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Alterations in Hepatic Microsomal Proteins of Mice Administered Mirex Orally

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The chlorinated aliphatic pesticide, Mirex has been used effectively to control fire ants which have become a severe problem to agriculture and health in the southern part of the United States. This compound has not only proven to be a persistent environmental contaminant (HOLDEN 1976), but is resistant to attack by microorganisms, mammalian detoxification mechanisms, and when photodegraded by sunlight, is transformed to two toxic monohydro derivatives, one of which is kepone (CARLSON et al. 1976).

Mirex persists in mammalian tissues where, for example, in the rhesus monkey, pharmacokinetic studies have revealed that Mirex has such a long biological half-life that even a 5-year projection was insufficient (WIENER et al. 1976, PITTMAN et al. 1976). Mirex accumulates in living systems, particularly mammals, because of its highly lypophilic nature and it undergoes little, if any biotransformation to more water-soluble derivatives (GIBSON et al. 1972, STEIN et al. 1976). In chronic toxicity studies, Mirex has been found eventually to bring about measurable biochemical alterations in mammalian species at concentrations as low as 1 ppm in the diet. For example, at a concentration of 1 ppm or higher, Mirex caused an increase in hepatic DNA synthesis of 130-150% in Charles River CD mice, and at higher concentrations, it stimulated mitochondrial respiration, increased coupling and decreased membrane permeability (BYARD et al. 1975). Mirex also produced hepatic enlargement and proliferation of the smooth endoplasmic reticulum (SER) of the parenchymal cell in many of the species studied (BYARD et al. 1975, BAKER et al. 1972, FABACHER and HODGSON 1976, GAINES and KIMBROUGH 1970, KENDELL 1974, MEHENDALE et al. 1977, FULFS et al. 1977). Liver enlargement and induction of the SER are not necessarily considered toxic lesions, since these effects are seen with a large number of compounds (SCHULTE-HERMANN 1974). However, many compounds, as is the case with Mirex, are suspect in the concomitant development of liver nodules after inducing liver enlargement and proliferation of the SER (SCHULTE-HERMANN 1974, BYARD et al. 1975, HOLDEN 1976, MEHENDALE et al. 1977). The implication of Mirex in tumor formation in several species (ULLAND et al. 1977) appears to be the primary motivation for the United States Environmental Protection Agency to limit considerably its agricultural uses (HOLDEN 1976).

The purpose of this communication is to report changes

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in hepatic microsomal protein patterns after feeding Mirex to mice for a period of 12 weeks.

MATERIALS AND METHODS

Mice (male, weanlings) were housed in plastic boxes and fed Wayne mouse laboratory diet containing 30 ppm Mirex. Diet and water were provided ad libitum. Mice of the same age receiving a control diet were otherwise treated identically to those fed Mirex. At sacrifice, livers were removed and weighed. The livers were either treated individually or were pooled into one group per analysis. The livers were finely minced with scissors, and approximately 200 mg samples were studied. Liver microsomal fractions were prepared by the method of BAILEY et al. (1974). The livers were processed immediately after sacrifice. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Pharmacia Gel Electrophoresis Apparatus (GE-4) with Pharmacia accessories. The gels were Pharmacia PAA 4/30 precast polyacrylamide gradient gels, and the buffer system consisted of 0.2% SDS, 0.5M urea, 0.1% mercaptoethanol, 0.4M Tris, 0.2M sodium acetate, 0.02M EDTA (pH - 7.4). The gels were then electrophoresed for 6.3 hours at 100 volts (14 ± 1^0). The gels were then stained with Coomassie Blue and scanned at 600 nm with a Beckman Model R112 densitometer with integrator-recorder.

RESULTS

As was found in studies previously reported from these laboratories, (BYARD et al. 1975), the 30 ppm Mirex-treated mice had livers that were found at autopsy to be grossly enlarged, weighing slightly more than twice those of the controls (4.16 g average for 7 experimental mice compared to 2.04 g for 6 control mice).

Isolation of liver microsomes, their solublization in SDS buffer and subsequent SDS-PAGE gave the results shown in Fig. 1. Here slots 1-3 and 7 contained several control liver microsomal preparations while slots 4-6 and 9 contained microsome preparations from livers of several mice fed 30 ppm Mirex. Slot 8 contained a bovine serum albumin standard (MW = 67,000). By closely observing Fig. 1, it can readily be seen that there were both quantitative and qualitative differences between the Mirex-treated and control liver microsomes. Of particular interest are the regions marked "A" and "B" in Fig. 1. It may be noted that region B contained much more highly stained material in the Mirex-treated mice than the control region B. These bands are known to be associated with the cytochrome P-450 system. Region A contained a band which stained to a much greater extent in the Mirex-treated mice than in controls. All other bands on the SDS-PAGE run gels appeared to have no Mirex-related differences.

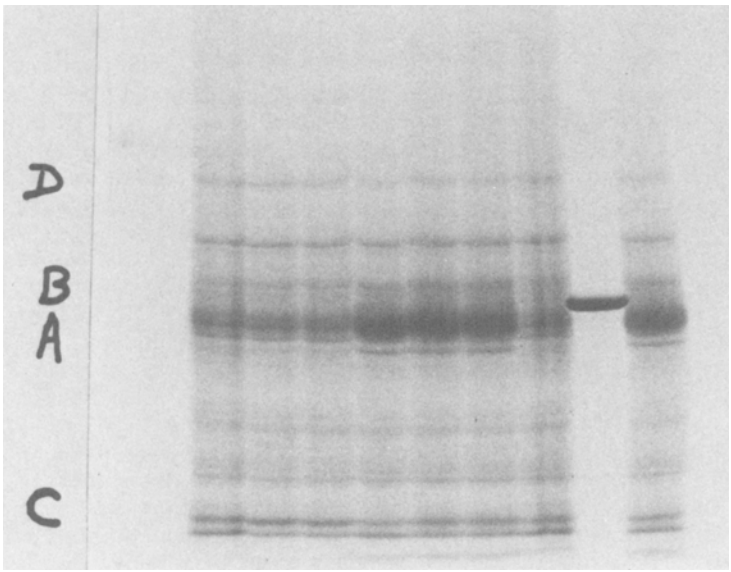

Fig. 1 SDS-PAGE of ribosome-free liver microsomes from Mirex (30 ppm) treated and control livers of mice. Approximately 8 to 11 µg of protein was applied to each slot. The bands migrate from top to bottom (negative to positive electrode). The top of the gel is the 4% polyacrylamide region and the bottom is the 30% polyacrylamide region. The slots are as follows: 1-3, and 7, controls, slots 4-6 and 9, experimentals (30 ppm Mirex) and slot 8, BSA standard (molecular weight ~67,000). The membranes were stripped by the pyrophosphate-citrate method described by BAILEY et al. 1974).

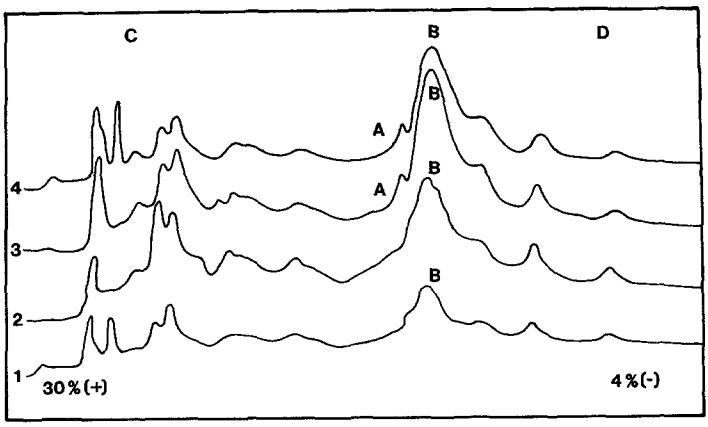

Fig. 2 Spectrophotometric scans (600 nm) of some of the SDS-PAGE runs shown in Fig. 1. (1) Control (8.24 µg of microsomal protein applied); (2) control; (10.94 µg of microsomal protein applied); (3), experimental treated with 30 ppm Mirex (9.06 µg of microsomal protein applied); (4) same as (3) with 10.28 µg of protein applied to the gel. The letters, A, B, C, D indicate regions of the scans discussed in the text. The bands migrate from the 4% region to the 30% region.

Fig. 2 presents spectrophotometric scans of some of the bands of Fig. 1. These scans show quantitative as well as qualitative differences between experimental and control microsomal proteins. The most important difference, as previously noted in Fig. 1, was the band designated in Fig. 2 as the "B" region. This band appeared considerably larger in the Mirex-treated liver preparations than it did in the corresponding controls. Calculating the magnitude of these bands from the area under the curve (estimated from the integrator associated with the spectrophotometric scanner), and the total amount of microsomal protein applied to the specific slot on the gel, it is found that the protein content of the experimental band B is about 1.6 times that of band B of the controls (ratio of 18.2 $\pm$ 0.5 to 11.5 $\pm$ 3.1).

The band designated as "A" in Fig. 2 is obviously more readily discernible in the Mirex-treated animals than in the controls. The same phenomenon was also noted in Fig. 1. It is questionable whether this band exists at all in the control microsomes. Some differences are also seen in the "C" region of the SDS-PAGE gels shown in Fig. 2. These differences did not appear to be Mirex-related and were not investigated further. Bands in the "D" region appeared to be identical in both experimental and control species.

DISCUSSION

SDS-PAGE studies on liver microsomes from mice treated with 30 ppm Mirex for 12 weeks were carried out to determine whether significant alterations in the microsomal proteins after Mirex treatment could be detected. It was hoped that this would give clues as to the nature of the effects that Mirex has on nodule formation in various species, particularly mice. Significant alterations in liver microsomal protein patterns as determined by employing SDS-PAGE have been reported by several investigators for both known carcinogens and compounds capable of affecting proliferation of the SER (CAMERON et al. 1976, HUANG et al. 1976, ALVARES and SIEKEVITZ 1973, WELTON and AUST 1974, and HAUGEN et al. 1976).

BAILEY et al. (1975) using a pyrophosphate citrate method to remove ribosomes from the SER were able to resolve 33-34 component bands on SDS-PAGE. Other investigators have found similar results (NEVILLE and GLOSSMAN 1971). Inspection of Fig. 1, indicates that the SDS-PAGE methods used here are capable of detecting at least 34 bands.

The results presented in Figs. 1 and 2 indicate that Mirex can induce signficant quantitative and qualitative changes in the liver microsomes of mice after treatment for 12 weeks at 30 ppm. The changes that are of significance and appear to be compound-related are seen in regions A and B of Fig. 1 and 2. These regions will be discussed separately.

Comparison with the results of other investigators (BAILEY et al. 1975, CAMERON et al. 1976) and from the position of the BSA standard on the gels (MW 67,000) indicates that region "B" of Figs. 1 and 2 contains proteins associated with the hepatic cytochrome P-450 and P-448 drug metabolizing system. The proteins have molecular weights in the range of 49,000 to 60,000, as determined by SDS-PAGE (BAILEY et al. 1975). When several species are treated with phenobarbital, a model compound for induction of the cytochrome P-450 system, these bands show higher intensity on SDS-PAGE (CAMERON et al. 1976), correlating positively with the ability of the compound to induce the microsomal enzymes (SCHULTE-HERMANN 1974, OESCH 1976, CONNEY and BURNS 1972, HUANG et al. 1976). Mirex also induces cytochrome P-450 and associated drug metabolizing systems as measured by various assay methods (BAKER et al. 1972, FABACHER and HODGSON 1976, GAINES and KIMBROUGH 1970, KENDELL 1974, BYARD et al. 1975, MEHENDALE et al. 1977). Effects of Mirex on inducing hepatic microsomal enzymes, when administered according to various protocols, have been abstracted from the literature and are summarized in Table 1. From these data it is clear that Mirex has a profound effect on inducing the drug metabolizing enzymes. From these data, it can also be seen that Mirex generally induces enzymes that are not related to its own metabolism (i.e., kepone appears to be the only Mirex metabolite, if there is any metabolism at all, (STEIN et al. 1976). The results presented in Fig. 1, (region B), are also in accord with the concept of a general induction of the microsomal enzyme system by Mirex, where region B appears to consist of a broad band of proteins. Similar results are seen in the same region (proteins of MW 49,000 - 60,000) for phenobarbital (CAMERON et al. 1976). Of particular interest to studies presented here, are the results of BYARD et al. (1975), which demonstrate that weanling mice fed 30 ppm Mirex for 10 weeks showed a 190% increase above controls for N-demethylase activity towards p-chloromethylaniline (Table I). This finding correlates satisfactorily with our observation (Fig. 2), that 30 ppm Mirex fed to mice for 12 weeks results in a 160 + 35% increase in the protein present in region B. Thus, determination of microsomal enzyme induction may serve as a rapid, routine, alternative technique in determining the ability of a given compound to induce the hepatic microsomal enzyme system. It also seems likely, due to recent advances in isolation and identification of enzyme activities of components of the microsomal cytochrome P-450 system, that individual components will be quantiated by electrophoretic gel techniques (HUANG et al. 1976, STANTON and KHAN 1976).

Perhaps the most significant observation concerning the effects of Mirex on hepatic microsomal enzymes is the appearance

of band "A" shown in Figs. 1 and 2 for the 30 ppm Mirex treated mice. This band moves ahead of the cytochrome P-450, P-448 enzyme system (band "B") and thus must have a molecular weight less than 50,000. Comparison of these results with those obtained by CAMERON et al. (1976) indicates that this band does not appear in SDS-PAGE of liver microsomes of rats treated with phenobarbital, 3-methylcholanthrene, benzo[α]pyrene or controls. The administration of these 3 compounds, however, does induce bands of higher molecular weight, with methylcholanthrene and benzo[α]pyrene inducing bands of still higher molecular weight than those induced by phenobarbital. CAMERON et al. (1976) have also presented evidence (SDS-PAGE) that 2-acetylaminofluorene 2-AAF) and diethylnitrosamine (DEN) induce microsomal bands in rat liver of lower molecular weight (i.e., less than 50,000) than those induced by phenobarbital. These two compounds each induce two bands in positions close to, if not identical to, the position of band A seen in Figs. 1 and 2. They also induce the same bands as induced by phenobarbital and presumably by Mirex (Region B of Figs. 1 and 2).

As pointed out by CAMERON et al. (1976) it is unlikely that these faster moving bands (near or equal to "A" of Fig. 1 and 2) induced by AAF and DEN (Mirex also) are protein contaminants, since they appear in both stripped rough and smooth endoplasmic reticulum membrane fractions.

Table 1. Summary of Previous Observations on the Induction of Hepatic Microsomal Enzymes by Mirex

Mirex Concentration	Enzyme System	% of Controls	Time of Exposure	Species	Age (Wt.)	Sex	Reference	Comments
5 mg/kg	P-450	100	14 days	Mice	6 Wks	M	BAKER et al (1972)	Diff. Spec Method
10 mg/kg	"	189 ± 100	"	"	"	"	"	"
25 mg/kg	"	270 ± 110	"	"	"	"	"	"
5 mg/kg	"	100	"	Rats	(101-140g)	F	"	"
10 mg/kg	"	100	"	"	"	"	"	"
25 mg/kg	"	158 ± 56	"	"	"	"	"	"
100 mg/kg	"	246 ± 33	"	"	"	"	"	"
1 ppm	Type 1 Binding	200	"	"	"	"	"	"
25 ppm	"	230	"	"	"	"	"	"
100 ppm	"	490	"	"	"	"	"	"
250 ppm	"	530	"	"	"	"	"	"
1 ppm	Type 2 Binding	170	"	"	"	"	"	"
25 ppm	"	208	"	"	"	"	"	"
100 ppm	"	860	"	"	"	"	"	"
250 ppm	"	698	"	"	"	"	"	"
10 ppm	O-demethylase	490	4+ days	Mice	4 days	M	FABACHER & HODGSON (1976)	(a)
10 ppm	O-demethylase	372	4+ days	Mice	4 days	F	FABACHER & HODGSON (1976)	(a)
10 ppm	N-demethylase	930	"	"	"	M	"	"
10 ppm	"	540	"	"	"	F	"	"
1 ppm	N-demethylase	130	70 Wks	Mice	Weanling	M	BYARD et al (1975)	(b)
5 ppm	"	110	60 Wks	"	"	"	"	"
15 ppm	"	250	25 Wks	"	"	"	"	"
30 ppm	"	190	10 Wks	"	"	"	"	"
90 ppm	"	320	1 Wk	"	"	"	"	"
1 mg/day	O-demethylase	226	7 days	Rats	Weanling	F	CHADWICK et al (1977)	(c)
1 mg/day	oxidative hydrolysis	412	"	"	"	"	"	"
1 mg/day	azo group reduction	261	"	"	"	"	"	"
1 mg/day	lindane hydroxylation	164	"	"	"	"	"	(d)

(a) Mothers fed Mirex for 4 days; pups continued to nurse.
(b) Parallel increases seen in Cy-P450 and 4-biphenylhydroxylase activities.
(c) Mirex was the most potent inducer of the 7 chlorinated pesticides studied.
(d) DDT was a more potent inducer of lindane hydroxylation than Mirex.

The fact that Mirex induces a band close or nearly identical to that induced by compounds undergoing N-hydroxylation (i.e., 2-AAF and DEN) raises a question as to whether Mirex is also capable of inducing such enzyme systems and with what consequences. The work of BYARD et al. (1975) (Table 1) would appear to indicate that Mirex is indeed capable of inducing N-hydroxylating enzymes. The consequence of this induction, its effects on metabolism of other chemicals, and its relation to liver nodule formation remain to be elucidated.

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