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Test for In Vivo Conversion of Mirex to Kepone

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In the course of an epidemiologic study of pesticide contents of human breast milk, it was suspected that low levels of the insecticide kepone were appearing in a few specimens. These had been submitted by persons whose exposure to kepone was most unlikely but who lived in regions of the South where some contact with the more extensively used mirex could have occurred. Kepone in the tissues of individuals exposed to mirex could be due either to absorption of environmentally generated kepone, or to conversion of absorbed mirex into kepone in the gut or body tissues. Significant conversion of mirex to kepone has been shown to occur in soil (CARLSON et al. 1976). Kepone is apparently one of many products formed in the process of mirex photodegradation (IVIE et al 1974), but it is not known whether kepone can be formed in the course of tissue metabolism of mirex. When mirex was fed to rats (GIBSON et al. 1972), large proportions were retained in body fat; of that excreted, essentially all was eliminated in the feces as unmetabolized mirex. Kepone was not sought in the tissues of these dosed animals.

Mirex is a stable organochlorine pesticide extensively used for over a decade to control fire ants in the deep South. Its toxicity for mammals is relatively low (GAINES and KIMBROUGH 1970). In common with many organochlorines, it does induce the drug-metabolizing enzymes of the liver (BYARD et al. 1975).

Kepone is another insecticide that is usually incorporated into bait. It is substantially more toxic to laboratory rodents than mirex (GAINES 1969). This toxicity became painfully manifest in humans at a kepone production plant in Hopewell, Virginia when workers were massively exposed in 1974 (TAYLOR et al. 1976).

Subsequent to the suspected detection of kepone in some breast milk samples, it has been determined that the original identification was probably erroneous. Even so, the extensive environmental distribution of mirex in past years, its known penetration into some seafood for human consumption (BORTHWICK et al. 1974, MARKIN et al. 1974), and its apparent presence in some specimens of human adipose tissue (KUTZ et al. 1974), justify an empirical determination of the convertibility of mirex to kepone in mammalian tissues.

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METHODS

Animal Dosing and Sample Collection:

Five Pitman-Moore minipigs weighing 11 to 15 kg were given 50 mg of pure mirex for 7 successive days. Mirex dissolved in corn oil was mixed into semisolid food (skimmed milk base with added casein, dextrose, minerals and vitamins) to accomplish dosing. Essentially all of the mixture was consumed by the pigs. Samples of blood (taken by jugular puncture), urine (drained from metabolic cages), and back fat (taken by open biopsy) were collected before dosing, and again on the day following the final dose. Allowing nine days after the final dose for any slow biotransformation of mirex in tissues, the animals were sacrificed and autopsied. All samples, including plasma, red cells, back fat, liver, kidney, and brain were analyzed for mirex and kepone. The animals exhibited an average 41% weight gain in the 16 day period of the experiment, which is approximately the average for the minipig in this stage of development.

Analytical Methods:

Methods of analysis for mirex and kepone were developed from published procedures for organochlorine pesticides in biological materials (THOMPSON 1977, BEVENUE et al. 1968, RADOMSKI and FISEROVA-BERGEROVA 1965).

Laboratories of the Epidemiologic Study Programs at Iowa and South Carolina collaborated in this study, analyzing the samples essentially in duplicate.

The Tracor-Model 220 gas chromatograph was fitted with 6 ft x ¼ in. OD glass columns packed with 4% SE-30/6% OV-210 coated on Chromosorb W, H.P. 80-100 mesh. Other parameters were: nitrogen flow 70 ml/min; injection port temp. 230°, column temp. 198°, detector temp. 210°C; polarizing voltage 18. Mirex eluted in about 20 min, kepone in about 10 min. Peak identification was based on standards injected just prior to sample injections

Purity of Mirex Fed:

The purity of the mirex fed was tested in two ways. Setting the attenuation of the electron capture detector so that 100 pg of kepone produced a deflection of 60% full scale, 1600 pg of benzene-dissolved mirex were injected. No deflection of the flat base line occurred at the retention time of kepone, thus excluding the presence of as much as 5 pg kepone. This was sufficient to establish that less than about 3 parts per thousand of kepone were present in the mirex.

A much more sensitive test was done by thin-layer chromatography on Eastman #6061 chromatographic plates, and Eastman Chromatogram Developing Apparatus #6071. A drop of mirex solution in hexane (containing 2 mg mirex) was applied to the outer margin of the plate alongside a drop containing 2 mg of kepone. The developing solvent (90% benzene, 10% isopropanol) was allowed to ascend 10 cm toward the center of plate, then the plate was allowed to air dry. It was sprayed with 0.01% Rhodamine B in 95% ethanol, and allowed to dry again. By visual observation, the kepone spot had advanced more rapidly than the mirex. The region ahead of the mirex spot corresponding in R_f value to the kepone was scraped up and poured into a disposable pipette which had been rinsed with hexane and plugged at the tapered neck with glass wool. Five ml of hexane were trickled through the pipette and collected, then concentrated to 0.5 ml. GLC analysis of 5 µl of this extract caused no deflection of the recording pen at the retention time of kepone. Based on the sensitivity of the GLC (10 pg would yield a 4:1 signal-to-noise ratio), this test precluded contamination of the mirex at a level greater than 0.5 ppm. On this basis, a pig could have received no more than about 175 ng kepone in the 350 mg dose of mirex.

Preparation of Samples for GLC Analysis:

In analyzing plasma for mirex, 2 ml were subjected to 2 h rotation with 6 ml hexane on a Roto-Rack. The extract was subjected to GLC analysis without cleanup. For kepone content, 1 ml of plasma was added to 20 ml of 0.1 N H_2SO_4, 12 ml benzene, and a magnetic stirring bar in a 125-ml stoppered flask. The mixture was stirred continuously at 50°C for 20 min, then cooled immediately. The mixture was then centrifuged and the benzene layer was transferred into a graduated test tube. Three successive benzene washes of the centrifugate, using vigorous agitation, were added to the first benzene extract. The benzene was then placed in a 40°C water bath and subjected to gentle stream of nitrogen until it was reduced to a volume convenient for injection into the gas chromatograph.

Red blood cells were thrice washed in saline, then subjected to the same dual analysis as plasma. Five ml portions of urine were similarly analyzed for mirex and kepone. Best recoveries of mirex from fortified samples were achieved by hexane extraction, ranging from 50-100%. Optimal recoveries of kepone were accomplished by the acid-benzene extraction. These amounted to 70-80% in the case of blood components, and 80-100% in the case of urine.

Two-tenths gram of fat were ground in a Duall tissue grinder in the presence of several ml petroleum ether. Mixture was centrifuged, and samples were subjected to GLC analysis directly, without concentration. (RADOMSKI and FISEROVA-BERGEROVA 1965).

One-half gram quantities of either liver or kidney were ground in Duall tissue grinders in several ml of acetonitrile. After centrifugation, supernatant was taken off by pipet, and tissue was re-extracted twice with additional volumes of acetonitrile. Twenty-five ml of 2% aqueous Na_2SO_4 were added to the collected volume of acetonitrile, and this mixture was agitated vigorously. Pesticide was then extracted into 3 serial additions of hexane and these were combined. Volume was reduced to 0.3 ml by a nitrogen stream, and the concentrate was applied to the top of a micro-Florisil column prewetted with hexane. Three eluate fractions were collected as follows: I: 12 ml of hexane, followed by 12 ml of 1% methanol in hexane; II: 12 ml of 1% methanol in hexane; III: 12 ml 5% methanol in hexane. Study of elution patterns demonstrated that essentially all of the mirex was recovered in fraction I, while kepone appeared almost entirely in fraction III. Eluates were concentrated by a stream of nitrogen for GLC analysis.

Analysis of brain tissue involved several steps in addition to those used for analysis of liver and kidney (THOMPSON 1977). Following acetonitrile extraction, addition of Na_2SO_4 solution, and extraction into hexane, the hexane extracts were concentrated to 0.5 ml. Three-tenths ml acetic anhydride and 0.3 ml pyridine were added. This mixture was incubated for 30 min at 60°C. Nine ml of 2% aqueous Na_2SO_4 were added to the cooled mixture. Pesticide was then extracted twice into 3 ml volumes of hexane. After concentration to 0.3 ml by a stream of nitrogen, the hexane extracts were subjected to Florisil column fractionation as described under analysis of liver and kidney. Eluates were concentrated and analyzed by GLC.

To test the adequacy of methods used to detect kepone, tissues and fluids from a 6 kg minipig fed 30 mg kepone 24 h prior to sacrifice were analyzed. The following concentrations of kepone, in ppm, were found:

Plasma	4.2	Urine	.01	Liver	25.7	Heart	28.3
Fat	6.0	Kidney	9.8	Spleen	22.1	Adrenals	196

Comparisons of peak height responses in diluted eluates with base-line noise levels from undiluted eluates (signal to noise ratios) indicated that detection of kepone would have been quite certain at minimum concentrations of 5 ppb in plasma and urine, and 25 ppb in fat and other tissues. Methods published subsequent to our own studies have shown that benzene is superior to aliphatic solvents for extraction of kepone from tissues (MOSEMAN et al. 1977, HODGSON et al. 1978). Using benzene instead of hexane as a partitioning solvent against acetonitrile, we confirmed a 3-fold improvement in recovery of kepone from kidney tissue, and a 46% increase in yield of kepone from liver tissue.

Our original methods employed benzene in the extraction of blood and urine for kepone, and therefore were optimal for its detection in these media.

The extraordinary concentration of kepone in the adrenal tissue of the kepone-dosed pig is noteworthy.

RESULTS

Ample absorption and penetration of mirex into tissues is documented by the data in tables 1,2 and 3, which represent average concentrations of mirex as determined by the two laboratories.

Mirex was absent from the blood, fat, and urine of 4 pigs before dosing, but appeared in minute concentrations in the backfat and blood of one animal. Contamination of sample containers may account for this result.

Blood mirex concentrations declined dramatically in the nine days after dosing, but backfat levels remained high, and may have increased slightly. This behavior indicated that mirex was excreted more rapidly than it moved from storage in adipose fat into the plasma. The distribution of mirex between plasma and red cells indicated substantial cellular penetration. Higher concentrations of mirex in liver than in kidney or brain were regularly observed. This may well be a reflection of strong binding of mirex to soluble liver protein (GAINES 1969).

Mirex was found in urine in amounts ranging from a trace to 0.2 ppm in the samples collected at the end of dosing. These small quantities could have resulted from contamination of the collection pan by spilled feed or feces. The results cannot, therefore, be taken as positive evidence of urinary excretion of mirex, although this may have occurred. Evidently, urinary excretion of unmetabolized mirex by minipigs must be very small, as it is in the rat (GIBSON et al. 1972).

No kepone was detected in any of the samples of plasma, red cells, urine, or fat tissues taken before dosing, at the end of dosing, or after dosing. Nor was any kepone identified in liver, kidney, or brain tissues analyzed at the end of the study. Taking into account the previously cited minimum detection levels for kepone in tissues, these results indicate that no more than 0.13 per cent of the 350 mgm dose of mirex could have been converted to kepone during this 16 day study (25 µg/kg x 18 kg final weight), unless 1) such conversion occurred in tissues that were not analyzed, or 2) the kepone was rapidly excreted. If no conversion occurred in the liver, it seems unlikely that other tissues would accomplish this oxidation. As kepone is storable in the tissues of man and rat (COHN et al.

TABLE 1

Concentrations of Mirex in Plasma Before Dosing, at the End of Dosing and Nine Days After Dosing, in ppm

	Before Dosing		At End of Dosing		9 Days After Dosing	
Pig No.	Plasma	RBC	Plasma	RBC	Plasma	RBC
1	.001	0	.47	.15	.04	.01
2	0	0	.28	.13	.04	.02
3	0	0	.26	.10	.04	.01
4	0	0	.40	.10	.04	.01
5	0	0	.30	.12	.03	.01
Averages	0	0	.34	.12	.04	.01

TABLE 2

Concentrations of Mirex in Backfat Before Dosing, At End of Dosing, and Nine Days After Dosing, in ppm

	Before Dosing	At End of Dosing	9 Days After Dosing
Pig No.			
1	0.2	28.4	33.6
2	0	48.7	42.7
3	0	43.5	47.2
4	0	32.3	42.8
5	0	45.9	41.3
Averages	0	39.8	41.5

TABLE 3

Concentrations of Mirex in Liver, Kidney and Brain Tissues Nine Days After Termination of Dosing, in ppm

	Liver	Kidney	Brain
Pig No.			
1	1.37	0.44	0.84
2	1.16	0.42	0.66
3	1.22	0.48	0.53
4	1.26	0.36	0.50
5	1.19	0.49	0.53
Averages	1.24	0.44	0.62

1976, GIBSON et al. 1972), it seems equally certain that significant amounts would have been retained in the pig tissues during the 16 days of observation. Because optimal methods for extraction and detection of kepone were applied to specimens of plasma and red cells, the percentage conversion of mirex to kepone that would have been detected by our study is probably much lower than the 0.13% level cited above.

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