MEASUREMENT OF INSECTICIDE EXPOSURE

Ether-Extractable Urinary Phosphates in Man and Rats Derived from Malathion and Similar Compounds

A. M. MATTSON and V. A. SEDLAK

Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education and Welfare, Savannah, Ga.

The urinary excretion of malathion-derived materials was studied to develop a chemical method which could be used as a measure of exposure. Following exposure to malathion, ether-extractable phosphatic compounds appear in the urine of man and rats. Quantitative determination was accomplished by a colorimetric method for phosphates. The amounts of these materials were proportional to dosage. Preliminary studies with other similar compounds were made. This method is useful in detecting and evaluating the degree of exposure to malathion. It is far more sensitive than measurement of cholinesterase inhibition.

MALATHION, *S*-[1,2-bis(ethoxycarbonyl)ethyl] *O*,*O*-dimethyl phosphorodithioate, is being used more widely as time goes on, especially in agriculture. A chemical method for analysis of body fluids or tissues which would give a measure of exposure to malathion would be very useful in studying exposure of workers. The excretion of malathion in urine was studied to find a method of analysis which might be correlated with the amount absorbed or ingested.

Reports have appeared concerning the excretion of malathion metabolites in the urine of various animals (*4*, *5*). Studies on mice using phosphorus-32–labeled malathion have shown that large amounts of the malathion-derived material are excreted in the urine, largely as compounds not extractable by chloroform (*5*). However, as the metabolism is complex, solvents other than chloroform or carbon tetrachloride might extract a portion of the metabolites. This presents a method for the extraction and colorimetric analysis of a portion of the malathion-derived materials excreted in the urine of man and rats, and application of this method to some studies of the excretion of malathion.

Urinary Excretion by Rats

Normal urine contains large amounts of inorganic phosphates with relatively small amounts of organic phosphorus compounds. Preliminary results using a colorimetric method showed that when technical malathion was given intraperitoneally to rats there was a definite increase in the amount of organic phosphorus compounds excreted in the urine. However, the increase in relation to the inorganic phosphate was not great enough to enable very accurate determinations to be made. Therefore it was necessary to study the separation of at least a portion of these organic phosphorus compounds before the malathion-derived material could be determined colorimetrically. To facilitate this study radioactive malathion labeled with phosphorus-32 was synthesized. This compound was synthesized as described by March *et al.* (*5*). The first step of the synthesis as described by these authors was eliminated by starting with phosphorus-32–labeled phosphorus pentasulfide. This material was obtained by irradiating phosphorus pentasulfide (technical grade, recrystallized twice from carbon disulfide) in the neutron pile at Oak Ridge for 4 weeks. Radioassay was done in solutions using a dipping Geiger tube. The initial activity of the malathion was 36 counts per second per γ per ml.

In the first experiment four rats were used. One male and one female were given 100 mg. per kg. of malathion intraperitoneally in peanut oil daily for 5 consecutive days, while one male and one female were given peanut oil only. Urine and feces were collected for each 24-hour period. A device was used to separate the feces from the urine during collection, but some cross contamination between feces and urine may have occurred. The urine was first assayed for the total amount of radioactive materials present (Table I). The per cent of the dosage so recovered for each 24-hour period ranged from 73 to 20 (average 46%) for the 5-day period of daily dosage of the animals. No great efforts were

Table I. Excretion of Malathion–Phosphorus-32 Derived Materials in Rat Urine Following Intraperitoneal Injection

Sample No.	Sex	Time Since Dosed, Hours	Urine Volume, Ml.	Total[a] Found by Radio-assay, Mg.	Daily Dose, %		Urinary Material Ether-Extractable, %
					Total in urine	Ether-extractable	
100 Mg. per Kg. per Day for 5 Consecutive Days							
1	M	24	6.9	14.5	73	43	59
	F	24	3.7	10.6	51	40	78
2	M	24	7.0	13.0	65	43	66
	F	24	4.0	8.5	45	33	73
3	M	24	5.5	6.7	34	16	47
	F	24	4.5	9.5	51	36	70
4	M	24	3.2	3.9	20	13	65
	F	24	3.2	5.4	29	20	69
5	M	24	7.5	10.0	50	32	66
	F	24	...	Lost	...	...	..
6	M	48	...	0.180	0.90	0.31	35
	F	48	...	Lost	...	...	..
7	M	72	9.0	0.110	0.6	...	..
	F	72	4.5	0.220	1.2	...	..
8	M	96	6.0	0.050	0.25	0.045	18
	F	96	5.0	0.100	0.53	0.14	26
25 Mg. per Kg. per Day for 5 Consecutive Days							
1	M	24	6.0	2.34	47	24	51
	F	24	4.5	1.65	37	14	38
2	M	24	5.5	2.13	43	27	63
	F	24	4.0	1.67	37	28	76
3	M	24	6.0	2.28	46	23	50
	F	24	6.0	1.79	40	30	75
4 & 5	M[b]	...	...	...	...	...	..
	F	...	...	...	...	...	..
6	M	48	5.0	0.028	0.6	0.1	17
	F	48	4.5	0.110	2.5	1.3	52
7	M	72	5.0	0.019	0.4	0.04	10
	F	72	5.0	0.050	1.1	0.4	36
8	M	96	5.0	0.015	0.3	...	..
	F	96	4.5	0.042	0.9	...	..
9	M	120	6.0	0.010	0.2	...	..
10	F	120	5.0	0.026	0.6	...	..

[a] Calculated as malathion equivalent.
[b] Daily injections continued for 5 days. Samples saved for other uses.

made to obtain quantitative recovery of all the urine; hence some of the variation in percentage recovery may have been due to losses in collection of the urine. However, the assay for total malathion metabolites established the presence of large amounts of these materials in the rat urine. Samples 6 (48 hours), 7 (72 hours), and 8 (96 hours) represent succeeding 24-hour periods of collection after cessation of treatment. The excretion falls off rapidly but was measurable after 96 hours.

Extraction of the urine using various solvents was then tried. Several samples of male and female urine were acidified and extracted with carbon tetrachloride. Any unchanged malathion would have been extracted by this solvent. The amount of such material found represented less than 1% of the dosage. As the amount was so small, the chances of detecting any unchanged malathion when smaller dosages were given would be remote. Therefore, most of the samples were not extracted with carbon tetrachloride. Ether, on the other hand, extracted large amounts of radioactive materials (Table I). The percentages of the daily dosage recovered by ether extraction of the acidified urine ranged from 43 to 13 for the treatment period. This ether-extractable material represented from 47 to 78% (average 66%) of the total malathion-derived material excreted in the urine. The samples collected during 24 to 48 hours (sample 6) and 72 to 96 hours (sample 8) show that ether-soluble malathion-derived materials are excreted for a considerable time after the last treatment.

Having shown that relatively large amounts of malathion-derived ether-extractable phosphatic materials were excreted in rat urine following a dosage at the 100 mg. per kg. level, the dosage level was lowered to 25 mg. per kg. The results with the lower level (Table I) are in general agreement with those found for the higher dosage. The percentage of total intake excreted in the urine was slightly lower but more consistent (ranging from 37 to 47% and averaging 42%). The samples collected for daily intervals starting 24 hours after treatment (samples 6, 7, 8, and 9; 48 to 120 hours) showed excretion of malathion-derived material in amounts detectable by radioassay. The proportion of ether-extractable materials (ranging from 38 to 75% and averaging 69%) for the period of daily treatment was in the same range as found for the higher level of intake. Thus the amount of ether-extractable phosphates would seem to be almost directly proportional to dosage at these two levels of dosage. The degradation of the malathion to materials not extractable by ether such as orthophosphate did not proceed to a markedly greater degree at the lower dosage level. The urine samples collected 48 and 72 hours after the last treatment showed some ether-extractable materials detectable by radioassay. However, by the colorimetric method used they approached or fell below the limits of detectability.

Orally administered malathion was excreted in the same way as the intraperitoneally injected malathion. Male and female rats given 100 mg. per kg. of phosphorus-32 malathion excreted a total of 24 and 48%, respectively, in the urine, of which 31 and 36%, respectively, was ether soluble.

Colorimetric determination of phosphates in the ether-soluble materials gave good correlation with the radioassay. Of greatest importance was the fact that ether extracts of urine from the control animals showed no detectable amounts of phosphate. This indicated that analysis for urinary ether-extractable phosphatic materials could be used as an index of malathion ingestion. Therefore methods for extraction and colorimetric determinations were developed and standardized.

Method of Extraction and Analysis

General Scheme for Extraction and Analysis. The urine samples were acidified and extracted with ether. Anhydrous sodium sulfate was used to remove traces of urine and water containing inorganic phosphates. The ether solution of extracted metabolites was treated with alkali to prevent losses during evaporation of the ether and then the solvent was removed. Water was added to the residue and aliquots were taken for oxidation by perchloric acid (*7*). Colorimetric determinations were usually made by the method of Fiske and Subbarow (*3*). Where greater sensitivity was desired the method of Ammon and Hinsberg (*1*) as modified by Chen, Toribara, and Warner (*2*) was used.

Reagents. Aminonaphtholsulfonic acid reagent, 0.2% of 1,2,4-aminonaphthol sulfonic acid, 12% sodium bisulfite, and 2.4% sodium sulfite.

Ascorbic acid solution (10%), Mallinckrodt U.S.P. ascorbic acid (store in refrigerator).

Ascorbic acid reagent. One volume of 6*N* sulfuric acid, 2 volumes of distilled water, and 1 volume of 2.5% ammonium molybdate are mixed; 1 volume of 10% ascorbic acid is added and mixed well.

This solution must be made freshly each day.

Extraction and Evaporation of Solvent. In this laboratory, an automatic extraction apparatus is used, which limited the maximum urine sample volume to 40 ml. When smaller volumes of urine are used, enough water is added to bring the total volume of urine and water to 40 ml. Two of these automatic extractors are used, enabling ten samples of urine to be extracted simultaneously. The urine is acidified with concentrated hydrochloric acid to pH 2.0 or slightly less. A volume of 40 ml. of ether (analytical grade) is added and the extraction is carried out. Some emulsions are formed, but ordinarily the two phases separated adequately. Any emulsions are carried along with the ether phase. Rapid agitation seems to give less emulsion than does the gentler shaking of the funnels. After the first extraction, the urine is drained off into a beaker. The ether phase is put into a 500-ml. glass-stoppered bottle. A $^{24}/_{40}$ ⧩ joint is placed in the mouth of the bottle. The urine sample is returned to the separatory funnel and extracted again with 40 ml. of ether. The urine phase is then discarded and the second ether solution is combined with the first ether solution.

This solution is dried by adding sufficient solid anhydrous sodium sulfate to break any emulsion present and to take up the water. Careful but vigorous shaking at this point is required to break emulsions and to remove traces of water. The use of the ⧩ joints allows more vigorous shaking than glass stoppers. The sodium sulfate is removed by filtering the ether through a glass wool plug inserted in the stem of a short-stemmed funnel. The filtrate is caught in another 500-ml. glass stoppered bottle. The bottle is rinsed with ether and filtered as before. The ether solution is washed once by adding 2 ml. of 0.1*N* hydrochloric acid and shaking the bottles vigorously. Anhydrous sodium sulfate is again used to absorb the water and the bottles are again shaken. The ether is filtered as before into 300-ml. flat-bottomed $^{24}/_{40}$ ⧩ flasks. Ten milliliters of absolute ethyl alcohol and 1 ml. of 0.1*N* potassium hydroxide are added. Several glass beads are added and the mixture in the flask is refluxed for 10 minutes using cold finger condensers. The ether is then boiled off on the steam bath until only a small volume remains in the flask. The remaining solvents are removed by means of a rotating evaporator until only a small amount of nonvolatile residue remains. A measured amount of deionized (phosphate-free) water is added. The amount of water to be added depends on the amount of phosphatic material expected. Usually 3 to 5 ml. of water are added and aliquots of 2 ml. are taken for oxidation.

Table II. Ether-Extractable Phosphates in Human Urine Following Oral Ingestion of Malathion

(Colorimetric analyses)

Sample No.	Urine Volume, Ml.	Accumulative Time after Ingestion, Hours	Accumulative Time after Ingestion, Min.	Ether Extraction: Malathion equivalent per sample, mg.	Ether Extraction: Urine, p.p.m.	Ether Extraction: Mg./hour	Dose, %	Accumulative % of recovery
58 Mg. Technical Malathion (0.84 Mg./Kg.)								
1	250	1	20	1.26	5	0.94	2.2	9
2	180	2	50	4.70	26	3.10	8.1	44
3	210	5	20	5.62	27	2.20	9.7	86
4	64	7	35	1.55	24	0.69	2.7	97
5	205	16	20	0.43	2	0.05	0.7	100
6	165	22	20	<0.08[a]	<0.5	...	...	...
		Total		13.56	...	...	23.4	...
11 Mg. of 99% Pure Malathion (0.16 Mg./Kg.)								
1	370	1	30	0.38	1.0	0.25	3.5	18
2	370	3		1.02	2.8	0.68	9.2	65
3	127	4	30	0.59	4.7	0.39	5.4	94
4	170[b]	6		0.14	0.8	0.09	1.3	100
5	235	7	30	<0.035[b]	<0.15	...	...	...
6	200	8	30	<0.030[b]	<0.15	...	...	...
		Total		2.13	...	...	19.4	...

[a] Not detectable, method of Fiske and Subbarow.
[b] Not detectable, method of Ammon and Hinsberg.

Table III. Fractionation by Solvents of Extractable Phosphatic Malathion-Derived Materials Found in Human Urine

Sample No.	Accumulative Time after Ingestion	% of Total Solvent-Extractable Material: CCl_4	$CHCl_3$	Ether
2	2 hours 50 min.	62.7	22.5	14.7
3	5 hours 20 min.	42.5	17.9	39.6
4	7 hours 35 min.	18.4	23.0	58.6

Oxidation and Colorimetric Determination. The aliquots are placed in test tubes (175 × 22 mm.). Perchloric acid (0.5 ml. of 60%) is added and the tubes are heated at 190° to 210° C. to evaporate the water and oxidize the organic material. Heating is conveniently done in an aluminum block into which holes to receive the test tubes have been drilled to a depth of 4 cm. When the samples have become colorless, the tubes are removed from the block and cooled.

Color development is usually carried out by the method of Fiske and Subbarow. To the oxidized sample 4.0 ml. of water are added. Then 0.2 ml. of the 5% ammonium molybdate reagent is added and mixed. This is followed by 0.2 ml. of the aminonaphtholsulfonic acid reagent. The color is allowed to develop for 15 minutes and then read at 700 mμ in a spectrophotometer using a 1-cm. cell. Determinations of unknowns are made by calculation using an extinction coefficient determined from malathion standards. The urine metabolites are calculated in terms of malathion. By this method an absorbance of about 0.1 will be found for 35 γ of malathion.

When the more sensitive Ammon and Hinsberg method is used according to the modification of Chen *et al.*, 4 ml. of water are added to the oxidized solution, then 4 ml. of the ascorbic acid reagent. The color is developed by incubation at 37° C. for 0.5 to 1 hour. Colorimetric measurements are made at 820 mμ in a spectrophotometer in a 1-cm. cell. A fourfold increase in sensitivity over the Fiske and Subbarow method can readily be obtained by this procedure.

Excretion by Humans. The method described was applied to human urine obtained from a male who ingested 58 mg. of technical malathion (0.84 mg. per kg.). This dose was below that capable of causing any detectable cholinesterase inhibition. Pre-exposure urine samples showed no detectable amounts of ether-extractable phosphates. The amount of ether-extractable phosphates rose quickly after dosage (Table II) and then decreased to the predosage level. A total of 23% of the dose was recovered after $16^1/_3$ hours and 97% of this was excreted in the first $7^1/_2$ hours. The rate in terms of milligrams per hour was greatest in the sample taken $1^1/_3$ to 3 hours after ingestion. A smaller dose of 11 mg. of malathion (0.16 mg. per kg.) gave similar results (Table II). The percentage recovery was similar. However, metabolites could be detected for a shorter time after ingestion, even though the more sensitive method for detection was used.

Dermal application to a human male

of malathion in a talc dust also produced ether-extractable phosphatic materials in the urine. When 100 mg. of malathion as a 1% dust was applied for 14 hours, 1% of the dose was recovered during this period. With higher amounts of malathion, the percentage recovery of metabolites from the urine was of the same order. No cholinesterase inhibition was detectable in any case.

Nature of Excretory Products. The malathion metabolites in rat urine contained only small amounts of carbon tetrachloride–extractable materials. The human urine, however, contained an appreciable amount of phosphatic material which was carbon tetrachloride–extractable. An experiment was done wherein the urine was extracted successively with carbon tetrachloride, chloroform, and ether. The results of the analysis by the colorimetric phosphate method (Table III) show a change in the composition of the metabolites with length of time after dosage. The carbon tetrachloride–extractable material decreases from 63 to 18% of the total solvent extractable compounds during a 5-hour period. This decrease is reflected in an increase in the material extracted by the ether from 15 to 59% of the total extracted.

The Norris, Vail, and Averell method (*8*) for malathion was applied to the carbon tetrachloride extracts. (This method will be referred to as the Norris method as contrasted to the organic phosphate method.) The Norris method gave a positive test with the carbon tetrachloride–extractable materials. Control samples had no interfering materials. This colorimetric method depends on the hydrolysis of malathion to dimethyl phosphorodithioate salt in alkaline solution. Any compounds which can be hydrolyzed to this material will give a positive reaction. Thus, in addition to malathion, either of the half esters, *S*-[(1-carboxy-2-carbethoxy)ethyl] *O,O*-dimethylphosphorodithioate or *S*-[(2-carboxy-1-carbethoxy)ethyl] *O,O*-dimethylphosphorodithioate is converted to the dimethylphosphorodithioate salt and therefore will give a positive test. The extractability of the half esters by carbon tetrachloride under the conditions used here is not known at present. Therefore, the carbon tetrachloride–extractable material could be malathion, one or both of the half esters, or other unknown compounds. Colorimetric organic phosphate determinations carried out on the same carbon tetrachloride extracts agreed with the results by the Norris method. Thus all of the carbon tetrachloride–extractable phosphatic material was detected by the Norris method.

The urine samples collected after the lower dosage (11 mg.) of malathion did not show the same results as at the higher dosage. From 23 to 27% of the ether-extractable material was found to be carbon tetrachloride–extractable in samples collected at 3 and $4^1/_2$ hours after dosage. However, this carbon tetrachloride–extractable material failed to give a positive color with the Norris method. There is apparently a variability in the nature of the excretory products which may be dependent on amount ingested. Thus the extraction by ether and determination of phosphatic materials are preferable to the Norris method, in that it determines a larger amount of materials for a longer period of time after ingestion of malathion and at lower concentrations of malathion ingestion.

Malathion is readily hydrolyzed by acid chiefly to *O,O*-dimethylphosphorothionic acid and by alkali chiefly to *O,O*-dimethylphosphorodithioate (*6*). It was thought that the ether-extractable urinary metabolites could be materials derived from one of the above dimethyl esters. As phosphorodithioate is rather unstable and is converted at least partially to the phosphorothionate, treating a rat with *O,O*-dimethylphosphorodithioic acid should produce ether-extractable phosphatic compounds in the urine if malathion degradation depended wholly on hydrolysis to these compounds. When *O,O*-dimethylphosphorodithioic acid was given intraperitoneally at the level of 100 mg. per kg. as the ammonium salt in water, the amount of ether-extractable material recovered was only 1% of the dose during the first 24 hours after dosage. This recovery, compared to that found for malathion, indicates that hydrolysis of malathion to the secondary dithio or thionophosphates is not responsible for the formation of the major portion of the ether-extractable urinary malathion metabolites.

Other Organic Phosphorus Insecticides. Some preliminary work was done using other organic phosphorus insecticides to determine whether appreciable amounts of ether-extractable phosphatic materials were excreted in the urine of rats following treatment with them. These compounds were given intraperitoneally in peanut oil to female and male rats and the urine was collected for two 24-hour periods.

The compounds tested were as follows:

I. 2,3-*p*-Dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate) (Delnav) (Hercules Powder Co., Inc., Wilmington, Del.), 10 mg. per kg.

II. *O*-(3-Chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate (Chlorthion) (Chemagro Corp., New York, N. Y.), 50 and 100 mg. per kg.

III. *O*-(2-Chloro-4-nitrophenyl)*O,O*-dimethyl phosphorothioate (Dicapthon) (American Cyanamid Co., Stamford, Conn.), 50 mg. per kg.

IV. *O,O*-Diethyl *O*-*p*-nitrophenyl phosphorothioate (Parathion), 2 mg. per kg.

V. *O,O*-Dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate (Dipterex) (Chemagro Corp., New York, N. Y.), 100 mg. per kg.

One of these compounds is a phosphorodithioate (I), three are phosphorothionates (II, III, IV), and one is a phosphonate (V). The phosphonate (V) was chosen because of its low mammalian toxicity. No detectable phosphatic materials were extracted by ether when V was given. Parathion is so toxic that only a small amount could be given to the rats. No urinary metabolites were detected by ether extraction when parathion was given. However, had the parathion been metabolized in a fashion similar to that of malathion the concentrations in the urine of ether-extractable materials should have been detectable. The phosphorodithioate (I) did show some excretion of ether-extractable phosphates in the urine but only to the extent of 3% for the male and 2% for the female. Such small amounts could possibly be due to impurities. The other two phosphorothionates (III and IV) also showed the excretion of some ether-extractable urinary products. For Chlorthion the recovery was 2% for both the male and female rats. The Dicapthon gave the highest results, with a recovery of 7% for the male and 1% for the female. None of these compounds approaches the recoveries found for malathion. The metabolism of malathion apparently differs from that of the other compounds tested. Nevertheless it is possible that excretion in man differs from that in rats with these latter compounds and that determination of urinary ether-extractable phosphates could be of value in the detection of exposure of man to various organic phosphorus compounds other than malathion.

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Received for review July 10, 1959. Accepted October 14, 1959. Division of Agricultural and Food Chemistry, 135th Meeting, ACS, Boston, Mass., April 1959.