will probably be high enough to displace all the magnesium, together with some calcium at the same time, thus leading to a poor separation. When the potassium resin is used, ionic exchange will take place between the K^+ ions on the resin and the NH_4^+ ions in the ammonium citrate buffer. The eluting solution will, therefore, contain both NH_4^+ and K^+ ions of total concentration of $0.025M$. As K^+ ions have a higher affinity for the exchanger than the NH_4^+ ions, the former will be preferentially adsorbed and will be responsible for the displacement of Mg^{++} and Ca^{++} ions on the resin. The concentration of these K^+ ions being less than $0.025M$ will be just enough to allow for separation by complex elution. Similarly, in the case of the sodium resin, NH_4^+ ions of a concentration less than $0.025M$ will be responsible for the separation of magnesium from calcium.

To explain why the separation gap is wider with the potassium than with the sodium resin, the concentration of the K^+ ions in the eluting solution passing through the potassium resin must be assumed to be less than that of the NH_4^+ ions present in the eluting solution passing through the sodium resin.

From the above, the effectiveness of separation seems to depend mainly on the concentration of the displacing ion. The smaller the concentration, the better will be the separation. This may explain why the separation gap is wider with the potassium than with the sodium resin.

The manganese remaining on the column was completely eluted with the magnesium but its concentration in the eluates was too small to interfere with the magnesium determination.

To determine the per cent of total cation eluted as a function of eluate volume, a solution containing 1 mg. of magnesium and 5 mg. of calcium was used. Magnesium was determined in the eluates by Titan yellow, as described before, while calcium was determined by the Unicam flame photometer. Results are shown in Figure 1.

In order to determine the effect of variations in the relative concentrations of magnesium and calcium on the efficiency of separation, a series of runs was made using solutions containing known amounts of magnesium ranging from 0.1 to 1 mg. and calcium from 0.5 to 5 mg. The magnesium-calcium ratio was also varied between the limits 1/50 to 2. In all cases, magnesium was eluted completely in about a 100-ml. volume, beginning approximately at 30 ml., and complete separation from calcium took place. However, the separation gap became gradually smaller with increasing amounts of calcium. For example, the gap with 0.5 mg. of calcium was about four times wider than that with 5 mg. of calcium as shown in Figure 2.

When the method was applied to soil extracts, it was necessary to bring the pH of the solution just above 7 by dilute potassium hydroxide solution before the addition of the ammonium citrate. On passing the solution through

the resin, it was observed that the colloidal organic matter in the soil extracts was not retained by the resin to any appreciable extent and passed almost completely in the effluent. The eluates containing the magnesium were clear and practically free from organic matter, thus allowing for the direct determination of magnesium by Titan yellow.

By assembling a number of columns, magnesium was determined in the acid extracts of some soils in a relatively short time and with a high degree of accuracy. The accuracy of determination was tested by the addition of calculated amounts of magnesium to the soil extracts and determination of the recovered magnesium. Results are shown in Table I. The recovery of the added magnesium was almost complete and the maximum error did not exceed 0.5%.

Literature Cited

(1) Abrahamczic, E., Mikrochemie ver. Mikrochim. Acta 33, 209 (1947).
(2) Bjerrum, N., Unmack, A., Kgl. Danske Videnskab. Selskab, Mat-fysi. Medd. 9, 1 (1929).
(3) Boyd, G. E., Schubert, J., Adamson, A. W., J. Am. Chem. Soc. 69, 2818 (1947).
(4) Cornfield, A. H., Pollard, A. G., J. Sci. Food Agr. 1, 357 (1950).
(5) Gillam, W. S., Ind. Eng. Chem., Anal. Ed. 13, 499 (1941).
(6) Mikkelsen, D. S., Toth, S. J., Prince, A., Soil Sci. 66, 385 (1948).
(7) Peech, M., English, L., Ibid., 57, 167 (1944).
(8) Pieters, H. A., Hanssen, W. J., Geurts, J. J., Anal. Chim. Acta 2, 241 (1948).
(9) Schubert, J., Richter, J. W., J. Am. Chem. Soc. 70, 4259 (1948).
(10) Tompkins, E. R., Ibid., 70, 3520 (1948).
(11) Tompkins, E. R., Khym, J. X., Cohn, W. E., Ibid., 69, 2769 (1947).

Table I. Magnesium in Soil Extracts

(P.p.m. of solution)[a]

| Soil No. | Magnesium | Add 100 P.P.M. Mg | | Error, % |
		Calcd.	Obs.	
1	162	262	263	+0.38
2	87	187	187	0
3	112	212	213	+0.47
4	142	242	242	0
5	300	400	402	+0.5

[a] Extraction was carried out by a sodium acetate–acetic acid buffer at pH 4.5.

PESTICIDE TOXICITY

Biological Activity of Several O,O-Dialkyl Alpha-Acyloxyethyl Phosphonates

B. W. ARTHUR[1] and J. E. CASIDA

Department of Entomology, University of Wisconsin, Madison, Wis.

INCREASING EMPHASIS is being placed on the development of insecticides of low mammalian toxicity. This selectivity requirement has been met with the organophosphate insecticides malathion,

[1] Present address, Department of Entomology, Alabama Polytechnic Institute, Auburn, Ala.

S - [1,2 - bis(ethoxycarbonyl)ethyl]O,O-dimethyl phosphorodithioate (18); Chlorthion, O-(3-chloro-4-nitrophenyl)-O,O-dimethyl phosphorothioate (13, 23); Trolene, O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate (11); and Dipterex, O,O-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (12).

Dipterex with its phosphonate bond differs in structure from most other phosphorus insecticides with phosphate, phosphorothiolate, and phosphorothioate groupings.

The low mammalian toxicity of Dipterex may be attributed to several factors: The lability of the cholines-

Knowledge of the mechanism of selective toxicity of insecticides is valuable in determining their safety in use and in developing new chemicals. Several O,O-dialkyl 2,2,2-trichloro-1-acyloxyethyl phosphonates and related derivatives were compared as to toxicity and anticholinesterase activity. O,O-Dimethyl 2,2,2-trichloro-1-*n*-butyryloxyethyl phosphonate was the most selectively toxic to houseflies of all the phosphonates studied. In vivo and in vitro metabolism studies with insects and rats showed that this phosphonate was hydrolyzed at the acyl group, the phosphorus-carbon bond, and possibly the phosphorus-oxygen-methyl bond. The initial site of in vivo hydrolysis appeared to be a major factor in the selective toxicity of this compound.

terase-inhibitor complex could facilitate a rapid reactivation of this essential enzyme (12); the rapid phosphonate hydrolysis by plasma and other esterases might prevent much of the anticholinesterase agent from reaching the final site of action (2); and possibly detoxification might occur through cleavage of an O-methyl ester linkage or alteration of the chloral portion of the molecule (22). Acetylation of the α-hydroxy group in the Dipterex molecule results in a phosphonate equally as toxic to some insects as Dipterex, but less toxic to mammals (2).

This study concerns the synthesis, and biological activity of several O,O-dialkyl 2,2,2-trichloro-1-acyloxyethyl phosphonates and related derivatives. Dipterex and O,O-dimethyl 2,2,2-trichloro-1-*n*-butyryloxyethyl phosphonate (designated by the authors as butonate) were prepared with a radiophosphorus label and their in vivo and in vitro metabolism investigated.

Materials and Methods

Synthesis of O,O-Dialkyl 2,2,2-Trichloro-1-acyloxyethyl Phosphonates and Related Compounds. The dialkyl α-hydroxyethyl phosphonates shown in Table I were prepared by the addition of chloral to equimolar dialkyl hydrogen

phosphites (5, 19). Carboxylic esters were synthesized by esterification of the α-hydroxyethyl phosphonates with equimolar acid anhydrides or acyl chlorides (2, 5). A single acyloxy derivative, butonate, was also prepared in very low yields by coupling α-chloro-β-trichloro-ethyl *n*-butyrate (10) with equimolar dimethyl phosphite and by the addition of methanol and phosphorus trichloride to the α-chloro-β-trichloroethyl *n*-butyrate intermediate in a 3 to 1 to 1 molar ratio.

The reaction mixtures were partially purified by adding water, extracting with *n*-hexane to recover the α-acyloxy-ethyl phosphonates and then with chloroform to recover the α-hydroxyethyl phosphonates. These separated fractions were then chromatographed on silica gel (2) and the acyloxyethyl derivatives eluted with hexane and the hydroxyethyl derivatives with chloroform. Elementary analyses of O,O-diethyl 2,2,2-trichloro-1-acetoxyethyl phosphonate purified in this way gave carbon, 29.36%; hydrogen, 4.43%; chlorine, 32.70%; and phosphorus, 9.29%. Theary ($C_8H_{14}O_5Cl_3P$) requires carbon, 29.33%; hydrogen, 4.31%; chlorine, 32.48%; and phosphorus, 9.46%. Thus purification on silica gel columns was adequate to remove gross impurities. The products were usually not distilled

because of possible thermal decomposition, especially with the higher acyloxy derivatives.

Characterization of other derivatives was based primarily on infrared spectra (6). On acylation of the α-hydroxy-ethyl phosphonates the phosphorus-oxygen vibration band at 8.10 microns was unchanged, the broad hydroxyl valency vibration band at 3.15 microns was lost, and a single strong carbonyl absorption band appeared at 5.70 microns. The phosphorus-oxygen-methyl vibrations at 9.60 microns were not affected except with the butyryl and higher derivatives, where a slight shift to 9.65 microns was noted. With the ethyl phosphonates, the bands appearing in this region at 9.80 to 9.85 and 10.20 to 10.22 microns were unaffected by acylation.

New peaks appeared on acylation of Dipterex at: 7.88 to 7.90 microns; 8.43 to 8.49 microns; 10.85 to 11.00 microns; 11.38 to 11.57 microns; 8.70 to 8.95 microns with the propionyl and higher analogs; 9.10 microns with the valeryl and higher analogs; 12.17 to 12.18 microns with the butyryl and lower acyl derivatives; 10.35 and 11.15 microns with the propionyl; 11.80 microns with the isobutyryl; and 10.42 microns with the isovaleryl derivative. With the acyl derivatives of diethyl Dipterex new bands appeared at: 7.90 microns; 11.70 microns with the acetyl and 11.80 to 11.85 microns with the higher acyl groups; 8.70 to 8.78 microns with the propionyl and higher analogs; 9.10 to 9.15 microns with the butyryl and higher derivatives; 8.38 and 11.00 microns with the acetyl; 11.18 microns with the propionyl; and 11.30 microns with the isovaleryl derivative.

Other phosphonates were prepared from the reactants shown in Table II. In this series, the α-hydroxy phosphonates were extracted from an aqueous medium into chloroform, and the tri-ester phosphonates extracted into *n*-hexane. The presence of phosphorus-containing materials in the organic solvent layer indicated partially successful reactions and infrared analyses of the residues showed an hydroxy absorption band for the α-hydroxy phosphonates,

Table I. Toxicity and Anticholinesterase Activity of Certain O,O-Dialkyl 2,2,2-Trichloro-1-hydroxy and- 1-acyloxyethyl Phosphonates

Substituted Groups (R₁)	$(CH_3O)_2P(O)CH\text{-}R_1$ $\mid$ CCl_3			$(C_2H_5O)_2P(O)CH\text{-}R_1$ $\mid$ CCl_3		
	Rat[a]	Housefly[a]	pI₅₀[b]	Rat[a]	Housefly[a]	pI₅₀[b]
OH	400	8	4.5	225	40	5.2
OC(O)CH₃	800	12	4.5	112	8	6.1
OC(O)CH₂CH₃	600	17	3.7	40	3	6.1
OC(O)(CH₂)₂CH₃	>3000	8	5.0	40	5	6.2
OC(O)CH(CH₃)₂	1075	65	3.8	40	3	6.3
OC(O)(CH₂)₃CH₃	>4000	32	3.9	40	6	6.2
OC(O)CH₂CH(CH₃)₂	>4000	30	3.9	135	8	6.1
OC(O)(CH₂)₄CH₃	>4000	30	3.9	300	14	5.8
OC(O)(CH₂)₅CH₃	>4000	90	3.9	300	30	5.7
OC(O)(CH₂)₆CH₃	>4000	90	3.9	300	35	5.6

[a] LD_{50}'s (mg./kg.) based on mortality 24 hours after subcutaneous treatment of rats or topical treatment of houseflies.

[b] pI_{50} = negative log of molar concentration to produce 50% ChE inhibition.

Table II. Toxicity and Anticholinesterase Activity of Certain Phosphonate Derivatives

Reaction Product from[a]	$(CH_3O)_2P(O)H\ +$			$(C_2H_5O)_2P(O)H\ +$		
	Rat[b]	Housefly[b]	pI_{50}[c]	Rat[b]	Housefly[b]	pI_{50}[c]
Cl₂HCCHO	1100	20	4.1	525	17	5.3
+ CH₃C(O)OC(O)CH₃	260	5	5.3	500	10	5.6
ClH₂CCHO	>2000	550	3.4	>2000	550	3.4
+ CH₃C(O)OC(O)CH₃	185	500	6.0	400	200	4.0
H₃CCHO	1250	2500	3.3	>2000	1500	3.3
+ CH₃C(O)OC(O)CH₃	>2000	>4000	3.4	>2000	>4000	3.4
Cl₃CCHO + ClP(O)(OCH₃)₂	375	10	4.6	...	...	...
+ ClP(O)(OC₂H₅)₂	85	100	4.2	...	...	...
+ ClC(O)N(CH₃)₂	625	75	5.4	...	...	...
+ ClSO₂N(CH₃)₂	625	20	4.6	...	...	...
+ ClC(O)CCl₃	>2000	20	4.6	...	...	...
+ ClC(O)CHCl₂	...	25	...	...	...	...
Br₃CCHO	150	3	5.7	15	9	6.8
+ CH₃C(O)OC(O)CH₃	75	1	5.1	7	2	7.4

[a] Reaction products represent n-hexane (substituted OH derivatives) or chloroform soluble (α-hydroxyethyl phosphonates) phosphorus containing materials of the reaction mixture and were not chromatographed on silica gel.

[b] LD_{50}'s mg./kg. based on mortality 24 hours after subcutaneous treatment of rats or topical treatment of houseflies.

[c] pI_{50} = negative log of molar concentration to produce 50% ChE inhibition.

Table III. Metabolism of Butonate and Dipterex in Several Biological Systems

Biological System	Per Cent Present as		
	Butonate	Dipterex	Hydrolyzed (P÷C)
Butonate			
Insect in vivo[a]			
Periplaneta americana (L.)	16.4	44.0	39.6
	15.8	61.5	22.7
Apis mellifera L.	65.0	3.7	31.3
Musca domestica L.	12.7	32.2	55.1
Rat, in vivo[b]			
Fat	17.9	44.6	37.5
Liver	2.1	3.6	94.3
in vitro[c]			
Liver homogenates, 1 hr.	54.1	28.2	17.7
Liver homogenates, 4 hr.	43.4	33.8	22.8
Kidney homogenates, 1 hr.	51.6	28.1	20.3
Kidney homogenates, 4 hr.	50.5	22.6	26.9
Human blood plasma[d]	65.1	0.9	34.0
Dipterex			
Periplaneta americana (L.)[a]	0.0	79.8	20.2
Rat liver homogenates, 1 hr.[c]	0.0	86.6	13.4
Rat kidney homogenates, 4 hr.[c]	0.0	81.5	18.5

[a] Cockroaches and honeybees treated topically at 500 γ/g. and houseflies at 200 γ/g.; after 2 hours the absorbed radiation was comparable to 390, 345, and 174 γ/g., respectively. Two separate determinations reported for cockroaches treated with butonate.

[b] Female white rats treated orally with 2000 mg./kg.; assay 4 hours later.

[c] 20% liver and kidney homogenates in saline solution (pH 7.2) containing 1000 p.p.m. insecticide.

[d] Human blood plasma (pH 7.2) containing 400 p.p.m. butonate incubated for 1 hour at room temperature.

which was not present in the spectra of the triesters. Lack of appropriate biological specificity did not warrant further characterization of these materials.

Synthesis and Characterization of Radioactive Dipterex and Butonate. Dimethyl hydrogen phosphite (service irradiation by the Atomic Energy Commission for 4 weeks at 7×10^{11} neutrons per sq. cm. per second) was distilled at 120° to 125° C. and 760 mm., and then reacted with equimolar chloral to form Dipterex in 83% yield (5, 19). Chromatography of the chloroform-soluble ma-

terials of the reaction mixture on silica gel with n-hexane, followed by chloroform elutrients, yielded more than 99% of the total radioactivity in the chloroform fraction. This major fraction was identical to known Dipterex in infrared absorption spectrum, and the total phosphorus (colorimetric analysis) and total radioactivity were present in the same compound based on chromatographic behavior, partition distribution between chloroform and water, dehydrochlorination rate (4, 20), and hydrolysis rate in alkali (2).

A portion of the radio-labeled Dipterex

was added to equimolar n-butyric anhydride to form butonate in 72% yield. The n-hexane-soluble materials of the reaction mixture were chromatographed on silica gel with an n-hexane elutrient which yielded a material with the partitioning properties and infrared spectrum identical to known butonate. The two radio-labeled phosphonates were over 99% chemically and radiochemically pure.

Biological Assays. Adult female houseflies, Musca domestica L., and mixed sexes of white rats were utilized in determining the relative toxicity of the phosphonates shown in Tables I and II. Methods for topical treatment of houseflies and LD_{50} determinations have been described (2). Serial dilutions were used with four replicates of 10 houseflies each and from three to six separate determinations were made depending upon the variability of results. Housefly LD_{50} values were calculated from the 24-hour mortality counts. Mammalian LD_{50}'s were determined 24 hours after subcutaneous administration of serial dilutions (2, 4, 8, 16, etc. mg./kg.) of the phosphonates in corn oil. Two white rats of approximately 200 grams each were treated at each dosage level, and from 10 to 20 additional rats were treated at the approximate LD_{50} value. Rat LD_{50}'s were estimated by gross inspection of the data.

In vivo Metabolism Studies. Insect metabolism of radioactive butonate was studied in the housefly; American cockroach, Periplaneta americana (L.); and honeybee, Apis mellifera L. Cockroaches and honeybees were treated topically with 500 γ of butonate in acetone per gram of insect, and houseflies with 200 γ per gram. About 20 grams of each insect species were used in the determinations. Two hours after treatment, the unabsorbed butonate was removed with acetone and then the insects were macerated in water with a Waring Blendor and the chitinous particles removed by filtration through cheesecloth. All chloroform-soluble materials were removed from the filtrate by extracting eight to 10 times with equal volumes of chloroform. The combined chloroform extracts were dried with anhydrous sodium sulfate, the solvent was removed, and the radioactive residue was chromatographed with 100 mg. each of nonradioactive butonate and Dipterex on silica gel using n-hexane followed by chloroform elutrients. Aliquots were removed from each chromatographic fraction for phosphorus determinations both colorimetrically (7) and radiometrically with a Geiger-Müller counter. The identical coincidence of the total phosphorus and total radioactivity, when plotted against volume of elutrient from the column, was used as the criterion for characterization of butonate metabolites

(Table III). Exhaustive partitioning of the radioactivity from the chloroform chromatographic fraction between chloroform and water (17) provided further evidence of the nature of this butonate metabolite. In vivo mammalian metabolism of butonate was also investigated. Female rats treated orally with 2000 mg. of radioactive butonate per kg. of rat were sacrificed 4 hours after treatment. The livers and fat were macerated in water and chloroform extracts of the aqueous medium were chromatographed on silica gel in an identical manner as described for insects.

In other metabolism studies, female white rats were treated orally with 200 or 2000 mg. of radioactive butonate or 200 mg. of radioactive Dipterex per kg. of rat. Metabolism cages (9) allowed for the separate collection of urine and feces at various intervals following treatments so that the total radioactivity and percentage insecticide hydrolysis could be determined. The ionized radioactive hydrolytic products remaining in the urine after chloroform extraction were chromatographed on an anion exchange resin (50 grams of Dowex 1 X8, 100- to 200-mesh) with nonradioactive dimethyl and mono-methylphosphoric acids. The materials were eluted from the resin using a hydrochloric acid elution gradient (7) of pH 3 to 0.

The chromatographic behavior of nonradioactive mono- and dimethyl-phosphoric acids was initially ascertained by determining the methoxyl (25) to phosphorus (1) ratio of the two peaks eluted. Characterization of the water-soluble metabolites was based on identical coincidence of the radioactivity with total phosphorus when plotted against volume of elutrient. The pH, partition distribution between chloroform and water, and response to a modified (2, 16) Fujiwara (15) test were also determined for each chromatographic fraction.

In vitro Metabolic Studies. Metabolism of butonate was studied in vitro with rat liver and kidney homogenates and with human plasma (Table III). One gram of rat liver or kidney was homogenized (26) in 5 ml. of saline solution buffered at pH 7.2 containing 1000 p.p.m. of radioactive butonate. The metabolites from duplicate assays were determined after 1 and 4 hours at 28° C. Radioactive butonate (400 p.p.m.) was added to human blood plasma (pH 7.2) and incubated at room temperature for 1 hour. Chloroform extracts of the aqueous tissue homogenates and plasma were chromatographed with nonradioactive butonate and Dipterex in the same manner as described for insects. The percentage hydrolysis of butonate (Table III) was calculated from the total radioactivity remaining

in the aqueous layer after eight to 10 chloroform extractions.

Hydrolysis of the two radio-labeled phosphonates, as a function of incubation time, was studied with human plasma buffered at pH 7.2 (2) and with rat liver and kidney homogenates.

Other Methods. Anticholinesterase activity was assayed by determining the inhibition of bovine erythrocyte acetylcholinesterase (Winthrop Laboratories, New York), estimating the residual acetylcholine colorimetrically (24). Silica gel chromatographic techniques were essentially those described by Arthur and Casida (2). Elutrients consisted of 500 ml. of n-hexane followed by 500 ml. of chloroform to elute butonate and Dipterex, respectively. The procedures used for the chromatographic separation of water-soluble metabolites of organophosphate insecticides on anion exchange resins were developed by Plapp and Casida (21). Infrared analyses were made with a Baird spectrophotometer using sodium chloride prisms and the phosphonates in 10% chloroform solutions.

Studies on the thermal stability of butonate were based on changes in chromatographic behavior and infrared spectra after heating the butonate in sealed ampoules or pyrolysis tubes for 1 to 24 hours at temperatures ranging from 100° to 180° C. Radioactive dimethyl phosphoric acid was prepared by alkaline degradation of radioactive Dipterex and characterized by chromatography on an anion exchange resin with nonradioactive carrier dimethyl phosphoric acid (21).

Results and Discussion

Selective Toxicity and Anticholinesterase Activity. O,O-Dimethyl 2,2,2-trichloro-1-acetoxyethyl phosphonate has been reported to be less toxic to mammals and equally as toxic to some insects as the α-hydroxyethyl phosphonate, Dipterex (2). This selectivity pattern of the acetoxy ester was substantiated and expanded with higher acyloxy esters of the dimethyl phosphonates (Table I). Of the dimethyl phosphonates, the n-butyryloxy ester or butonate was more selectively toxic to houseflies than any of the other acyloxy derivatives. The toxicity of butonate to mammals is equal to that reported for other organophosphates of low mammalian toxicity such as malathion (13, 18), Chlorthion (13, 23), and Trolene (11). The propionoxy ester and the structural isomer of butonate, the isobutyryloxy ester, had rat to fly toxicity ratios much lower than butonate. A decrease in toxicity to both houseflies and rats resulted with derivatives containing more than four carbon atoms in the acyl group.

In general, diethoxy phosphonates were more potent in vitro anticholinester-

ase agents than the dimethoxy phosphonates (Table I). There was no direct correlation for all the phosphonates studied between the anticholinesterase activity in vitro and the toxicity to flies and rats. Similarly in dialkyl homologs of Dipterex (3) there was no direct correlation between toxicity to flies and in vitro anticholinesterase activity where cockroach heads were used as the source of the enzyme. However, the anticholinesterase activity of the diethoxy phosphonates was generally related to the toxicity of these compounds to flies (Table I). This general relationship holds for most organophosphates except for certain phosphoramides and most phosphorothioates which are metabolized in vivo to more active anticholinesterases (8). Butonate was the most active anticholinesterase agent and the most toxic to flies of the acylated dimethoxy phosphonates.

The toxicity and anticholinesterase activity of several other phosphonate derivatives are shown in Table II. Although these reaction products were not purified by chromatography on silica gel and may represent mixtures of materials, certain generalizations are evident concerning their biological activity. With the α-hydroxyethyl phosphonates there was a decrease in toxicity with substitutions on the β-carbon in the order of CBr_3 > CCl_3 (Table I) > $CHCl_2$ > CH_2Cl > CH_3. Acetylation of these compounds usually resulted in a slight increase in anticholinesterase activity and toxicity. However, purified samples of these derivatives might yield somewhat different biological results.

In vivo and in vitro Metabolism of Radioactive Butonate and Dipterex. The chromatographic identical coincidence of the total phosphorus and total radioactivity when plotted against volume of elutrient from silica gel and partitioning properties of the chloroform eluate fraction showed that butonate was at least partially deacylated at the α-carbon atom to form Dipterex. This debutyrylation and phosphonate (phosphorus ÷ carbon) hydrolysis of butonate in several biological systems is shown in Table III. Houseflies and cockroaches were more efficient in the conversion of butonate to Dipterex than honeybees, but the honeybees were killed more rapidly by butonate than were the houseflies and cockroaches with the excessive dosages used in these studies. This high per cent phosphonate hydrolysis in honeybees suggests a greater comparative ability of these insects to hydrolyze the phosphonate bond than to deacylate butonate. An in vitro enzymatic deacylation of the acetoxy ester by housefly and cabbageworm heads has been reported (2).

In mammals, both in vivo and in vitro deacylation of butonate were

demonstrated (Table III). Liver and kidney homogenates were about equally effective in metabolizing butonate by both phosphonate hydrolysis and deacylation. The rapid in vivo dissipation of radio-labeled butonate from the fat of rats necessitated sacrificing animals 4 hours after oral administration of 2000 mg. of butonate per kg. of rat, in order to obtain enough radioactivity for chromatograms. During this short metabolism period, the radioactive materials occurring in the fat were largely in the form of Dipterex and hydrolysis products, and more than 94% of the total radioactive material in the livers was hydrolyzed with only 3.6% having the chromatographic behavior and solubility properties of Dipterex.

Human blood plasma esterases detoxify butonate largely through cleavage of the phosphonate bond (Table III and Figure 1). Less than 1% of radiolabeled butonate was deacylated by plasma esterases at a time when 34% was accounted for as ionized metabolites (Table III). The rapid hydrolysis of phosphonates relative to certain phosphates in the presence of human plasma has been reported (2). The more rapid hydrolysis of butonate than Dipterex, by plasma esterases (Figure 1), is further evidence that butonate undergoes phosphonate hydrolysis as well as deacylation.

Dipterex is not biologically dehydrochlorinated to form the more active vinyl phosphate, O,O-dimethyl 2,2-dichlorovinyl phosphate (DDVP) (2, 20), as the vinyl derivative is more stable in biological systems than Dipterex and yet no DDVP could be isolated as a Dipterex metabolite (2). Butonate was even less stable than Dipterex in biological systems and chromatography failed to show any DDVP as a butonate metabolite. Thus butonate was not deacylated to Dipterex and then dehydrochlorinated to the vinyl phosphate.

The radioactivity in the urine of rats treated with butonate and Dipterex was more than 99% as ionized water-soluble metabolites, based on partition distribution of the radioactivity between chloroform and water. Chromatography of these radioactive hydrolytic products
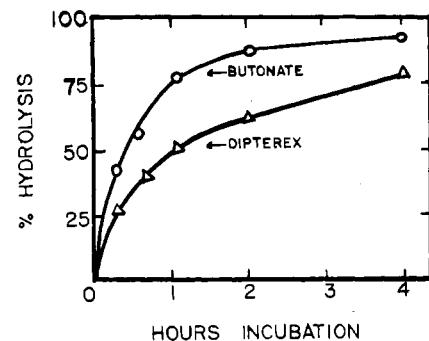

Figure 1. Detoxification rate of Dipterex and butonate by human plasma

with nonradioactive dimethyl and monomethyl phosphoric acid carriers from an anion exchange resin showed that from 50 to 70% of the radioactivity was dimethyl phosphoric and the remaining 30 to 50% was a hydrolyzed metabolite which eluted from the column with stronger acid. The chromatographic peaks of dimethyl phosphoric acid and the second hydrolyzed metabolite from the column were at pH 1.6 and 0.8, respectively. Radioactive materials occurring in the urine at 2, 4, and 16 hours after treatment with either insecticide showed the same chromatographic behavior and yielded similar proportions of the metabolites. When the urine metabolites separated by ion exchange were chromatographed individually, they were found to be pure. The urine metabolites could not be separated on the basis of partition distribution at neutral or acid conditions or by precipitation with heavy metals.

The radioactive hydrolyzed metabolite fractions isolated from the urine of butonate- and Dipterex-treated rats were oxidized and responded negatively to the Fujiwara (15) test, which indicated that neither metabolite fraction was associated with a trichloro-containing group. Evidence that the major metabolic pathway of Dipterex in the cow was not by phosphonate hydrolysis, but probably through cleavage of the O-methyl ester linkage or alteration of the chloral portion of the molecule, has been reported (22). The possibility that ·dimethyl phosphoric acid, which formed in vivo by phosphonate hydrolysis, might then undergo further metabolic changes was investigated. Radioactive dimethyl phosphoric acid formed by alkaline hydrolysis of Dipterex was administered orally to rats and the radioactivity for the first 3 days following treatment was recovered completely as the administered chemical. These data suggest that the dimethyl phosphoric acid from the urine of Dipterex- or butonate-treated rats was formed by hydrolysis of the phosphonate bond, and that the second hydrolyzed metabolite was probably formed by cleavage of the O-methyl ester linkage and that the trichloro-containing group might also have been altered before excretion.

Thermal Stability of Butonate. Butonate undergoes thermal degradation after 24 hours' exposure at temperatures of 100° C. or higher, based on changes in infrared absorption spectra. Butonate at 180° C. for this period of time left a black, chloroform-insoluble residue. The spectral changes associated with thermal degradation were decreases in intensity and broadening of the bands in the regions of 3.38, 5.70, 7.90, 9.60, 10.85, and 11.57 microns with the change in the 5.70-micron band being the most sensitive criterion for thermal degradation.

About 65% thermal decomposition of butonate occurred at 175° C. for 1 hour, based on the chromatographic behavior of the phosphorus-containing materials and infrared analysis of the eluate fractions. None of the degradation products recovered from a silica gel column (2) on elution with chloroform (0.6% of total phosphorus) or with methanol (64.5% of total phosphorus) showed hydroxyl absorption bands corresponding to Dipterex. The thermal instability of similar phosphonates has been reported by Fields (14). Butonate can be distilled for purification at 129° C. and 0.5 mm., but care must be taken to keep the pot temperature and distillation time at a minimum. The thermal degradation products are more toxic than butonate to rats.

Literature Cited

(1) Allen, R. J. L., Biochem. J. 34 858 (1940).
(2) Arthur, B. W., Casida, J. E., J. Agr. Food Chem. 5, 186 (1957).
(3) Babers, F. H., Mitlin, N., J. Econ. Entomol. 48, 430 (1955).
(4) Barthel, W. F., Alexander, B. H., Giang, P. A., Hall, S. A., J. Am. Chem. Soc. 77, 2424 (1954).
(5) Barthel, W. F., Giang, P. A., Hall, S. A., Ibid., 76, 4186 (1954).
(6) Bellamy, L. J., "The Infrared Spectra of Complex Molecules," Wiley, New York, 1956.
(7) Bock, R. M., Ling, N-S., Anal. Chem. 26, 1543 (1954).
(8) Casida, J. E., J. Agr. Food Chem. 4, 772 (1956).
(9) Comar, C. L., "Radioisotopes in Biology and Agriculture," McGraw-Hill, New York, 1955.
(10) Deodhar, G. W., J. Indian Chem. Soc. 11, 83 (1934).
(11) Dow Chemical Co., 1956, personal communication.
(12) DuBois, K. P., Cotter, G. J., Arch. Ind. Hyg. Occupational Med. 11, 53 (1955).
(13) DuBois, K. P., Doull, J., Deroin, J., Cummings, O., Ibid., 8, 350 (1953).
(14) Fields, E. K., U. S. Patent 2,579, 810 (Dec. 25, 1951).
(15) Fujiwara, K., Sitz. Nat. Ges. Rostock. 6, 33 (1916).
(16) Giang, P. A., J. Agr. Food Chem. 2, 1281 (1954).
(17) Heath, D. F., Lane, D. W. J., Llewellyn, M., J. Sci. Food Agr. 2, 69 (1952).
(18) Johnson, G. A., Fletcher, J. H., Nolan, K. G., Cassaday, J. T., J. Econ. Entomol. 45, 279 (1952).

(19) Lorenz, W. (to Farbenfabriken Bayer A.-G.), U. S. Patent 2,701,225 (Feb. 1, 1955).
(20) Mattson, A. M., Spillane, J. T., Pearce, G. W., J. AGR. FOOD CHEM. 3, 319 (1955).
(21) Plapp, F. W., Casida, J. E., Department of Entomology, University of Wisconsin, Madison, Wis., 1957, unpublished results.

(22) Robbins, W. E., Hopkins, T. L., Eddy, G. W., J. Econ. Entomol. 49, 801 (1956).
(23) Schrader, G., Angew. Chem. 66, 265 (1954).
(24) Shell Development Co., 1956, personal communication.
(25) Siggia, S., "Quantitative Organic Analysis via Functional Groups," Wiley, New York, 1949.

(26) Umbreit, W. W., Burris, R. H., Stauffer, J. H., "Manometric Techniques and Tissue Metabolism," 2nd ed., Burgess Publ. Co., Minneapolis, Minn., 1949.

FLUORINE TOLERANCE OF LAMBS

Effect of Various Levels and Sources of Fluorine in the Fattening Ration of Columbia, Rambouillet, and Targhee Lambs

LORIN E. HARRIS, MILTON A. MADSEN, DELBERT A. GREENWOOD, J. LEGRANDE SHUPE, and ROBERT J. RALEIGH

Utah Agricultural Experiment Station, Utah State University, Logan, Utah

Fluorine is generally present in almost all feeds consumed by livestock. Low levels can be ingested for indefinite periods without economic loss, but it is toxic when ingested in amounts above the critical level—the degree of toxicity depending on amount and length of time over which it is consumed. Forages with high concentration of fluorine are often found in areas where certain industrial processes expel fluorides into the air. Hay containing up to 160 p.p.m. of fluorine on a dry basis, when mixed with 50% grain, to make a ration containing 112 p.p.m. of fluorine on a dry basis, can be fed to fattening lambs for 14 weeks without causing measurable bad effects upon feed lot performance.

COMPREHENSIVE REVIEWS of the toxicity of fluorine by De Eds (1), McClure (5), Roholm (13), Peirce (11), Greenwood (3), Mitchell and Edman (7), and Phillips et al. (12) state that fluorides are widely distributed in soil, rocks, water, and plants, and, under certain conditions, the concentrations are high enough to affect animal and human nutrition adversely.

Most of the investigation on the toxicity of fluorine has been with man, cattle, rats, and swine—relatively little has been reported on fattening lambs. Studies reported by Slagsvold (15), Velu (17), and Roholm (13) established that fluorosis occurred in sheep when they consumed water or feed containing high levels of fluorine. Hobbs et al. (4) reported that fattening lambs, receiving 200 p.p.m. of fluorine in the diet, consumed slightly less feed and gained slightly less weight than animals receiving 100 p.p.m. or less of fluorine in their diet.

Peirce (10) reported a 3-year study in which feed consumption, growth, and health were not affected when 120 mg. or less of fluorine per day were consumed. Shrewsbury et al. (14) reported that sheep receiving 6.0 mg. of fluorine per kg. of body weight per day showed decreased grain consumption and depressed growth.

Experimental

Ninety weanling wether lambs of equal numbers of Columbia, Rambouillet, and Targhee breeds were used in the trial. After being weighed, the lambs were separated, according to breed and weight, into groups of 10. The nine groups of 10 lambs each were allotted at random to the treatments in Table I. The three lambs on each treatment were fed in a pen together. The average initial weight was 31.7, 32.5, and 32.7 kg., for Columbia, Rambouillet, and Targhee, respectively.

A pre-experimental ration consisting of alfalfa hay, water, salt, and a mineral mixture of salt and dicalcium phosphate (containing 80 p.p.m. of fluorine) was fed for a week prior to the trial. During this period, the lambs were treated with phenothiazine for internal parasites and vaccinated for entero-toxemia and contagious exanthema.

At the beginning of the trial, the wool on each lamb was sheared from about a 5-inch square area posterior to the shoulder. In the center of this area, the wool was clipped close with size 00 electric clippers. Two 3 X 3 cm. squares were tattooed in this area. At the end of the trial, the wool was clipped from the tattooed areas on each sheep and used to determine wool production during the trial.

The trial was conducted for 14 weeks. Throughout the trial the lambs were fed

to maintain the designated fluorine levels based on hay consumption (Table I). Five levels ranging from that in normal hay to 160 p.p.m. of fluorine were fed. Two sources of fluorine were used: one from sodium fluoride added to normal hay and the other from hay containing a fluoride residue from the stacks of an industrial plant. Only hay with a 55-p.p.m. fluorine residue was obtainable; therefore, for the 80- and 160-p.p.m. levels it was necessary to add some sodium fluoride to the diet. During the first 4 weeks of the experiment the grain was gradually increased and the hay decreased. The different fluorine levels were maintained by using varying proportions of two-grain mixtures, one containing sodium fluoride and the other free of sodium fluoride; and by varying the amounts of hay with a normal fluorine content and hay containing a high fluorine residue.

At the start of the fifth week the lambs were fed a diet consisting of alfalfa hay, 50%; barley, 26.5%; wheat, 10%; dried beet pulp, 10%; omalass (dried cane molasses product manufactured by VyLactos Laboratories, Inc., Des Moines, Iowa), 2.5%; sodium chloride, 0.5%; and dicalcium phosphate 0.5%; and remained on it throughout the balance of the trial. Sufficient sodium fluoride replaced barley to give the fluorine levels outlined in Table I. Ninety-five per cent of the ration was fed in