

CLINICAL OBSERVATIONS AND HISTOPATHOLOGICAL STUDIES
OF TWO ORGANIC PHOSPHORUS COMPOUNDS IN RABBITS

by

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INTRODUCTION

Parasites, both internal and external, are of great importance to the workers in veterinary, medical, and agricultural fields because of their adverse effects on health, productivity, and quality of animals and crops, which result in a great economic loss, the accurate estimate of which is difficult to make.

There are approximately 300 kinds of internal parasites of economic importance to the livestock industry. Together, they cause an estimated annual loss of about \$400,000,000 which represents a conservative estimate since it does not include many hidden losses. Insect pests, as well, have an equally great share in depriving the livestock industry of an income estimated at more than \$500,000,000 per year. Insects also occupy an important place in the field of public health by their role as vectors in transmitting diseases from one animal to another, from man to man, and from animal to man.

The Eber's Papyrus - the oldest medical document, dating from about 1500 B.C. - mentions the use of pomegranate against intestinal worms. This fact demonstrates dramatically the early recognition of the essentiality of parasite control. Parasitism is essentially a herd or flock disease rather than one of individual animals. Due to the abundance in number and kind of parasites, their control is a complex problem. By modern standards, eradication is the only rational goal which, in most cases, is unfeasible in practice--if not impossible. Sanitation and medication are the keynotes of parasite control, which, if applied

strongly must ultimately spell eradication. This would be reflected by an increase in the income of the livestock industry. The control of zoonoses will also be achieved to a great extent.

A properly planned program for eradication has to take into consideration parasite-host relationships, life cycle, source of infection and means of transmission, epizootology and bionomics, and many other factors. The attack is planned to destroy the parasite when it is most vulnerable. Antiparasitic chemicals are powerful weapons in this attack because they are generally immediate in effects, economical, and simple. During the past few decades thousands of chemical compounds were synthesized and tried as parasiticides. Some of them, like phenothiazine, promise to be very efficient against at least a few species of internal parasites. Search for agents for the control of arthropod parasites led to the synthesis of chlorinated hydrocarbons, which, for a time at least, appeared to be the ultimate answer. However, it was soon discovered, to the great disappointment of scientists, that new variants of insects resistant to the chlorinated hydrocarbons were rapidly occurring.

In 1934 Gerhard Schrader (42) of Farbenfabriken Bayer, began a pioneering work, results of which were published in 1947. He synthesized a new group of compounds now known as organic phosphorus compounds. They represent a novel class of pharmacological agents, and their study has led to far-reaching discoveries about the biochemistry of the nervous systems of vertebrates and invertebrates. These compounds were used as

insecticides and were found to be very effective. They can also be used as systemic insecticides, and it was during the study of systemic insecticides in animals that their potentiality as parasiticides was discovered.

Each year a number of organic phosphorus compounds are being synthesized, and trials are run to determine their toxicity to the hosts and parasites, their efficiency as parasiticides, and other related features. Dimethoate (American Cyanimide 12880) and Ruelene (Dowco 109) are two new organic phosphorus compounds which are being investigated as possible parasiticides for use in domesticated animals. The object of this experiment was to observe the clinical symptoms and to study the gross and histopathological changes produced when toxic doses of these drugs are administered.

REVIEW OF LITERATURE

The action of the organic phosphorus compounds is due primarily to their interference with normal functions of the autonomic nervous system. Wescoe (62) discussed the nature of the "chemical mediator" in the function of the autonomic nervous system and explained the important role of acetylcholine. Riker (53), Holmstedt (24), and Whittaker (63) described the mechanism of physiological hydrolysis of acetylcholine by the enzyme cholinesterase.

Specificity of esterases for various substrates, and their classification have been considered in detail by Whittaker (63),

Koelle (30), Metcalf (42), and Holmstedt (24).

Michel (45) described a method to determine the cholinesterase level of plasma and erythrocytes by measuring the variation of pH due to hydrolysis of the substrate by the enzyme. Robbins et al. (54) described a colorimetric method to determine the cholinesterase level of bovine whole blood. Radeleff and Woodard (50) have studied the cholinesterase activity of normal blood of cattle and sheep.

Koelle and Friedenwald (32) developed a technique to localize the esterases histochemically which was applied by Bergner and Durlacher (2) and Bergner and Bayliss (3) to detect fatal poisoning by cholinesterase inhibitors. Koelle (30) modified his technique by using various substrates and varying their concentration to localize the different types of esterases in various tissues.

The mechanism of inhibition of the cholinesterases by organic phosphorus compounds has been explained by Metcalf (49), Fukuto (17), Koelle (30), and Riker (53). A discussion regarding the relationship between the structure of the compound and the nature of inhibition of the esterase is found in the review of "Anticholinesterase drugs" by Koelle and Gilman (31) and in the paper "Pharmacology of Organophorus Cholinesterase Inhibitors" by Holmstedt (24). This author also has described the role of phosphorylphosphatases in the reversal of cholinesterase inhibition by organic phosphorus compounds.

An exhaustive account of pharmacological actions of organic phosphorus compounds has been given by Koelle and Gilman (31) and by Holmstedt (24).

A number of workers are engaged in determining the suitability of organic phosphorus compounds as parasiticides in domestic animals and poultry.

Galvin et al. (18) reported that Bayer 21/199 was effective against helminths in the abomasum and small intestine of cattle and sheep. Herlich and Porter (20) used the same drug at a dosage of 25 mg/kg and found it to be highly effective against species of genera *Hemonchus*, *Ostertagia*, *Trichostrongylus*, *Cooperia*, *Trichuris*, and *Capillaria*. Herlich and Johnson (19) reported the result of critical tests carried out to determine the anthelmintic activity of Dow ET-57. Ruelene gave excellent control against *Hemonchus*, *Trichuris*, *Ostertagia*, *Oesophagostomum*, and *Cooperia* infection in cattle and sheep. It was found to be ineffective against *Nematodirus* and *Bunostomum* (1, 60).

Douglas et al. (10) tested Dowco 105 as an anthelmintic in sheep as a single oral dose calculated at 200 mg/kg and 75 mg/kg. An 89 percent reduction in egg count was noted in the group receiving higher dosage, and 68 percent in those treated with a lower dosage compared to a reduction of 18 percent in the untreated group. Higher dosage showed 91 percent efficiency against abomasal worms, and 98 percent against intestinal worms. Dorney and Todd (8) observed that Dow ET-57, at a dosage of 200 mg/kg showed anthelmintic activity against *Hemonchus*, *Ostertagia*,

Strongyloides, and Nematodirus in sheep. Douglas and Baker (9) found that Ruelene at a dosage of 200 mg/kg was highly efficient against Ostertagia, Trichostrongylus axei, T. vitrinus, and Nematodirus. This drug also was found to be effective against Haemonchus, Cooperia, and Oesophagostomum.

Levine et al. (35) administered orally, eight new experimental organic phosphorus compounds to horses to find their effect on the development of strongyle larvae in feces. They observed that seven compounds gave encouraging results.

Levine et al. (36), in another experiment, used 18 compounds to test their activity against Strongyloides stercoralis in dogs. Only one compound appeared to eliminate nematodes from one dog.

Schad, Allen, and Samson (59) found that Dow ET-57, at a dosage rate of 100 mg/kg, was ineffective in controlling sheep tapeworm Thysanosoma. Ruelene (1) also was found to be ineffective against Moniezia species in cattle and sheep.

Efficacy of organic phosphorus compounds in the control of cattle grub is being extensively investigated. Turner and Gaines (61) reported satisfactory control with Dowco 109 administered at 15 mg/kg as a single oral dose. DeFoliart et al. (7) observed no grub control when Ruelene was used as a spray in 0.5 percent concentration. Drummond and Graham (13) used Ruelene at 25 mg/kg orally and also by the intravenous route and observed grub control varying between 76 and 99 percent. Roth and Eddy (57) investigated the relationship between the time of dosage and duration of cattle grub control. Observation of Knapp et al.

(27) confirm the findings of DeFoliart (7) that the drug is more effective orally than as a spray.

Hewitt et al. (22) noted that Dimethoate as a single oral dose at 2.5 mg to 40 mg/kg and a single intramuscular dose of 5 and 10 mg/kg killed 86 to 100 percent second instar hyperderma larvae in the back of treated cattle within one week after treatment. Ten milligrams per kilogram was found to be the minimum dose uniformly highly effective against third instar larvae. Single oral or intramuscular treatment at 10 and 20 mg/kg killed 50 percent or more of the first instar larvae in the gullets of five calves within 4 to 25 days after treatment. Turner and Gaines (61) reported that Dimethoate was unsatisfactory in controlling cattle grub at a dosage of 8 and 15 mg/kg. Drummond (12) observed that an adequate margin between effective and toxic dosage of Dimethoate for cattle does not seem to exist.

Many other compounds also have been tried as systemic insecticides for cattle grub control, and their efficacy reported (38, 39, 40, 41, 44, 52, 56, 57, 58).

Peterson et al. (46) used Dow ET-57 as a 50 percent emulsion in drench, the dose being calculated at 100 mg/kg. They found that a single dose at this level was effective against only the first instar larvae of sheep head grub. Peterson et al. (47), in another experiment, used Dimethoate orally as a single dose at 5 to 100 mg/kg and intramuscularly in a dosage varying from 20 to 80 mg/kg. They found that the overall percentage of *Oestrus ovis* larvae killed was 97. Drummond (11) reported that

in sheep and goats, a single oral dose of Dimethoate at 50 mg/kg was lethal to the lone star tick and at 25 mg/kg it was lethal to stable flies and screw worms.

Kraemer (33) and Knapp and Krause (28) used Bayer's 21/199 in the control of ectoparasites of poultry. They observed an effective control of the ectoparasites. Hoffman (23) tested 12 different compounds as sprays at 0.05 percent, 0.1 percent, and 0.25 percent concentration. He noted that Dimethoate at 0.25 percent concentration gave a 100 percent lice control for 20 days. Ruelene at 1.0 percent concentration gave similar results. Bigley et al. (4) have, as well, found Malathion and Ronnel effective against poultry mites and lice.

When organic phosphorus compounds are administered in large doses, they produce toxic symptoms. Fukuto (17), Metcalf (42), and Koelle and Gilman (31) have explained the mechanism of toxic action by cholinesterase inhibitors and discussed the relationship of structure to toxicity.

Ruelene (1) at 200 mg/kg caused severe toxic symptoms in cattle whereas some sheep tolerated a dose of 600 mg/kg although deaths occurred even at 400 mg/kg. Two goats given 500 mg/kg died shortly after treatment.

Brady et al. (5) studied selective toxicity and animal systemic effectiveness of Dimethoate, using the rabbit as the experimental animal and the bedbug as the test insect. They found that there was 100 percent mortality among bedbugs feeding on rabbits which were given Dimethoate orally at 50 mg/kg one

hour earlier. When Gulf Coast ticks were allowed to feed on these rabbits, 97 percent mortality was noticed among the ticks. Residual systemic effectiveness of Dimethoate lasted for two hours with a 100 percent death in bugs.

Roberts et al. (55) found that an unknown metabolite was detectable in the blood of cattle which received Dimethoate intramuscularly, intravenously, or orally. This metabolite was 40 times more toxic to the stable fly than Dimethoate. Both the toxic metabolite and the Dimethoate could not be detected after 24 hours. The toxicity lasted longer when Dimethoate was administered orally. Dauterman et al. (6) and Kaplanis et al. (26) studied the metabolism of Dimethoate in cattle. Krueger et al. (34) have published their findings on relationship between metabolism and toxicity of Dimethoate in insects and mice.

Hewitt et al. (21) found that Dimethoate caused the death of sheep in less than 24 hours at a dose of 100 mg/kg. Three calves were given an oral dose of 80 mg/kg and all three died. The same dose intramuscularly produced death.

Radeleff and Woodard (49) studied the toxicological effects of Dow ET-57. Galvin, Bell, and Turk (18) have published their observations on toxicological studies of Bayer 21/199.

Radeleff et al. (50) and DuBois et al. (14) have published their observations on toxicology of Parathione. The toxic dose varies according to the compound and species of animal in which the compound is used. In the same species, the dose varies according to the sex. DuBois et al. (14) and Holmstedt (24)

have published data showing the relation of sex to toxic dose.

Radeleff and Woodard (50) studied the symptoms of poisoning in animals with organic phosphorus compounds. They used 18 different compounds in five different species. The mode of administration also was variable. They noted that irrespective of the animal and the mode of administration, the symptoms are essentially similar. Williams and Hickcox (64) have reported the symptoms observed in Army animals due to poisoning with the nerve gas Sarin.

Holmstedt (24), Koelle and Gilman (31), Radeleff (51), and Williams and Hickcox (64) described the treatment in the event of poisoning due to cholinesterase inhibitors.

Radeleff et al. (51), Radeleff and Woodward (50), Holmstedt (24), and Williams and Hickcox (64) described the lesions observed at necropsy on animals that died of organic phosphorus poisoning.

MATERIALS AND METHODS

Experimental Animal

The rabbit was selected as the experimental animal during this study. Rabbits were obtained from local breeders. Animals selected for the experiment were healthy and active. An attempt was made to use both males and females at the same dose level although it was not the intention to determine specifically the variation in susceptibility due to sex. Age was not taken into consideration while selecting the rabbits.

Feed was withdrawn from the rabbits which were to receive Ruelene, on the evening prior to the morning on which the drug was to be administered. After administration of Ruelene, feed was put back in the cages. Rabbits treated with Dimethoate had food in their cages throughout the experiment.

The rabbits were weighed one-half hour before the administration of the compound, and the weights were recorded in pounds. Weight in pounds was converted into kilograms for the purpose of computing the amount of total dose of the compound.

Drugs Used

Two organic phosphorus compounds were used during this study.

Dimethoate. Dimethoate or American Cyanamide 12880 is chemically defined as O,O -dimethyl S- α -mercapto-N-methylacetamidodithiophosphate. This compound was supplied in a 50 cc vial as a 45 percent solution for parenteral administration.

Ruelene. Ruelene is the trademark of Dow Chemical Company for an organic phosphorus compound chemically defined as 4-tert-butyl-2-chlorophenyl methyl methyl-phosphoramidate. This compound was supplied for oral administration as a wettable powder containing 25 percent of the active ingredient.

It was not the intention to determine the LD₅₀ for rabbits of the compounds used. The dosage was increased until death was caused due to the toxic action of the compound.

A rate of dose of the active ingredient in milligrams for one kilogram of body weight (mg/kg) was chosen, and the total dose

of drug was computed for the weight in kg of the animal used. Table 1 shows the weights of rabbits and the rate of dose of Dimethoate while Table 2 shows the weights of rabbits and the rate of dose of Ruelene administered.

Method of Administration

Dimethoate. After computing the total dose of the active ingredient in mg for the body weight of the experimental rabbit, the volume in cubic centimeters (cc) of the 45 percent solution containing the total dose was found by dividing the weight of the active ingredient by 450. This volume was calculated correct up to the second decimal. The solution was drawn into a 1 cc syringe calibrated in hundredths of a cc and injected intramuscularly in the thigh.

Ruelene. After computing the total dose of the active ingredient in mg for the body weight of the rabbit, the weight of the wettable powder to be administered was found by multiplying the weight of the active ingredient by 4. The powder was weighed on a balance, correct up to one-tenth of a mg, and was suspended in 20 to 50 ml of water, according to the quantity of the drug, so that it could be administered by means of a syringe.

The experimental rabbit was anesthetized by intravenous administration of Halatal (Jen-Sal); the abdomen was shaved and cleaned with 80 percent alcohol. Then an incision was made on the middle, through skin, fascia and peritoneum about an inch behind the xiphoid cartilage, to expose the stomach. The stomach

was fixed by means of an artery forceps, and the suspension of the drug was injected into the stomach. Penicillin was instilled in the abdominal cavity through the incision for antisepsis, and the incision was closed with interrupted sutures.

During this study, individual rabbits were administered a single dose of one compound; however, three rabbits received three doses of Dimethoate at an increasing rate until death occurred. Four rabbits had received Ruelene before they were given Dimethoate, which was administered after apparent recovery from the effects of Ruelene was observed. Two rabbits were given two doses of Ruelene each, at increasing rates.

Observations

Observations were made after administration of the compound for any detectable symptoms of toxicity. Temperature was recorded in the morning and evening. The effect of the drug on the appetite was noted. Weights were recorded in the case of some rabbits which showed severe toxic reaction. Hourly observations were made in case of rabbits receiving the fatal dose.

Rabbits that survived the administration of the compounds were euthanised after a varied-time interval, by an intracardiac injection of saturated solution of magnesium sulphate, and a necropsy was performed. Gross appearance of the viscera was noted. Sections of tissues were collected in 10 percent buffered formalin, and embedded in paraffin. Sections were stained with Harris' hematoxylin and alcoholic eosin (37) for histological

Table 1. Weights of rabbits, dosage of Dimethoate, and severity of toxic symptoms.

Rabbit: No.	Sex	Weight (kg)	Dose (mg/kg)	Date	Toxicity	Remarks
2	--	2.83	125	1-19-1960	Moderately severe symptoms noticed. Complete recovery on 1-22-1960	Had received Ruelene at 75 mg/kg on 1-29-1960. Euthanized on 1-29-1960
3	Male	2.04	150	1-30-1960	Severe toxic symptoms. Died within 6 hours	
4	Female	4.99	210	1-30-1960	Rapid onset of severe toxic symptoms. Died within 6 hours	
5	Male	2.27	130	2- 1-1960	Severe toxic symptoms for 3 days	
		2.03	145	2- 9-1960	Severe symptoms for 3 days	
		1.81	160	2-13-1960	Severe symptoms. Died after 12 hours	
6	Female	3.86	140	2- 1-1960	Severe symptoms for 2 days	
		3.86	150	2-11-1960	Severe symptoms for 2 days	
		3.52	160	2-16-1960	With severe symptoms. Died within 4 hours	
7	Female	4.34	140	2-17-1960	Severe symptoms for 1 day	
		3.97	150	2-27-1960	Severe symptoms for 3 days	Had received Ruelene at 100 mg/kg on 2-1-1960
		3.41	160	3- 5-1960	Very severe symptoms. Died within 2 hours	
8	Male	3.18	160	3- 5-1960	Severe symptoms. Died 6 hours after injection	Had received Ruelene at 112.5 mg/kg on 2-3-1960
13	Male	3.29	128	3-22-1960	Gradual development of severe symptoms. Died 41 hours after	Ruelene at 500 mg/kg on 2-25-1960 Ruelene at 750 mg/kg on 3-15-1960

Table 2. Weights of rabbits, dosage of Ruelene, and severity of toxic symptoms.

Rabbit No.	Sex	Weight (kg)	Rate of dose (mg/kg)	Date of administration	Toxicity	Remarks
1	--	4.26	50	12-14-1959	No toxic symptoms	Euthanised on 12-22-1959
2	--	2.83	75	12-22-1959	No toxic symptoms	Given Dimethoate on 1-14-1960
7	Female	4.42	100	2- 1-1960	Mild toxic symptoms for 3 days. Normal on fifth day	Given Dimethoate on 2-27-1960
8	Male	2.49	112.5	2- 3-1960	Mild toxic symptoms for 3 days	Given Dimethoate on 3-5-1960
9	Female	3.74	125	2- 8-1960	Mild symptoms for 3 days. Normal on fourth day	Euthanised in extremis after 28 hours
		3.18	750	3- 5-1960	Very severe toxic symptoms	
10	Female	3.17	150	2-13-1960	Mild symptoms for 3 days. Showed onset of fever on fifth day	Euthanised on 3-3-60. Showed pneumonia and pyopericardium
11	Female	3.18	250	2-18-1960	Mild symptoms for 2 days. Normal on third day	Euthanised on 2-27-1960
12	Male	3.63	375	2-23-1960	Severe toxic symptoms. Did not show complete recovery	Euthanised on 3-8-1960
13	Male	3.63	500	2-25-1960	Severe toxic symptoms. Normal on sixth day	Given Dimethoate on 3-22-1960
		3.41	750	3-15-1960	Very severe toxic symptoms. Normal on sixth day	
14	Male	3.06	625	2-27-1960	Severe symptoms. Normal on fifth day	Euthanised on 3-21-1960
15	Male	3.06	875	3-19-1960	Very severe symptoms. Normal on seventh day	Not euthanised to observe recovery
16	Female	3.74	875	3-22-1960	Very severe symptoms. Death after 19 hours	
17	Female	2.75	1000	3-24-1960	Lost accidentally	
18	Male	2.27	937.5	3-26-1960	Very severe symptoms. Normal on fourth day	Not euthanised to observe recovery
19	Male	2.95	1000	3-29-1960	Very severe symptoms. Death after 5 hours	
20	Female	3.41	937.5	3-31-1960	Very severe symptoms. Normal on eighth day	Not euthanised to observe recovery
21	Male	3.52	1000	3-31-1960	Very severe symptoms. Death after 7 hours	
22	Female	3.29	937.5	4- 2-1960	Very severe symptoms. Normal on seventh day	Not euthanised to observe recovery
23	Female	2.72	1000	4- 2-1960	Very severe symptoms. Death after 28 hours	

parasympathetic nerves were sectioned.

All of the preganglionic fibers of the autonomic nervous system are cholinergic; that is, they produce acetylcholine at their terminals when stimulated. All ganglia, therefore, contain acetylcholine. In general, all postganglionic fibers of the parasympathetic system are cholinergic. Besides, the postganglionic sympathetic fibers that innervate the sweat glands in some species (man and cat) are cholinergic, as also are probably some vasodilator fibers. Adrenal may be considered as a ganglion, and the nerve fibers supplied to the adrenal medulla are, like all preganglionic fibers, cholinergic. Although not a part of the autonomic nervous system, the nerves supplied to the skeletal muscle are cholinergic. Evidence shows that all cholinergic fibers are capable of synthesizing acetylcholine.

The heart, bronchi, stomach, intestines, salivary glands, sex glands, and bladder receive nerves from the parasympathetic system. Table 3 shows the action of parasympathetic nerves on the effector cells.

It has been noted earlier that the nerves supplied to the skeletal muscle are cholinergic. However, acetylcholine is without effect on striated muscle fiber if applied directly. It activates the muscle through the excitation of the motor end-plate. The mechanism of the action of acetylcholine on a receptor structure was studied through the observations made during the stimulation of the muscle by excitation of the motor end-plate. This is valid with respect to the actions of acetylcholine on other effector cells.

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Table 3. Table showing effects of stimulation of parasympathetic nerve on the effector organs (after W. Clarke Wescoe).

Effector	Action of parasympathetic
Eye - iris	Constriction (ex)
Salivary gland	Secretion (ex)
Bronchioles	Contraction (ex)
Heart	Slowing (in)
Gastrointestinal tract	
Muscle wall	Constriction (ex)
Sphincters	Relaxation (in)
Urinary bladder	
Sphincter	Relaxation (in)
Fundus	Contraction (ex)

ex = Excitatory
in = Inhibitory

The chemical structure and a strong cationic charge endow acetylcholine with its affinity for the motor end-plate. Union of acetylcholine with the motor end-plate results in dissipation of the normal potential difference existing across the end-plate membrane, and there develops a localized activity potential characteristic of excitation at this region, called the end-plate potential. This potential undergoes a spatial decay from the region of the end-plate, and during the decay some degree of depolarization of the surrounding muscle membrane results. As the potential at the end-plate starts building up, the reduced resistance of the muscle membrane permits the formation of the characteristic action potential of striated muscle, which is then propagated without decrement along the muscle fiber. As a consequence, the muscle responds with contraction.

The action of acetylcholine on the neuromuscular junction is excitatory. If the concentration of acetylcholine is excessive, there is an extensive discharge of membrane potential, and the usual regenerative electrochemical process acting to restore the membrane potential is prevented. Under these circumstances, an initial brief excitatory phase is followed by loss of function. This is the mechanism of depolarization paralysis of neuromuscular function or ganglionic transmission. It is characteristically produced by any chemical which interferes with the mechanism of physiological hydrolysis of acetylcholine when liberated at the synaptic junctions subsequent to the transmission of an impulse.

The biochemical functions of acetyl choline have not been conclusively determined. As described above, it was thought to serve as the chemical mediator for nerve impulse transmission between neural synapses or from nerves to muscles and glands. A more recent viewpoint is that acetylcholine is associated with the production of nerve action potential by controlling the permeability of the nerve membrane to ions, and may exercise a similar function in controlling the permeability of the erythrocyte envelope (42).

Inhibition of Cholinesterase by Drugs

Although many chemicals can inhibit cholinesterase in vitro, there are relatively few compounds which act in vivo, producing pharmacological effects that are directly attributable to

inactivation of cholinesterase. Chemically, there are three principal types of compounds that exhibit these actions in vitro and in vivo: (1) alkyl or aryl carbamate derivatives of several organic materials; (2) alkyl or aryl phosphates and alkyl fluorophosphates; and (3) quaternary ammonium ions.

The mechanism of inhibition by some of these drugs is similar to that which precedes the hydrolysis of acetylcholine by the enzyme. Quaternary ammonium compounds will combine at the anionic site of the enzyme protein as acetylcholine does, and thereby act as competitive inhibitors. Since the enzyme has another active center, the esteratic center, which has an affinity for the carbonyl group, drugs having a structure similar to that of acetylcholine inhibit the enzyme by combining with the esteratic center.

Some of the inhibitors of cholinesterase do not form a permanent union with the enzyme, and the inhibition is temporary. This is exemplified by neostigmine. Fluorophosphates, which belong to the organic phosphorus compounds, inactivate the esteratic center permanently by a stoichiometric reaction between the halogen and the enzyme center.

Most of the organic phosphorus compounds are powerful anticholinesterases. In fact, they owe their parasitocidal action to their capacity to inhibit cholinesterase which leads to an unrestricted accumulation of acetylcholine at the sites where it is produced. The speed and magnitude of such a disastrous occurrence can be appreciated from in vitro measurements in which 1 mg of a purified cholinesterase affects the hydrolysis of about 75 gm

of acetylcholine per hour.

During the past ten years a group of enzymes, capable of hydrolyzing phosphorus esters, has engaged the attention of workers. This group of enzymes is designated as phosphorylphosphatases. Their specificity is not yet definitely ascertained. They have been identified in various tissues such as adrenal gland, liver, kidney, testis, spleen, heart, lung, brain, muscle, skin, and plasma.

The plasma enzyme has received much attention. It was found that this enzyme was capable of hydrolyzing not only the esters of phosphorus but also phosphorylated esterases, thus uncoupling the combination between the cholinesterase and the organic phosphorus compound, and releasing the cholinesterase for its physiological function of hydrolysis of acetylcholine. This action of the enzyme acts as the natural detoxifying mechanism. By adaptive production of phosphorylphosphatase, the insects can as well develop a resistance to these erstwhile potent insecticides.

Cholinesterase and Its Pharmacological Significance

In 1926 Loewi and Navrahl (64) discovered in the frog's heart, the presence of an enzyme capable of blocking "Vagustoffe" rapidly. Stedman and co-workers (64) suggested the term cholinesterase for this enzyme which regulates the physiological activity of the acetylcholine by catalyzing its hydrolysis rapidly.

The enzyme protein is considered to have two active centers. The first is called the anionic site, for it is the point at which

the cationic head of acetylcholine combines electrostatically. The second center is referred to as the esteratic site, for it is at this site that the chemical union between the enzyme and the substrate occurs. However, this is only an intermediate complex with which water reacts, and as a consequence, the ester link is activated and hydrolyzed, resulting in denaturation of substrate.

Several types of esterases which are capable of hydrolyzing acetylcholine have been found in the tissues of man and animals. Galehr and Plattner (64) observed, in 1928, a difference in choline-splitting activity of serum and whole blood. The latter showed greater activity quantitatively, and this was explained by assuming that in addition to serum, the erythrocytes also contained an esterase. It was further noted that erythrocytic cholinesterase differs from the serum cholinesterase in that the former was able to hydrolyze acetyl-B-methylcholine and was sensitive to changes in pH and salt concentration. Presence of esterases capable of hydrolyzing acetyl choline along with other substrates were soon detected in many tissues.

Investigations of substrate specificities of acetylcholine-splitting enzymes from various sources and their sensitivity to different inhibitors led Mendel and Rudney (65) to propose a general classification of enzymes of this type. Included under the term "Pseudocholinesterases" were the enzymes from pancreas, serum, and other tissues which hydrolyzed acetylcholine, benzoylcholine, and several noncholine esters, but not acetyl-B-

methylcholine and which exhibited maximum activity in the presence of high concentrations of acetylcholine. The term "True Cholinesterase" was reserved for the enzymes of erythrocytes and nervous tissue which acted upon only esters of choline including acetyl-B-methylcholine but not benzoylcholine, and which were inhibited by high concentrations of acetylcholine.

The pharmacological significance of the above studies lies in the fact that endogenously liberated acetylcholine, at the low concentration in which it is present in the body, apparently is hydrolyzed exclusively by means of acetylcholinesterase. Consequently, anticholinesterase drugs produce their typical cholinergic effects when this type of enzyme is inhibited beyond a certain threshold. This fact was not fully appreciated prior to the studies of alkyl phosphates, which were shown to be capable of producing practically complete inactivation of serum cholinesterase without causing any significant symptomatology. Therefore, the fact that a compound is capable of inhibiting serum cholinesterase does not imply that it produces its pharmacological effects by this mechanism.

Increasing uses of organic phosphorus compounds have occasioned the need for determination of the degree of cholinesterase inhibition in animals treated with such compounds. The cholinesterase activity of the erythrocytes may be employed as an index of activity of acetylcholinesterase in other tissues to determine the toxicity of the compound used.

Several methods for determination of cholinesterase activity, including manometric, electrometric, and titrimetric techniques, are available. Metcalf (56) applied the calorimetric reaction of Hestrin for acetylcholine to the measurement of human erythrocytic and serum cholinesterase activity. This method is suitable to determine rapidly, a low degree of cholinesterase activity after inhibition. A small volume of blood is needed. Since a direct measurement of unhydrolyzed substrate is obtained, the results are precise and reproducible.

Pharmacology of Organic Phosphorus Compounds

The compounds of phosphorus that are being developed differ from one another in chemical composition and structure, and also in efficiency as parasiticides. However, since most of them inhibit the cholinesterase to a greater or lesser extent, the toxic symptoms produced by them follow similar patterns. Di-isopropyl-fluorophosphate (DFP) was first synthesized in 1932 and has been extensively studied since. Magur and Bodansky (31) observed that when human subjects were exposed to low concentrations of DFP, no toxic symptoms were noticed although serum cholinesterase was completely inactivated. This surprising finding was explained by recognizing that acetylcholinesterase of nervous tissue and erythrocytes is less susceptible to inhibition by DFP than the cholinesterase of plasma.

The actions of alkyl phosphates on autonomic effector cells are due to the irreversible inhibition of cholinesterase. The

responses observed are similar to those seen when cholinergic nerves are stimulated but the effects are of longer duration since the physiological hydrolysis of choline is no longer possible.

Eye. An intense miosis develops within a few minutes following exposure to the vapors of alkyl phosphates or topical application of solutions. Following a single instillation of DFP in normal human eyes, the spasm of accommodation lasts for days, and the miosis persists for weeks. A decrease in intraocular tension accompanies the miotic action of DFP. The effects observed on topical application of DFP are not seen following the systemic administration. Miosis is prominent in experimental animals only when lethal doses are administered.

Lung. DFP causes constriction of bronchial muscles and increased secretion of bronchial glands. These effects of alkyl phosphates are very prominent in animals receiving large doses, and contribute significantly to the lethal effects of these compounds.

The respiratory symptoms are, however, not entirely of local action. By recording the action potentials of phrenic and intercostal nerves, Krivoy et al. (24) showed that respiratory paralysis by DFP included an important central component. Wright (66) analyzed central and peripheral components of respiratory failure produced by cholinesterase inhibitors, and found that neuromuscular transmission was first impaired.

Gastrointestinal Tract. The actions of DFP on the gastrointestinal tract in animals are prominent. There is an increase in the tone of the intestinal muscle as well as the rate and amplitude of contraction.

Duodenal activity is extremely susceptible to the influence of cholinesterase inhibitors. Doses producing respiratory effects caused a spastic contraction of the duodenum within one to two minutes. These effects were observed all over the intestinal canal, and can be antagonized by atropine.

Cardiovascular System. Small doses of DFP do not produce prominent effects on the cardiovascular system. With large doses the blood pressure falls progressively to shock levels. A complete A-V block occurs. The cardiovascular effects are prevented by atropine.

Miscellaneous. The effects of alkyl phosphates on other autonomic effector cells have not been studied in detail but certain observations are made in the course of studies of the general systemic actions of this group of anticholinesterases. DFP enhances the secretion of the submaxillary gland, and sweat glands also are stimulated. Salivation is prominent in animals receiving lethal doses. Ninety percent cholinesterase must be inactivated before spontaneous salivation begins. Sensitization to nerve stimuli occurs when 50 percent of the enzyme is inhibited. Dogs receiving DFP over long periods exhibit urinary incontinence.

Autonomic Ganglia. The mechanism of transmission of nerve impulses at the ganglionic synapses is, as yet, a matter incompletely understood. While practically all investigators believe that acetylcholine plays a role in the metabolism of autonomic ganglia and is in some way related to synaptic transmission, beyond this point, agreement ceases. Most of the pharmacological actions of the anticholinesterase drugs at this site can be interpreted on the basis of their acting only as enzyme inhibitors, and with the full acceptance of acetylcholine as the chemical mediator of transmission.

Striated Muscle. When DFP was administered intra-arterially to normal subjects, fasciculations and motor weakness developed; the degree and duration of which depended upon the dose. Larger doses (2.0 mg) caused a pronounced paresis, and strength returned over a period of 11 weeks. If, however, the drug was administered by a route other than intra-arterial, comparatively larger doses were needed to produce similar effects. This was due to the uptake of drug by the cholinesterase of plasma and other tissues.

The effect of DFP on patients of myasthenia gravis was entirely different. Normally, in these patients, the muscles show a progressively declining response to motor nerve stimulation or administration of acetylcholine. Administration of DFP corrected this, and the response of the muscle became similar to that of a normal individual without DFP. The effects were noticed within 15 minutes after intra-arterial administration, and were

maintained for days.

Central Nervous System. In 1945, Feldberg (15) reviewed literature on the actions of anticholinesterase agents at central sites, and stated, "The present position of the theory of acetylcholine as central transmitter is all but settled." Much information has been obtained since then by the use of alkyl phosphates which exhibit more prominent central actions. This is probably due to their high lipoid solubility and the rapidity with which they gain access to nervous tissue and reduce the cholinesterase activity of the brain to a critical level.

Application of DFP to the brain was found to produce tonic and clonic convulsions which continued without interruption. It appeared that animals die of central rather than peripheral actions of these drugs when administered in lethal doses.

An electro-encephalogram indicates increased electrical activity of the brain. DFP also increases spinal cord activity in humans.

Toxicology of Organic Phosphorus Compounds

The fact that continuous activity goes on at most cholinergic synapses despite a gross appearance of quiescence, explains the progressive onset of symptoms when toxic doses of the organic phosphorus compounds are administered to animals. After inhibition of the cholinesterase, tonic activity leads to the accumulation of a sufficient quantity of acetylcholine

to manifest overfunction at several structures concerned. The symptoms of poisoning, therefore, are those noticed when excessively large amounts of acetylcholine are continuously injected.

Alkyl phosphates can cause death by three different mechanisms: (1) excessive stimulation of the autonomic effector cell, usually called the muscarinic effect; (2) stimulation followed by paralysis of striated muscle, called nicotinic effect; and (3) central stimulation followed by depression, called the central action.

Muscarinic Effects. In order of their appearance they are: anorexia and nausea, followed by vomiting, abdominal cramps, sweating, salivation, and in some cases by pupillary contraction. In severe cases of poisoning, diarrhea, tenesmus, involuntary defecation and urination, pallor, pinpoint nonreactive pupils, blurred vision, excessive bronchial secretion, respiratory difficulty, and pulmonary edema with cyanosis ensues.

Nicotinic Effects. Twitching of the eyelids and tongue are early symptoms. This is followed by fasciculation of facial muscles, and the rest of the muscles are progressively involved so that general fasciculations and weakness result. High concentration of acetylcholine rapidly results in muscular paralysis, and death occurs when respiratory muscles become involved.

Central Effects. Symptoms manifested as a result of central involvement are giddiness, uneasiness, restlessness, anxiety, and

in severe cases, ataxia, drowsiness, and finally coma.

Poisoning due to most of the organic phosphorus compounds gives rise to symptoms which follow more or less those described above. It must be noted, however, that they vary in different species of mammals. Variation also is due to the compound involved.

Radeleff and Woodard (50) studied the general symptoms observed in poisoning by phosphorus compounds. In their experiments they used cattle, calves, sheep, goats, and turkeys. Eighteen compounds were administered orally and 17 compounds were used externally as spray or dip. Their findings are as follows: poisoned animals showed excessive salivation; saliva being of a watery consistency. The animals breathed with their mouths open and with greatly exaggerated respiratory movements. They walked stiff-legged and wandered about restlessly. Fasciculation of all skeletal muscle was present. Eventually, exhaustion forced the animal to lie down. As death approached, there were pulmonary rales, and the animal grunted softly. Death appeared to occur by suffocation. Only with the highest doses have convulsions been seen.

In acute poisoning, lesions are never outstanding and pathognomonic. In many cases, findings are negative. Lesions, when they appear, may be hemorrhages of varying sizes on the heart, lungs, or gastrointestinal canal, which are not consistent in their location. Lungs may be congested and often are

edematous and heavy. Frothy exudate may be present in the bronchi and trachea. If the animal has been affected over a prolonged period, pneumonia may be present.

In another experiment, these authors studied the toxicology of Dow ET-57, and found that this drug produced symptoms which resembled those due to chlorinated phenols, in the beginning. Later, however, the symptoms were those typical of a cholinesterase inhibitor.

DuBois et al. (14) studied the toxicity of parathion in dogs, cats, mice, and rats. The toxic symptoms conformed, in general, to those described above. They noted, besides, that male rats were more resistant to the drug than the females. They further found that injections of stilbestrol in males increased their susceptibility to parathion while injection of testosterone in females decreased their susceptibility. Table 4 shows the relation of sex and lethal dose of some of the organic phosphorus compounds.

Chronic Poisoning. Physiological effects of a chronic reduction of cholinesterase have been studied in dogs, cats, rats, and monkeys by the administration of DFP over a period of six months (31). Animals in which cholinesterase activity was only moderately depressed exhibited few signs and symptoms and no changes occurred in the formed elements of the blood or in the blood chemistry. When dogs were given doses sufficient to elicit nicotinic and muscarinic responses, and such doses were repeated twice

Table 4. Acute oral LD50 values of some organic phosphorus compounds for white rats. (After Holmstedt, B.)

Compound	Oral LD50 mg/kg	
	Male	Female
Malathion	1375	1000
Chlorothion	880	980
Dipterex	630	--
Diagidon	108	76
DDVP	80	56
EPN	36	7.7
Parathion	15	3.6

weekly for several months, functional disturbance of smooth and striated muscles occurred which persisted after the drug was discontinued. The first effect on striated muscles was the appearance of fasciculations in the tongue. These then spread to other muscles. Muscle weakness of hind legs eventually leading to paralysis followed within three months and showed no significant improvement when the drug was discontinued.

Regeneration of Cholinesterase

A point of great importance in the toxicology of phosphorus compounds is the irreversibility of the cholinesterase inhibition. Although some reactivation may occur, repeated chronic absorption or severe acute poisoning reduces the levels of cholinesterase enzymes of blood and tissues to 10 to 20 percent of normal, and the rate of regeneration is equivalent to the time required for

synthesis of new enzyme proteins.

Hunt and Ricker (25) investigated the time required for regeneration of cholinesterase of the muscle and the brain in 32 cats subjected to chronic poisoning with DFP. They found that regeneration of the muscle cholinesterase is most rapid, being complete in two weeks. The brain cholinesterase regenerated more slowly. By one month it had reached 69 percent, and in the nerves, 85 percent of their normal values.

The regeneration of enzyme protein apparently takes place at each of the sites at which the enzyme naturally occurs, and the rate at which it is reformed is a function of the tissue concerned. After severe depletion of cholinesterase, the non-specific enzymes in the blood serum of man returned to normal in about two weeks. These enzymes are generated by liver, and reflect the capacity for protein synthesis of this organ. Restoration of brain cholinesterase requires nearly three months. The seeming parallelism between the recovery of erythrocytic and brain cholinesterase is coincidental, and the regeneration of erythrocytic cholinesterase actually relates to the rate of hematopoiesis.

Rate of regeneration of erythrocytic and brain cholinesterase has been studied in rats that were exposed to Sarin. The evidence obtained in these studies showed that recovery of erythrocytic cholinesterase was delayed by one or two days as compared to other tissues. Rate of regeneration of erythrocytic cholinesterase was found to be approximately 4.4 percent per day

for the first 15 days and it decreased subsequently to about 0.8 percent per day. From these rates, by calculation, complete recovery time was found to be 48 ± 6 days, a figure considerably lower than the one stated earlier. Regeneration of brain cholinesterase was found to be slower than the erythrocytic regeneration. During the first 15 days the rate was found to be about 3.7 percent per day, and it dropped to 0.3 to 0.4 percent, according to the estimates. Calculated time for complete recovery appeared to be about 140 ± 31 days (24).

Atropine relieves the muscarinic effects produced by the anticholinesterases, whereas magnesium is effective in counteracting the nicotinic effect to a great extent. Atropinization is the method of treatment which affords immediate relief from toxic symptoms. A knowledge of the rate of regeneration of cholinesterase, therefore, gives an indication of the extent and duration for continuing atropinization in poisoning with organic phosphorus compounds.

Discovery of phosphorylphosphatases stimulated the search for a drug which could be used in the treatment of poisoning by organic phosphorus compounds. In 1951, Jandorf (24) found that hydroxylamine acted rapidly and smoothly with organophosphorus inhibitors and detoxified the compounds. In 1953, Wagner-Jauregg et al. (24) found that compounds known as hydroxamic acids are effective reactivators. During the same year, Wilson and Meislich (65) reported that DFP-inactivated cholinesterase could be regenerated successfully by treatment with dilute solutions of

certain hydroxamic acids in vitro. In 1955, Davies (24) introduced oximes, particularly pyridine-2-aldoxime methiodide (PAM) as the reactivator. Intravenous injections of 5 mg/kg of PAM into cats and dogs poisoned by intravenous injections of about LD50 of either Savin or Tabuin produces comparatively rapid and complete recovery of the ability of muscles to maintain a response to tetanic stimulations.

OBSERVATIONS ON RABBITS TREATED WITH DIMETHOATE

Clinical Observations

Rabbits that were given sublethal doses of Dimethoate exhibited muscular weakness and contraction of the pupils as the first signs, noticeable within three hours. These signs were followed by an onset of diarrhea and salivation; also muscular twitching was observed along with a rapid, panting type of respiration accompanied by rales. Muscles soon became flacid so that the animal was not able to move, and was lying in the cage exhausted, with its head resting on the floor. Food and water remained untouched. These signs subsided gradually and the rabbit appeared normal on the third or fourth day. The severity, time of onset, and duration of toxic symptoms varied with the dose and the individual.

When lethal doses of Dimethoate were administered, the same signs were observed except that they appeared earlier and were of a more severe character. Muscular fasciculations were seen four to six hours after injection. Death occurred, apparently due to

respiratory failure, two to twelve hours after the injection.

Rabbit No. 6, a female, aborted four days after the second injection was given. Rabbit No. 5 showed suppurative pneumonia in the right lung, probably metastatic in origin, from an abscess on the left flank.

Necropsy Findings

In rabbits that died of acute toxicity, the heart was found to have stopped in a diastolic state, all the chambers were full of blood, and the coronary vessels were engorged.

The larynx, trachea, and bronchi were severely congested, and in most cases contained a thick mucous discharge, which in a few cases was blood stained. The lungs were emphysematous and hyperemic. Collapse and hemorrhage were occasionally seen around the hilus and near the borders.

The pyloric portion of the stomach was firmly contracted. The small intestines were hyperemic and contained only a small quantity of mucous fluid. The cecum was filled with ingests in all animals, and the colon and rectum were firmly contracted and contained a few mucous clots. The liver, kidneys, and spleen were hyperemic. The urinary bladder was found to be distended in a few animals, but in others it was found to be empty.

The cerebrum and cerebellum were severely congested.

Histopathological Observations

Heart. The myocardial vessels were congested, and the majority of the sections revealed cloudy swelling of the muscle fibers. Myocardial necrosis was seen in one case.

Trachea. Submucous blood vessels were severely congested in all specimens, and hemorrhage was observed in one section.

Lungs. Severe congestion and marked emphysema were constant findings in all cases, and areas of collapse also were present. Alveolar hemorrhage and edema were observed in a few specimens. Suppurative broncho-pneumonia was seen in rabbit No. 5.

Skeletal Muscle. Cloudy swelling of the fibers and loss of striations were observed in some of the bundles of thigh muscles from the site of inoculation of Dimethoate.

Stomach. Gastric mucosa was congested, and ulceration of the gastric mucosa also was observed in rabbits which were given Ruelene before they were treated with Dimethoate. In the case of rabbit No. 8, death was caused by a perforated gastric ulcer.

Small Intestine. Acute hyperemia of the mucosal vessels was present in all cases. Exfoliation of the epithelium of the villi was observed in some cases. In the case of rabbit No. 8, an ulcer, extending to the muscularis externa, was seen in the duodenum.

Large Intestine. Acute congestion of the mucous membrane was seen in all sections, and hemorrhage was observed in two sections.

Liver. Acute congestion and cloudy swelling was a constant observation, and mild fatty changes were seen in the liver of rabbit No. 6.

Spleen. Acute congestion was present.

Kidney. Acute congestion and cloudy swelling of the cells of convoluted tubules was observed. Glomeruli were swollen and occupied the entire Bowman's capsule.

Reproductive Organs. Except for hyperemia, no other changes were observed in either male or female reproductive organs.

Cerebrum. Acute congestion and edema were present. Mild gliosis and cuffing were seen in rabbits Nos. 5 and 6. These lesions probably were not due to the toxic action of the drug because rabbit No. 5 had developed metastatic suppurative pneumonia, and rabbit No. 6 had aborted. Sections stained with Gallocyanin did not reveal degenerative changes in neurons.

Cerebellum. Acute congestion and edema were observed.

Medulla oblongata. Acute congestion.

Spinal Cord. Acute congestion.

OBSERVATIONS ON RABBITS TREATED WITH RUELENE

Clinical Observations

Rabbits treated with varying doses of Ruelene at the rate of 50 mg/kg and 75 mg/kg did not show toxic symptoms whereas stiffness of hind quarters, and anorexia for two or three days after the treatment were observed in rabbits treated with doses ranging between 100 mg/kg and 150 mg/kg. One rabbit, No. 8, showed

panting type of respiration and rales when treated with a dose at the rate of 112.5 mg/kg.

Rabbits that received the compound at dose level, ranging between 250 mg/kg to 750 mg/kg, manifested severe symptoms. The first noticeable symptoms were slight pupillary contraction and stiffness of the limbs. These signs were followed by salivation and diarrhea, after which lacrimation also was noticed. Respiration was rapid and of the panting type. Salivation became profuse usually four hours after administration of the compound. Distinct rales were heard and the rabbits passed clots of mucin along with diarrhoeic stools. Muscular twitching was noticed in animals receiving the higher doses. Complete recovery was apparent within a period varying between three to six days. Weight loss up to 0.45 kg was observed in severe cases.

One female died at the dose level of 875 mg/kg and another at 937.5 mg/kg although two other females survived doses at 937.5 mg/kg. One thousand mg/kg was found to be the dose which caused death in every male and female.

The symptoms observed in animals treated with a lethal dose were: constriction of pupils (Plate I); salivation (Plate II); lacrimation, rapid and panting type of respiration with rales, a fall in rectal temperature (Plate III); diarrhea which was followed by passage of gelatinous mucus clots, muscular twitching and fasciculations, paralysis, extreme prostration (Plate IV); convulsions, and death. Death occurred within 5 to 28 hours after administration of the compound. (All Plates are in the Appendix.)

The onset of toxic symptoms, severity duration, and time of death varied with individual rabbits even at the same dose level. One rabbit, No. 10, developed suppurative pneumonia and pyopericardium. Pasteurella was isolated in pure culture from the pus in the pericardial cavity.

Necropsy Findings

Dilatation of the heart was observed in all rabbits (Plates V and VI). Trachea and bronchi showed severe congestion and contained a sticky, mucous discharge which, in a few cases, was hemorrhagic. The lungs showed marked emphysema and severe congestion (Plate V). Hemorrhage was noticed in a few cases, around the hilus.

The stomach showed a firmly contracted pylorus, and usually contained ingesta. When the stomach was emptied, the mucous membrane was found to be coated with a sticky mucus. Hemorrhage and ulceration also were observed on the mucous membrane. The small intestines were hyperemic, and in the duodenum, ulcerations were noticed in a few cases. Intussusception of the small intestine was observed in two animals (Plate 7). The cecum was always filled with ingesta. The large intestines were empty and contracted. In a few rabbits the urinary bladder was distended with urine. The liver, kidney, and spleen showed hyperemia. The meninges and brain were severely congested.

Histopathological Observations

Trachea. Severe congestion of the submucous vessels was a constant finding. Hemorrhage also was noticed in a few cases.

Lungs. Severe congestion and acute alveolar emphysema were constant lesions (Plate VIII). Collapse of a few lobules also was noticed. Hemorrhage was observed in a few sections. Edema was noticed in the case of rabbits Nos. 16, 17, and 23, of which Nos. 16 and 23 were autopsied about four hours after death. Bronchial hemorrhage was observed in some sections.

Heart. Severe congestion and cloudy swelling were observed in all cases. Myocardial hemorrhages were observed in a few cases.

Diaphragm. The diaphragmatic muscle showed moderate swelling of fibers and loss of striations in some muscle bundles.

Salivary Gland. The salivary gland showed the alveoli in a resting stage.

Stomach. The gastric vessels showed severe congestion. The mucous membrane was necrotic, and ulcerations were present (Plate X). In a few sections the ulcer was found to extend up to the muscularis externa while in one, perforation was seen.

Small Intestine. Severe congestion was observed in mucosa and submucosa in all sections. Epithelium of the villi was found to be exfoliated. Ulceration was seen in two cases (Plates IX, XII, and XIV).

Large Intestine. Congestion of mucosal vessels was the change observed in all sections; in a few sections the glands

showed hyperactivity (Plate XI).

Liver. Acute congestion and cloudy swelling was observed.

Spleen. Congestion was observed in every section.

Kidney. Acute congestion was present in all cases. Convoluted tubules showed cloudy swelling. Glomerular tufts were swollen and occupied almost the entire Bowman's capsule. In one rabbit, infarction of the posterior pole of the left kidney was seen. This was not considered to be related to the action of the compound.

Reproductive Organs. Acute congestion was noticed in all cases.

Cerebrum. Acute congestion and edema was found in all cases. Sections stained with Gallocyenin did not reveal degenerative changes in neurons (Plate XIII).

Cerebellum. Acute congestion and edema were observed.

Medulla Oblongata. Acute congestion was observed.

Spinal Cord. Showed acute congestion.

DISCUSSION

From the observations made during this study it was found that the lethal dose of Dimethoate for rabbits was approximately 160 mg/kg, although one rabbit died after being treated at the rate of 150 mg/kg. Ruelene was found to cause death, without any exception, when the dose was calculated at the rate of 1000 mg/kg. Two females died after receiving Ruelene at a lower dose level of 750 mg/kg and 875 mg/kg, respectively, but two females survived

the dose calculated at 937.5 mg/kg. None of the males died at a dose lower than 1000 mg/kg. Thus, it was found that of the two compounds used, Dimethoate apparently was more toxic than Ruelene. Absence of fatalities in the males by doses of Ruelene below the level of 1000 mg/kg, and less severe symptoms for a shorter duration, as compared to females, gave an impression of the males being comparatively less susceptible to Ruelene than the females. More experimental data must be obtained before this aspect is decided.

The symptoms of poisoning produced by both the compounds were very similar and were typically those produced by inhibition of cholinesterase. Individual variation was observed as to the time after which the first symptoms were noticed. The severity of the symptoms, their duration, rate of recovery, and the fetal termination also were found to be variable in the individual rabbit.

The first noticeable signs were the nicotinic effects involving the skeletal muscles. However, when large doses were given, it was found that the nicotinic effects and the muscarinic effects occurred almost simultaneously. Symptoms indicative of nervous involvement appeared later.

Constriction of the pupils was noticeable as one of the first muscarine-like effects of the compounds on cholinergic nerves of the iris. Lacrimation also was evident, subsequent to the excitatory effect as a result of inhibition of cholinesterase.

The respiratory symptoms comprising rapid, panting type of breathing, rales, and dyspnea are of a complex nature.

Involvement of respiratory muscles causes the dyspnea in the early stage and an increased inspiratory discharge occurs in the phrenic nerve, according to Wright (66) who analyzed central and peripheral components of respiratory failure produced by anticholinesterase compounds. The constriction of the bronchial muscles and progressive accumulation of the bronchial secretion further aggravate the respiratory distress. Paralysis of the respiratory muscles and failure of the respiratory center may together precipitate the ultimate respiratory failure. The effects on the respiratory apparatus are important since they may precipitate a nonclinical respiratory disease into a severe respiratory disease in animals receiving sublethal doses, a fact to be considered while animals are being treated with these compounds. This was observed in case of rabbit No. 10 which developed pneumonia and pyopericardium after treatment with a single dose of Ruelene.

Salivation is another muscarine-like effect of cholinesterase inhibitors. As the symptoms progress, the salivation is profuse and the saliva flows out of the mouth owing to inability of the animal to swallow. The respiratory distress may be aggravated further by aspiration of the profuse salivary secretion.

Increased peristalsis was evident in the initial stages, by the frequent passage of feces of normal consistency in the beginning, and this was followed by the stools being coated with mucus and a gradual change in their consistency. This indicated an increased intestinal secretion. Tenesmus was evident and the animal

passed only gelatinous mucus clots, indicative of a complete emptying of the colon and rectum and possibly of a spasm of the caeco-colic valve.

The nicotine-like effects on skeletal muscles were evident by a stiffness in their earliest stage. Involuntary muscular twitching and generalized fasciculations followed. Progressive loss of tonus of the muscles followed, which became very flabby, and ultimately paralysis occurred. The sequence of these symptoms is in conformity of the known facts about neuromuscular transmission. The partial inhibition of the acetylcholinesterase of muscles in early stages of the poisoning causes an increased tonus exhibited by stiffness. However, the progressive accumulation of acetylcholine, owing to progressive inhibition of cholinesterase, leads to a permanent loss of the end plate potential and cessation of the usual neuromuscular transmission leading to depolarization paralysis. These effects are very important in respect to respiratory muscles, paralysis of which leads to respiratory failure and death.

Symptoms referable to the involvement of the central nervous system consisted of ataxia, and were followed by convulsions. The role of acetylcholine in the function of the central nervous system has not been completely understood. The central effects of anticholinesterase compounds, as studied by the electrical activity of the cortex, comprise desynchronization, convulsions, and finally quiescence of the cerebral cortex. Respiratory and probably cardiac centers also are affected as can be surmised

from the respiratory disturbances and general hyperemia.

Abortion was encountered in one female, whereas dead embryos were encountered in another female. This observation probably is of some significance but more information needs to be collected for a proper understanding of the precise hazards involved in this regard.

In spite of the manifestation of toxic symptoms of a very severe degree, two males and two females survived a dose calculated at the rate of 937.5 mg/kg. The first indication of recovery was cessation of salivation. The respiration improved gradually and the animal was able to raise its head. Water was taken after about 48 hours. The animal was able to move after 72 hours but was easily exhausted even after slight exertion. Food was consumed in small quantities and stools returned to apparently normal consistency. Normal intake of food was resumed seven days after the administration of Ruelene, and complete recovery was observed after 10 days.

At necropsy, the heart was found to be in a diastolic state and all the chambers contained blood. The coronary vessels were engorged. Histopathological examination of myocardium revealed acute congestion, and hemorrhage also was noticed in two cases. These findings were strongly suggestive of cardiac failure in the terminal stages of the poisoning.

The trachea and bronchi were always found to be severely congested and contained varying amounts of mucous discharge. Hemorrhage was observed in two cases. Marked emphysema and acute

congestion was conspicuous in the lungs, and hemorrhage was noticed in a few cases near the hilus and along the borders. The bronchial constriction, together with the impediment for expiration by the increased secretions, explain the occurrence of emphysema. Edema of the lungs was observed in the case of rabbits which were necropsied four or more hours after death. In other cases, it was absent or insignificant.

The liver, spleen, and kidney showed hyperemia at necropsy which also was seen at the histological examination of these organs. Hyperactivity of the glands and severe congestion were observed in the stomach and intestines. The small intestines, colon, and rectum were found to be empty, but the cecum always was found to be filled with ingesta. The colon and the rectum were firmly contracted. The pylorus was firmly contracted, and the stomach was full with ingesta. In rabbits which were given Ruelene intragastric, the mucous membrane was covered with an excessive quantity of mucin, and hemorrhage and ulceration also were observed. These findings could possibly be attributed to the irritant effects of the compound. Ulceration was encountered in the duodenum in some cases but not in any portion below that level. Intussusception of the small intestine, probably owing to the violent peristalsis produced by the compound, was observed at necropsy in two rabbits treated with Ruelene.

Male and female reproductive systems showed congestion at necropsy. Histological examination showed normal spermatogenesis in the sections of testes and failed to reveal any lesion in

ovaries. A reference already has been made to the occurrence of abortion in one female.

Acute congestion was noticed in the meninges and the brain. Histological examination of brains from rabbits that died of acute poisoning revealed edema in the cerebrum, and hemorrhage also was noticed in two cases. Moderate cuffing and foci of gliosis were noticed in the case of rabbits Nos. 5 and 6 which were treated with three successive doses of Dimethoate. These lesions were not considered of any significance since rabbit No. 5 had metastatic suppurative pneumonia, and rabbit No. 6 had aborted during the experiment.

When the symptoms, autopsy findings, and histological observations were considered together, the cause of death appeared to be a cardiac failure and possibly a simultaneous respiratory failure as well. Emphysema in the lungs undoubtedly causes strain on the heart which is already affected by the cholinergic action of the compounds. Besides, the depression of the cardiac center also has to be taken into consideration. Cardiac failure as the cause of death was justified by the findings of the widespread passive congestion and the diastolic state of the heart observed at necropsy.

SUMMARY

Dimethoate, an organic phosphorus compound, was administered to eight rabbits, intramuscularly; the doses varying between 125 mg/kg and 160 mg/kg.

The rabbits treated with the higher dosage died, manifesting symptoms characteristic of cholinesterase inhibition.

Ruelene, an organic phosphorus compound, was administered to rabbits intragastrically; the doses varying between 50 mg/kg and 1000 mg/kg. Rabbits receiving doses less than 100 mg/kg did not show any evidence of toxicity. Moderate toxicity was observed in rabbits treated with doses between 112.5 mg/kg to 650 mg/kg. Severe toxic symptoms occurred in rabbits treated with doses between 875 mg/kg and 937.5 mg/kg. Rabbits treated with 1000 mg/kg died of poisoning, showing symptoms typical of cholinesterase inhibition. Complete recovery was observed after a period of six to ten days in cases of rabbits treated with doses as high as 937.5 mg/kg.

Emphysema of the lungs, dilation of the heart, and general congestion suggestive of cardiac failure were the constant findings at necropsy.

The cause of death due to the poisoning with compounds studied, is cardiac failure possibly accompanied by respiratory failure.

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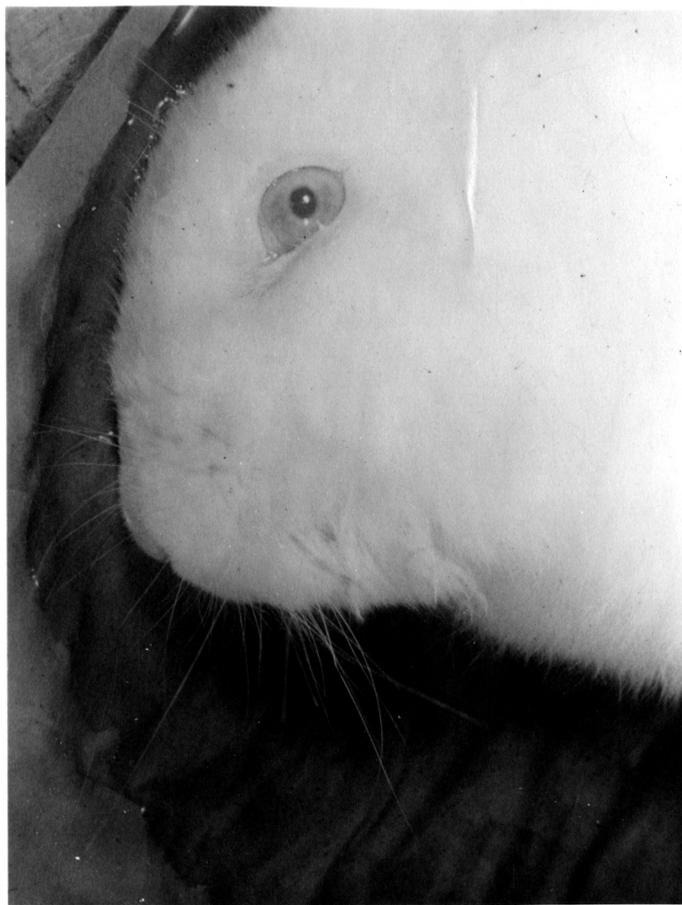
APPENDIX

EXPLANATION OF PLATE I

Rabbit treated with Ruelene at 1000 mg/kg.

Note constriction of pupil.

PLATE I



EXPLANATION OF PLATE II

Rabbit treated with Ruelene at 1000 mg/kg,
showing saliva flowing from mouth.

PLATE II



EXPLANATION OF PLATE III

Rabbit treated with Ruelene at 1000 mg/kg.

Note diarrheic stools.

PLATE III



EXPLANATION OF PLATE IV

Rabbit treated with Ruelene at 1000 mg/kg,
showing extreme prostration and paralysis.

PLATE IV



EXPLANATION OF PLATE V

Lungs and heart of a rabbit that died after treatment with Ruelene at 1000 mg/kg.

- A. Heart shows dilation and severe hyperemia of coronary vessels.
- B. Lungs are emphysematous.

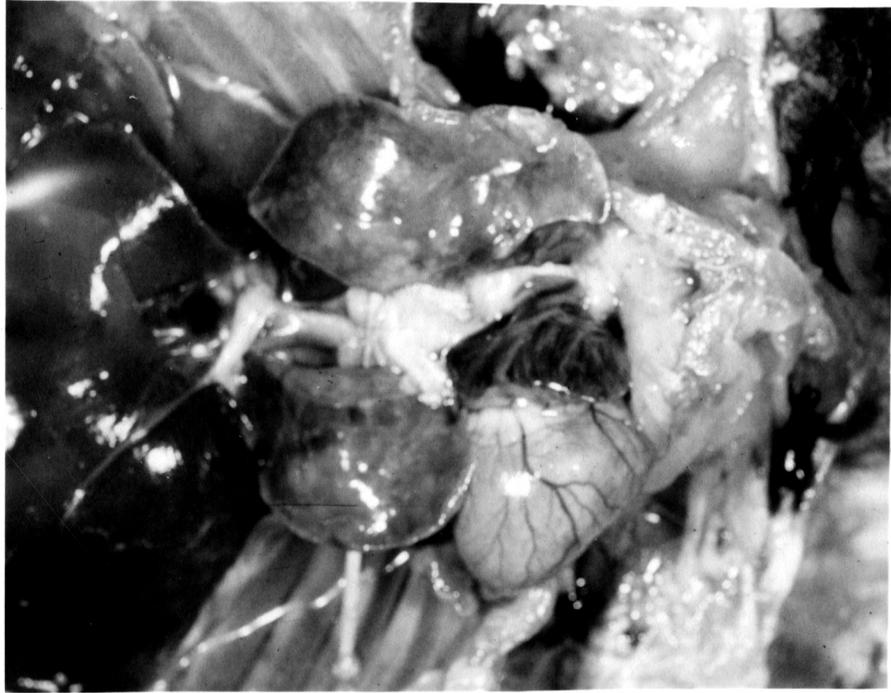
PLATE V



EXPLANATION OF PLATE VI

Lungs and heart of a rabbit that died after treatment with Ruelene at 1000 mg/kg. The heart is turned to show dilated auricles.

PLATE VI



EXPLANATION OF PLATE VII

Mesentery and intestine, showing intussusception
and hyperemia.

PLATE VII



EXPLANATION OF PLATE VIII

Section of lung. Note the marked emphysema
and rupture of alveoli.

PLATE VIII



EXPLANATION OF PLATE IX

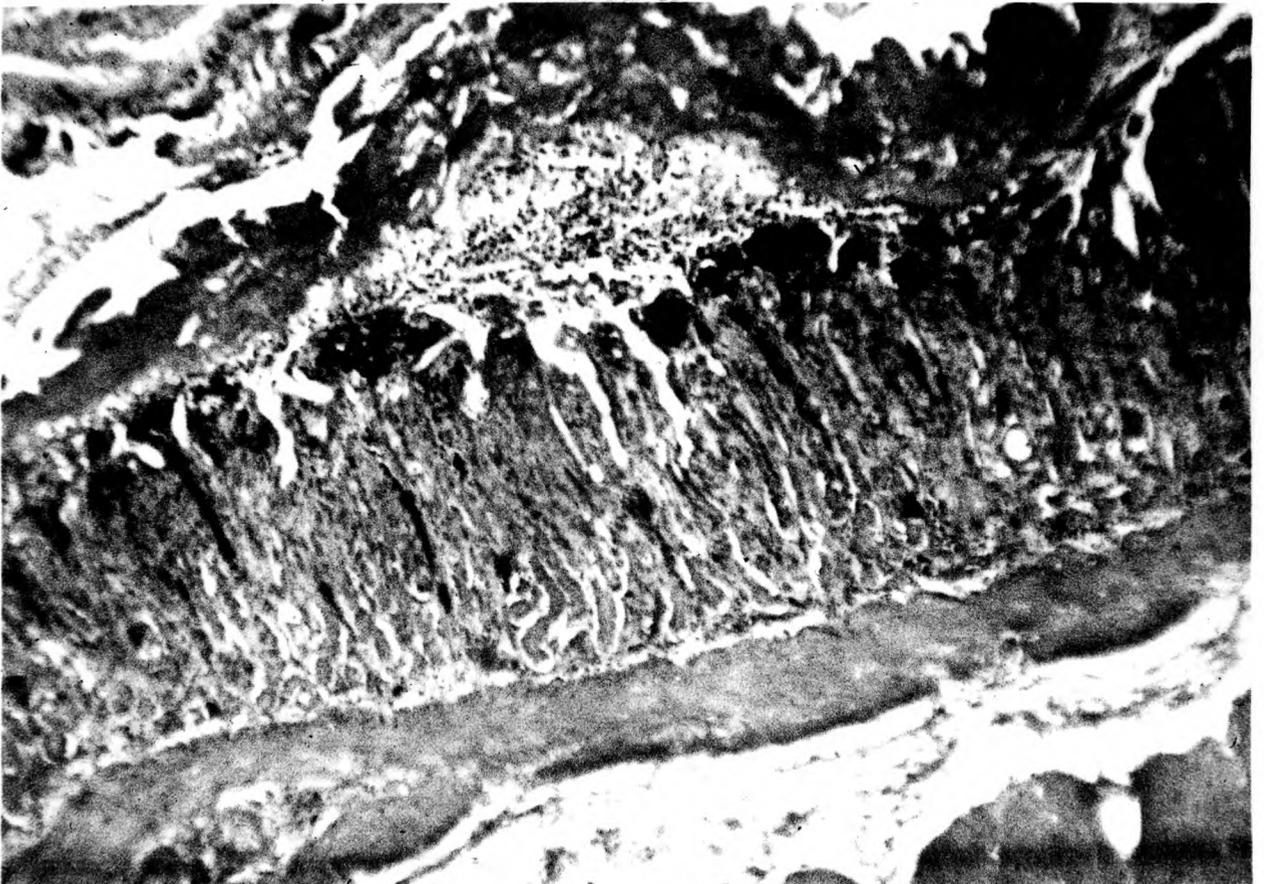
Section of duodenum showing ulceration extending to the muscularis externa.

EXPLANATION OF PLATE X

Section of stomach showing severe congestion of mucosa and hemorrhage.



PLATE X



EXPLANATION OF PLATE XI

Section of colon showing hyperactivity of the mucous glands.

EXPLANATION OF PLATE XII

Section of duodenum showing severe congestion and destruction of the villi (low power).

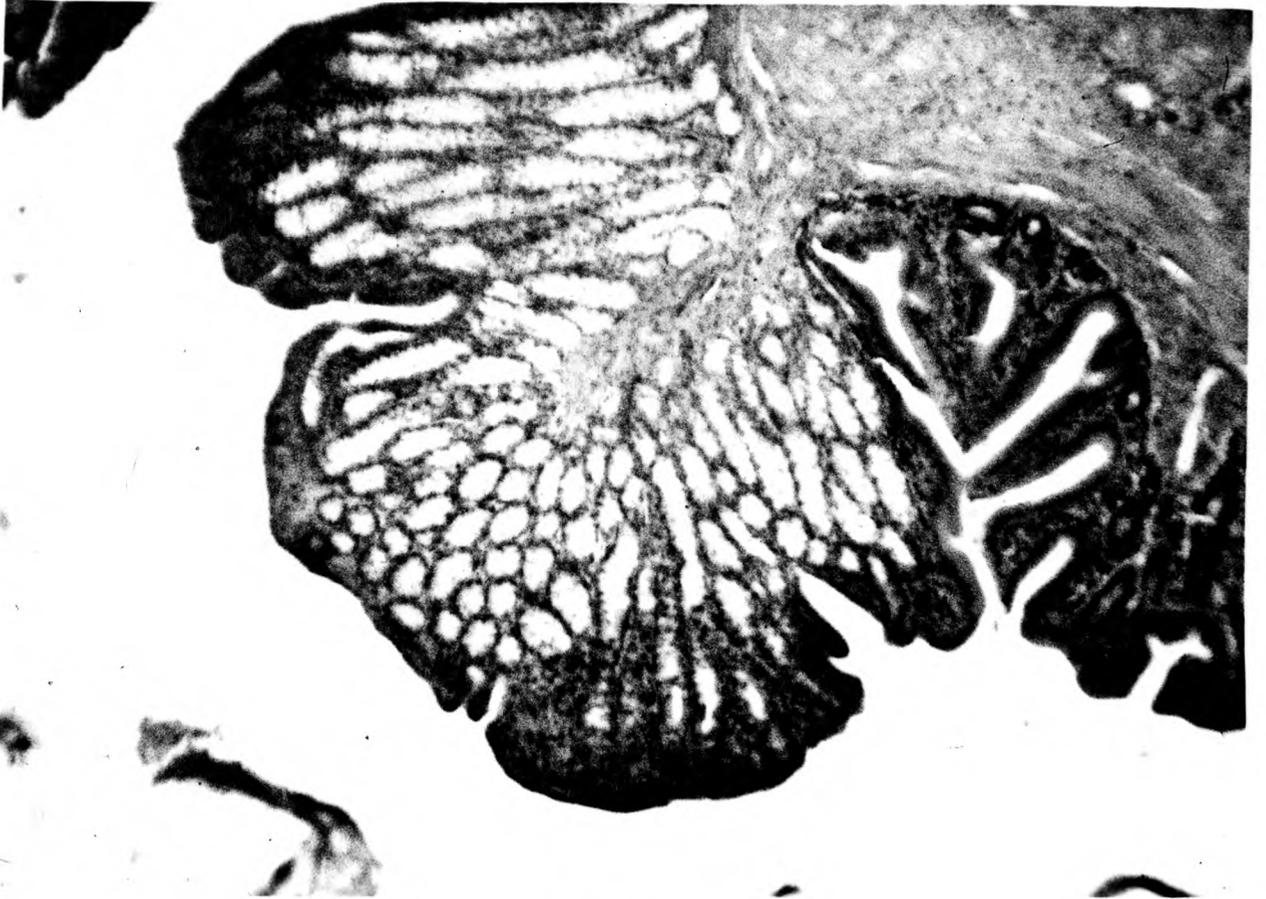
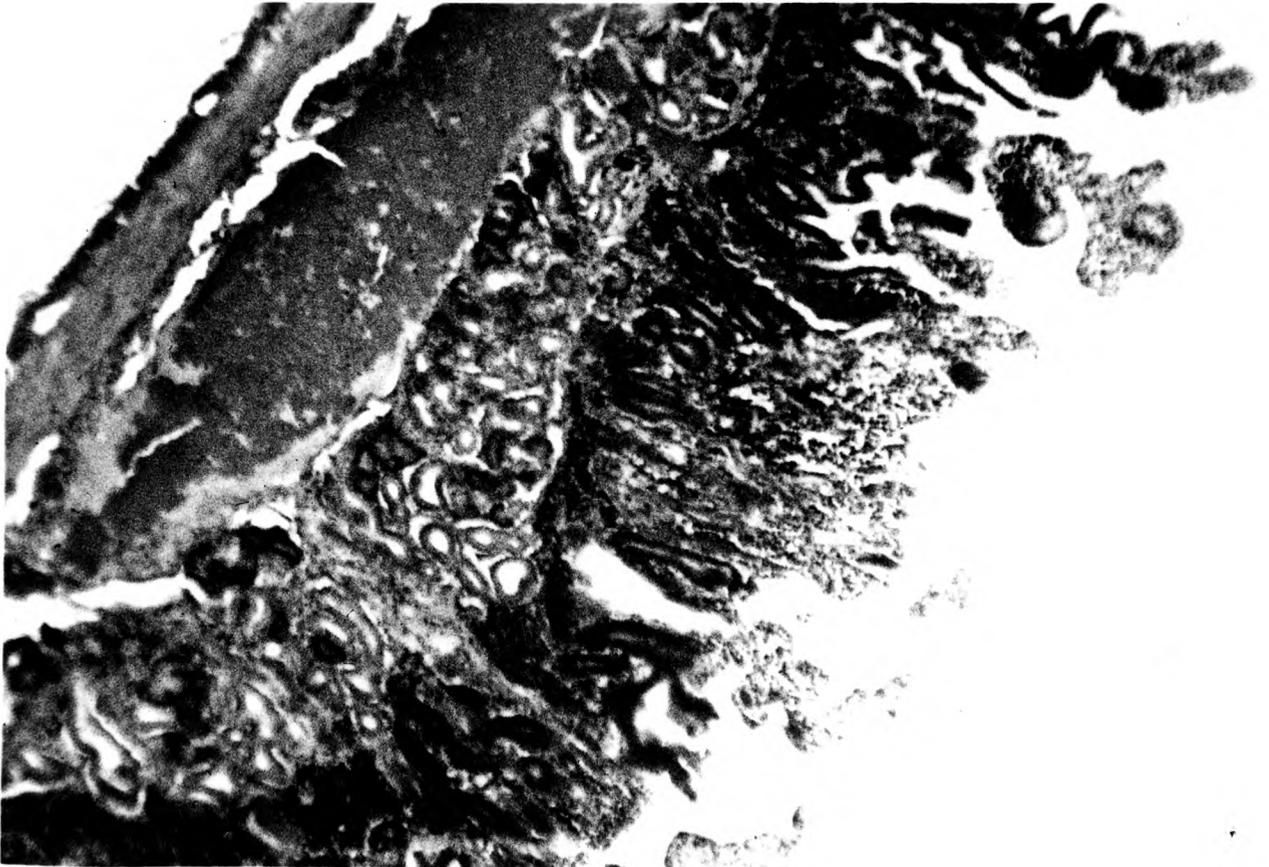


PLATE XII



EXPLANATION OF PLATE XIII

Section of cerebrum showing a congested vessel and edema in the surrounding tissue (high power).

EXPLANATION OF PLATE XIV

Section of duodenum showing severe congestion and destruction of the villi (high power).

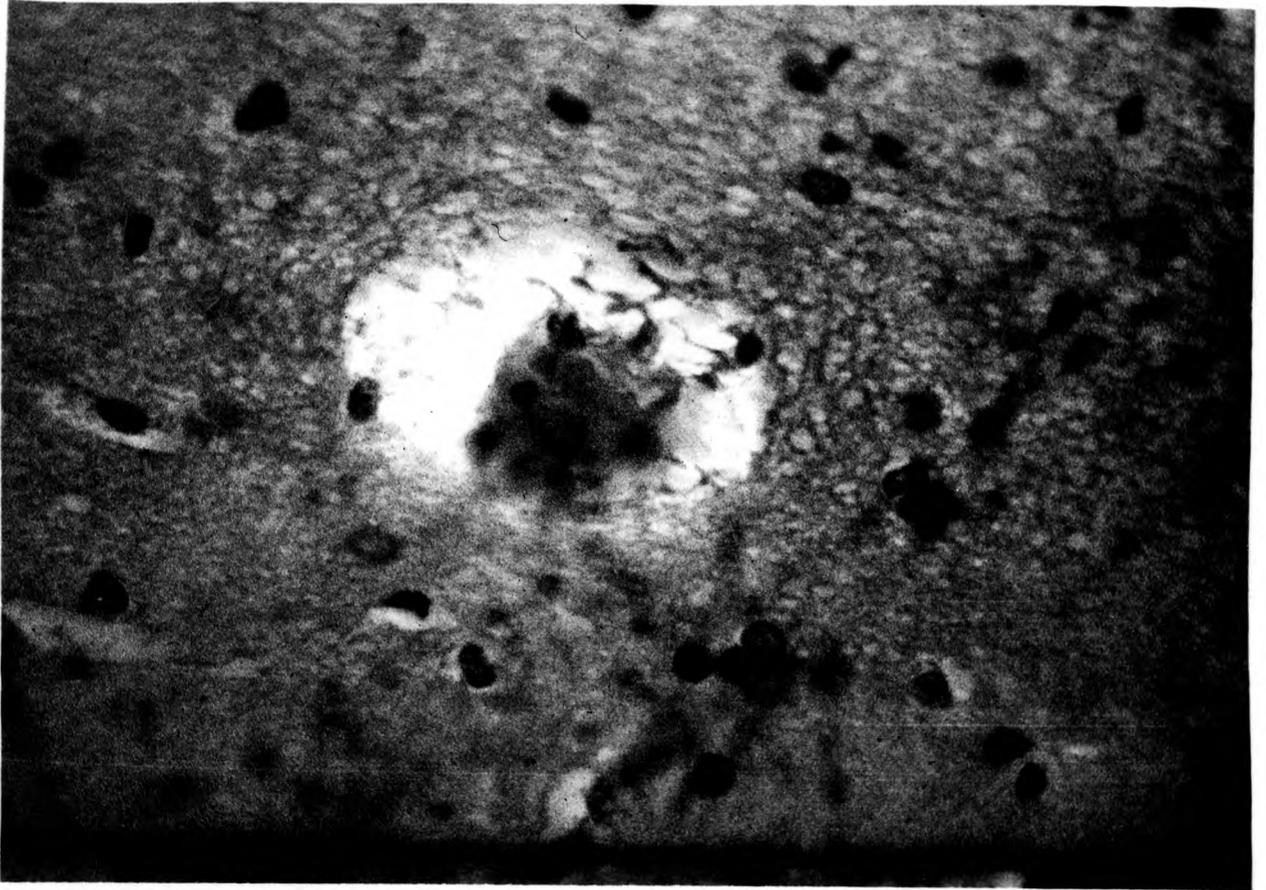
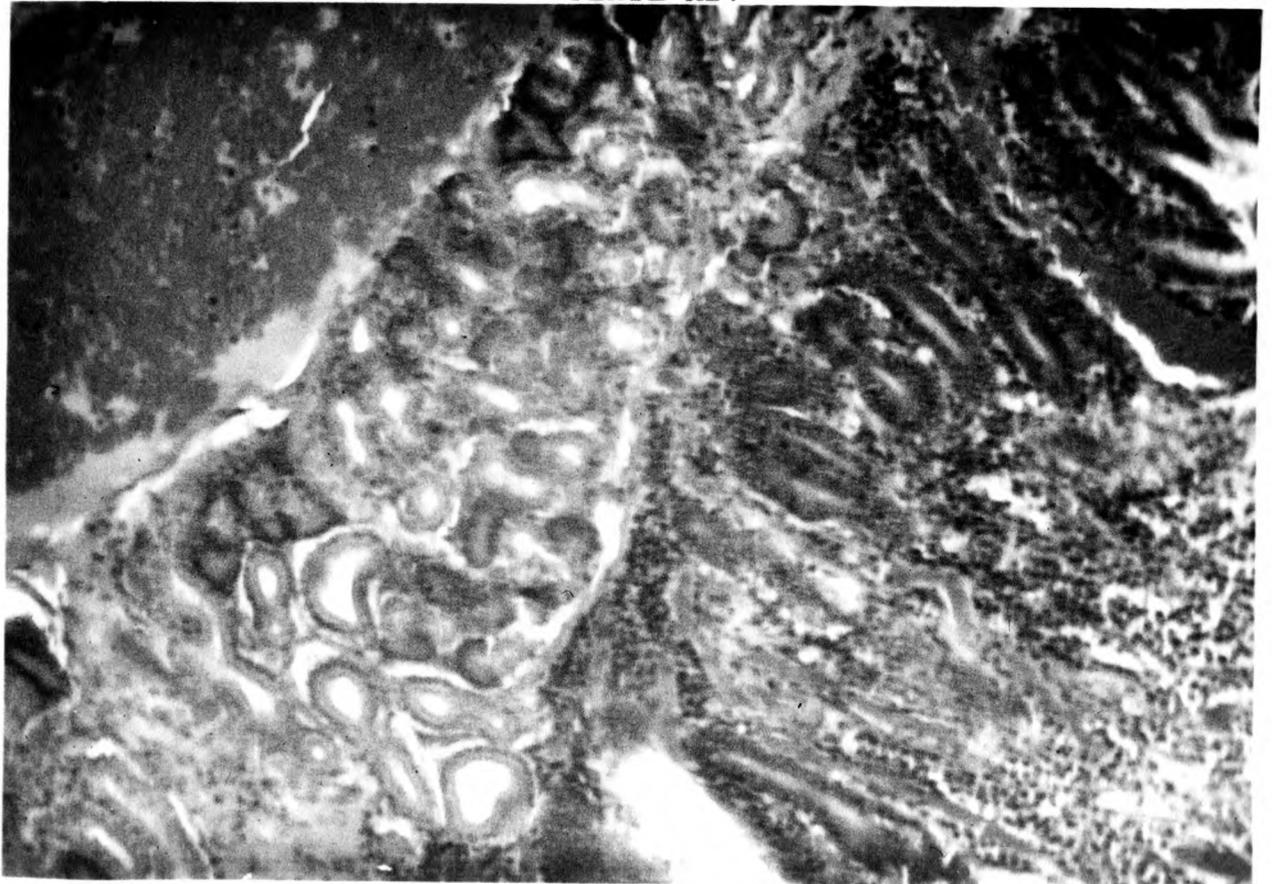


PLATE XIV



CLINICAL OBSERVATIONS AND HISTOPATHOLOGICAL STUDIES
OF TWO ORGANIC PHOSPHORUS COMPOUNDS IN RABBITS

by

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B.Sc.(Vet.), Bombay University, India, 1955

AN ABSTRACT OF A THESIS

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Organic phosphorus compounds are being utilized in recent years for use in the livestock industry as systemic insecticides and anthelmintics. The parasitocidal action of these compounds is due to their capacity to inhibit the cholinesterase enzymes of the parasites, leading to a severe disturbance of the functions of the autonomic nervous system and death. An identical action of these compounds occurs in vertebrate hosts which manifest severe toxic effects when larger doses of the compounds are administered.

Dimethoate, a product of the American Cyanamide Company, and Ruelene, a product of the Dow Chemical Company are two organic phosphorus compounds which are being studied as to their efficacy as systemic insecticides and anthelmintics. This study was undertaken to observe the toxic symptoms produced by these compounds and to study gross and histological changes in tissues and organs.

The experimental animal chosen for this study was the rabbit. Dimethoate was administered intramuscularly as it was supplied as a solution for parenteral use. An aqueous suspension of Ruelene, which was supplied as a wetttable powder, was administered intragastrically after a laparotomy.

Initial doses of the compounds were based on the body weight of rabbits and were chosen at random and the doses were increased until death was produced due to the toxic action of the compounds. Rabbits were observed after administration of the compounds, and the symptoms recorded.

Rabbits that were treated with sublethal doses of the compounds were euthanized at varying intervals, and necropsy was performed to study the gross lesions. Tissues from various organs were collected in 10 per cent buffered formalin for histological study. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections of nervous tissue were stained with Gallocyanin to study degenerative changes in the nerve cells.

Four rabbits that were given sublethal doses were not destroyed in order to study the pattern of recovery from the toxic effects.

When lethal doses were administered, the clinical symptoms manifested were characteristic of the inhibition of cholinesterase, and consisted of disturbances of respiratory, digestive, muscular, and central nervous systems.

Rapid and panting type of breathing with moist rales were the prominent respiratory symptoms. Anorexia, profuse salivation, diarrhea, and tenesmus were the important digestive disturbances. Involuntary muscular twitching, fasciculations, and ultimately paralysis were observed in the muscular system. Ataxia and convulsions were the symptoms referable to the central nervous system. Marked constriction of pupils and extreme prostration were other symptoms which are of diagnostic value. Weight loss was observed in all.

The most significant necropsy findings were: severely congested trachea containing sticky mucous discharge; marked

emphysema and severe congestion of lungs; dilated heart; congestion of intestines and other abdominal viscera; and severe congestion of the brain and spinal cord.

Necropsy findings indicated the cause of death to be cardiac failure.