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INHIBITION OF MITOCHONDRIAL Mg^{2+} ATPase ACTIVITY IN ISOLATED PERFUSED RAT LIVER BY KEPONE

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Abstract—Hepatic mitochondrial Mg^{2+} ATPase (MATPase) activity was determined in isolated perfused rat liver preparations. Perfusion for 1–4 hr with 30% rat blood did not affect either the native or DNP (10^{-4} M)-stimulated MATPase activity. Mitochondrial preparations obtained from perfused livers were sensitive to added kepone: first, addition of kepone (10^{-6}M) resulted in total abolishment of DNP-stimulated MATPase activity; second, over and beyond the abolishment of DNP-stimulated activity, part of the native MATPase activity was also inhibited by kepone (10^{-6} M). Furthermore, a higher concentration of kepone (10^{-5} M) inhibited the native MATPase activity in a dose-related manner. Liver perfusion with 10^{-4} M kepone in the blood perfusate resulted in a similar abolishment of DNP-stimulated, as well as inhibition of native, MATPase activity. The requirement for a higher concentration of kepone in the perfusion system is because of the partial loss of intracellular kepone from the liver into the 9000 *g* supernatant fraction which would have been otherwise available for the inhibition of MATPase activity in the intact liver. These results suggest that interference of energy metabolism in the liver may bear a cause-effect relationship to the previously reported kepone-induced impairment of biliary excretory function.

Kepone and mirex are structurally related carbon-caged organochlorine compounds (Fig. 1) used in controlling leaf-eating insects and fire ants respectively [1–3]. Biotransformation of kepone has not been reported, but mirex is resistant to biodegradation [4] and both compounds interfere with normal reproduction [5] and cause hepatic carcinomas in experimental animals [6, 7]. Other untoward effects of these compounds include liver hypertrophy and a proliferation of endoplasmic reticulum associated with an increase in the mixed-function oxidases [8–11] and inhibition of LDH in the muscle tissue [12]. Pre-exposure to these compounds induces impairment of hepatobiliary function which appears to be due to interference with the transport of substances from the hepatocytes to the bile canaliculi [13, 14].

Kepone was shown to be a potent inhibitor of ATPase activities in fish brain [15]. Na^+-K^+ ATPase, involved in the active transport across cell membranes [16, 17], and oligomycin-sensitive Mg^{2+} ATPase, involved in the oxidative phosphorylation [18, 19], have been shown to be inhibited by a variety of chlorinated hydrocarbon pesticides [20–26]. We have recently observed that kepone inhibits hepatic mitochondrial Mg^{2+} ATPase activity *in vitro* as well as after pre-exposure of rats to this pesticide [27].

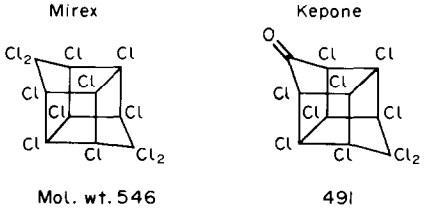

Fig. 1. Structures of mirex and kepone.

The purpose of the present investigation was to study the effect of kepone on mitochondrial Mg^{2+} ATPase activity in the isolated perfused rat liver. Since pre-exposure to kepone as well as addition of kepone *in vitro* to isolated perfused rat liver preparations effectively suppressed biliary excretion of imipramine metabolites [28], and possible implication of hindered energy production and/or utilization, it was of interest to study the effect of kepone on energy production and utilization in the liver. Although microsomal Na^+-K^+ ATPase activity might be more directly involved in the transport of substances across the bile canaliculi [16, 17], the effect of kepone on mitochondrial Mg^{2+} ATPase was studied in order to determine if the kepone effect was manifested at the site of energy production itself. Isolated perfused rat liver preparations were utilized in these investigations.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River) were maintained in animal facilities away from any known inducers. The bedding used for these animals was made from corn cobs. Rats used to collect blood weighed 250–300 g, whereas the animals used as liver donors weighed 200–250 g. Kepone (decachloro-pentacyclo-decone-4-one) was obtained from Allied Chemical Corp., Baltimore, MD. Eumlphor used to solubilize kepone in these experiments was a gift of GAF Corp. (New York, NY) and has been used in our laboratory for solubilizing other compounds [14, 29]. All other chemicals used for the ATPase assay were obtained from Sigma Chemical Co., St. Louis, MO.

The details of the perfusion technique and apparatus used in this study were reported by Mehen-

dale [29]. Kepone, at the desired concentration added to the perfusate (blood) as solution, was dissolved in Emulphor–ethanol (1:1, v/v) and this solution was mixed with 8 parts of water. For control perfusion, only Emulphor solution was added to the perfusate. The perfusion time was 1 hr and 4 hr for both control and kepone experiments. The length of perfusion and the addition of Emulphor solution were without any effect on ATPase activity. At the end of the perfusion the liver was removed, homogenized and fractionated as described below.

The liver tissues were homogenized in sucrose solution (containing 0.32 M sucrose, 1 mM EDTA and 10 mM Imidazole buffer, pH 7.5) by using ground glass homogenizer. The heavy and light mitochondrial fractions were obtained by centrifugation [30]. The homogenate was centrifuged at 750 *g* for 10 min to remove nuclei and cellular debris. The supernatant was centrifuged at 9000 *g* for 20 min and the pellet (heavy mitochondria) was resuspended in sucrose solution. Fresh (unfrozen) mitochondrial suspension was used for DNP-stimulated Mg^{2+} ATPase activity studies.

ATPase activity was measured by a continuous method [31, 32] which had been previously compared with the discontinuous method [33]. A 3-ml reaction medium contained 4.5 mM ATP, 5 mM Mg^{2+}, 135 mM imidazole–HCl buffer (pH 7.6), 0.2 mM NADH, 0.5 mM phosphoenol pyruvate, 0.02% bovine serum albumin, approximately 9 units of pyruvate kinase and 12 units of lactic acid dehydrogenase. A 100-μl mitochondrial fraction was used with a protein content of 60–70 μg. When used, DNP was present at a concentration of 1×10^{-4} M. Absorbance changes in the reaction mixture were measured at 340 nm using a Beckman Acta III recording spectrophotometer with the temperature controlled at 37°. The change in O.D. at 340 nm over a period of 10 min was used in calculating the specific activity. Enzyme activities were expressed as μmoles P_i mg^{-1} protein hr^{-1}. Protein was determined by the method of Lowry *et al.* [34] using bovine serum albumin as standard.

Mg^{2+} ATPase was delineated into oligomycin-sensitive (mitochondrial) and oligomycin-insensitive Mg^{2+} ATPase activities by adding 5×10^{-6} M of oligomycin (based upon a combined mol. wt of 401.20 from 15% oligomycin A and 85% oligomycin B) in ethanol to the reaction mixture. The volume of ethanol used was 1 μl in a 3-ml reaction mixture. At this concentration, oligomycin is a potent inhibitor of mitochondrial ATPase activity [35]. Although data are not presented here, it was routinely noted that the oligomycin-insensitive Mg^{2+} ATPase activity (5.44 ± 0.47 μmoles P_i mg^{-1} protein hr^{-1}) was not affected by kepone both *in vitro* and in isolated perfused liver experiments.

RESULTS

Liver perfusion had no effect on the native mitochondrial Mg^{2+} ATPase activity. Further, mitochondrial Mg^{2+} ATPase activity of unperfused livers and the activity of livers perfused for either 1 or 4 hr were indistinguishable from each other. The results in Fig. 2 show that addition of kepone *in vitro* significantly

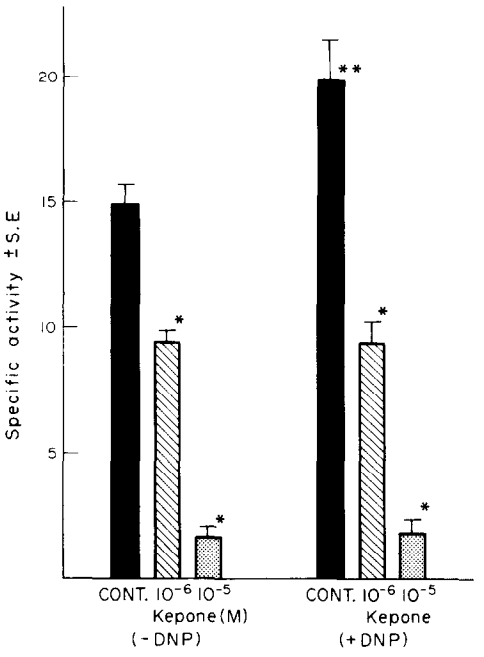

Fig. 2. Effect of kepone on oligomycin-sensitive (mitochondrial) Mg^{2+} ATPase activity in isolated perfused rat liver. Livers were perfused with 30% rat blood for 4 hr. At the end of the perfusion, livers were homogenized and fractionated as described in the text. Where indicated, DNP (10^{-4} M) and kepone were added to the reaction mixture. Each bar represents the mean specific activity of four (N = 4) different liver preparations. Inhibition of enzyme activity by kepone was significant (a single asterisk indicates $P < 0.005$) and stimulation by DNP was also significant (a double asterisk indicates $P < 0.025$). The specific activity of oligomycin-insensitive MATPase in these preparations was 5.44 ± 0.47 μmoles $Pi \cdot mg^{-1}$ protein $\cdot hr^{-1}$ and was unaffected by kepone.

inhibits the oligomycin-sensitive Mg^{2+} ATPase activity in mitochondria obtained from perfused control livers. Addition of DNP (10^{-4} M) to the mitochondria results in a 30 per cent stimulation of the native Mg^{2+} ATPase activity. This DNP-stimulated activity was totally suppressed by the inclusion of 10^{-6} M kepone in the incubation assay medium (Fig. 2). In addition, a part of the native activity was also inhibited by kepone. Increasing the concentration of kepone to 10^{-5} M resulted in a further increase in the inhibition, indicating a dose-related response.

Livers were perfused with two concentrations of kepone in the perfusate for 1 and 4 hr, in order to determine if kepone taken up by the liver would be inhibitory to Mg^{2+} ATPase activity. The results are illustrated in Fig. 3. Kepone, at a concentration of 2×10^{-5} M in the perfusate, was without any significant effect on the mitochondrial Mg^{2+} ATPase activity after the perfusion experiment (Fig. 3A). However, when the kepone concentration was increased to 5×10^{-4} M, a decrease in the enzyme activity was evident after perfusion for 1 hr (Fig. 3B). In view of the much higher inhibition of Mg^{2+} ATPase activity by the addition of kepone *in vitro* to the mitochondrial preparation at lower concentrations (Fig. 2), it was argued that a longer perfusion time might be necessary to allow for kepone to be distributed and bound to the mitochondria in the intact perfused

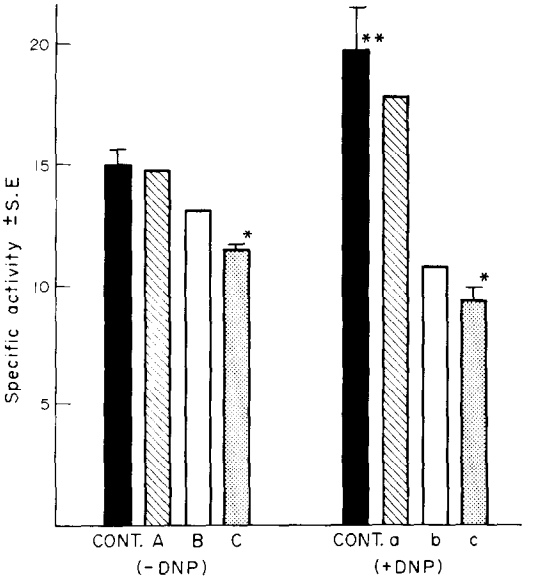

Fig. 3. Oligomycin-sensitive (mitochondrial) Mg^{2+} ATPase activity in kepone-perfused rat liver. The concentration of kepone and perfusion time are as follows: (A, a) 2×10^{-5} M kepone perfused for 1 hr; (B, b) 5×10^{-4} M kepone perfused for 1 hr; and (C, c) 5×10^{-4} M kepone perfused for 4 hr. At the end of perfusion, the livers were homogenized and fractionated as described in the text. ATPase activities were determined in the absence (−DNP) and presence of DNP (+DNP) in the reaction mixture. Each bar represents the mean of four different liver preparations except in A, a and B, b, where two liver preparations were used. Inhibition by kepone was significant (a single asterisk indicates $P < 0.001$) and the control enzyme activity was stimulated by DNP significantly (a double asterisk indicates $P < 0.025$). The specific activity of oligomycin-insensitive MATPase in these preparations was 5.44 ± 0.47 μmoles Pi·mg^{-1} protein·hr^{-1} and was unaffected by kepone.

liver. A significant decrease in the enzyme activity was observed when the perfusion time was increased to 4 hr (Fig. 3C). Similarly, the DNP-stimulated activity was not affected after 1 hr of perfusion with a low concentration of kepone (Fig. 3a) and a significant inhibition was observed after perfusion with the higher concentration of kepone both at 1 and 4 hr perfusion times (Fig. 3b and 3c).

A relatively higher concentration of kepone was required in order to demonstrate the effect of kepone in the perfused liver. Loss of kepone from the liver in the supernatant fraction after centrifugation to obtain the mitochondrial pellet might explain the requirement for a relatively higher concentration of kepone. In order to establish this possibility, Mg^{2+} ATPase activity was determined using the whole homogenate of the liver perfused for 4 hr with and without kepone. A 35 per cent inhibition of mitochondrial Mg^{2+} ATPase activity was observed when the liver was perfused with 5×10^{-4} M kepone for 4 hr (Table 1). This level of inhibition of mitochondrial ATPase activity in the whole homogenate is consistent with the idea that reduced inhibition observed with the isolated mitochondria might be a result of loss of kepone in the supernatant after fractionation. The results in Table 1 also demonstrate that DNP-stimulated Mg^{2+} ATPase activity was also

Table 1. Oligomycin-sensitive (mitochondrial) Mg^{2+} ATPase activity in whole homogenate of kepone (5×10^{-4} M) perfused rat liver*

Kepone (M)	Perfusion time (hrs)	Oligomycin-sensitive Mg^{2+} ATPase† − DNP	+ DNP
0	4.0	6.80	8.20
5×10^{-4}	4.0	4.45 ± 0.21	3.46 ± 0.36

* Livers obtained from male rats were perfused with 30% rat blood. The desired amount of kepone was added to the perfusate as Emulphor solution 30 min after the initial period of equilibration of the liver preparation. Only Emulphor vehicle was added to the control liver preparations. Liver was homogenized after the 4-hr perfusion and Mg^{2+} ATPase activity was determined. Where indicated, the desired amount of DNP was included in the medium. Results are averages of two experiments for control and three for kepone experiments.

† Specific activity = μmoles P_i mg^{-1} protein hr^{-1}.

inhibited in the whole homogenate obtained from livers perfused with 5×10^{-4} M kepone. Inhibition of the native mitochondrial Mg^{2+} ATPase activity by added kepone could be demonstrated in the whole liver homogenates (Table 2). Furthermore, stimulation of the enzyme activity by addition of DNP as well as suppression of this DNP-stimulated activity by kepone, could be demonstrated in the whole homogenates of the perfused livers.

Since the above results indicated that a part of kepone was washed out in the 9000 *g* supernatant fraction during mitochondrial fractionation, additional experiments were carried out as follows. A mitochondrial preparation was divided into two tubes. To one was added kepone (5×10^{-6} M) and the other received a 20-μl vehicle only. Mg^{2+} ATPase was assayed in the mitochondria in both tubes. After the first assay, the mitochondria were resuspended in the original volume of buffer and recentrifuged. The 9000 *g* supernatant was discarded at first washing.

Table 2. Effect of kepone on oligomycin-sensitive (mitochondrial) Mg^{2+} ATPase activity in whole homogenate of isolated perfused rat liver*

Kepone (M)	DNP (10^{-4} M)	Oligomycin-sensitive Mg^{2+} ATPase Sp. act.†	Per cent change
0	0	6.80	
10^{-6}	0	4.49	−33.9
10^{-5}	0	0.61	−91.0
0	+	8.20	+20.5
10^{-6}	+	5.53	−32.6
10^{-5}	+	0.81	−90.1

* Livers obtained from male rats were perfused with 30% rat blood. After the initial 30-min equilibration of the liver preparation, only the Emulphor vehicle was added to the control liver preparations and Emulphor solution of kepone was added to the perfusate of kepone experiments. At the end of a 4-hr perfusion, the liver was homogenized and used for Mg^{2+} ATPase assay. Where indicated, DNP was added to the incubation mixture. Results are averages of two experiments.

† Specific activity = μmoles P_i mg^{-1} protein hr^{-1}.

Table 3. Reversibility of kepone inhibition of oligomycin-sensitive Mg^{2+} ATPase activity in rat liver mitochondria by washing*

Condition	Sp. act. ± S.E. (μmoles P_i mg^{-1} protein hr^{-1})	Per cent inhibition
Control	25.8 ± 1.47	
Kepone (5×10^{-6} M)	0.77 ± 0.16†	97
First wash	6.07 ± 0.87†	76
Second wash	11.4 ± 2.22†	56

* A 1-ml rat liver mitochondrial preparation (9000 g pellet) which was quick frozen in liquid nitrogen, containing about 2.4 mg protein, was incubated with 5×10^{-6} M kepone for 3 min. An aliquot of 100 μl was used for enzyme assay and the remaining protein was diluted ten times with 0.32 M sucrose solution and centrifuged at 9000 g for 20 min. The pellet was resuspended in 0.9 ml of sucrose solution (I wash). Two hundred μl was used for protein and enzyme activity determinations. The remaining 0.7 ml was diluted with 9.3 ml sucrose solution and centrifuged again at 9000 g for 20 min. The pellet was resuspended in 0.7 ml sucrose solution (II wash) and protein and enzyme activities were determined. This procedure was repeated with three different rat livers, and standard errors of the means were calculated.

† Significant to $P < 0.01$ when compared to control activity.

Resuspended mitochondria were assayed for Mg^{2+} ATPase activity. This procedure was repeated once more to obtain mitochondria after a second wash. Enzyme preparations from three individual livers were processed as above. The results of these experiments (Table 3) indicate that increments of kepone were successively lost from mitochondrial preparations after resuspension and centrifugation, as judged by inhibition of Mg^{2+} ATPase as the end point. These results confirm that part of kepone taken up by the liver is lost during preparative procedures, which would explain the necessity for a higher concentration of kepone to be able to demonstrate inhibition of Mg^{2+} ATPase activity in mitochondria from kepone-perfused liver preparations.

DISCUSSION

To our knowledge, mitochondrial Mg^{2+} ATPase activity has not been examined in perfused liver preparations prior to these investigations. The demonstration that mitochondrial Mg^{2+} ATPase activity is unaltered even after 4 hr of perfusion is consistent with the contention that viable organ perfusion preparations can be obtained for use as models in biochemical studies [29].

Although the inhibition of Mg^{2+} ATPase by kepone can be demonstrated in kepone-perfused liver, two difficulties are encountered in this regard. First, only a portion of kepone added to the blood perfusate is taken up by the liver. Second, a part of whatever quantity of kepone is taken up by the liver, is lost during subsequent tissue homogenization and centrifugation and is removed along with the 9000 g supernatant. Indeed, the increased inhibition of Mg^{2+} ATPase observed in the whole homogenates of kepone-perfused livers compared to the mitochondrial preparations of the same livers is suggestive of the above observation. Moreover, the results of successive rewashings (Table 3) of kepone-treated mitochondria confirm these observations.

The results demonstrate that kepone is a potent inhibitor of oligomycin-sensitive (mitochondrial) Mg^{2+} ATPase activity in isolated rat liver mitochondria. This finding is in agreement with the results of Desaiah and Koch [15] using fish brain. It is of interest to compare kepone and oligomycin with respect to the qualitative and quantitative aspects of inhibition of Mg^{2+} ATPase activity. Like oligomycin, kepone is inhibitory to DNP-stimulated Mg^{2+} ATPase activity. Oligomycin was shown to inhibit Mg^{2+} ATPase activity in mitochondria by 100 per cent at a concentration of 10^{-7} M [18, 19, 35, 36], whereas kepone produced a 100 per cent inhibition of the enzyme activity at 10^{-5} M in rat liver. The difference in concentration required for both compounds may well reflect differences in the potency of the two compounds or, on the other hand, differences in permeability or access to the site of action. Kepone has a cage-type carbon structure with ten carbons whose ten valences are occupied by chlorines and two by an oxygen to form a carbonyl group. Although the structure of oligomycin is not established, oligomycin B is known to have a very complex chemical formula, $C_{15}H_{72}O_{12}$, and to contain three double bonds in a conjugated diene, a separate unsaturated carbonyl structure, four secondary hydroxyl groups and probably a lactone [37]. Although both compounds differ greatly in their chemical structure, results reported here suggest that kepone has a mode of action similar to that of oligomycin on Mg^{2+} ATPase in rat liver mitochondria. Sensitivity of the soluble oligomycin-sensitive mitochondrial ATPase to oligomycin is known to be conferred by a lipo-protein complex called oligomycin sensitivity conferring protein (OSCP) [38, 39]. Further experimental evidence would be required in order to determine if sensitivity of mitochondrial ATPase to kepone is conferred by a similar mechanism.

Pre-exposure of rats to kepone results in impaired hepatobiliary function [13]. Biliary excretion of the metabolites of the tricyclic antidepressant imipramine was markedly suppressed by pre-exposure to kepone. This effect was also demonstrated by adding kepone prior to the addition of imipramine metabolites to the perfusate of isolated perfused liver preparations [28]. The demonstration that kepone added to the perfused liver preparations effectively inhibits DNP-stimulated Mg^{2+} ATPase as well as the native Mg^{2+} ATPase activities indicates that kepone interferes with energy production in the intact liver. Although microsomal Na^+-K^+ ATPase may be more directly involved in the transfer of substances across the cell membranes to the bile canaliculi, inhibition of ATP production might be expected to adversely affect the transport of substances across cell membranes. Since Na^+-K^+ ATPase activity in fish brain preparations was shown to be sensitive to kepone [15], it is likely that hepatic microsomal Na^+-K^+ ATPase would also be sensitive to kepone. The inhibition of hepatic mitochondrial Mg^{2+} ATPase activity by pre-exposure of animals to kepone [27] and after liver perfusion with 5×10^{-4} M kepone is consistent with the observations that similar treatments

result in impaired hepatobiliary function [28]. In view of the above observations, aberration of energy metabolism appears to be an attractive mechanism for kepone-induced modification of hepatobiliary function and deserves further attention.

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