Table I. Recoveries of NAA from Apples

Added, P.P.M.	% Recovery 283 mμ	370 mμ
0.15	102.7	64.7
0.15	90.7	...
0.25	91.2	75.6
0.25	85.2	89.6
0.50	99.0	85.6
0.75	92.0	80.1

differences in these steps of the method will produce correspondingly small differences in the column separation. Adoption of this technique makes it possible to collect the NAA in the smallest practical volume of eluate, and the check values are also kept to a minimum. In the experiment described, all of the NAA was contained in the 50-ml. fraction except in the case of a single pair of samples and corresponding check and recovery. In that instance, where a small amount of NAA appeared in the last 10-ml. fraction, the values obtained for both fractions were added together.

Analysis. Ultraviolet. Dissolve each fraction of each sample in 5 ml. of $CHCl_3$. Measure the absorbance of these solutions with a Beckman DU spectrophotometer in a 1-cm. cell at 283 mμ with $CHCl_3$ as a reference.

Colorimetric. Add 5 ml. of 0.1% $NaNO_3$ in concentrated H_3PO_4 (prepare by heating to dissolve the salt) to the evaporated residue of each fraction. Nitrate the fractions by heating on a steam bath with occasional swirling for 10 minutes. Allow the samples to cool about 15 minutes, and read at 370 mμ in a 1-cm. cell using the $NaNO_3$-H_3PO_4 solution as a reference. The yellow color is stable for at least 2 hours. A check and recovery are run with each pair of samples.

Standard Curve. Pipet 0, 2, 5, 10, and 15 ml. of a standard NAA solution containing 5 μg. per ml. into a series of 50-ml. beakers and make up to 15 ml. with water. Evaporate to dryness on a steam bath and analyze as described for the samples. This technique corrects for any error introduced by the addition of different volumes of water. Any losses caused by evaporation at 100° C. would also be corrected for.

Table II. Analysis of Field Treated Samples

Harvest Date	Interval	Residue, P.P.M. 283 mμ	370 mμ
20 P.P.M. Application			
9/19[a]	1 hr.	0.0	0.0
		0.0	0.1
9/20	24 hr.	0.0	0.0
		0.1	0.1
9/29	10 days	0.0	0.0
		0.0	0.0
200 P.P.M. Application			
9/26[a]	1 hr.	0.5	0.3
		0.6	0.2
9/27	24 hr.	1.2	1.1
		0.8	0.7
10/3	7 days	0.0	0.1
		0.0	0.1

[a] Application date.

Results and Discussion

Apple trees were treated with the normal spray concentration of 20 p.p.m. for drop control. A separate tree was sprayed at 10 times the normal rate or 200 p.p.m. to measure the disappearance of NAA from the fruit more accurately.

Table I shows recoveries of NAA added to fruit before extraction. For the ultraviolet determination, checks averaged 0.095 p.p.m. with a standard deviation of 0.032. For the colorimetric determination, checks averaged 0.134 p.p.m. with a standard deviation of 0.024.

Table II shows the results of these two experiments. Where 20 p.p.m. of NAA was applied, the residue after 1 hour is less than 0.1 p.p.m. Where 200 p.p.m. was applied, in 7 days the residue of NAA had declined to below 0.1 p.p.m.

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INSECTICIDE METABOLISM

The Detection of Dieldrin Metabolites in Human Urine

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Dieldrin (1,2,3,4,10,10-hexachloro-6,7 - epoxy - 1,4,4a,5,6,7,8,8a-octahydro - 1,4-*endo-exo* - 5,8 - dimethanonaphthalene) has found extensive use in this country and in many other areas of the world as a means of controlling insects of both agricultural and communicable disease importance. The toxicity of the insecticide to spraymen (*10*) and the limited knowledge of its storage (*2, 15*) and elimination (*8*) by humans indicate a need for further studies on the metabolic fate of the compound.

The conversion of aldrin (1,2,3,4,10,10-hexachloro-1,4,,4a,5,8,8a-hexahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene) to dieldrin in mammals was first observed by Barnes as reported by Winteringham and Barnes (*16*), and the conversion was later confirmed by others (*1*). Using houseflies, Brooks (*5*) not only demonstrated the metabolism of aldrin to dieldrin and isodrin to endrin, but also obtained data that indicated further degradation of endrin to its known keto rearrangement product (*3*). Indication of the possible metabolic breakdown of dieldrin itself was reported as early as 1953 by Kunze and Laug (*13*). Using a bioassay technique, these workers were able to detect a toxic material different from the parent compound in both the kidneys and the urine of male rats fed dieldrin in their diets. Further details of this work have not been published. Winteringham and Harrison in 1959 (*17*), using a topical application of sulfur-35 analog of dieldrin on houseflies, detected unidentified metabolites of the compound by paper chromatography in both the body and the excreta of the flies.

In the early work on the toxicity of dieldrin to spraymen, Blazquez and Bianchini (*4*), using a bioassay technique, determined the presence of a toxic material in the blood of men exposed to dieldrin and speculated on the possible elimination of this compound in the bile, feces, and urine. Fletcher (*8*), using the knowledge obtained from investigations on the storage of DDT and the elimination of DDA in man (*12*), determined, by the total

The diphenylamine–zinc chloride colorimetric procedure for the determination of dieldrin in fat has been adapted to dieldrin-derived material in human urine. Application to the urine of men with occupational exposure to dieldrin has given evidence of excreted metabolites not present in the urine of unexposed persons. Study of the excreted unidentified material has involved colorimetric analysis, adsorption chromatography, paper chromatography, and microcoulometric gas chromatography. Dieldrin is eliminated in human urine as at least two neutral, polar, chlorinated metabolites. Unchanged dieldrin is not excreted. However, dieldrin, or a material having the same retention time, can be detected in urine by microcoulometric gas chromatography, through the possible thermal conversion of a dieldrin conjugate to the free insecticide.

chlorine method, the presence of dieldrin or a dieldrin-derived material in the urine of spraymen exposed to the pesticide. His technique involved the isolation of the excreted compound from a 24-hour urine sample. The isolated material was reduced with metallic sodium in isopropyl alcohol, and the chlorides formed were determined by a Volhard titration. The procedure, although giving valuable information, lacks both specificity and sensitivity.

On investigating the possibility that the material in the urine of dieldrin-exposed individuals could be detected using the diphenylamine–zinc chloride method (*7*), we obtained a color reaction of an intensity suitable for colorimetric determination. Urine of unexposed persons submitted to the same analysis gave no color development (*11*).

The present paper is limited to a description of the detection of dieldrin-derived material in human urine and to a very brief consideration of the possible correlation of this material with dieldrin exposure. The methods used involve colorimetric analysis and column, paper, and microcoulometric gas chromatography (*6*). Although the metabolites have not been identified, we believe that the following presentation will contribute to an understanding of the dynamics of dieldrin metabolism and excretion by humans and will possibly permit the development of a simple chemical test for the determination of the extent of absorption of dieldrin.

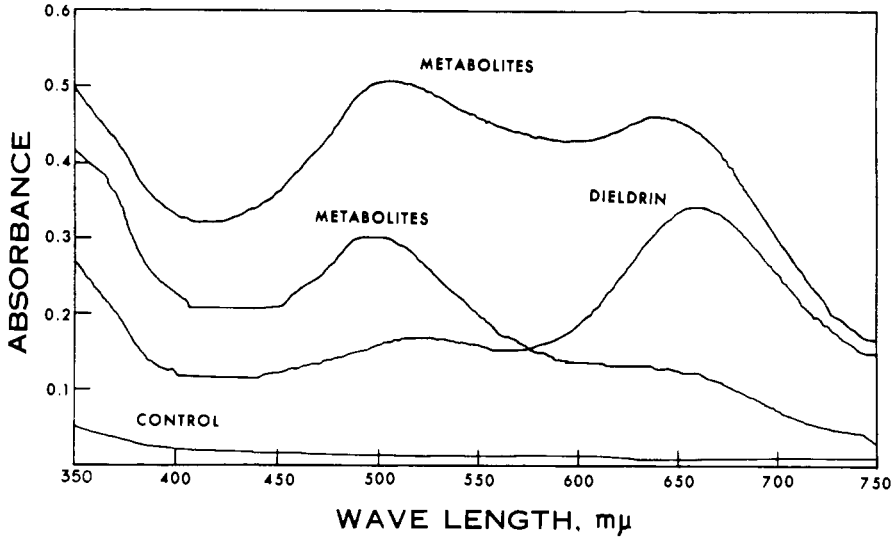

Figure 1. Spectra of material extracted from human urine and subjected to dieldrin colorimetric analysis

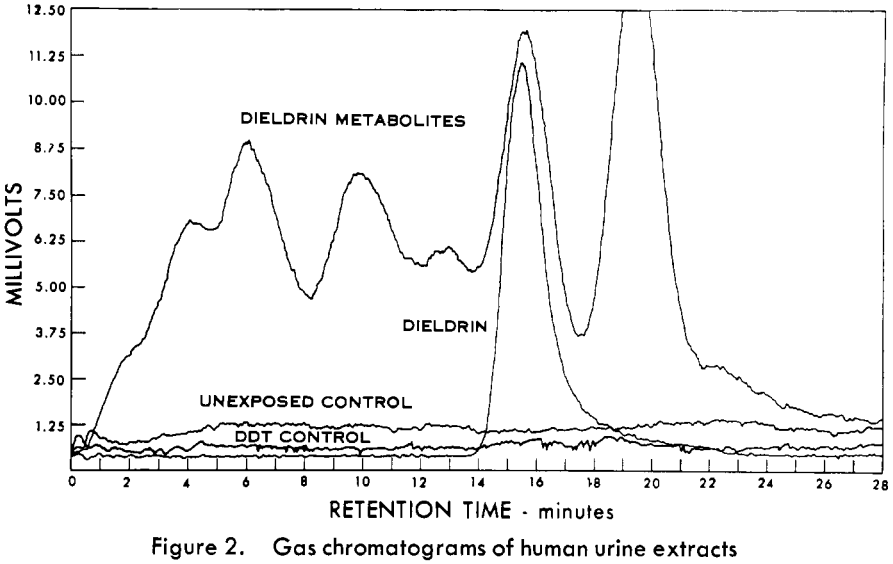

Figure 2. Gas chromatograms of human urine extracts

Procedure

Extraction. The urine specimens used in this study were obtained from men with occupational exposure to dieldrin and from men with no known exposure. Formaldehyde was used as a preservative. The specimens were refrigerated until ready for analysis. One hundred milliliters of the urine was placed in a ground-glass–stoppered bottle, and 50 ml. of purified redistilled *n*-hexane was added. The mixture was shaken vigorously, and the phases were allowed to separate. This operation was repeated three times. The lower urine phase was then aspirated. If any emulsions formed, they were broken by aspirating as much of the urine as possible and then adding 1 or 2 ml. of 95% ethanol. The sample was then gently swirled, the phases were allowed to separate, and the urine was aspirated. The organic solvent extract was washed once with 50 ml. of water, twice with 10 ml. of 2% sodium hydroxide, and once again with 25 ml. of water. The aqueous layers were aspirated after each washing. The final extract was dried with sodium sulfate, and a 40-ml. aliquot was pipetted into a test tube. The sample was evaporated to dryness with a gentle stream of clean air in a 40° C. water bath. The residue obtained was then ready for color development by the diphenyl-

amine–zinc chloride method or for other proposed studies.

Colorimetric Analysis. Two milliliters each of a solution containing 0.25% diphenylamine in *n*-hexane and a solution containing 0.25% zinc chloride in diethyl ether were added to the residue in a test tube. The samples were evaporated to complete dryness in the 40° C. water bath using a gentle stream of air. The residue was fused by placing the tubes in an aluminum heating block at 205° ± 5° C. for exactly 3.0 minutes. The tubes were removed and cooled in running tap water. The colored reaction products were dissolved in 4.0 ml. of a solvent mixture composed of 20% acetic anhydride in glacial acetic acid.

The visible spectrum of the colored complex was determined between 700 and 350 mμ using a recording spectrophotometer. Since the color faded 15 to 20% during the first hour, it was of prime importance that the spectra be recorded within a relatively short and specified time. The regions of main interest were at 650 and 500 mμ. A diphenylamine–zinc chloride reagent blank gave no color development and was used as a reference in determining each spectrum.

This procedure was applied to the urine of persons with occupational exposure to dieldrin as a means of establishing the presence of the metabolites. Urine samples shown to contain relatively high amounts of the degradation products were then used as a source of the metabolites for further studies involving adsorption, paper, and gas chromatography.

Adsorption Chromatography. Adsorption grade and neutral alumina were both used in an attempt to make chromatographic comparisons between the properties of dieldrin and those of the material isolated from urine of men with occupational exposure to dieldrin.

Chromatographic columns (1 × 33 cm.) were packed with the appropriate alumina to a height of 10 cm. The alumina was confined by a plug of glass wool. The material to be chromatographed, contained in 5 ml. of *n*-hexane, was transferred quantitatively to the column. The column was then eluted successively with *n*-hexane solutions containing increasing amounts of diethyl ether. Each eluate was collected as one fraction, evaporated to dryness, and subjected to the colorimetric procedure.

Paper Chromatography. An aliquot representing about 90 ml. of urine was taken from each *n*-hexane extract and used directly for paper chromatography. The technique employed, with the exception of the developing solvent system, was essentially that described by Mitchell (*14*) for the separation of chlorinated hydrocarbon insecticides. A solution composed of 80% methanol, 15% water, and 5% glacial acetic acid was found to give good resolution and satisfactory R_f values. Mineral oil was used as the immobile phase. The chromatogram was developed, sprayed with silver nitrate chromaogenic reagent, and exposed to ultraviolet light, and the R_f value of each spot was calculated.

Gas Chromatography. Twenty-microliter aliquots of various concentrated *n*-hexane extracts representing about 90 ml. of urine were injected into the microcoulometric gas chromatograph described by Coulson *et al.* (*6*). A 6-foot coil of $^1/_4$ inch o.d. aluminum tubing packed with 30/60-mesh acid-washed Chromosorb B containing 20% by weight of high vacuum silicone grease was used as the partitioning column. The temperature of the column was maintained at 215° C., and a nitrogen carrier-gas flow rate of 60 ml. per minute was employed. Oxygen was used in the combustion phase of this technique, and the quartz column was maintained at 800° C.

The hydrochloric acid liberated during the chromatographic-combustion procedure was titrated coulometrically. A constant resistance of 512 ohms was used. The resulting voltage changes due to changes in current during titration were plotted against retention time by means of a potentiometric recorder.

Results and Discussion

Extraction. The extraction procedure described above gave 90 to 95% recovery of dieldrin added to human urine. When this procedure was applied to the urine of exposed individuals, 90% of the *n*-hexane extractable and colorimetrically detectable metabolites appeared in the first extraction. Successive extractions were therefore eliminated.

Earlier work established that the degradation products of dieldrin were equally recovered from acidic, neutral, or alkaline urine. Hydrolysis of the urine with sodium hydroxide or hydrochloric acid by autoclaving at 18 to 20 p.s.i. above atmospheric pressure for 1 hour did not result in an increased recovery of the metabolites. However, extraction under acid conditions or after hydrolysis resulted in an interfering color development if the extraction was not followed by an alkaline wash.

Further studies on the nature of the excreted dieldrin-derived material established it as a neutral compound. A *n*-hexane extract of acidified urine was fractionated into neutral, acidic, and phenolic components, and each fraction was subjected to colorimetric analysis. Only the neutral fraction was found to give a characteristic color development having a spectrum similar to that shown on Figure 1 for the metabolites.

Colorimetric Analysis. Urine samples from spraymen in Ecuador and Panama, and from workers in chemical manufacturing and formulating plants in the Netherlands and in Colorado and Georgia, and from unexposed persons have been analyzed colorimetrically for dieldrin-related material. Figure 1 shows the typical spectra obtained with the metabolites of dieldrin and the spectra obtained with control urine and with dieldrin added to control urine. An alkali-washed *n*-hexane extract of urine from individuals exposed to DDT gave a spectrum identical to that given by control urine.

The spectra of the metabolites were obtained from material extracted from the urine of two different spraymen. The variation in the ratio of the absorbance at 650 mμ to the absorbance at 500 mμ for the two extracts is characteristic of all samples containing dieldrin-derived material. This variation is evidence that at least two materials are present whose concentration ratios vary from sample to sample, causing the differences in the spectra.

Attempts at quantitation are not valid at this time, since the metabolites have not been separated chemically nor identified. However, an empirical basis for comparison of the amount of excreted material is possible and may yield useful information. For this purpose, only the absorbance at 650 mμ was considered, and the results were calculated in terms of dieldrin.

Thus, calculated as dieldrin, the highest concentration of dieldrin-derived material observed in a single sample was 3.4 p.p.m., and the lowest value obtained was 0.1 p.p.m. Fletcher (*8*), using a different method, found an average of 0.4 to 1.1 p.p.m. in the urine of each of four spraymen in a program in which the average daily dermal exposure was 1.8 mg. of dieldrin per kilogram of body weight (*9*). Control urines in the present study have been uniformly negative. However, because dieldrin-derived material has not been detected in the urine of many men known to have exposure, it seems very likely that a low level of excretion of dieldrin metabolites may escape detection by the present method.

The exposure histories of workers we have studied are not sufficiently detailed to permit a conclusion about the relationship between the degree and duration of exposure to dieldrin and the concentration of derivative material in the urine, or about the relationship of metabolites in the urine and the occurrence of illness. No sample has been received from anyone reported to have experienced severe poisoning at any time. Several workers who sent samples reported that they had suffered symptoms (insomnia, nervousness, dizziness, tremor, jerking of an arm or leg) that may have represented mild poisoning as well as

Table I. Behavior of Dieldrin and Metabolites in Alumina Columns

	Total Absorbance at 650 Mμ, %			
	Neutral Alumina		Adsorption Alumina	
Eluates[a]	Metabolites	Dieldrin	Metabolites	Dieldrin
n-hexane	0.0	0.0	0.0	0.0
20% ether	3.8	90.7	3.5	91.6
25% ether	28.8	3.9	27.6	3.5
30% ether	9.8	2.9	17.4	2.2
50% ether	18.2	1.9	19.9	1.7
Pure ether	39.4	0.6	31.6	1.0

[a] A total volume of 120 ml. of each eluate was collected and subjected to colorimetric analysis.

Table II. Paper Chromatography of Extracts of Human Urine

	R_f Values of Spots		
Extracts	A	B	C
Dieldrin[a]	0.10	0.45	...
Metabolites[b]	0.10	0.32	0.77
Control urine	0.11	...	...

[a] Extract of control urine to which 2 p.p.m. of dieldrin had been added.
[b] Extract of urine from spraymen exposed to dieldrin.

other symptoms of more doubtful cause. However, according to the reports, all of the ailments had occurred several weeks or months before the samples were taken, so there is no way to relate a single illness to the amount of dieldrin-derived material in the urine.

Adsorption Chromatography. The data shown in Table I support the evidence from colorimetry that at least two dieldrin-derived materials are eliminated in the urine. This is indicated by the increased recovery of metabolites with the 25% ether in *n*-hexane and the diethyl-ether eluents. Larger volume of eluents and collection of smaller fractions might possibly lead to a more satisfactory resolution of the components.

Since a more polar solvent is needed to elute the metabolites than to elute dieldrin, the data prove that the dieldrin-derived material is not unchanged dieldrin. This conclusion is confirmed by the fact that the spectra of the color developed by the metabolites contained in the second and third eluates have only one maximum absorption peak. This peak appears at 500 mμ and not at 650 mμ, the wavelength of maximum absorption characteristic of the dieldrin color complex.

Paper Chromatography. The results of the paper chromatography studies are summarized in Table II. A material having an R_f value of 0.10 appeared in all samples chromatographed and is considered to be a normal constituent of urine. The R_f values of the two spots obtained with the extract of urine from spraymen exposed to dieldrin are significantly different from the R_f value given by dieldrin added to control urine. The data confirm the findings that at least two metabolites are excreted, neither of which is unchanged dieldrin.

Gas Chromatography. Figure 2 shows the typical gas chromatograms obtained with recrystallized dieldrin and with the alkali-washed *n*-hexane extracts of urine from unexposed individuals, DDT-exposed volunteers, and dieldrin-exposed spraymen. The DDA present in the urine of the volunteers was removed, of course, by the alkali wash used in the technique for dieldrin-derived material.

The history of exposure to dieldrin and the chemical basis of the microcoulometric titration establishes the metabolites detected in urine of spray men as chlorinated material derived from dieldrin. The metabolites are not derived from possible ingestion of DDT in food nor from occupational exposure to this insecticide. The DDT control chromatogram establishes this point.

The greater number of apparent metabolites detected by gas chromatography, as compared with the number found by the other techniques used, may be due to better resolution by gas chromatography or to the occurrence of a thermal-chemical reaction in the chromatographic column that results in further conversion of the metabolites. If a dieldrin conjugate is present, it may be thermally split in the column with the release of free dieldrin. The occurrence of such a reaction may be involved in the detection by gas chromatography of what appears to be dieldrin in the urine of spraymen, since unchanged dieldrin was not found by the other techniques described.

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