Bovine Metabolism of Organophosphorus Insecticides: Significance of Rumen Fluid with Particular Reference to Parathion

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Bovine rumen fluid hydrolyzed many organophosphate insecticides. Oxidation reactions in the rumen fluid were of little importance and reduction reactions were of very great significance in metabolizing these compounds. A greater proportion of the *p*-nitrophenyl phosphates were recovered as *p*-amino derivatives than with their phosphorothioate analogs because of the much greater hydrolysis rate of the phosphorothioates than the phosphates in the rumen juice. The rate of reduction of radioactive parathion in the bovine rumen fluid in vivo was very similar to the rate with stagnating rumen juice in vitro. Parathion, paraoxon, amino parathion, and amino paraoxon were circulating in the blood and secreted in small amounts in the milk. Amino parathion constituted a major excretory metabolite of parathion in cows along with diethyl phosphoric and phosphorothioic acids. The toxicological significance of these findings are considered in relation to the toxicity of the various derivatives.

ORGANOPHOSPHORUS INSECTICIDES ingested by ruminating animals are exposed to potential metabolic attack by the rumen fluid for a period of time depending on the animal and the chemical. Many microorganisms are active in hydrolyzing (*1*, *18*) and oxidizing (*1*) certain organophosphate esters. In a study initiated and carried on concurrently with the experiments reported here, Cook (*8*) found that bovine rumen fluid was active in metabolism of certain phosphate insecticides in vitro through both hydrolysis and reduction reactions.

The metabolic fate of parathion has been studied in insects, rats, rabbits, dogs, goats, cows, monkeys, and men, and the findings have been adequately reviewed (*5*, *14*). Parathion is oxidized in vivo to paraoxon, which is the active anticholinesterase agent in poisoning. Parathion is not stored in the fat or other animal tissues. The identified mammalian excretory products of parathion are *p*-nitrophenol, *p*-aminophenol, and *p*-aminophenylglucuronide—the ratio of these depending on the experimental animal, the dose, and the route of administration.

Parathion can be fed to cattle at high levels without causing depression of their blood cholinesterase activity or the appearance of detectable parathion in the milk (*9–11*, *19*). However, a potential anticholinesterase agent appeared in the milk of goats fed parathion (*25*). No parathion, free *p*-nitrophenol, or free *p*-aminophenol were present in the jugular blood, urine, and milk of cows fed high levels of parathion (*19*). However, a parathion metabolite in the urine was diazotizable without a preliminary reduction step; it yielded *p*-aminophenol and glucuronic acid on acid hydrolysis; and appeared to be *p*-aminophenylglucuronide, except that it could not be crystallized. Pankaskie, Fountaine, and Dahm (*19*) concluded that *p*-nitrophenol, formed from hydrolysis of parathion in cattle, was apparently reduced to *p*-aminophenol, conjugated to an appreciable extent with glucuronic acid, and excreted in this conjugated form. Their results do not rule out the possibility that *O,O*-diethyl *O*-*p*-aminophenyl phosphorothioate (amino parathion) might be a major excretory metabolite and *p*-aminophenylglucuronide of lesser quantitative importance. This point is subject to reinvestigation at this time because of the demonstration by Cook (*8*) that parathion was reduced to amino parathion by bovine rumen fluid in vitro.

This investigation concerns the significance of rumen microorganisms in the metabolism of organophosphorus insecticides with particular reference to parathion.

Methods

Synthesis of Parathion Derivatives. Radioactive *O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate (parathion) was prepared by the method of Hein and McFarland (*15*) from irradiated phosphorus trichloride (AEC service irradiation at Oak Ridge for 4 weeks at 7 × 10 inch neutrons per sq. cm. per second flux). The technical radioactive parathion was purified by partition chromatography (Figure 1) with celite–iso-octane–methanol (*4*) and was identical in infrared absorption spectrum with known parathion. The yield of purified radioactive parathion from irradiated phosphorus trichloride was 52%.

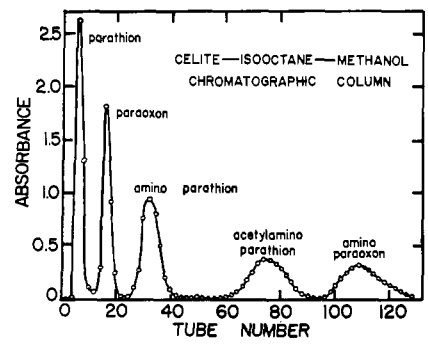

Figure 1. Chromatographic separation of parathion and certain possible metabolites

O,O-Diethyl *O*-*p*-aminophenyl phosphate and phosphorothioate (amino paraoxon and amino parathion) were prepared by reduction of paraoxon and parathion with zinc and hydrochloric acid and purified by partitioning systems designed to separate the aminophenyl derivatives or their hydrochlorides from the nitrophenyl compounds. Chromatography and infrared spectra on the products showed that the paraoxon and parathion had been completely destroyed or removed on purification. Less than 0.012% of parathion was present in the amino parathion based on fly bioassay, and less than 0.01% of paraoxon was present in the amino paraoxon based on anticholinesterase activity (Table I). Reaction of the amino parathion with acetic anhydride gave complete conversion to *O,O*-diethyl *O*-*p*-acetylaminophenyl phos-

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Table I. Comparison of Partitioning Properties and Biological Activity of Organophosphorus

	Parathion	Paraoxon	Amino Parathion	Amino Paraoxon
	Per Cent Partitioning into Organic Phase			
Hexane/2% HCl	100	78	2	0
$CHCl_3$/2% HCl	100	91	79	9
$CHCl_3$/pH 10 buff.	100	92	98	96
	LD_{50}			
Housefly, topical, 24 hr.	0.48	0.46	4000	4200
White rat, oral, 48 hr.	4	4	450	1000–1500
	pI_{50}			
Human blood ChE	4.86	8.10	2.68	3.99

Table II. Recovery of Some Organophosphates Incubated with Rumen Juice, Based on Total Phosphorus Determined Colorimetrically

	Organophosphate	Fresh or Boiled[a]	Per Cent Recovery after Hours Indicated 1	3	6	12	24
1.	$(C_2H_5O)_2P(S)(OC_2H_5)$	Fresh	...	87	70	65	46
2.	$(C_2H_5O)_2P(S)(SC_2H_5)$	Fresh	...	87	62	52	46
3.	$(C_2H_5O)_2P(O)OP(O)(OC_2H_5)_2$ (TEPP tech.)	Fresh	40	21	16	14	9
4.	$(C_2H_5O)_2P(S)OP(S)(OC_2H_5)_2$ (sulfotepp, tech.)	Fresh	...	89	70	65	53
5.	$(C_2H_5O)_2P(S)SP(S)(OC_2H_5)_2$	Fresh	...	79	68	50	23
6.	$(C_2H_5O)_2P(S)SCH_2SC_2H_5$ (Thimet, 99%)	Fresh	71	68	66	50	44
7.	$(C_2H_5O)_2P(S)SCH_2CH_2SC_2H_5$ (Disyston tech.)	Fresh	69	64	58	53	42
8.	$(C_2H_5O)_2P(O)SCH_2CH_2SC_2H_5$ $(C_2H_5O)_2P(S)OCH_2CH_2SC_2H_5$ (demeton, tech.)	Fresh	80	77	74	70	66
9.	$[(C_2H_5O)_2P(S)S]_2$-2,3-*p*-dioxane (Delnav, tech.)	Fresh	67	66	64	55	40
10.	$(CH_3O)_2P(S)SCH(CH_2C(O)OC_2H_5)C(O)OC_2H_5$	Fresh	82	44	40	33	24
		Fresh	80	60	50	36	29
	(malathion, 98%)	Boiled	90	89	83	80	74
11.	$(CH_3O)_2P(S)SCH_2C(O)NHCH_3$ (Dimethoate, 99%)	Fresh	100	98	95	94	92
		Boiled	100	100	97	93	92
12.	$(C_2H_5O)_2P(O)SCH_2CH_2N(C_2H_5)_2$ (R-6199 as free base, 99%)	Fresh	83	78	62	51	45
13.	$(CH_3O)_2P(S)O\phi$-Cl_3-2,4,5 (Trolene, 99%)	Fresh	95	95	91	86	87
		Boiled	94	94	89	89	89
14.	$[(CH_3)_2N]_2P(O)OP(O)[N(CH_3)_2]_2$ (schradan, tech.)	Fresh	...	97	94	87	83
15.	$(C_2H_5O)_2P(O)OCH{=}CHCl$ (OS-1836, tech.)	Fresh	...	91	86	81	69
16.	$(CH_3O)_2P(O)OC{=}CHC(O)OCH_3$ CH_3 (Phosdrin, tech.)	Fresh	...	96	91	79	72

[a] All at 250 p.p.m.—except malathion at 50 p.p.m. and Trolene at 20 p.p.m.—with average of 3 to 5 replicates. Following sources for organophosphates are acknowledged: 1, 2, and 9, Hercules Powder Co.; 3, 4, and 14 Victor Chem. Works; 5, 6, 10, and 11, Am. Cyanamid Co.; 7 and 8, Chemagro Corp.; 12, Imperial Chem. Ind. Ltd.; 13, Dow Chem. Co.; and 15 and 16, Shell Development Co.

phorothioate (acetylamino parathion) based on chromatography and an infrared spectrum. The partitioning properties between *n*-hexane and 2% hydrochloric acid for this acetylamino parathion were intermediate between parathion and amino parathion (Table I), with 64% of the acetylamino derivative appearing in the hexane phase.

In Vitro Studies with Stagnating Bovine Fluid. Bovine rumen fluid was obtained from an alfalfa hay–fed cow fitted with a permanent rumen fistula. All samples for any series of experiments were drawn from the same cow 1 hour after the morning feeding. The rumen fluid was filtered through a double thickness of cheesecloth and adjusted to pH 6.0 with hydrochloric acid, and the insecticide was introduced within 30 minutes after the rumen sample was taken. Other portions of rumen juice were boiled 15 minutes, cooled, and adjusted to pH 6.0 to determine the effect of boiling on the microbial and other enzyme activity.

For hydrolysis studies (Table II), several organophosphate compounds were incubated at a final concentration of 250 p.p.m. with 500-p.p.m. Triton X-155 (Rohm & Haas), for various periods of time at 40° C. The unhydrolyzed organophosphates were extracted from the rumen juice with 3.0 ml. of chloroform, and 1.0 ml. of the chloroform extract was analyzed colorimetrically for total phosphorus. For zero-time determinations the chloroform was added to the organophosphate prior to adding the rumen fluid and the results

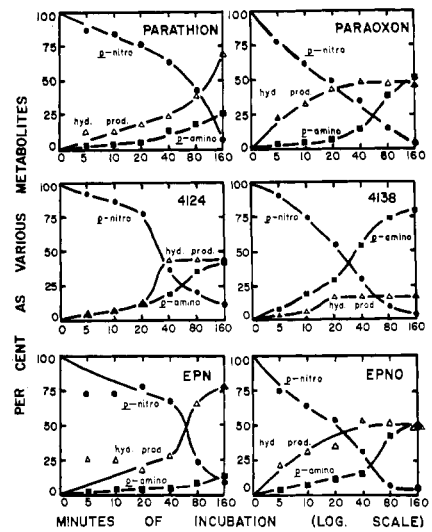

Figure 2. Comparison of in vitro reduction and hydrolysis rates of certain *p*-nitrophenyl phosphates and phosphorothioates by stagnating bovine rumen fluid (compounds at 300 p.p.m., based on total phosphorus determined colorimetrically)

for the hydrolysis studies were expressed as the per cent of total phosphorus recovered in the chloroform, after various incubation times, compared with the zero-time analyses. In the case of nitrophenyl and aminophenyl phosphates and phosphorothioates, the rumen juice was adjusted to pH 9.9 to 10.1 by adding 0.2 ml. of 3*M* sodium carbonate solution before extracting with chloroform.

The possibility of oxidation reactions in the stagnating rumen fluid was investigated with *O,O*-diethyl *S*-(ethylthio)-methyl phosphorodithioate (Thimet). The chloroform-extractable phosphorus compounds from rumen juice, incubated for various periods of time with Thimet, were further fractionated, by partitioning between hexane and acetone water and by chromatography (*4*), to ascertain if oxidation had occurred at the phosphorothioate or thioether groups.

For studying the reduction of nitrophenyl phosphorothioates (Figure 2), the incubation mixtures were prepared in the same way as for hydrolysis studies except that 5.0 ml. of rumen fluid and a final concentration of 300 p.p.m. of organophosphate were utilized with incubation times up to 160 minutes at 40° C. At the end of each incubation period, 0.10 ml. of concentrated (37%) hydrochloric acid was added to each tube to make the fluid 2.0% (v./v.) with respect to concentrated hydrochloric acid, and it was then extracted with 5.0 ml. *n*-hexane. The tube was centrifuged to aid separation of the phases and 1.0 ml. of the hexane layer was analyzed for total phosphorus, this reading representing the unreduced and unhydrolyzed nitrophenyl phosphorothioate. Three milliliters of the acid rumen fluid

were then transferred to a clean tube, adjusted to pH 9.9 to 10.1 by adding 1.0 ml. of 3*M* sodium carbonate and extracted with 3.0 ml. chloroform. Analysis of 1.0 ml. of the chloroform layer for total phosphorus gave the amount of aminophenyl phosphorothioate and analysis of 1.33 ml. of the aqueous phase allowed determination of the hydrolysis products. Zero-time incubation samples were analyzed in the same way except that the hexane was added prior to the rumen juice. This analytical procedure is based on the partitioning properties for parathion and amino parathion shown in Table I.

A modified procedure was used for studying nitrophenyl phosphates (Figure 2) as paraoxon is incompletely extracted into hexane from a 2% hydrochloric acid solution (Table I). After incubation, the rumen juice was adjusted to pH 9.9 to 10.1 by adding about 0.3 ml. of 3*M* sodium carbonate solution and extracted with 5.0 ml. of chloroform. The aqueous alkaline phase, 1.06 ml., was analyzed to determine the hydrolysis products. Three milliliters of the chloroform phase were then transferred to another tube and partitioned with 3.00 ml. of 2% hydrochloric acid. Analysis of 1.00 ml. of the chloroform phase gave the amount of nitrophenyl phosphate, and analysis of 1.00 ml. of the aqueous acid phase allowed determination of the aminophenyl phosphate as its hydrochloride salt. The per cent of the various organophosphate products was then calculated from the zero-time analyses and from known partitioning properties of the compounds (Table I).

Boiling the rumen fluid destroyed almost all activity in reducing the hydrolyzing nitrophenyl phosphates and phosphorothioates and over 90% of the activity in hydrolyzing malathion. Rumen juice diluted up to 1 to 3 with water was still active in reducing parathion but a 1 to 10 dilution was without activity. About 75% of the activity in reducing parathion was found in the particulate fraction from a mild centrifugation.

Metabolism of Parathion by a Lactating Cow. A 6-year-old, 500-kg. Hereford cow with a rumen fistula was placed in a metabolism stall, which allowed separate collection of urine and feces. Water, grain, and alfalfa hay were provided. Radioactive parathion was administered orally in gelatin capsules at 6.7 mg. of parathion per kg. of body weight. A similar 6.7-mg.-per-kg. dose was again administered 5 days later.

Just prior to treating with the first parathion dose, a rumen juice sample was taken and to it was added 2 p.p.m. of radioactive parathion at the same time the radioactive parathion was administered to the cow. A 24-hour comparison was made of the rate of conversion of parathion to amino parathion and hydrolysis products in stagnating bovine rumen juice (in vitro) *vs.* rumen juice in vivo (Figure 4). The partitioning procedure used to fractionate the radioactive compounds in the rumen juice was the same as that described above. The total radioactivity in samples of rumen juice, jugular blood, milk, urine, and feces was determined to calculate the total parts per million of parathion equivalents present (Figure 3). The radioactivity was then fractionated by extracting the sample with an equal volume of chloroform, evaporating the chloroform, and partitioning the residue between hexane and 2% hydrochloric acid to evaluate the per cent of parathion plus paraoxon, amino parathion plus amino paraoxon, and hydrolysis products (Table IV indicates results with milk and Figure 5 with blood). Duplicate urine samples from 0 to 3 and 38 to 47 hours after treatment were adjusted to pH 10 and extracted twice with equal volumes of chloroform. The aqueous phase was chromatographed with carrier phosphoric, diethyl phosphoric, and diethyl phosphorothioic acids on an ion exchange resin (*20*).

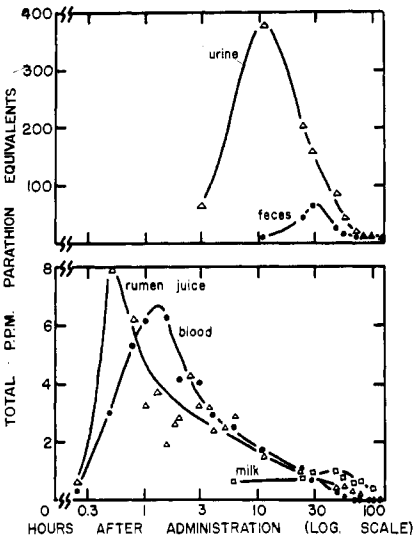

Figure 3. Rate of absorption and distribution of parathion and metabolites following oral administration to a fistulated cow of 6.7 mg. of radioactive parathion per kg. of body weight

After administering the second 6.7-mg.-per-kg. dose of parathion, larger biological samples were taken for characterization of the radioactive metabolites (Table III). Blood, rumen juice, and urine were adjusted to pH 10 and then extracted twice with equal volumes of chloroform. To aid in extractions, the blood was diluted to 5% with distilled water before adding the chloroform and the pH was not adjusted with the milk. Milk samples, 100-ml., were homogenized in a Waring Blendor with 200 ml. of chloroform, the mixture was centrifuged, and the chloroform extract was dried with anhydrous sodium sulfate.

Twenty-gram fat biopsy samples were extracted by homogenizing with 40 ml. of water and 80 ml. of chloroform in a Waring Blendor, centrifuging, and drying the chloroform extract. The chloroform extract of blood, rumen juice, and urine was suitable after evaporation for chromatography, but a further cleanup was needed with the fat and milk samples. The residue remaining on evaporation of the chloroform extracts of fat and milk was partitioned between equal volumes of hexane and acetonitrile to leave the fat in the hexane phase and recover 93% or more of the radioactivity in the acetonitrile phase. The acetonitrile was then removed at reduced pressure. To the residue from chloroform or acetonitrile extractions was added a mixture of 25 mg. each of nonradioactive parathion, paraoxon, amino parathion, and acetylamino parathion.

Following chromatography on a celite–iso-octane–methanol column, the fractions eluted were located by radio-

Table III. Nature of Parathion Metabolites Present after Oral Administration to a Cow of 6.7 Mg. per Kg. of Radioactive Parathion

Biological Fluid	Hr. after Treatment	P.P.M. Expressed as Parathion Equivalents[a] Parathion	Amino parathion	Paraoxon	Amino paraoxon	Hydrolysis products
Rumen juice	0.5	10.0	7.5	0.0	0.0[b]	0.28
	2.0	2.7	8.3	0.0	0.0[b]	0.34
	6.0	0.69	2.6	0.0	0.0[b]	1.6
Blood	0.5	0.18	0.32	0.29	0.12	3.0
	2.0	0.14	0.15	0.14	0.27	4.3
	6.0	0.07	0.04	0.04	0.42	2.7
Milk	6.0	0.04	0.11	0.07	0.61	0.79
Urine	0–24	1.2	170	0.0	8[c]	239

[a] P.p.m. as hydrolysis products is based on radioactivity that could not be extracted from the aqueous phase into chloroform; p.p.m. of other metabolites is based on column chromatography with celite–iso-octane–methanol of the chloroform-soluble metabolites.

[b] Up to 5% of a polar metabolite of parathion was present in these samples, which may have been amino paraoxon, but its properties were not determined.

[c] This urine was extracted for recovery of amino derivatives and yielded amino parathion with no definite absorption bond of P=O at 1260 cm.$^{-1}$

Table IV. Parathion Metabolites Present at Various Times after Oral Administration of 6.7 Mg. per Kg. of Radioactive Parathion

Time, Hr.	P.P.M. Expressed as Parathion Equivalents: Parathion + paraoxon	Amino parathion + amino paraoxon	Hydrolysis products
	Milk		
6	0.057	0.35	0.26
24	0.020	0.23	0.53
47	0.032	0.026	0.92
55	0.015	0.010	0.77
71	0.012	0.011	0.52
77	0.006	0.002	0.53
	Fat Biopsies		
2	0.097	0.19	0.48
6	0.074	0.14	0.57
24	0.028	0.017	0.78

active and total phosphorus analyses. The radioactive peaks eluting from the columns were then separately partitioned between hexane and 2% hydrochloric acid. The identity assigned to the radioactive metabolites was based on chromatographic behavior compared with nonradioactive known compounds and partitioning properties. The total radioactivity in the fat biopsy samples was inadequate for chromatography so only partitioning studies were made in this case (Table IV).

The cholinesterase activity of the whole blood was determined (*6*) on each of the blood samples drawn for radioactive analysis.

Metabolism of Parathion and Amino Parathion by Rats. Three 200-gram male white rats were treated with 10 mg. per kg. of parathion and three with 10 mg. per kg. of amino parathion. Corn oil solutions of the radioactive chemicals were administered with stomach tubes and the rats were held for 48 hours in individual metabolism cages (*7*) to allow separate collection of urine and feces. The total radioactivity excreted in the urine within 0 to 24 and 24 to 48 hours was determined, and the 0- to 24-hour urine was fractionated by the procedure described for the cow urine.

Another group of rats was similarly treated with amino parathion or parathion; they were sacrificed after 2 hours, and their livers were removed for analysis. Each of the six livers was homogenized with 70 ml. of chloroform in a Waring Blendor and extracted with 70 ml. of water in a separatory funnel. The chloroform phase was separated, dried, and its radioactive content was compared with that in the aqueous phase. The partitioning of the radioactive materials in the residue, after evaporating the chloroform, was determined between hexane and 2% hydrochloric acid.

Miscellaneous Studies. Infrared spectra were obtained on the chloroform-soluble metabolites after incubation of parathion, paraoxon, and *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN), at 500 p.p.m., in a liter of rumen juice for 24 hours at 28° C. The rumen juice after incubation was adjusted to pH 10 and extracted with chloroform, the chloroform was evaporated, the residue was partitioned between hexane and 2% hydrochloric acid, the acid phase was separated and adjusted to pH 10, and the metabolites were recovered in chloroform. After removing the chloroform, infrared spectra obtained on the residues were identical to ones for amino parathion, amino paraoxon, and *O*-ethyl *O*-*p*-aminophenyl phenylphosphonothionate (amino EPN prepared by reduction of EPN with zinc and hydrochloric acid) except for a small per cent of carbonyl impurities. Chromatographic purification of the parathion metabolite removed this impurity and yielded an infrared spectrum superimposable on one for known amino parathion. The 0- to 3-hour urine sample for the first dose of radioactive parathion to the cow was similarly fractionated and yielded an infrared spectrum of amino parathion.

The stability of amino parathion and parathion were studied in vitro in the presence of bovine rumen juice, bovine plasma, and aqueous alkali. Parathion and amino parathion were incubated with bovine rumen juice at 250 p.p.m. and 40° C., and aliquots taken at different times were analyzed as previously described. For studying the hydrolysis rate in cow plasma, 70 γ of radioactive parathion or radioactive amino parathion and 250 γ of Triton X-115 were placed in a series of tubes and 0.30 ml. fresh heparinized cow plasma was added. The tube contents were mixed and incubated at 40° C. for various times up to 12 hours before extraction. Then 2.7 ml. of water and 3.0 ml. of chloroform were added and partitioned, and the radioactivity in each phase was determined.

For studying the stability in alkali, radioactive parathion and amino parathion were dissolved in ethyl alcohol and added to 0.1*M* aqueous sodium carbonate to yield a 20-p.p.m. concentration of the phosphorothioates in 5% of ethyl alcohol and 95% of 0.1*M* sodium carbonate. This solution read 11.0 on a pH meter. At frequent time intervals up to 8 days of incubation at 28° C., 3.0-ml. aliquots were removed and partitioned with 3.0 ml. of chloroform, and the radioactivity in each layer was determined.

Partition chromatography with a celite–iso-octane–methanol column (*4*) permitted separation of parathion, paraoxon, amino parathion, amino paraoxon, and acetylamino parathion as shown in Figure 1. Ion exchange chromatography (*20*) was used to study the hydrolysis products of parathion in urine. Chromatography with known carrier materials was used for all studies on characterization of radioactive metabolites.

Total phosphorus was determined as reduced phosphomolybdate following oxidation of the organophosphate with perchloric acid (*2*). Radioactive determinations were made with an end-window G-M tube by counting 1.00-gram or 1.00-ml. aliquots and correcting for isotope decay and self-absorption. Infrared spectra were obtained with 10% chloroform solutions of the organophosphates with a Baird IR spectrophotometer and sodium chloride prism. The anticholinesterase activity of parathion derivatives was determined potentiometrically with 30% whole human blood (*6*). LD_{50} figures for houseflies (*Musca domestica* L.) were obtained by a described procedure (*16*) and rat LD_{50} values were determined with 150- to 250-gram male white rats 48 hours after administering corn oil solutions of the organophosphate with a stomach tube.

Results and Discussion

In Vitro Studies with Stagnating Bovine Rumen Fluid. Many organophosphates were susceptible to hydrolysis by the bovine rumen fluid in vitro. Of the insecticides studied, other than nitrophenyl derivatives, TEPP and malathion were the most readily hydrolyzed and Trolene and Dimethoate were the most stable (Table II). Boiling the rumen juice definitely reduced the hydrolytic activity on malathion but did not affect the low degree of hydrolysis occurring with Trolene and Dimethoate. Trolene underwent hydrolysis at both the phosphorus-oxygen-methyl bond and the phosphorus - oxygen - phenyl bond (*21*). Thimet only underwent hydrolysis in rumen fluid with no oxidation to the sulfoxide, sulfone, or phosphorothiolate derivates and parathion was not oxidized to paraoxon. However, a slight degree of oxidation (or hydrolysis after isomerization) by rumen juice has been reported for the phosphorothioate group in either *O*-hydrogen *O*-methyl *O*-(2,4,5-trichlorophenyl) phosphorothioate or *O*,*O*-dimethyl phosphorothioate (*21*).

The reduction of nitrophenyl phosphate and phosphorothioate insecticides by rumen juice can occur as rapidly as or more rapidly than the hydrolysis reactions. With parathion, the per cent yield of amino parathion in rumen juice varied with the concentration of parathion as indicated by a 62% recovery after 2 hours with 2 p.p.m. (Figure 4), and a 25% recovery after 2.7 hours

with 300 p.p.m. (Figure 2). After 24 hours with 500 p.p.m. of parathion, paraoxon, or EPN, in a liter of rumen juice, 73% of the paraoxon was recovered as amino paraoxon, 53% of the parathion as amino parathion, and 1.8% of the EPN as amino EPN with the remaining materials being accounted for as hydrolysis products. The loss of parathion and formation of a directly diazotizable metabolite in rumen juice was confirmed by analysis after various incubation times using the Averell-Norris procedure (*3*) with and without the reduction step.

The formation of amino parathion, amino paraoxon, and amino EPN by rumen fluid was also confirmed by column chromatography and infrared spectra on the metabolites. Based on the partitioning system described earlier and the change of activity between fresh and boiled rumen juice, reduction was also demonstrated with the following nitrophenyl phosphates: *O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate (methyl parathion), *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate (EPNO), *O*-ethyl *O,O*-*bis*-*p*-nitrophenyl phosphorothioate (E3500), *O*-(3-chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate (Chlorthion), *O*-(2-chloro-4-nitrophenyl) *O,O*-dimethyl phosphorothioate (Am. Cyanamid 4124), and *O*-(2-chloro-4-nitrophenyl) *O,O*-dimethyl phosphate (Am. Cyanamid 4138). The comparison of the reduction and hydrolysis rates for parathion, paraoxon, EPN, EPNO, 4124, and 4138 are shown in Figure 2. With all the nitrophenyl phosphate and phosphorothioate insecticides over 90% of the compound was metabolized within 3 hours on incubation at 300 p.p.m. with rumen juice in vitro. There was always a greater recovery of amino derivative from the phosphates than for the phosphorothioates after 1 or more hours of incubation because of the much greater hydrolysis rate of the phosphorothioates than of the phosphates by the rumen juice. Methyl parathion yielded more amino derivative than parathion, and parathion more than EPN. The chloro substituents in Chlorthion and 4124 reduced the efficiency in conversion to the amino analogs compared with methyl parathion lacking these substitutions.

The rapid reduction of nitrophenyl phosphates and phosphorothioates in stagnating bovine rumen fluid is not surprising as the reduction of nitrates to hydroxylamine and ammonia by sheep rumen fluid has been previously reported (*17*). The findings on hydrolysis and reduction of organophosphate insecticides in vitro with stagnating rumen juice are not necessarily of significance in the rumen juice in the cow where passage through the digestive tract, mixing of the rumen contents, and absorption rates are also involved.

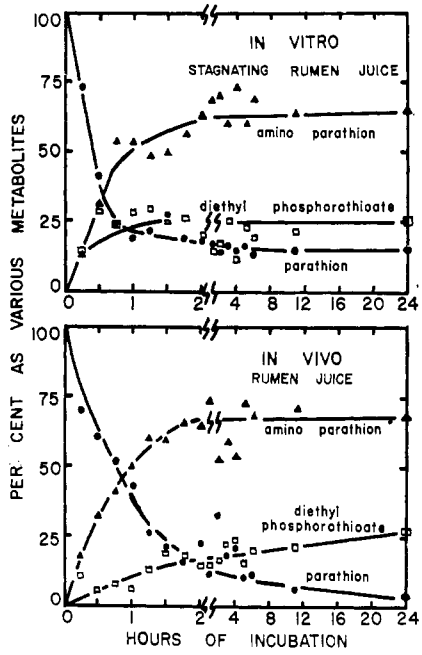

Figure 4. Comparison of the rates of radioactive parathion reduction and hydrolysis by stagnating of bovine rumen fluid in vitro and by the rumen fluid in vivo in a fistulated cow (parathion concentration 2 p.p.m. in vitro and 0.2 to 8 p.p.m. in vivo)

In Vivo Studies on Parathion Metabolism. Parathion reduction by the rumen fluid appeared to be of definite significance when studied in vivo with a lactating cow. The total parts per million of parathion and metabolites from an oral dose reached peaks at 0.5 hours in rumen fluid, 1.5 hours in jugular blood, 11 hours in urine, 29 hours in feces, and 47 hours in milk (Figure 3). When the metabolism of parathion in stagnating and in vivo rumen juice were compared (Figure 4), very similar rates were evident with only slightly faster reduction and hydrolysis of parathion in vitro, but a more complete destruction of the parathion within 24 hours in vivo. The absorption of parathion from the rumen was slow enough, compared with the rate of the reduction to amino parathion, to allow a large portion of the ingested dose to be metabolized in the rumen. Less than 0.1% if any of the radioactivity in the rumen juice was as paraoxon (Table III) indicating that parathion was only reduced or hydrolyzed in the rumen fluid in vivo and not oxidized before absorption.

The cow blood contained parathion, paraoxon, amino parathion, and amino paraoxon as well as a large proportion of hydrolysis products. The amino derivatives became increasingly important as circulating parathion metabolites with increasing time after treatment (Figure 5, Table III). No depression of the blood cholinesterase activity occurred from either of two doses of 6.7 mg. of parathion per kg. with 5 days between treatments.

Within 5 days after treatment, 0.81% of the administered parathion dose was secreted in the milk. The metabolites in the milk were the same as those in the blood. Within the first 24 hours after treatment, amino parathion and amino paraoxon were the major parathion metabolites other than hydrolysis products in the milk (Table IV) with amino paraoxon predominating (Table III). The very small amounts of parathion and paraoxon recovered from the milk confirm the observations of Dahm and coworkers who reported that 1 or 5 p.p.m. in the feed (*10*, *11*) or even up to 32 mg. of parathion per kg. of body weight per day (*19*) gave less than 0.2 p.p.m. of parathion residues in the milk, and of Cook and Sykes (*9*) who found that cows fed up to 50 p.p.m. of parathion in the whole diet secreted less than 0.02 p.p.m. of parathion equivalents in the milk. The isotope experiment reported here shows that much larger amounts of amino parathion and amino paraoxon are secreted in the milk than parathion and paraoxon. Low levels of parathion and/or paraoxon and amino parathion and/or amino paraoxon also appeared in fat biopsy samples (Table IV). The amino parathion and amino paraoxon were rapidly lost from the cow so that little was recovered from the milk and fat except during the first 24 hours after treatment.

Within 5 days after feeding the radioactive parathion to the cow, 43% of the administered dose was excreted in the urine and 24% in the feces. About 70% of the radioactivity in the urine within the first 5 days was amino parathion, which accounted for about 30% of the administered dose. In another experiment, 40.7% of the radioactivity in the urine in the first 24 hours after treatment was amino parathion and 1.8% was amino paraoxon (Table III). Very small amounts of parathion were excreted in the urine during the first 24 hours (Table III) and this excretion of parathion continued for 3 days, but no paraoxon was ever recovered from the urine. Amino parathion was characterized as a parathion metabolite in the 0- to 3-hour urine by an infrared spectrum obtained on the purified metabolite. Diethyl phosphoric acid constituted 7% of the hydrolysis products in the 0- to 3-hour urine and 26% in the 36- to 47-hour urine and the remaining 93% and 74%, respectively, were predominately if not entirely diethyl phosphorothioic acids.

A proposed metabolic pathway for the phosphorus portion of the parathion molecule following oral administration to a cow is shown in Figure 6. Pankaskie, Fountaine, and Dahm (*19*) did not demonstrate amino parathion or

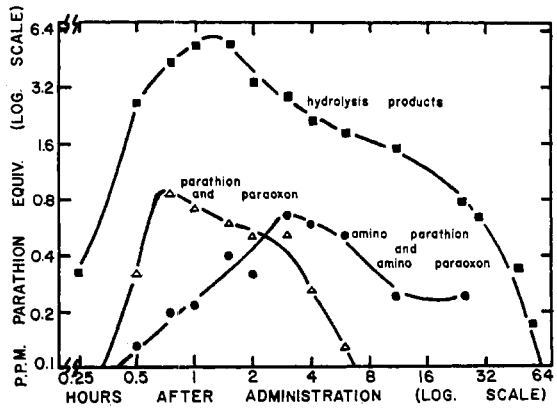

Figure 5. Nature of parathion metabolites in jugular blood following oral administration of 6.7 mg. of radioactive parathion per kg. of body weight

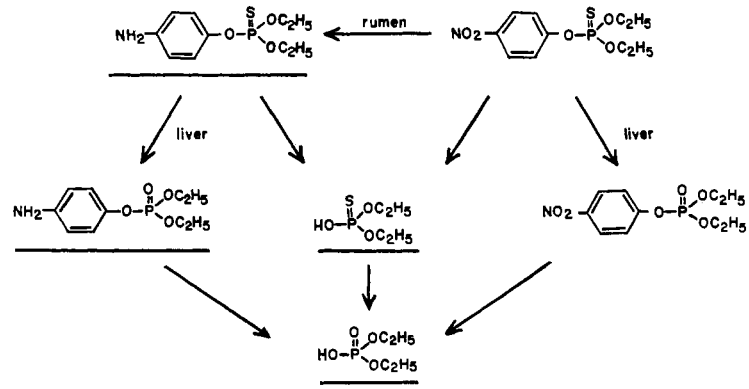

Figure 6. Proposed metabolic pathway for parathion administered orally to cattle with the metabolites excreted in the urine underlined

amino paraoxon in cows fed parathion, but did show the absence of *p*-nitrophenyl derivatives in the blood, milk, and urine, and the presence of a *p*-aminophenyl conjugate, probably *p*-aminophenylglucuronide, in the urine. The excretory metabolites of parathion in cattle would therefore appear to be amino parathion, diethyl phosphoric acid, diethyl phosphorothioic acid, *p*-aminophenylglucuronide and small amounts of parathion and amino paraoxon. The possibility also exists that a portion of the *p*-aminophenol was conjugated to *p*-aminophenyl sulfuric acid before excretion (*24*).

The metabolism of parathion and amino parathion in rats was quite different from that in the cow. Parathion was more rapidly degraded and excreted by rats. With a 10-mg.-per-kg. oral dose, 66% of the parathion and 68% of the amino parathion were excreted as metabolites in the urine within the first 24 hours, with an additional 5% excretion in each case during the second 24-hour period. The radioactivity excreted in the urine within the first 2 days with parathion was 99.5% hydrolysis products with an indication based on partitioning that the remaining 0.5% might be amino parathion. With amino parathion 96.3% of the excreted radioactivity in the urine was as hydrolysis products with the remaining 3.7% being amino parathion with a possible trace amount of acetylamino parathion based on partitioning properties. When the livers of rats treated with parathion and amino parathion were removed 2 hours after treatment and fractionated, higher total levels of radioactivity were found with the amino parathion rats than with the parathion-treated animals. Twenty-two per cent of the parathion in the liver was hydrolyzed and the remainder partitioned predominately as for known parathion. With amino parathion, 41% of the material in the liver was hydrolyzed and the unhydrolyzed portion partitioned as if some of it had been converted to acetylamino parathion. Free *p*-nitrophenol is excreted in the urine of parathion-poisoned nonruminating mammals (*4, 14*) but conjugated *p*-aminophenol in the urine of cattle (*19*). The differences in metabolic pathway would appear primarily to be due to the ability of the rumen organisms to reduce parathion to amino parathion.

Other Considerations. The *p*-amino derivatives were much less biologically active than the *p*-nitro derivatives of parathion and paraoxon (Table I). With the anticholinesterase assay employed, parathion was 100 times more active than amino parathion, and paraoxon was 12,880 times more active than amino paraoxon. The nitro derivatives were almost 10,000 times more toxic to houseflies than the amino derivatives and about 100 to 300 times more toxic to white rats. The poisoning symptoms in rats treated with amino parathion and amino paraoxon were very slow in appearing, compared with parathion and paraoxon, and were not typical of an anticholinesterase action.

Of the analytical procedures available for parathion and paraoxon (*13*), many do not determine the amino parathion and amino paraoxon because of extraction losses, inability of these compounds to enter into colorimetric determinations of the nitrophenol group, low anticholinesterase activity, and low toxicity to flies for bioassays. Yet the low toxicity to rats of amino parathion and amino paraoxon compared with that of parathion and paraoxon (Table I) indicate that their residues are of far less toxicological significance than the nitrophenyl phosphates and phosphorothioates.

Parathion was much less stable to hydrolytic attack than amino parathion. The alkaline hydrolysis constant for parathion was about four times greater than for amino parathion over an 8-day period in 0.1*M* sodium carbonate. In bovine plasma in vitro, the parathion was again hydrolyzed nearly four times as fast as amino parathion based on comparison of the hydrolysis constants for determinations up to 24 hours. In bovine rumen juice, the rate difference was even greater with the hydrolysis constant being 19 times greater for parathion than amino parathion for the first 5 to 7 minutes of reaction with the parathion hydrolysis rate then rapidly dropping to only about 1.3 times that of amino parathion for the 7- to 40-minute interval of incubation. The change in hydrolysis rate of parathion in rumen fluid was probably due to its conversion to amino parathion which was more resistant to hydrolysis.

The metabolism of organophosphates other than parathion by bovine rumen fluid may also be of significance in their toxicity to cattle, nature of the excreted metabolites, and residues in tissues and milk. This would depend on their rate of degradation in the rumen compared with their rate of absorption from the digestive tract. Absorption figures from the bovine digestive tract are only directly available for parathion, but can be interpreted from the rate of appearance and persistence in the blood with diazinon (*22*), L 13/59 (*23*), Trolene (*21*), Phosdrin (*6*), and Dimethoate (*12*). Of these materials the attack by the rumen microorganisms would appear to be of the greatest significance with parathion. Hydrolysis of TEPP and malathion in the rumen might also be of significance as would the reduction of other nitrophenyl phosphate and phosphorothioate insecticides by the rumen fluid.

Acknowledgment

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FUNGICIDE RESIDUES

Determination of Phygon Residues on Food Crops

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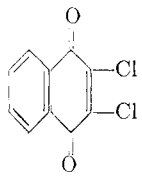
A colorimetric method of analysis for microgram quantities of residual Phygon on food crops is based upon the reaction of Phygon with dimethylamine to produce an intense orange color. The method is sensitive to 1 γ per ml. and detectable to 0.5 γ per ml., and it has been applied to five food crops. Eighteen untreated samples charged at 0.4 to 1.0 p.p.m. have given recoveries of 85 to 101% averaging 91% recovery. Interference values obtained from untreated samples, in terms of parts per million of Phygon, have averaged 0.02 p.p.m. with a high of 0.12 and a low of —0.02 p.p.m. The color reaction is specific for Phygon.

THE FUNGICIDAL PROPERTIES of Phygon were discovered by the United States Rubber Co.'s General Laboratories, Passaic, N. J., in 1940 (*7, 8*).



Considered one of the most potent organic fungicides (*5*), Phygon gives outstanding control of apple scab, brown rot of stone fruits (*4*), botrytis, late blight of tomatoes (*2*), celery blight, potato seed piece rot, and many other plant diseases caused by fungi (*3, 9*). It has been granted a residue tolerance of 3 p.p.m. under the Miller Amendment for use on apples, celery, peaches, and tomatoes (*6*).

The reaction between Phygon and dimethylamine to produce an orange color is taken from a colorimetric method, described by Burchfield and McNew (*1*), wherein analysis is made for fractions of a per cent of Phygon on seed. In order to adapt the above color reaction from fractions of a per cent of Phygon on seed to fractions of a part per million of Phygon on food crops, several modifications were made.

The aqueous acetone medium for color development used by Burchfield and McNew was replaced with an anhydrous benzene medium because acetone readily extracts colored plant matter from most crops, thereby presenting considerable background interference, whereas benzene extracts almost none. Also, the sensitivity of the method was increased by use of a considerably larger sample and by employment of 10-cm. cells in place of 1-cm. cells for spectrophotometric measurements.

The reaction between Phygon and anhydrous dimethylamine in a benzene medium to produce an orange color takes place almost instantly and is stable for 20 to 30 minutes (Figure 1). The orange color, with an absorption maximum at 495 mμ (Figure 2), is specific to Phygon. Standard curves of the orange color follow Beer's law. Natural plant components dissolved from the crop by the solvent form very weak yellow or green interference coloration with dimethylamine. Interferences obtained from untreated samples, in terms of parts per million of Phygon, have averaged 0.02 p.p.m., with a high of 0.12 and a low of —0.02 p.p.m. Typical recovery data of the crops analyzed are given in Table I.

Experimental

Processing of Sample. Place a 500- to 1000-gram sample of the crop and a volume of benzene in milliliters equal to 1/2 the sample weight in a suitable glass jar, protecting the contents from the lid by a piece of cellophane or polyethylene sheet. Roll the jar on an automatic rolling device for 15 minutes to dissolve the Phygon from the surfaces of the sample. Decant the benzene and dry over anhydrous sodium sulfate for 10 minutes. Filter the benzene through a fast filter paper and retain a pint for analysis. Analysis should be carried out the same day the samples are processed, as Phygon tends to decompose slowly in crop washes when stored.

Special Reagent. Phygon, purified. Filter a saturated benzene solution of technical Phygon through an alumina chromatographic column; concentrate benzene filtrate to 1/4 its volume, chill, collect crystalline precipitate of Phygon on a sintered glass funnel, and air dry.

Procedure. Transfer 38 ml. of the filtered solvent wash to a ground glass–stoppered 50-ml. mixing cylinder. Di-