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Interactions of Kepone with Rabbit Muscle Lactate Dehydrogenase

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The inhibition of rabbit muscle lactate dehydrogenase catalyzed oxidation of lactic acid by the polychlorinated hydrocarbon pesticide, Kepone, was investigated with respect to the documentation of direct interactions of the pesticide with this enzyme in the absence of the coenzyme, NAD. The slow loss of catalytic activity of this dehydrogenase in 20% ethanol is greatly increased by the presence of micromolar concentrations of Kepone, and the rate enhancement observed was proportional to the concentration of Kepone added. The suggestion that the Kepone effect results from a direct interaction with the enzyme, resulting in a destabilization of protein structure, is supported by the protective effects observed with NAD and NADH. Using fluorescence quenching techniques, no evidence was obtained for complex formation between Kepone and 3-aminopyridine adenine dinucleotide. In addition, the properties of the Kepone inhibition of rabbit muscle lactate dehydrogenase did not change when the chemical structure of the pyridinium and purine moieties of the coenzyme were varied. These observations add further support to a proposed direct interaction of Kepone with the enzyme as a mode of inhibition by the pesticide.

The polychlorinated hydrocarbon pesticide, Kepone (decachloro-1,3,4-metheno-1*H*-cyclobuta[6*d*]pentalen-2-one), has been shown in in vitro studies to be an effective inhibitor of certain pyridine nucleotide requiring dehydrogenases. In early studies of the Kepone inhibition of beef liver glutamic dehydrogenase (EC 1.4.1.3) (Freedland and McFarland, 1965), a degree of specificity was attributed to the Kepone inhibition since several other chlorinated hydrocarbons were observed to be ineffective as inhibitors of this enzyme. However, saturated solutions of Kepone were employed and K_i values for Kepone were not determined. More recently an effective inhibition of lactate dehydrogenase (EC 1.1.1.27) by Kepone was observed by Hendrickson and Bowden (1975, 1976a), and evidence was presented (Hendrickson and Bowden, 1976a) to indicate that the structurally related, fully chlorinated pesticide mirex inhibited the lactate dehydrogenase in a similar fashion. Studies of Kepone inhibition of lactate dehydrogenase were extended by Anderson and Noble (1977) to include studies of the effects of the pesticide on homologous lactate dehydrogenase isozymes from rabbit, beef, pig, and chicken. In these studies, Kepone was observed to be an effective inhibitor of several M_4 isozymes while within the limits of solubility, no inhibition of H_4 isozymes was detected. The isozymic preference shown would again argue for a more selective inhibitory process and as in earlier studies, mirex appeared to function in a similar fashion. Although ethanolic solutions are required to solubilize Kepone and mirex, the low concentrations of these pesticides required to obtain the effects observed with lactate dehydrogenases compare favorably with those readily available through tissue accumulation of these compounds, indicating a possible physiological significance to these types of interactions.

Kepone and mirex have also been observed (Anderson et al., 1977) to be effective inhibitors of bovine mitochondrial, porcine mitochondrial, and porcine cytoplasmic malate dehydrogenase (EC 1.1.137). Mixed inhibition with respect to substrates and coenzymes was observed in the reactions catalyzed by these enzymes. In these studies, however, an additional important observation was made in that at concentrations inhibitory to the bovine mitochondrial enzyme, Kepone induced an accelerated rate of inactivation of the enzyme by *N*-ethylmaleimide. The data obtained suggested the possible importance of an enzyme unfolding or dissociation process promoted by the binding of Kepone. Since these effects were obtained in the absence of substrates or coenzymes, a more direct interaction between Kepone and the enzyme was indicated. A different mechanism, one requiring complex formation between mirex and pyridine nucleotides was recently suggested (Hendrickson and Bowden, 1976a) for the inhibition of lactate dehydrogenase. Since this appears not to be the case with Kepone inhibition of malate dehydrogenases (Anderson et al., 1977), additional studies of the Kepone inhibition of lactate dehydrogenases were indicated.

MATERIALS AND METHODS

Crystalline rabbit muscle lactate dehydrogenase, NAD, NADH, nicotinamide hypoxanthine dinucleotide, thionicotinamide adenine dinucleotide, and L(+)-lactic acid, lithium salt were obtained from Sigma Chemical Company. A sample of Kepone was generously supplied by Allied Chemical Company and was recrystallized twice from *n*-hexane prior to use. 3-Aminopyridine adenine dinucleotide was prepared chemically from NAD as described by Fisher et al. (1973). After purification by ion-exchange chromatography on Dowex-1-formate, the dinucleotide was desalted by gel filtration on a Sephadex

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G-10 column. 2,2′-Dithiopyridine was obtained from Aldrich Chemical Company.

Lactate dehydrogenase catalyzed reactions were studied in 3-mL reaction mixtures at 25 °C. Initial velocities were determined spectrophotometrically at the appropriate wavelength for reduction of the coenzyme molecule being studied. The wavelengths employed were 340 nm for the reduction of NAD, 340 nm for the reduction of nicotinamide hypoxanthine dinucleotide and 395 nm for the reduction of thionicotinamide adenine dinucleotide. Spectrophotometric measurements were made on a Beckman Acta M VI recording spectrophotometer. Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorimeter. pH measurements were made with a Radiometer PHM52 pH meter with a type 202 C glass electrode. First-order rate constants were calculated using the equation $k_1 = 0.693/t_{1/2}$. Half-lives were determined from semilogarithmic plots of rate data.

RESULTS

In previous studies (Anderson and Noble, 1977) rabbit muscle lactate dehydrogenase was shown to be inhibited reversibly by low concentrations of Kepone. The type of inhibition was mixed with respect to lactate and noncompetitive with respect to NAD, and K_i values in the range of 6–9 μM were determined. As expected from the predominant M-subunit composition of the rabbit muscle LDH preparation, the rabbit homologous M_4 LDH exhibited the same properties with respect to Kepone inhibition (Anderson and Noble, 1977). The inhibition of these enzymes by Kepone was studied in 10% ethanol to provide the greater solubility needed to evaluate higher concentrations of the pesticide. In reversibility experiments, these enzymes were incubated in 10% ethanol containing 30 μM Kepone with no loss of catalytic activity over a 10-min period (Anderson and Noble, 1977). This indicated no time-dependent reaction of Kepone with these enzymes and that the enzymes were stable in 10% ethanol. In earlier studies of LDH isozymes (Anderson et al., 1974), 20% ethanol was found to facilitate inactivation by *N*-alkylmaleimides presumably through a loosening of tertiary structure resulting in a greater accessibility of essential sulfhydryl groups. In the present study, such structural changes induced by 20% ethanol were investigated with respect to the possible facilitation by Kepone interactions with rabbit muscle LDH.

Rabbit muscle LDH (8 μg) was incubated at 25 °C in 3-mL reaction mixtures containing 66 mM potassium phosphate buffer, pH 7.6, and 20% ethanol. At timed intervals, 0.1-mL aliquots of this incubation mixture were transferred to a LDH assay mixture containing 50 mM Tris–HCl buffer, pH 8.1, 0.63 mM NAD, 42 mM lithium lactate, 10% ethanol, and initial velocities of NAD reduction were determined spectrophotometrically at 340 nm. A slow loss of enzyme activity was observed over the 60-min incubation period (Figure 1, line 1), indicating a $t_{1/2}$ of 113 min. When Kepone was added to the incubation mixture, the loss of lactate dehydrogenase activity was accelerated (Figure 1) and the half-lives of enzyme inactivation were observed to be 92, 49, and 37 min at 15, 30, and 45 μM Kepone, respectively. The first-order rate constants for these inactivation processes were 0.0075, 0.014, and 0.019 min^{-1} for 15, 30, and 45 μM Kepone, respectively. Since this inactivation of lactate dehydrogenase appeared related to structural changes in the enzyme, the effects of stabilization of enzyme structure through coenzyme binding was investigated. Lactate dehydrogenase (8 μg) was incubated with 30 μM Kepone as described above alone and in the presence of either 1.9 mM NAD or 0.13 mM NADH. Both oxidized and reduced forms of the coenzyme protected the enzyme against inactivation by 30 μM Kepone (Figure 2). The presence of 1.9 mM NAD provided 72% protection decreasing the rate of inactivation from a half-life of 37.5 to 134 min. The presence of 0.13 mM NADH provided 60% protection corresponding to an increase in half-life from 37.5 to 92.5 min. It should also be noted that these concentrations of oxidized and reduced coenzyme also protected the enzyme against the slow loss of catalytic activity in the presence of 20% ethanol alone.

Since the possibility existed that the Kepone induced inactivation of lactate dehydrogenase could arise through thiohemiacetal formation with an essential sulfhydryl group of the enzyme, it is important to note that the presence of 0.1 M mercaptoethanol in the incubation mixture had no effect on the rate of inactivation by Kepone. Also, the available sulfhydryl groups of rabbit muscle lactate dehydrogenase were determined using

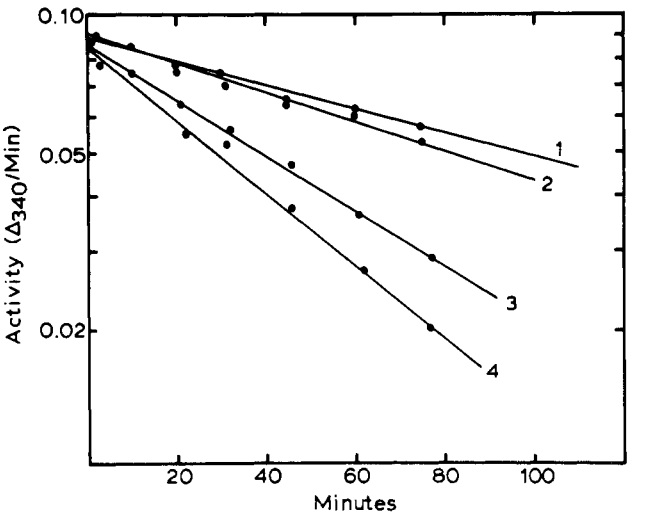

Figure 1. Inactivation of rabbit muscle lactate dehydrogenase by Kepone. Incubation mixtures contained 66 mM potassium phosphate buffer, pH 7.6, 20% ethanol, 8 μg of crystalline lactate dehydrogenase, and Kepone as indicated in a total volume of 3 mL. Line 1, no Kepone; line 2, 15 μM Kepone; line 3, 30 μM Kepone, and line 4, 45 μM Kepone.

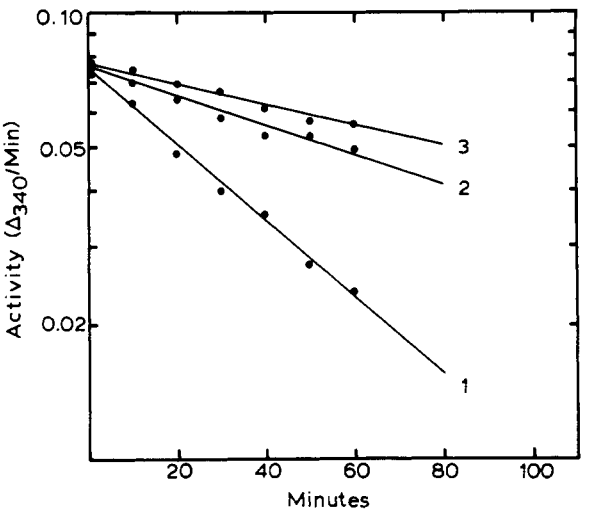

Figure 2. Protection of Kepone inactivation of rabbit muscle lactate dehydrogenase by NAD and NADH. Incubation mixtures contained 66 mM potassium phosphate buffer, pH 7.6, 20% ethanol, 8 μg of enzyme, 30 μM Kepone in a total volume of 3 mL. In addition, line 1 contained no added coenzyme, line 2, 0.13 mM NADH, and line 3, 1.9 mM NAD.

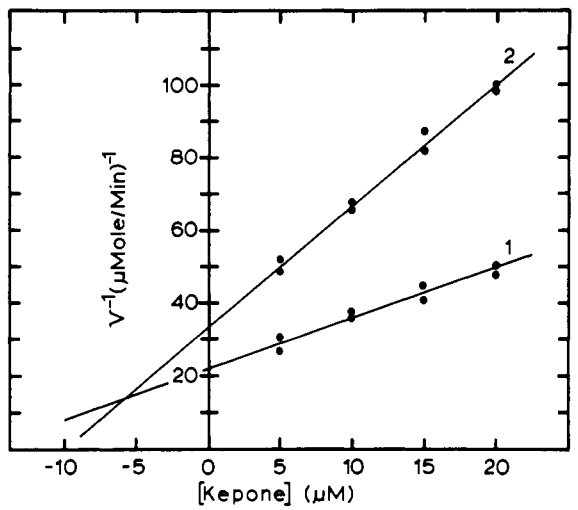

Figure 3. Inhibition of rabbit muscle lactate dehydrogenase as a function of Kepone concentration. Reaction mixtures contained 50 mM Tri-HCl buffer, pH 8.1, 10% ethanol, 1.34 mM nicotinamide hypoxanthine dinucleotide, 1.2 μg (0.9 IUB units) of rabbit muscle lactate dehydrogenase, two lactate concentrations, 1.67 mM (line 1) and 3 mM (line 2), and inhibitor varying from 0–20 μM in a total volume of 3 mL.

2,2′-dithiopyridine as described by Grassetti and Murray (1967), and no change in free sulfhydryl groups of the enzyme was detected during 90% inactivation by 30 μM Kepone. Six determinations of free sulfhydryl groups were performed during the course of this reaction.

In a set of experiments identical with those depicted in Figure 1, Kepone was shown to inactivate the pig lactate dehydrogenase, M_4 isozyme.

In considering the recently suggested possibility (Hendrickson and Bowden, 1976a) that the inhibition of lactate dehydrogenases by Kepone could arise from the prior nonenzymatic formation of a Kepone–coenzyme complex, several experiments have been performed. The first experiment involved the quenching of fluorescence of the pyridine nucleotide coenzyme analogue, 3-aminopyridine adenine dinucleotide. This compound which contains a fluorescing pyridinium base has been shown to be very sensitive to charge-transfer complex formation with halogens resulting in the quenching of fluorescence of the 3-aminopyridine moiety (Fisher et al., 1973). A 5×10^{-4} solution of 3-aminopyridine adenine dinucleotide in 50 mM Tris–HCl buffer, pH 8.1, and 10% ethanol was excited at 331 nm and the fluorescence at 420 nm was recorded. This fluorescence emission was then studied in the presence of five concentrations of Kepone ranging from 5 to 30 μM, and no change in the fluorescence of this compound was observed.

A second set of experiments was designed to investigate the effects of changing the structure of the pyridine nucleotide coenzyme on the ability of Kepone to inhibit lactate dehydrogenase. The rabbit muscle lactate dehydrogenase catalyzed oxidation of lactate was studied in 3-mL reaction mixtures containing 50 mM Tris–HCl buffer, pH 8.1, 10% ethanol, 1.34 mM nicotinamide hypoxanthine dinucleotide, two concentrations of lactate, 1.67 and 3 mM, and five concentrations of Kepone ranging from 0 to 20 μM. The initial velocity measurements obtained spectrophotometrically at 340 nm plotted according to Dixon (1953) are shown in Figure 3. An inhibitor dissociation constant (K_i) for the Kepone inhibition observed was calculated to be 5.9 μM. An identical experiment using the true coenzyme, NAD at 0.63 mM, gave a K_i value for Kepone inhibition of 6.25 μM. A similar result was obtained when the pyridine nucleotide coenzyme used was modified in the pyridine moiety of the molecule. In this case, the rabbit muscle lactate dehydrogenase catalyzed oxidation of lactate was studied in 3-mL reaction mixtures containing 50 mM Tris–HCl buffer, pH 8.1, 10% ethanol, 50 mM lactate, two concentrations of thionicotinamide adenine dinucleotide, 140 and 70 μM, and five concentrations of Kepone ranging from 0 to 20 μM. The reduction of thionicotinamide adenine dinucleotide was followed spectrophotometrically at 395 nm. A K_i value of 6.3 μM was obtained for the Kepone inhibition of this reaction. The Kepone inhibition of lactate dehydrogenase using the three different functional coenzyme molecules was studied in each case in triplicate, and the average K_i values for Kepone inhibition are listed in Table I.

Table I. Kepone Inhibition of Rabbit Muscle Lactate Dehydrogenase

Coenzyme	K_i,[a] μM
Nicotinamide adenine dinucleotide	5.9
Nicotinamide hypoxanthine dinucleotide	6.0
Thionicotinamide adenine dinucleotide	6.2

[a] Average values of experiments run in triplicate.

DISCUSSION

Rabbit muscle lactate dehydrogenase is effectively inhibited by low concentrations of the polychlorinated hydrocarbon pesticide, Kepone. This inhibition has been adequately documented in several studies (Hendrickson and Bowden, 1975; Anderson and Noble, 1977); however, the mechanism by which Kepone exerts its inhibitory effect on this enzyme needs further clarification. In recent studies of the inhibition of lactate dehydrogenase by mirex (Hendrickson and Bowden, 1976a) and by other polychlorinated hydrocarbons (Hendrickson and Bowden, 1976b), a mechanism for inhibition was proposed which involved the prior nonenzymatic formation of a NADH–pesticide complex supported by increases in the absorption spectrum of NADH in the presence of various polychlorinated hydrocarbons. Under the conditions used to demonstrate spectral changes in the presence of mirex (Hendrickson and Bowden, 1976a), preliminary studies in this laboratory measuring the fluorescence emission of NADH in the presence of mirex indicate considerable lightscattering at the 30 μM concentration of the pesticide. A 30 μM solution of mirex in 95% ethanol did not absorb in the wavelength region from 250 to 400 nm. The same concentration of mirex in 10% ethanol was turbid to the naked eye and yielded the same absorbance characteristics reported by Hendrickson and Bowden (1976a). The changes in the spectrum of NADH in the presence of 30 μM mirex observed by Hendrickson and Bowden (1976a) could conceivably be related to turbidity effects. These authors (Hendrickson and Bowden, 1976a) indicate some difficulty in characterizing the nature of the proposed complex between polychlorinated hydrocarbons and NADH and suggest that it would be helpful to study interactions between the oxidized coenzyme, NAD, and polychlorinated hydrocarbons where complex formation would be more likely since such interactions have been shown to be more favorable (Kosower, 1965). It was speculated (Hendrickson and Bowden, 1976a) that a NAD–pesticide complex could be of importance in the inhibition of H_4 isozymes of lactate dehydrogenases which preferably catalyze the conversion of lactate to pyruvate. In this respect, Anderson and Noble (1977) have reported that Kepone and mirex do not inhibit lactate dehydrogenase H_4 isozymes at the limit of solubility in 10% ethanol

or 10% dimethylformamide. Also, it has been reported (Anderson et al., 1977) that mirex and Kepone do not inhibit the reactions catalyzed by yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, and yeast 6-phosphogluconate dehydrogenase under conditions where effective inhibition of lactate dehydrogenase M_4 isozymes and malate dehydrogenases occurs.

It is felt that Kepone and mirex inhibition of lactate and malate dehydrogenases results from the direct interactions of these pesticides with the enzymes themselves. This is indicated (Anderson et al., 1977) by the enhancement of the rate of maleimide inactivation of malate dehydrogenases in the absence of either oxidized or reduced coenzyme. In fact, the presence of coenzyme protected the malate dehydrogenase from the Kepone and mirex effects, presumably through the stabilization of the tertiary or quaternary structure of the enzyme. In the present study, similar effects were observed with Kepone on the loss of catalytic activity of rabbit muscle lactate dehydrogenase in 20% ethanol. As shown in Figure 1, the presence of Kepone induces a faster rate of inactivation of the enzyme, an effect observed in the absence of coenzyme. The presence of either oxidized or reduced coenzyme stabilizes the dehydrogenase structure, protecting the enzyme against the Kepone effect (Figure 2).

The possibility of the nonenzymatic formation of a pyridine dinucleotide–Kepone complex was investigated by studying the fluorescence of 3-aminopyridine adenine dinucleotide. This fluorescing dinucleotide has been shown to be a sensitive indicator of charge-transfer complex formation with halogens (Fisher et al., 1973) which results in the extensive quenching of the fluorescence of the pyridinium moiety of this compound. At concentrations causing effective inhibition of lactate and malate dehydrogenases, no quenching of the fluorescence of 3-aminopyridine adenine dinucleotide was observed, suggesting no appreciable complex formation.

Since hemiketal formation with Kepone has been demonstrated (McBee et al., 1956), thiohemiketal formation might be suspected in interactions of Kepone with enzymes containing essential sulfhydryl groups. In this respect, it is important to note that during the Kepone enhanced inactivation of rabbit muscle lactate dehydrogenase no change in the number of free sulfhydryl groups of the enzyme was observed.

Additional evidence for the lack of coenzyme complex formation in the inhibition of rabbit muscle lactate dehydrogenase by Kepone was obtained in studies involving pyridine dinucleotides of varying chemical structure. The K_i values for Kepone inhibition of lactate dehydrogenase did not vary when either thionicotinamide adenine dinucleotide or nicotinamide hypoxanthine dinucleotide was substituted for the native coenzyme (Table I). This indicates that the chemical properties of both the pyridinium and purine moieties of the coenzyme molecule can be varied without affecting the type and effectiveness of the Kepone inhibition. These observations in conjunction with those discussed above add further support to the proposed direct interaction of Kepone with lactate dehydrogenase as a mode of inhibition of this enzyme.

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