Table II. Comparative Residue Values for CIPC in Bin-Treated Potatoes

Sample	Residue, P.P.M. Electron affinity	Residue, P.P.M. Colorimetric
Medium II	4.6	5.6
Medium VI	3.5, 3.5	4.1
Medium VII	3.4	3.8
Medium VIII	2.6	2.5

p.p.m. of added monuron (*C*) (the peak is at about 5.5 minutes), and untreated grapes (*D*).

The potatoes were treated in their storage bins to control sprouting. Figure 3 shows chromatograms of purple raspberries with 0.1 p.p.m. of diuron added (*A*), untreated raspberries (*B*), carrots with 0.1 p.p.m. of linuron added (*C*), and untreated carrots (*D*). The peak in the case of each herbicide appears at approximately 6 minutes.

The recoveries of the herbicides are listed in Table I. Acetone solutions of the herbicides were added to the crops in the Waring Blendor. For the recovery of the herbicides added above the 0.1 p.p.m. level, the acetone strip solutions were appropriately diluted before partitioning into hexane or benzene. The method is sensitive to about 0.02 p.p.m. of any of the herbicides. This concentration would yield peak heights equal to at least a 5% full-scale deflection with a 10-μl. injection. Table II lists the comparative residue values obtained by electron affinity and colorimetric (*1*) analysis of potatoes treated in bins with CIPC to control sprouting. Analysis of diuron in raspberries which received a foliar-soil application of 3 and 6 pounds per acre of the active herbicide (as an 80% wettable powder) in April and May and were harvested in July, 1962, showed no detectable residues.

All glassware used in the procedure was cleaned in dichromate–sulfuric acid solution and thoroughly rinsed. This is necessary to remove any oxidizable organic contaminents which can cause reduction of bromine and iodine. Stock and standard solutions of the herbicides must be stored in clean glassware. Alkaline or acid contaminants can frequently initiate hydrolysis to the anilines. The solutions may then turn yellow or reddish brown owing to oxidation and polymerization reactions leading to quinone-type compounds.

A constant temperature of 130° C. for 1 hour during hydrolysis and bromination is essential. Each flask must then be removed and immediately cooled, neutralized, and partitioned into hexane.

At constant flow rate and temperature, the brominated anilines of the above herbicides show the following order of increasing retention time: monuron < diuron = linuron < CIPC. This order would be expected from the molecular weights of the brominated anilines assuming ortho and para bromination. In strongly acid solution, the NH_3^{+1} ion can exist which is very weakly meta-directing for bromination. The *m*-bromoanilines of these herbicides would, however, (based on molecular weight) indicate the following order of increasing retention times: CIPC < diuron = linuron < monuron. This is the exact reverse of the experimentally determined retention times. Apparently, the initial presence of the electrophilic chlorine substituents on the aniline ring sufficiently reduce the basic strength of the amino group to prevent formation of the NH_3^{+1} group in the acetic-sulfuric acid hydrolysis solution. The amino group would therefore exist to promote ortho and para bromination.

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INSECTICIDE METABOLISM

The Metabolism of Dimethoate by Vertebrate Tissues

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THE INSECTICIDE DIMETHOATE [*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate] is of interest as a compound of low mammalian toxicity with systemic activity in animals and plants. It is metabolized initially in lactating cows and male rats (*3*) and in sheep (*2*) to give primarily dimethoate acid (*O,O*-dimethyl *S*-carboxymethyl phosphorodithioate), somewhat less *O,O*-dimethyl phosphorodithioate, and various other products (*3*). Dimethoate is broken down much more rapidly in the mouse than in the housefly or American cockroach, and this difference has been suggested to be the cause of its selectivity (*7*). It is 70 times more toxic to the cockroach and 325 times more toxic to houseflies than it is to mice (*7*).

The term "selectophore" was introduced (*9*) in 1960 to apply to a chemical group whose presence in a molecule conferred selectivity, usually by permitting an enzyme which is particularly effective in one class of animals to degrade the poison to ineffective products. The validity of such a concept has been amply shown for the case of the carboethoxy group in malathion [*O,O*-dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorodithioate], which is hydrolyzed more rapidly in mammals than in insects at a carboethoxy group to yield the corresponding carboxyacid, which is a poor anticholinesterase because of its anionic character. Is the amide group of dimethoate a selectophore? Evidence in favor of the hypothesis is that EPN (ethyl *p*-nitrophenyl phenylphosphorothionate) and TOCP (tri-*o*-cresyl phosphate) synergized the toxicity of dimethoate to mice (*10*), and this has been shown to constitute good evidence that carboxyesterase or carboxyamidase is responsible for low toxicity (*10*).

Let us define "selectophore enzyme" as the enzyme which operates upon the selectophore. It is the purpose of this paper to determine whether an amidase is the selectophore enzyme for dimethoate, and to establish its location

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The ability of vertebrate tissues to degrade dimethoate in vitro was investigated. Dimethoate was degraded rapidly in rat liver but very little in other rat tissues. The degrading activity of the livers of other species was, in descending order: rabbit, sheep, dog, rat, cattle, hen, guinea pig, mouse, and pig. The activities were related to the toxicity of dimethoate. Dimethoate acid was the only product from sheep liver; O,O-dimethyl phosphorodithioate was the only product from guinea pig liver. Rat and mouse liver gave both products. In sheep liver, the degradation was by a microsomal amidase, some of whose properties are described.

and properties in order to prepare for future work on its purification and determination of its substrate specificity. This last is the major need, for only when specificity information is available can one reliably suggest what amide substituents can be permitted if the chemist proposes to design an organophosphate containing an amidic selectophore.

Materials and Methods

Animals. Mice (30 to 39 grams), albino females from Rolfsmeyer Farms, Madison, Wis.; rats (280 to 450 grams), females from Holtzman Co., Madison, Wis.; rabbits, 2 years old (2.9 kg.), female, and 3 months old (1.8 kg.), female; guinea pigs, 7 months old (650 grams), female, and 10 months old (800 grams), female; hens, 22 months old (3.5 kg.); dogs, 4 months old, male; pigs, 1 month old, male, and 10 months old, male; sheep, 7 to 10 months old, mixed, sexes; cattle, castrated male calves. The animals were obtained locally, except mice and rats.

Dimethoate Derivatives. The following derivatives were obtained from American Cyanamid Co.: potassium *O,O*-dimethyl phosphorothioate $(CH_3O)_2P(O)SK$; potassium *O,O*-dimethyl phosphorodithioate $(CH_3O)_2P(S)SK$; dimethoate acid, $(CH_3O)_2P(S)SCH_2COOH$; dicyclohexylamine salt of desmethyl dimethoate $[(C_6H_{11})_2NH\cdot HS](CH_3O)P(O)SCH_2CONHCH_3$; dimethoxon, $(CH_3O)_2P(O)SCH_2CONHCH_3$; and dimethoate.

Synthesis. Phosphorus-32 dimethoate was synthesized and purified essentially as described by Dauterman *et al.* (*3*). However, the ammonium salt of *O,O*-dimethyl phosphorodithioate was prepared by passing ammonia gas into the free acid product in toluene. After removal of solvent and excess ammonia in vacuo, 2 ml. of water was added, and the original procedure was then followed. The material was purified by silicic acid chromatography (*3*) and its identity confirmed by infrared analysis. In the two syntheses that were run, yields were 41 and 57%, and specific activities were 18.7 and 10.0 mc. per gram.

Phosphorus-32 dimethoate acid was synthesized biochemically; as shown below, liver from castrated male sheep hydrolyzes dimethoate at only the C—N bond to give dimethoate acid. Thirty-two milligrams of purified P^{-32}-dimethoate, 36 μc. per gram, were dissolved in 200 ml. of chilled 20% homogenate of castrated male sheep liver in 0.1*M* potassium phosphate buffer, pH 7.4. The dimethoate concentration was $1.4 \times 10^{-4}M$. The homogenate was incubated for 30 minutes in a water bath at 38° C. After cooling, it was shaken with 150 ml. of chloroform, centrifuged, and the aqueous phase was shaken again with 150 ml. of chloroform to remove unreacted dimethoate. Calculations showed that more than 99% of unreacted dimethoate should be removed by the chloroform and 99% of dimethoate acid should remain in the aqueous phase at this step.

The aqueous phase was acidified to pH 0.5 by adding 2*N* hydrochloric acid dropwise and was then extracted with 150 ml. of chloroform twice. The combined chloroform phases were reduced to a volume of 20 ml. under vacuum and were extracted with 20 ml. of water. An aqueous solution of dimethoate acid was thus obtained. The yield was 19%. Specific activity was 38 μc. per gram. The compound was identified by cochromatography with known dimethoate acid on paper using the potassium acetate buffer–isobutyl alcohol system.

Degradation of Dimethoate by Tissues. Ten per cent tissue homogenates were prepared in 0.1*M* potassium phosphate buffer solution using an ice-cooled Omni-Mixer for 1 minute. One milliliter of an aqueous solution of radioactive dimethoate was added to 14 ml. of homogenate, to give a final concentration of $6.4 \times 10^{-6}M$. After incubation at 38° C. for 30 minutes, the sample was shaken with 20 ml. of chloroform for 2 minutes, and centrifuged at 4800 × G for 20 minutes. The radioactivity of the aqueous layer was counted using a liquid-counting Geiger tube (efficiency 10.1%). In each case, homogenates shaken with chloroform were used as a control solution in order to correct for the amount of dimethoate remaining in the aqueous layer.

Degradation of Dimethoate Acid in Liver. One milliliter of $1.12 \times 10^{-4}M$ P-32-dimethoate acid aqueous solution was added to 14 ml. of chilled 10% homogenate of fresh liver in 0.1*M* potassium phosphate buffer, pH. 74 and incubated at 38° C. for 30 minutes. After cooling, the homogenate was shaken with 15 ml. of chloroform and centrifuged. The aqueous phase was acidified to pH 1.0 by adding 1.7 to 2.0 ml. of 5*N* hydrochloric acid, and shaken with the same volume of chloroform. The radioactivities of both phases were counted after centrifuging. A homogenate shaken with chloroform before incubation with P-32 dimethoate acid was used as a control.

Paper Chromatography of Degradation Products. Whatman No. 1 filter papers were soaked in 0.2*M* potassium acetate buffer solution, pH 3.6, and were dried at room temperature. A 5:1 mixture of isobutyl alcohol and 0.2*M* acetate buffer solution, pH 3.6, were used as a mobile solvent. After tissue homogenates were shaken with chloroform, 10 μl. of aqueous layer was applied to the paper. The spots of radioactivity were detected by the Vanguard 880 Automatic Chromatogram Scanner. The position of added nonradioactive metabolites, used for identification purposes, was found by color development of phosphorus by the Hanes-Isherwood method (*5*).

Results

Partitioning of Dimethoate and Related Compounds. Partition coefficients of dimethoate and related compounds that were potential metabolites were examined in a chloroform-water system. The compounds were equilibrated in equal volumes of chloroform and 0.1*M* tris buffer, pH 7.4, and phosphorus was determined by the molybdenum blue method (*7*) or radioactivity was counted in both layers. The partition coefficient of dimethoate, expressed as $CHCl_3/H_2O$, pH 7.4, was 41.5; dimethoate acid was 2.7×10^{-3}; *O,O*-dimethyl phosphorodithioic acid was less than 1.4×10^{-5}; and *O,O*-dimethyl phosphorothioic acid was less than 2.2×10^{-6}. Thus, one can calculate that 97.6% of dimethoate goes into the chloroform layer, whereas 99.8% of dimethoate acid, and 99.9% of *O,O*-dimethyl phosphorodithioic acid and *O,O*-dimethyl phosphorothioic acid remain in the aqueous layer. The

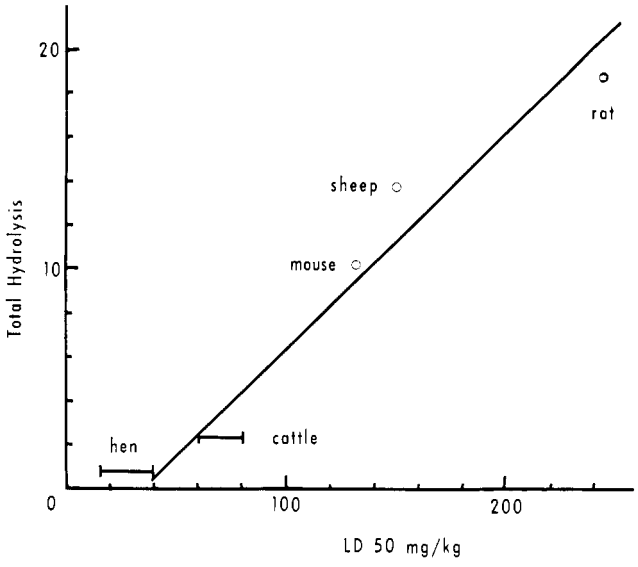

Figure 1. Correlation between total hydrolysis of dimethoate and LD_{50}

Total hydrolysis expressed as *A* times *B*, where *A* = liver per cent in the body; *B* = dimethoate hydrolyses μg. per 1 gram wet liver per 30 minutes

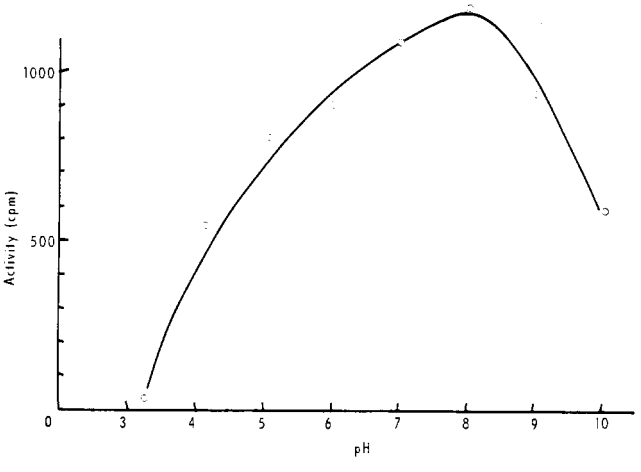

Figure 2. Effect of pH on amidase activity of liver from castrated male sheep

Exposed at pH given at 38° C. for 10 minutes, then assayed at pH 7.4. Values for activity are those in the aqueous phase after chloroform extraction

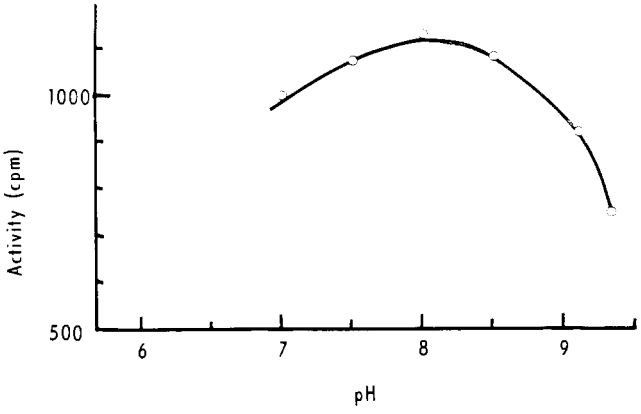

Figure 3. Effect of pH of assay on amidase activity of liver from castrated male sheep

Assayed at given pH. Values for activity are those in the aqueous phase after chloroform extraction

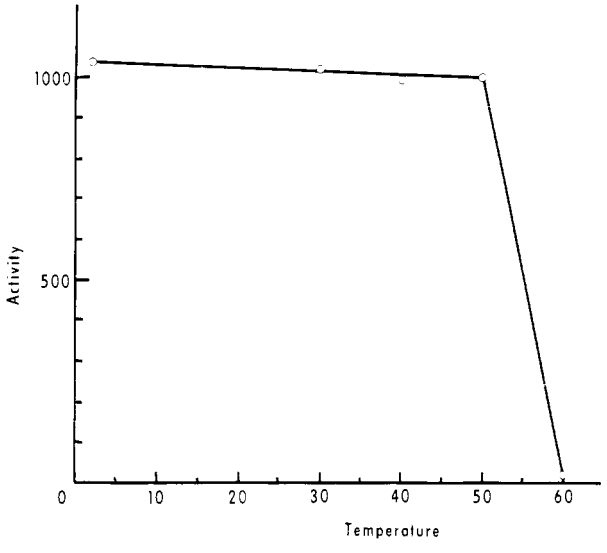

Figure 4. Effect of heat treatment on amidase activity of liver from castrated male sheep

Values for activity are those in the aqueous phase after chloroform extraction, expressed in c.p.m.

coefficient of dimethoate acid, $CHCl_3$/-H_2O,pH 1.0, was 2.76.

When dimethoate in chloroform-treated 10% tissue homogenates was partitioned, 93.1% was in the chloroform, and 6.9% was in the aqueous layer, so the tissue homogenate slightly affected the partitioning of dimethoate. The average total recovery of radioactivity from 10% tissue homogenates was 98%.

Degradation in Vertebrate Tissues. Table I shows that dimethoate was hydrolyzed readily by rat liver, slightly by lung, muscle, and pancreas, and not at all by brain, spleen, and blood. The differences in degradation of dimethoate by liver from several other vertebrate was examined. Table II shows that the livers from rabbit, sheep, and rat degraded dimethoate to a larger extent than those from the other species. In the case of the rat, however, there was considerable variation in degradation individually, with a range of from 1 to 13.2 μg. per gram in 30 minutes.

The LD_{50} values for dimethoate vary greatly among species. In examining the role of variations in liver hydrolysis, account was taken of the variation in liver proportions. Values for liver as per cent of whole body were measured for mouse (5.5%), and hen (0.27%), and were taken from the literature (*4*, *11*) for sheep (1.25%), cow (1%), and rat (4.5%). Since dimethoate is degraded to a large extent in the liver, the value—(amount of dimethoate degraded per 1 gram of wet liver) × (percentages of liver in the body)—was considered to represent the ability to degrade dimethoate by the animal. This value showed roughly linear correlation with LD_{50} in the five species for which an LD_{50} was available (Figure 1).

Nature of the Metabolic Products. R_f values of dimethoate and related compounds are shown in Table III. The potassium phosphate buffer solution used to prepare tissue homogenate did not affect the R_f values. However, when more than 50 μg. of homogenate was spotted, an R_f lower than the pure compound was sometimes found.

After incubation with livers from all mammals tested up to two radioactive

spots were found, which were identified on the basis of cochromatography with known compounds as dimethoate acid and *O,O*-dimethyl phosphorodithioic acid.

The metabolic pathway of dimethoate in the liver from three species of mammals (rat, sheep, mouse, and guinea pig) was dependent on species, sex, and concentration of dimethoate (Table IV). Thus, in the rat, more degradation took place at the C—N bond than at the S—C bond at high concentration of dimethoate ($10^{-3}M$), but the reverse occurred at the low concentration ($10^{-6}M$). The castrated male sheep and female guinea pig did not show this concentration dependence. The former gave only dimethoate acid, and the latter gave only *O,O*-dimethyl phosphorodithioic acid as a metabolite. The female mouse gave two metabolites, whose ratio was dependent on concentration of dimethoate, but this was not so apparent as in the case of rat (Table IV).

Degradation of Dimethoate Acid in Liver. *O,O*-dimethyl phosphorodithioate was found in the liver of all mammals examined except in that of castrated male sheep. The possibility that it might be produced from dimethoate acid, derived from hydrolysis of dimethoate at the C—N bond, was examined in liver from the female rat and female guinea pig.

O,O-dimethyl phosphorodithioate does not partition into chloroform at all from water at pH 1, but 73.4% of dimethoate acid partitions into chloroform (see above). Table V shows that the same amount of dimethoate acid partitioned into chloroform from incubated liver homogenates as from control. This shows that dimethoate acid was not further degraded in vitro. Confirmation by chromatography was not possible because of the low specific activity of the dimethoate acid at this time.

Subcellular Distribution of Amidase. Ten per cent liver homogenates from castrated male sheep in $0.1M$ potassium phosphate buffer, pH 7.4, which contained $0.25M$ sucrose were prepared using an ice-cooled glass and plastic Potter-Elvejhem homogenizer. The homogenates were centrifuged in a Lourdes VA2 centrifuge at 0° C. Nuclei, whole cell, and debris were precipitated at 880 × G for 10 minutes, mitochondria at 9800 × G for 20 minutes, and microsomes at 68,000 × G for 1 hour. The latter two fractions were washed by resuspending in buffer and recentrifuging. The activity of the four fractions in degrading dimethoate was assayed using labeled dimethoate as a substrate: 3% of hydrolytic activity was found in nuclei, 6% in mitochondria, 60% in microsomes, and 31% in the supernatant.

The nature of the hydrolytic products from sheep liver microsomes was examined by cochromatography with known metabolites on an ion-exchange column, using the procedure of Dauterman *et al.* (*3*) and cochromatography on paper. Only dimethoate acid was found (Table IV). In other experiments, 10% of liver homogenate from castrated male sheep, rat, and mouse were prepared and centrifuged by the same procedure except that nuclei, whole cells, debris, and mitochondria were not separated. As the results in Table VI show, most hydrolytic activity was found in rat liver microsome as in the case of the sheep, but unlike the mouse.

Table I. Degradation of Dimethoate by Rat Tissues in Vitro

Tissue	Dimethoate Degraded, μg./Gram Wet Tissue/30 Min.
Liver	12.3
Lung	0.43
Muscle	0.19
Pancreas	0.10
Brain	0
Spleen	0
Blood	0

Table II. Degradation of Dimethoate by Liver of Various Species

Animal	Sex	Number of Expts.	Av. of Dimethoate Hydrolyzed, μg./Gram Wet Liver/30 Min..	Std. Error
Rabbit	F	2	12.2, 10.7	...
Sheep	F	1	10.5	...
	M (castrated)	4	10.8	±1.6
Dog	M	1	5.9	...
Rat	F	10	4.9	±1.1
Cattle	M (castrated)	2	3.9, 1.7	...
Hen	F	2	2.7, 2.7	...
Guinea pig	F	2	1.8, 2.6	...
Mouse	F	7	1.9	±0.53
Pig	M	2	1.3, 1.2	...

Table III. R_f Value of Dimethoate and Related Compounds in Isobutyl Alcohol–Acetate System

Compound	R_f
$(CH_3O)_2P(S)SCH_2CONHCH_3$	0.90
$(CH_3O)_2P(O)SCH_2CONHCH_3$	0.75
$(CH_3O)_2P(S)SCH_2COOH$	0.48
$(HS)(CH_3O)P(O)SCH_2CONHCH_3$	0.40
$(N_3O)(CH_3O)P(S)SCH_2CONHCH_3$	0.29
$(CH_3O)_2P(S)SH$	0.10
$(CH_3O)_2P(O)SH$	0.06
H_3PO_4	0

Table IV. Metabolites from Mammalian Liver and Liver Microsomes

Animal	Sex	Concn. of Dimethoate[b]	Metabolite %[a] From whole liver $(CH_3O)_2P(S)SCH_2COOH$	From whole liver $(CH_3O)_2P(S)SH$	From liver microsomes $(CH_3O)_2P(S)SCH_2COOH$	From liver microsomes $(CH_3O)_2P(S)SH$
Sheep	F	A	50	50	...	..
		B	100	Trace	...	..
	M (castrated)	A	100	0	100	0
		B	100	0	100	0
Rat	F	A	15	85	50	50
		B	70	30	75	25
Guinea pig	F	A	0	100	...	..
		B	0	100	...	..
Mouse	F	A	45	55	50	50
		B	65	35	55	45

[a] Values are given only to the nearest 5% due to approximate nature of the evaluation procedure—i.e., measuring areas under curves on scans of paper chromatograms.
[b] A = $6.4 \times 10^{-6}M$; B = $6.4 \times 10^{-3}M$.

Table V. Absence of Degradation of Dimethoate Acid in Liver[a]

Animal	Sex	% Dimethoate Acid Partitioned into $CHCl_3$ from Water, pH 1.0
Blank[b]		73.4
Guinea pig	Female	74.9 (sample)
		73.3 (control)
Rat	Female	74.8 (sample)
		72.7 (control)

[a] If hydrolysis had occurred, partitioning of radioactivity into chloroform would be proportionately reduced, since *O-O*-dimethyl phosphorodithioate does not partition into chloroform at pH 1.
[b] Partitioned without the homogenate.

Table VI. Subcellular Distribution of Hydrolytic Activity

Fraction	Activity, % Rat (F)	Sheep[a] Castrated (M)	Mouse (F)
Nuclei, debris, and mitochondria	29	19	35
Microsomes	54	53	22
Supernatant	17	28	43

[a] Values for sheep are from a different series of experiments from those reported in text for fractionation of sheep liver into four fractions.

Metabolites from microsomes of rat and mouse were cochromatographed with known compounds on paper (Table IV). The rat gave two metabolites, and the proportion of dimethoate acid produced was higher than in whole liver. The mouse also gave two metabolites, but there was no apparent difference in proportions compared with whole liver (Table IV).

Properties of Crude Sheep Liver Amidase. The liver from castrated male sheep was used for these experiments because all of its hydrolytic activity was by amidase action (Table IV). To study the effect of pH, 15 ml. of a 10% homogenate in water was prepared, the pH adjusted, and the preparation was exposed at 38° C. for 10 minutes. The pH was then adjusted to 7.4 and the homogenate made up to 18 ml. The pH adjustment was carried out in an ice-cooled beaker by adding 0.1*N* hydrochloric acid or 0.1*N* sodium hydroxide dropwise. The results (Figure 2) showed that the enzyme was most stable at pH 8.0. The activity decreased to 50% at pH 4.8 and 10.0.

In another study designed to determine the optimum pH for assay, 10% homogenates were prepared in 0.05*M* tris buffer at different pH's, and assayed at those pH's. The optimum pH for assay was 8.0 (Figure 3).

To study the effect of heat, 10% homogenates in 0.1*M* phosphate buffer pH 7.4 were prepared and exposed at various temperature for 30 minutes and then assayed as usual. The enzyme was considerably stable up to 50° C. (Figure 4).

The values for the Michaelis constant, *Km*, were calculated by the method of Lineweaver and Burk (*8*). The values were 6.37 (±0.21, standard error) $\times 10^{-3}M$ for whole liver, and 1.55 (±0.015, standard error) $\times 10^{-4}M$ for microsomes. These values are significantly different by the *t* test at the 1% probability level.

Discussion

The results shown in Figure 1 suggest that the degradation of dimethoate in liver is a controlling factor in dimethoate toxicity. However, it cannot be generally concluded that amidase action controls hydrolysis. The contribution of amidase varies widely according to sex and species. Since it also varies with the in vitro concentration, it is not possible to determine what in vitro concentration is most appropriate to estimate in vivo conditions. A dose of 50 mg. per kg., if uniformly distributed, would give a tissue concentration of $2 \times 10^{-4}M$. But uniform distribution is extraordinarily unlikely. A similar dependence of pathway upon concentration in vitro was shown by Hodgson and Casida (*6*) for degradation of DDVP (dimethyl dichlorovinyl phosphate) by rat liver.

Fortunately, in castrated male sheep, amidase action is the only hydrolytic pathway. From Figure 1, we may therefore consider that an amidase controls toxicity in the castrated sheep, and may be called a selectophore enzyme. This amidase is present primarily in the liver microsome, and appears to be a fairly stable enzyme, as judged by the effects on it of heat and pH.

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