed in a cuvet (1.0 cm) and allowed to equilibrate 5–10 min (see Results section). The reaction was started by adding 1 IUB unit of lactate dehydrogenase (0.1 ml) to obtain a final reaction volume of 3.0 ml. The initial velocity of the reaction was measured in optical density (OD) units per minute and converted to millimoles per liter of NADH consumed per minute (using the value 6.2×10^3 OD per mol of NADH). Experimental data were then plotted according to the methods of Dixon (1953) and of Lineweaver and Burk (1934) to determine the apparent inhibition constant K_i'. Two methods were used in order to clearly determine the type of inhibition.

UV Absorbance and *CD Studies.* A Beckman DB spectrophotometer and a Jasco J20 spectropolarimeter were used to take the uv absorbance spectra and circular dichroism (CD) spectra of NADH in the presence and absence of Mirex and ethanol.

Time-Dependence Studies. When reaction was started with pyruvate, NADH, or enzyme, reaction cuvets were prepared as described for the activity assay. Twelve reactions were started at 1-min intervals. Data were plotted as length of equilibration period of unreacted mixture vs. initial velocity. When NADH and Mirex in buffer–ethanol were used to start the reaction, cuvets were prepared as described but without ethanol. Reactions were started with aliquots removed from the NADH–Mirex solution at 1-min intervals, and the "length of equilibration" plotted as the abscissa was the age of the NADH–Mirex solution.

RESULTS

Effect of Ethanol. The reaction mixtures were adjusted to 10% ethanol in order to solubilize Mirex in the assay system, since this compound is very insoluble in aqueous systems (Spencer, 1968). To determine what effect this level of ethanol had on the active site of lactate dehydrogenase, assays were run in the presence of oxamate, a well-studied competitive inhibitor of lactate dehydrogenase (Novoa et al., 1959) and a structural analogue of pyruvate. The K_i' for oxamate was determined in the presence and absence of ethanol. In both cases, inhibition was competitive and K_i' was unchanged at 0.04 mM, indicating that ethanol does not interfere with the active site of the enzyme. In addition ethanol has no effect on the K_m of pyruvate at the 10% level.

When lactate dehydrogenase and ethanol were incubated together and the reaction was started with pyruvate or NADH, a plot of initial velocity vs. length of incubation period was linear; however, activity of the enzyme with ethanol dropped as low as 30% of the activity without ethanol. This effect of ethanol on lactate dehydrogenase has been reported by George et al. (1969). When reaction was started by addition of enzyme to a mixture containing all other components, or when ethanol was added along with NADH or pyruvate to start the reaction, inhibition was constant at approximately 15%; therefore, at this level of ethanol the degree of activity losses are at a minimum. The initial velocity in the presence of ethanol is corrected for ethanol at this level of inhibition. This assay system is similar to the solvent used by McCorkle and Yarbrough (1974) for the assay of succinate dehydrogenase in the presence of Mirex and solvent.

In the case of addition of enzyme alone, the plot of equilibration period vs. velocity was nonlinear in the presence of ethanol but with a constant and reproducible profile which was unaffected by the addition of Mirex, indicating that Mirex and ethanol inhibitions do not interfere with one another, Hendrickson and Bowden (1975). Addition of NADH or pyruvate with ethanol to start the reaction yields a linear plot. In both cases, the velocities after 5–7 min were identical. In the presence of inhibitor, velocity decreased proportionally as concentration of the inhibitor increased and after the initial equilibration period, velocity was the same by both methods. For purposes of simplicity, reactions for initial velocity determinations were started by addition of enzyme alone.

Inhibition of Lactate Dehydrogenase by Mirex. K_i' for Mirex with respect to pyruvate was 0.02 mM (Hendrickson and Bowden, 1975) and with respect to NADH was 0.03 mM. In both cases inhibition was competitive. Because lactate dehydrogenase is a tetramer and considering the structural nature of Mirex, it would be more credible than otherwise to expect some type of secondary kinetic effects in this system; this was indeed the case. Figure 2 shows that as Mirex concentration increases and as substrate concentration drops, plots are no longer linear, indicating some secondary effects of Mirex at levels above 0.047 mM on the enzyme.

Time-Dependent Association of NADH with Mirex. The results of this experiment are shown in Figure 3. When Mirex and NADH are incubated together with buffer and ethanol, and this mixture is used to start the reaction, initial velocity decreases with the length of the incubation period, dropping to a minimum after 5–7 min. This indicates a time-dependent association of NADH and Mirex, such as the formation of some type of complex.

Effect of Mirex on Uv Absorption Spectra of NADH. In Figure 4, it can be seen that at 340 nm there is no change in the character of NADH absorbance in the presence of ethanol or Mirex and ethanol. However, at 260 nm, although no shift was apparent, NADH absorbance was quenched in the presence of Mirex; i.e., Mirex and

Figure 3. Time-dependent association of NADH with Mirex. Experimental conditions: [pyruvate] = 1.3 mM; [NADH] = 0.18 mM; [Mirex] = 0.01 mM. Reactions were carried out in 0.1 M phosphate buffer (pH 7.5) at 25 °C. Initial velocity (V_i) is expressed in millimoles of NADH consumed per minute.

Figure 4. Effect of Mirex on uv absorption of NADH. Experimental conditions: [Mirex] = 0.03 mM; [NADH] = 0.18 mM; [ethanol] = 10% (v/v), in 0.1 M phosphate buffer (pH 7.5). Mixtures were allowed to equilibrate 5-10 min: NADH ± ethanol (—); Mirex (···); NADH + Mirex (-·-·).

NADH absorbances are not additive, indicating association of the two molecules.

Effect of Mirex on CD Spectra of NADH. In the presence of 20% ethanol, some quenching occurred at 260 nm. Addition of Mirex caused slight additional quenching, but more significantly, caused shifting at this point to a lower wavelength, shown in Figure 5.

DISCUSSION

The reaction in which lactate dehydrogenase catalyzes the reduction of pyruvate to lactate has been studied in depth, and its mechanism, shown in eq 1, has been de-

$$\text{enzyme} \begin{cases} \overset{\pm\ \text{NADH} + \text{H}^+}{\nearrow} \text{enzyme-NADH} + \text{H}^+ \xrightleftharpoons{\pm\ \text{pyruvate}} \text{enzyme-}\overset{\text{H}^+}{\text{NADH}} \,\|\, \text{pyruvate} \\ \overset{\pm\ \text{NAD}^+}{\searrow} \text{enzyme-NAD}^+ \xrightleftharpoons{\pm\ \text{lactate}} \text{enzyme-NAD}^+ \,\|\, \text{lactate} \end{cases} \quad (1)$$

scribed in detail (Holbrook and Gutfreund, 1973). This mechanism forms the basis for our speculations concerning

Figure 5. Effect of Mirex on CD spectra of NADH. Three solutions were scanned: NADH (0.96 mM) in 0.1 M phosphate buffer (pH 7.5); NADH + 20% ethanol in buffer; and NADH + ethanol + Mirex (0.08 mM). Increased ethanol concentration was necessary to ensure Mirex solubilization. Phosphate buffer was used as a baseline, since Mirex alone caused no change in the region scanned.

the mode of inhibition of lactate dehydrogenase by Mirex.

The standard definition of competitive inhibition is the competition between an enzyme's natural substrate A and an inhibitor A* for the active site of that enzyme. A* is usually a structural analogue of the natural substrate (Mahler and Cordes, 1971). However, this is not the only reason for competitive inhibition; because the inhibitory compound under consideration has no immediately apparent structural resemblance to pyruvate or to NADH, it appears that some alternate mechanism is employed, rather than direct competition. One such mechanism involves complexation or other association (or actual reaction) of the inhibitor with the substrate or coenzyme. Equations 2a and 2b illustrate this case for lactate de-

$$\text{pyruvate} \xrightleftharpoons{\pm\ \text{I}} \text{pyruvate-I} \xrightleftharpoons[*]{\pm\ \text{enzyme-NADH}} \text{etc.} \quad (2a)$$

$$\text{NADH} \xrightleftharpoons{\pm\ \text{I}'} \text{NADH-I}' \xrightleftharpoons{\text{enzyme}} \text{etc.} \quad (2b)$$

hydrogenase, where I and I' represent hypothetical inhibitors. The reaction at points indicated by an asterisk in eq 2 is impaired by the association of inhibitor with the substrate (eq 2a) or coenzyme (eq 2b).

Based on the preceding mechanisms and on the results of our kinetic determinations, we would like to propose the mechanism represented in eq 3a and 3b for the reversible

$$\text{NADH} \xrightleftharpoons{\pm\ \text{Mx}} \underset{\text{Mx}}{\underset{\cdot\cdot\cdot\cdot}{\text{NADH}}} \xrightleftharpoons[\text{A}]{\pm\ \text{enzyme}} \text{enzyme-}\overset{\text{Mx}}{\overset{\cdot\cdot\cdot\cdot}{\text{NADH}}} \rightleftharpoons \text{etc.} \quad (3a)$$

$$\text{enzyme-}\overset{\text{Mx}}{\overset{\cdot\cdot\cdot\cdot}{\text{NADH}}} \xrightleftharpoons[\text{B}]{\pm\ \text{pyruvate}} \text{enzyme-}\underset{\text{pyruvate}}{\overset{\text{Mx}}{\overset{\cdot\cdot\cdot\cdot}{\text{NADH}}}} \rightleftharpoons \text{etc.} \quad (3b)$$

inhibition of lactate dehydrogenase by Mirex (Mx) at low concentrations (less than 0.05 mM). The association of Mirex with NADH we will call an "attraction complex", represented by four dots. At point A in eq 3a, association of NADH with Mirex hinders complexation of enzyme with NADH. At point B in eq 3b, reaction of pyruvate and the enzyme–NADH complex to form the ternary complex would similarly be hindered by the association of Mirex with NADH. Complexation of protein and pesticide has been predicted by several groups (Wilson et al., 1971; Matsumara and O'Brien, 1966). However, our data are not complete enough at this point to determine if there is also

a binding of Mirex to the enzyme molecule in our postulated "attraction complex". However, unless protein–hydrocarbon binding caused some change in protein structure, competitive inhibition would occur with respect to both the coenzyme and the substrate; in eq 3a this is due to interference of hydrocarbon with coenzyme availability, and in eq 3b it is caused by blocking of the active site for pyruvate formed normally by the enzyme–NADH complex. Our results show competitive inhibition at both points (3a and 3b).

Because of the high stability of Mirex under the conditions of our experiments (Spencer, 1968), any reaction resulting in a covalent bond is very unlikely. Although the absence of any major spectral changes rules out the formation of an actual charge-transfer complex (Kosower, 1965), changes in both uv absorption spectra and CD spectra in the presence of low levels of Mirex indicate weak complex formation of some type. In uv studies, the ratio NADH:Mirex was varied from 3 to 6; in CD studies, this ratio was approximately 12. Changes were particularly noticeable in the CD spectra where a weak shift was observed at 260 nm. Also, Figure 3 shows a time-dependent association of NADH and Mirex.

Many organic compounds which contain nitrogen can act as electron donors in the formation of charge-transfer complexes; for example, aliphatic amines, pyridine, piperidine, and hexamethylenetetramine can serve as such donors (Andrews and Keefer, 1961). The nicotinamide ring of NADH, a pyridine analogue, readily forms charge-transfer complexes with halogens (Kosower, 1965). The formation of the expected complex with a monohalogen acceptor, for example, could be easily followed spectrophotometrically (Rose, 1967). However, in the case of a highly chlorinated compound such as Mirex, steric effects would hinder complete complex formation, although the tendency of NADH and Mirex to associate would result in an "association" or "attraction complex". Studies where the coenzyme exists as the pyridinium ion or NAD^+ would probably be helpful in determining the extent of association of the two compounds, since the pyridinium ion more readily enters into such complexes than does the reduced form (Kosower, 1965). It is interesting to consider at this point the consequences of association of NAD^+ and inhibitor in the case of the H_4 isozyme of lactate dehydrogenase. The H_4 form is found in heart muscle and catalyzes the transformation of lactate to pyruvate. In this case, the coenzyme is already in the pyridinium or oxidized form, and if the preceding speculations hold true, the enzyme in this form is even more susceptible to NAD^+–inhibitor complexation effects than the M_4 isozyme. This question is currently under investigation in our laboratory.

The adenine portion of NADH cannot be ignored; a donor molecule with more than one available coordination site can associate with more than one halogen molecule, and the aromatic heterocyclic nitrogen rings of adenine appear to be a donor also. However, interference in the nicotinamide region would certainly result in a change in the initial velocity, since it is that loss–acceptance of hydrogens by the nicotinamide moiety which is the actual contribution of NADH to the enzymatic reaction, but we do not rule out an adenine–Mirex complex as part of the inhibitory process.

A general survey of the effect of a number of pesticidal hydrocarbons (Hendrickson and Bowden, 1973) showed competitive inhibition in this system by 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT), 1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene (dieldrin), and its isomers. All are high molecular weight chlorinated hydrocarbons, and although the actual mechanism of association may differ with the structure of the inhibitor we consider it likely that association or complexation with NADH may well be the in vitro mode of inhibition of all of these. This has been indicated by Byczkowski (1973) who noted that DDT and its metabolites inhibit NADH-dependent enzymes, and by others who have observed the effect of similar compounds on glutamate dehydrogenase (NADH related) (Freedland and McFarland, 1965) and on succinate dehydrogenase (FADH related) (McCorkle and Yarbrough, 1974). Other researchers have predicted that formation of complexes with metabolites and tissue components may be the mode of action of certain pesticides (Ross and Biros, 1970; Haque et al., 1969; Wilson et al., 1971; Matsumara and O'Brien, 1966). A combination of the two mechanisms discussed above, i.e. pesticide association with protein and association of cofactor with pesticide, is not unlikely from both our data and from the literature evidence presented.

Whether this in vitro inhibition effect is indeed the same mechanism operating in vivo, or whether in vivo inhibition is a combination of this and other effects (membrane directed, genetic), is unknown. This question and the possibility of a generalized effect of similar compounds on other NADH- and FADH-dependent enzymes are currently under investigation in our laboratory.

LITERATURE CITED

Abston, P. A., Yarbrough, J. D., *J. Agric. Food Chem.* **22**, 66 (1974).
Andrews, L. J., Keefer, R. M., *Adv. Inorg. Chem. Radiochem.* **3**, 112 (1961).
Byczkowski, J., *Arch. Toxikol.* **31**, 137 (1973).
Dixon, M., *Biochem. J.* **55**, 170 (1953).
Freedland, R. A., McFarland, L. Z., *Life Sci.* **4**, 1735 (1965).
George, H., McMahan, J., Bowler, K., Elliott, M., *Biochim. Biophys. Acta* **191**, 466 (1969).
Gertig, H., Nowaczyk, W., Gniadek, M., *Diss. Pharm. Pharmacol.* **22**, 253 (1970).
Haque, R., Coshow, W. R., Johnson, L. F., *J. Am. Chem. Soc.* **91**, 3822 (1969).
Hendrickson, C. M., Bowden, J. A., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **32**, 235 (1973).
Hendrickson, C. M., Bowden, J. A., *J. Agric. Food Chem.* **23**, 407 (1975).
Holbrook, J. J., Gutfreund, H., *FEBS Lett.* **31**, 157 (1973).
Kosower, E. M., *Prog. Phys. Org. Chem.* **3**, 81 (1965).
Lineweaver, H., Burk, D., *J. Am. Chem. Soc.* **56**, 658 (1934).
Mahler, H. R., Cordes, E. H., "Biological Chemistry", 2nd ed, Harper and Row, New York, N.Y., 1971, pp 267–324.
Matsumara, F., O'Brien, R. D., *J. Agric. Food Chem.* **14**, 36, 39 (1966).
McCorkle, F. M., Yarbrough, J. D., *Bull. Environ. Contam. Toxicol.* **11**, 364 (1974).
Novoa, W. B., Winer, A. D., Glaid, A. J., Schwert, G. W., *J. Biol. Chem.* **234**, 1143 (1959).
Rose, J., "Molecular Complexes", Pergamon Press, Oxford, 1967, p 28.
Ross, R. T., Biros, F. J., *Biochem. Biophys. Res. Commun.* **39**, 723 (1970).
Spencer, E. Y., Guide to Chemicals Used in Crop Protection, 5th ed, Publication 1093, Canada Department of Agriculture, Ottawa, Information Canada, 1968, p 239.
Wilson, W. E., Fishbein, L., Clements, S. T., *Science* **171**, 180 (1971).

Received for review April 30, 1975. Accepted November 18, 1975.