Effects of Parathion and Malathion Separately and Jointly upon Rat Esterases in Vivo

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Effects of parathion and malathion in acute and subacute doses upon several rat esterases in vivo were studied. The effects of the two compounds were qualitatively similar in depression and recovery of the esterases. Combinations of malathion and parathion each in subacute dosage were antagonistic with respect to externally visible symptoms. After acute doses the effects were at least additive on the basis of both symptoms and esterase inhibition. Two procedures of tissue homogenization were compared to evaluate the significance of previous reports that the standard homogenization technique of tissues from poisoned animals gave inhibition values which were too high. Inhibition of acetylcholine hydrolysis was found to be consistent with the two techniques, whereas a slight difference occurred with o-nitrophenylacetate under the experimental conditions described.

RECENTLY it has been shown that certain combinations of organophosphate insecticides when administered to mammals are more toxic than anticipated from the "theoretical sum" of their individual effects (*7, 9, 10, 14*). This might result if one compound affected the rate of detoxification of the other, or if the specificity in blocking the physiologically important sites involved varied with the compounds. As the organophosphates display their most potent effects as antiesterase agents, a broad approach of studying esterase inhibition patterns in vivo was used in the initial survey reported here. The short-term processes of esterase depression and recovery in rats treated with organophosphates have been examined. Previous investigations have dealt mainly with long-term processes, where esterase resynthesis as well as recovery is involved (*8*).

In mammals the toxic effects of most organophosphates are primarily attributable to inhibition of cholinesterases and subsequent processes. However, studies with insects (*12*) have shown that other esterases may be severely inhibited by organophosphates, and it has been suggested that it may be the inhibition of these other esterases which causes death (*4*). Comparable studies with mammals have not hitherto been carried out in vivo. This paper deals with effects upon cholinesterases (ChE) as assayed with acetylcholine (ACh), and other esterases hydrolyzing triacetin (TA), *o*-nitrophenyl acetate (NPA), and diethyl maleate (DEM). Each substrate may be hydrolyzed by several esterases in a particular tissue homogenate (*1, 2*), but it was feasible to assay only total tissue preparations, and this limitation must be considered in interpreting the results.

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Materials and Methods

Albino male rats (Holtzman Farms, Madison, Wis.), 180 to 200 grams, were lightly anesthetized with ether and injected intraperitoneally with the organophosphates. The solutions for injection were prepared by dissolving the insecticides in minimal acetone and making up to volume with corn oil (U.S.P.), so that 1 µl. of solution per gram of rat was administered. All combinations were injected simultaneously from the same syringe.

The experimental rats were decapitated and the blood was collected in 0.1 ml. of 2% sodium heparin (2000 U.S.P. units per ml.) per ml. of blood. The blood was centrifuged for 20 minutes at 10,800 × G and at 2° C. After the plasma had been removed, the corpuscles were washed once with buffer (0.15*M* sodium chloride, 0.04*M* magnesium chloride, 0.025*M* sodium bicarbonate) and centrifuged for 10 minutes at 6775 × G and at 2° C. The other tissues were chilled immediately after removing them from the body, weighed, and homogenized in glass homogenizer tubes. Two types of homogenization and dilution media were used: For the "unprotective" homogenization technique bicarbonate buffer and for the "protective" technique the appropriate substrate solution in its final concentration were employed.

ACh was dissolved directly in bicarbonate buffer to yield a 1*M* stock solution. NPA and DEM were dissolved in 95% ethyl alcohol to make 1*M* stock solutions. The 0.1*M* solutions were made by mixing 1 ml. of the stock solution with 0.3 ml. of Triton X-100 (Rohm and Haas Co.) and making up to 10 ml. with bicarbonate buffer. The 1*M* stock solutions of TA were made in the same way, the 0.1*M* solutions like those of NPA and DEM, but without emulsifier. Dilutions were made fresh for every experiment. The substrates yielded true solutions or finely dispersed, stable emulsions with the buffer tissue suspensions; the formulation media alone gave no carbon dioxide evolution with tissue homogenates.

The esterase assays were carried out by the Warburg manometric technique at 37.5° C. The tissue preparation (0.2 ml.) was in the side arm; the substrate in buffer (1.8 ml.) was in the main compartment. During preparation the vessels were kept in an ice-water mixture; then the flasks were attached to manometers, gassed (5% carbon dioxide in nitrogen) and equilibrated, and the tissue preparation was tipped in. Starting 5 minutes later—"zero time"—seven readings were taken at 5-minute intervals.

Parathion (99% *O,O*-diethyl *O-p*-nitrophenyl phosphorothionate) and malathion [96% *O,O*-dimethyl *S*(1,2-bis ethoxycarbonyl ethyl) phosphorodithioate] were donated by the American Cyanamid Co.

The results reported are average values from three or more determinations, the individual replicates varying only slightly from the mean during the entire experimental period.

Table I. Esterase Activity in Various Tissue Preparations from Untreated Rats Homogenized by the Unprotected Technique

Tissue	Tissue Wet Weight/ Flask, Mg.	Substrate	Substrate Concn., M	CO_2/30 Min., μl.	Enzyme Activity, μl. CO_2/Mg. Tissue
Brain	50	ACh	0.05	132	2.64
	50	NPA	0.03	343	6.86
	50	TA	0.02	172	3.44
Corpuscles	200	ACh	0.05	58	0.29
	50	NPA	0.03	242	4.85
	50	TA	0.02	175	3.5
Kidney	1	NPA	0.01	215	215
	2	TA	0.02	61	30
	50	DEM	0.02	193	3.85
Liver	1	NPA	0.01	215	215
	2	TA	0.02	99	49
	10	DEM	0.02	83	8.3
Plasma	200	ACh	0.05	51	0.25
	50	NPA	0.03	400	8
	200	TA	0.02	174	0.87
	200	DEM	0.02	279	1.39

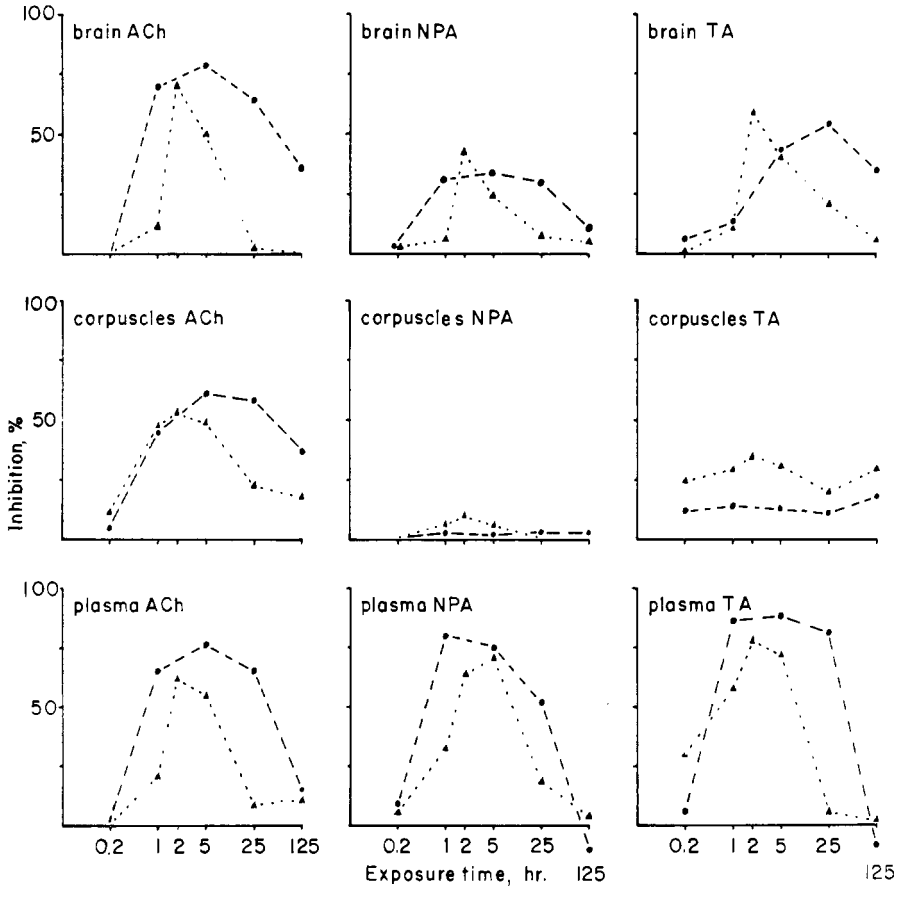

Figure 1. Effect of parathion and malathion, given singly at ALD/2 to rats, upon esterases

Table II. Per Cent Inhibition of Normal Hydrolytic Activity at Maximum and after 125 Hours' Exposure

(Unprotective homogenization)

Tissue	Parathion ALD/2	Parathion ALD/4	Malathion ALD/2	Malathion ALD/4
Maximal Inhibition				
Brain				
ACh	80	60	70	37
NPA	33	28	43	18
TA	55	59	59	18
Corpuscles				
ACh	62	64	53	56
Plasma				
ACh	76	79	61	33
NPA	80	68	71	48
TA	88	85	79	67
Inhibition 125 Hours after Treatment				
Brain				
ACh	35	34	0	7
NPA	11	14	5	3
TA	35	32	5	0
Corpuscles				
ACh	36	25	18	14
Plasma				
ACh	16	48	11	11
NPA	−8	0	5	0
TA	−10	6	1	15

Results and Discussion

Without Inhibitors. The carbon dioxide evolution was proportional to homogenate concentration under the experimental conditions (Table I). Whereas usually the activity-time relationship was linear—i.e., the carbon dioxide liberation per 5-minute reading interval was constant from the first to the sixth interval—this was not the case with NPA: In all reaction systems containing NPA, the carbon dioxide evolution rate dropped such that 25% of the total gas per 30 minutes was evolved in the first 5-minute reading interval and only about 10% in the sixth interval. This effect was observed in systems prepared by the method of unprotective and protective homogenization, from treated and untreated rats. It was not due to the formulation constituents (ethyl alcohol and Triton X-100) nor to inadequate substrate. Possible explanations might be that some kind of product poisoning occurred or that the pH decreased abnormally during assay (*6*).

Single Inhibitors. The acute lethal dose—i.e., the minimum dose which invariably was lethal to all of the 10 rats used in the ultimate determination—was established. The depression recovery curves of a number of esterases after parathion and malathion treatment were established using half of this acute lethal dose (ALD/2) which produced severe symptoms, but allowed the animals to survive. The ALD/2 for parathion was 5 mg. per kg., for malathion 500 mg. per kg. The results (Figure 1) show that the degree of tissue sensitivity to treatment with either compound was of the same order; however, considerable quantitative differences occurred.

The curves for depression and recovery of esterases after treatment with parathion and malathion are resultants of: the distribution pattern of each toxicant—i.e., their activation products—in the body; reaction with the esterases; degradation of the toxicants; and reversal of the enzyme blockage. Synthesis of new esterases is probably negligible in the time periods involved in this study (*8*). With malathion,

metabolism and excretion occur very rapidly and at least seven metabolites are formed in the mouse and hen (*13*). Degradation of malaoxon [*O,O*-dimethyl-*S*-(1,2-bis ethoxy carbonyl) phosphorothiolate] by mammals takes place not only in the liver and kidney, but in various other organs (*14, 15*). The degradation of malathion in the rat has been attributed to the hydrolytic activity of an enzyme system in liver which has been provisionally termed "malathionase" (*7*). The rate of inhibition of different esterases in vitro is known to vary greatly with the organophosphate used as an inhibitor (*16*).

Bergmann (*5*) concluded that many esterases have esteratic sites very similar to those of cholinesterase; as these are the sites attacked by organophosphates and as the curves in Figure 1 are qualitatively comparable, it appears likely that similar recovery processes are involved.

Malathion and parathion were next administered individually to rats at their ALD/4 dosages. Neither induced any visible symptoms, but both caused esterase inhibition (Table II). The esterase inhibition with parathion was similar at the ALD/2 and the ALD/4 dosages, whereas with malathion the lower dosage gave generally less esterase inhibition. The times for maximal esterase inhibition were approximately the same for the ALD/2 and ALD/4 dosages.

Combined Inhibitors. Administration of malathion and parathion simultaneously at their individual ALD/4 dosages produced no symptoms nor any significant inhibition of esterase activity. This effect is interpreted as antagonism, because at this level the compounds individually affected the esterases (see above and Table II).

Application of both compounds each at ALD/3 resulted in symptoms which were more severe than from either compound at the ALD/2, and led to death within an hour after injection (Table III).

If the responses were simply additive, one would expect that the effects of administering the compounds jointly each at ALD/3.5 should be somewhat greater than those produced by either at ALD/2. Table III shows that the effects were at least additive based upon the severity and persistence of symptoms. Therefore, this combination was selected for studying effects upon esterases (Figure 2). A rough comparison (an exact comparison cannot be made because of the differing dose levels) of Figures 1 and 2 shows that the effects of the combined dosages are intermediate between either compound alone at ALD/2. Once more, there is a striking discrepancy between the effects as judged by visible symptoms and by esterase inhibition, indicating that the degree of esterase inhibition does not necessarily parallel the severity of symptoms.

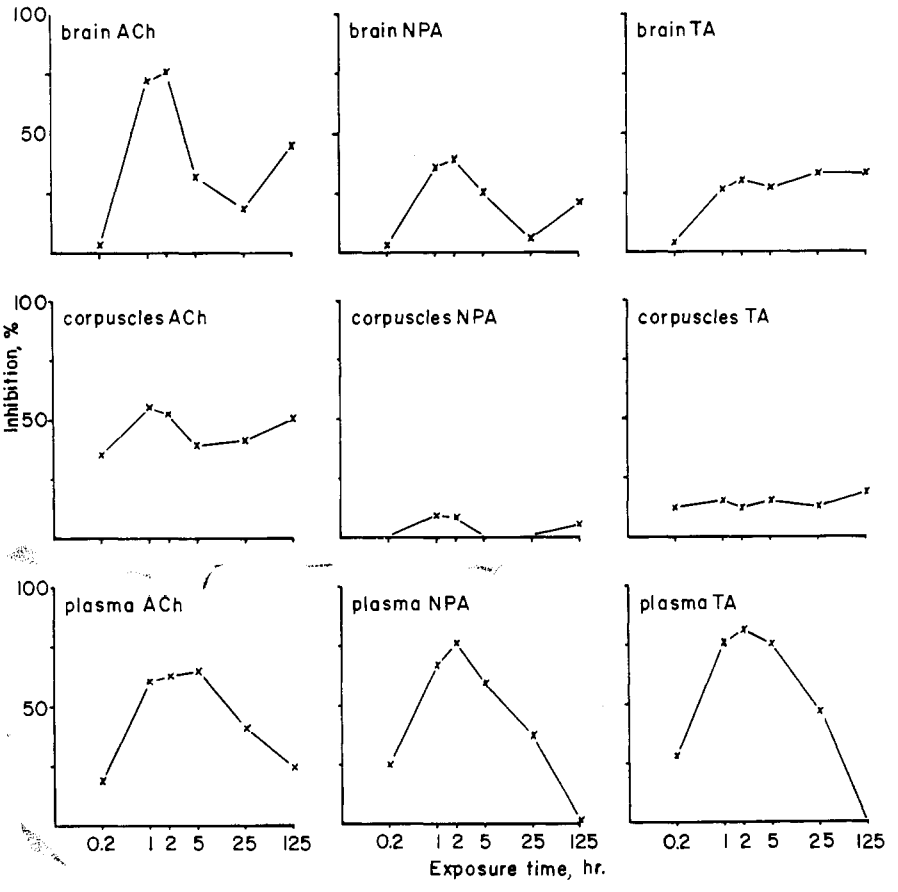

Figure 2. Effect of simultaneous administration of parathion and malathion each at ALD/3.5 upon rat esterases

Table III. Symptoms Following Treatment with Parathion and Malathion[a]

(Beginning and end of intoxication stages in hours after injection intraperitoneally, 4 rats per dosage)

Conditions	Malathion, ALD/2		Parathion, ALD/2		Malathion + Parathion, Each ALD/3.5		Malathion + Parathion, Each ALD/3	
	Start	End	Start	End	Start	End	Start	End
Hypersalivation	0.4	1.8	0.5	2.8	0.2	23	0.1	0.9
Facial muscular twitches	1	2	0.8	3	0.4	19	0.2	0.9
Muscular twitches of whole body	1.2	3.8	1	7.5	0.5	20	0.2	0.9
Apathy (including diarrhea, rough hair)	1.5	4	2	7	0.5	54	0.2	0.9
Death	...		...		...		0.9	

[a] No symptoms with malathion plus parathion each at ALD/4.

Artifacts in Preparing Tissue Homogenates. In poisoned animals certain amounts of organophosphates (and/or their active antiesterase metabolites) may be present at the time of sacrificing, which come into contact with the enzyme only during homogenization and subsequent stages of preparation (*3, 4, 11*). In order to prevent such an artifact, van Asperen (*4*) homogenized the tissues not in buffer (as it is usually done), but in the appropriate substrate solution, thus protecting the enzyme from further reaction with excess of inhibitor. This technique is termed the protective method, whereas the method of homogenization in buffer is called the unprotective method.

Tissues from untreated rats prepared by protective or unprotective homogenization yielded the esterase activities shown in Figure 3. The activity values differed considerably with the two methods. In the case of ACh, it is well known that inhibition occurs at high substrate concentrations. The protective technique was therefore examined in more detail. Experiments were carried out with additional substrate added 5 minutes before zero time or to the homogenization-reaction medium. The design and results of this experiment are given in Table IV. The values obtained with different concentrations of ACh and brain gave the characteristic concentration-activity relationship with

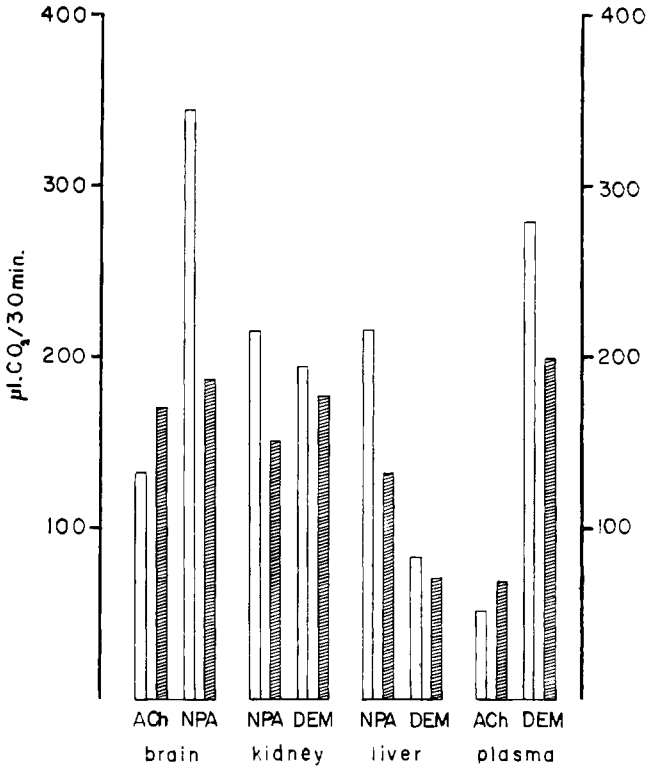

Figure 3. Comparison of unprotective and protective homogenization techniques

Left. Unprotective homogenization
Right. Protective homogenization
Tissues from untreated rats (protection of plasma consisted in diluting it to the desired concentration in substrate solution)

Table IV. Modified Homogenization Methods

(ACh; brain from untreated rats)

Conditions	Molarity	μl. CO_2/ 30 Min.
1. Protective homogenization in 0.05*M* substrate		
a. No additional substrate		182
b. Extra substrate added 5 min. before zero time to total	0.06	185
	0.08	173
	0.1	154
2. Protective homogenization in indicated substrate concentration, no additional substrate	0.05	203
	0.08	168
	0.1	127
3. Unprotective homogenization in buffer, substrate concentration for reaction	0.05	139

Table V. Unprotective and Protective Homogenization with Treated Rats

(5 mg./kg. parathion intraperitoneally)

	Time of Exposure to Parathion, Hr.	Protective		Unprotective	
		μl. CO_2/30 min.	Inhibition, %	μl. CO_2/30 min.	Inhibition, %
Brain NPA	0	187	0	343	0
	0.2	170	9	332	3
	1	150	20	201	41
	5	120	36	190	45
	25	152	19	240	30
Brain ACh	0	169	0	132	0
	0.2	172	−2	134	−1
	1	50	70	41	69
	5	37	78	29	78
	25	59	65	48	64

a substrate optimum for both procedures of homogenization at 0.05*M*. In addition to this proof that the substrate concentration in the protective procedure was sufficient and optimal, the rate of carbon dioxide evolution was constant for the 30-minute reaction period (with the exception of NPA as previously mentioned).

After the validity of the methods had been established comparatively with tissue homogenates from untreated rats, experiments were run with tissues from rats treated intraperitoneally with 5 mg. per kg. of parathion. Two examples of the generally analogous results are given in Table V. The differences of the absolute activity values were noted above. It is clear that in the case of ACh as substrate, the method of homogenization—whether protective or unprotective—does not have any influence upon the inhibition values, if these are based upon the definition "per cent inhibition of normal activity" (columns 4 and 6, Table V). However, in the case of NPA as substrate, some protection was observed, the maximal difference between both methods being at 1 hour of exposure to parathion. The patterns of NPA inhibition were, however, substantially the same with both methods, in spite of the greatly reduced absolute activity which resulted from the protective technique in tissue homogenates containing no free inhibitor.

The differences between the activity values depending merely upon the method of preparation of tissues from unpoisoned animals cannot be explained from the data presented.

The difference between van Asperen's (*4*) and these experimental results might be explained by the fact that he has worked with an insecticide [DDVP (*O,O*-dimethyl *O*-2,2-dichlorovinyl phosphate)] which is a potent inhibitor per se whereas the thiono compound used in this study (parathion) needs conversion into its oxygen analog in order to become a potent ChE inhibitor. As this conversion requires time, thus giving an opportunity for metabolic detoxification, free esterase inhibitor may not have accumulated in amounts sufficient to be affected by competition with the substrate added in the protective method.

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Received for review February 2, 1959. Accepted October 7, 1959. Approved for publication by the Director of the Wisconsin Agricultural Experiment Station. Research partially supported by a grant from the U. S. Public Health Service, National Institutes of Health.

HERBICIDE TOXICOLOGY

Toxicology of Dalapon Sodium (2,2-Dichloropropionic Acid, Sodium Salt)

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Dalapon sodium, a plant growth regulator, is low in acute oral toxicity to laboratory animals, and cattle tolerate large doses without serious effect. Dogs receiving 100 mg. per kg. per day for 1 year and rats given 50 mg. per kg. per day for 2 years exhibited only slight, statistically significant increases in average kidney weights. Rats receiving 15 mg. per kg. per day for 2 years and dogs at 50 mg. per kg. per day for 1 year showed no significant differences from controls. There were small amounts of dalapon found in rat and dog tissues, and in the milk of lactating female rats as determined by chemical analyses. Reproduction and lactation were not affected in rats receiving diets equivalent to 150 mg. per kg. per day. Dalapon sodium presents no unusual handling problems in the field. These data provide a basis for judging the safety of the various residues likely to occur when this herbicide is used as directed on edible produce, or on soil in which food crops are grown.

DALAPON SODIUM is herbicidally active on grasses and cattails at relatively low rates. Controlled laboratory investigation has establishd that this herbicide is readily absorbed and translocated by active grass foliage. It is also absorbed by roots following soil application (*1*). As dalapon sodium may be used effectively for selective control of grasses in certain crops, the following studies were designed to obtain information from experimental animals upon which to base conclusions regarding the safety of any residues occurring in or on edible portions of such crops. Data were desired also for the purpose of making recommendations for safe handling of the material.

Material

Dalapon sodium is a white to tan-white, free-flowing, water-soluble powder with a melting point range of 193° to 197° C. The technical grade material was used in the acute oral studies. By chemical and infrared analyses of the original acid, and by hydrolysis assay techniques on the sodium salt, it was determined that this preparation had the following approximate composition:

83 to 85% 2,2-dichloropropionic acid, sodium salt
6% related chloropropionic acids, sodium salts
1% pyruvic acid, sodium salt
8% sodium chloride
2% undetermined

The sample used in both the subacute and chronic feeding studies was obtained from a large batch produced in May 1953. Twelve 1-pound bottles were sealed and stored under refrigeration, and a particular bottle was not opened until needed. Each time, a 25-gram sample was analyzed. Special attention was given to moisture content as an index of hygroscopy and to sodium pyruvate as an index of hydrolysis. These analytical results indicated that the 12 pounds of material had the following approximate composition and did not change significantly during the storage period:

65% 2,2-dichloropropionic acid, sodium salt
16% related chloropropionic acids, sodium salt
2% pyruvic acid, sodium salt
5% sodium chloride
5% water
7% undetermined

Acute Toxicity

Single oral doses of dalapon sodium dissolved in water were administered by intubation to five animal species in the following concentrations: rats: 10 and 50%; mice: 16%; guinea pigs: 10 and 50%; rabbits: 20%; New Hampshire red chickens: 30%. A total of 130 rats, 15 mice, 18 guinea pigs, 16 rabbits, and 10 chicks were divided into groups and received single doses ranging from 1.0 to 15.8 grams per kg. of body weight. All surviving animals were observed until it was certain that they had fully recovered—usually about 2 weeks. The acute oral LD_{50} with its 19/20 confidence limits was calculated for the five different animal species according to the "Moving Average" method of Thompson (*5*). The results are presented in Table I. Animals that died succumbed within a period of 2 hours to 1 day following administration. No gross pathological changes were found and the principal autopsy findings were a large quantity

Table I. Acute Oral LD_{50} Values for Dalapon Sodium

Species	Sex	LD_{50} 19/20 Confidence Limits, G./Kg.
Rat	Male	9.33 (8.46 to 10.3)
	Female	7.57 (6.88 to 8.35)
Mouse	Female	>4.6
Guinea pig	Female	3.86 (2.76 to 5.43)
Rabbit	Female	3.86 (2.30 to 6.50)
Chick	Mixed	5.66