Brit. J. Pharmacol. (1964), 23, 295–304.

NEUROLOGICAL DISRUPTION PRODUCED IN HENS BY TWO ORGANOPHOSPHATE ESTERS

BY

R. L. BARON AND H. JOHNSON

From the Division of Pharmacology, Bureau of Biological and Physical Sciences, Food and Drug Administration, U.S. Department of Health, Education and Welfare, Washington, D.C., U.S.A.

(*Received January* 16, 1964)

A histological and enzymatic examination was made of the neurological disruption produced in hens by two organophosphate esters. Intraperitoneal administration of DEF (tributyl phosphorotrithiolate) and Merphos (tributyl phosphorotrithioite) produced central and perpheral nervous system lesions accompanied by clinical signs of ataxia similar to those seen following administration of tri-*o*-cresyl phosphate. Histological examination (utilizing the Marchi stain) showed the occurence of spinal cord disruption before the onset of clinical ataxia. Oral administration of DEF and Merphos did not induce signs of peripheral weakness. However, severe lesions in the spinal cord and sciatic nerve were prominent. A discussion of the occurrence of central and peripheral nerve disruption either in the presence or absence of clinical ataxia is presented. Enzymatic examination of the effect of DEF on spinal cord and brain esterases at various intervals following administration showed a pattern of esterase inhibition similar to that found after tri-*o*-cresyl phosphate, dyflos and other organophosphates. Some prolonged inhibition is believed due to the extent of initial involvement rather than to selective prolonged inhibition.

The delayed neurological effects of certain organophosphorus esters have recently been the subject of extensive study. These neurologically-active compounds have been reported to disrupt myelin (demyelination) of the central and peripheral nervous system in several vertebrate species, including man. From the initial reports on adulterated alcoholic beverages showing tri-*o*-cresyl phosphate to be the primary cause of paralysis, various organophosphorus esters including aryl, alkyl, and fluorophosphates have been shown to induce similar effects (reviewed by O'Brien, 1960; Baron, 1962; Heath, 1961; Davies, 1963). The study of the distribution of central and peripheral nervous system lesions, originally described for tri-*o*-cresyl phosphate by Barnes & Denz (1953), has been extended by other workers to include fluorophosphates (Lancaster, 1960) and a metabolite of tri-*o*-cresyl phosphate (Baron & Casida, 1962).

Tributyl phosphorotrithiolate (DEF) and tributyl phosphorotrithioite (Merphos; Folex) were initially shown by Casida Baron, Eto & Engle (1963) to induce clinical signs of ataxia in hens following a series of intraperitoneal injections. As these two organophosphorus compounds were the first alkyl esters shown to induce signs of delayed neurological ataxia in hens, it was desirable to investigate these effects further.

METHODS

Treatment and evaluation of chickens

White leghorn hens (24 months of age) averaging 2.0 kg in weight were used in all experiments. DEF and Merphos were administered as corn oil solutions in multiple daily doses by oral intubation or intraperitoneal injection. Hens treated with DEF (100 mg/kg) by intraperitoneal injection daily for 10 days were killed at various intervals after the initial treatment, for manometric analyses of brain and spinal cord esterases. Control animals were evaluated histologically and manometrically following multiple doses of corn oil. Daily examinations were made of the onset and severity of neurological signs, and body weights were recorded initially, weekly and at the time of death.

Histology

After anaesthesia with pentobarbitone sodium (45 mg/kg) the chickens were perfused through the aorta with 200 ml. of 0.9% saline followed by 200 ml. of 10% formalin in saline. In all cases the entire spinal cord and one or both sciatic nerves from the level of the greater trochanter to the knee were dissected and fixed in 10% formalin for 24 hr. Representative cross-sections of the cervical, lumbar, and lumbo-sacral areas of the spinal cord and the sciatic nerve were further fixed for 24 hr. in 10% formalin. The Swank-Davenport modification of the Marchi stain was used as the indicator of nerve disruption. Sections were removed from the staining media, dried, imbedded in gelatin and cut as frozen sections. The sections, cleared through alcohol and xylene, were mounted in a synthetic mountant and examined.

Esterase analyses

Hens were killed by decapitation at selected intervals following the initial dose of DEF. The brain and spinal cord were quickly removed, chilled and homogenized with Teflon-glass homogenizers at 4° C in a bicarbonate buffer. Analyses of brain and spinal cord esterase activity were performed manometrically as previously described (Baron & Casida, 1962). Final substrate concentrations of 0.01 M-acetylcholine chloride, methacholine chloride, propionylcholine *p*-toluenesulphonate or butyrylcholine *p*-toluenesulphonate were prepared directly in the bicarbonate buffer. Emulsions of 0.01 M-triacetin (glyceryl triacetate), tripropionin (glyceryl tripropionate), tributryrin (glyceryl tributryrate), phenyl acetate and phenyl butryrate were prepared with the aid of 1% Triton X-100 (Rohm & Haas Co.) before the addition of the bicarbonate buffer. Enzymatic activity was based on the initial rate of carbon dioxide evolution corrected for nonenzymatic hydrolysis and enzyme activity due to endogenous substrate.

RESULTS

Clinical and histological responses of hens to multiple administrations of DEF and Merphos are summarized in Tables 1 and 2, respectively. These data include the time elapsed from the initial treatment until the onset of signs and an evaluation of the final condition of the birds by the following criteria: I: distinct weakness with the ability to rise and stand; II: weakness with the ability to rise but sitting upright most of the time; III: ataxia with maintenance of an upright sitting position; and IV: ataxia and inability to maintain an upright position.

Hens treated with DEF (twenty-six of the twenty-seven tested), killed 10 to 60 days after the initial intraperitoneal injection, showed distinct clinical signs of peripheral weakness. Of the thirteen hens treated with Merphos (daily intraperitoneal injections), four showed peripheral weakness. Daily oral doses of DEF of 100 mg/kg and above were not well tolerated and the mortality rate was high. However, administration of 50 mg/kg for up to 15 days by this route was tolerated. No hen

TABLE 1

CLINICAL AND PATHOLOGICAL EVALUATION OF HENS TREATED WITH DEF

* I, distinct weakness with the ability to rise and stand ; II, weakness with the ability to rise but sitting upright most of the time ; III, ataxia with maintenance of an upright sitting position ; and IV, ataxia and inability to maintain an upright position. † – = normal ; 0=artifact ; 1=questionable positive staining ; 2=slight positive staining ; 3=moderate positive staining ; 4=heavy positive staining ; and 5=severe positive staining. Ip=Intraperitoneal

Toxic agent and route	Dose (mg/kg)	No. of treatments	Time			Pathology†			
			Maintained (days)	To onset of symptoms (days)	Final condition*	Sciatic nerve	Cord		
							Cervical	Lumbar	Lumbosacral
DEF, i.p.	100	3	3	—	—	—	—	—	—
		3	3	—	—	—	0	0	0
		5	5	—	—	—	—	—	—
		5	5	—	—	—	1	—	—
		5	5	—	—	—	0	2	—
		5	10	—	—	1	—	—	1
		5	15	11	II	3	—	3	3
		5	15	11	II	3	1	3	3
		5	20	11	III	3	3	3	3
		5	21	13	IV	3	4	4	3
		5	21	13	IV	3	4	3	4
		5	30	11	III	—	3	2	3
		5	60	11	III	—	2	1	1
		9	9	—	—	—	1	2	1
		10	10	10	I	—	1	0	1
		10	11	11	<I	—	1	1	1
		10	14	12	<II	—	—	—	—
		10	15	10	II	3	1	1	3
		10	15	10	II	4	3	3	3
		10	20	10	III	3	3	3	3
		10	21	12	IV	3	4	4	4
		10	21	12	IV	2	4	4	4
		10	21	13	III	1	4	4	3
		10	21	14	IV	4	4	4	3
		10	22	10	IV	4	3	3	2
		10	42	13	IV	3	4	4	4
		15	21	13	III	3	4	4	4
		15	21	13	III	2	4	5	4
	50	5	43	14	II	3	4	4	3
		10	36	14	IV	3	5	4	4
		10	43	14	II	3	4	4	4
		15	43	14	II	3	4	4	4
		15	43	14	III	2	3	3	3
DEF, oral	50	5	44	—	—	3	5	5	4
		5	44	—	—	3	5	5	5
		10	44	—	—	3	5	4	5
		10	44	—	—	2	4	3	5
		10	44	—	—	4	4	3	4
		10	47	—	—	2	2	3	3
		15	44	—	—	3	5	5	5
	100	4	44	—	—	4	5	4	5
		5	60	—	—	—	1	1	1
	150	5	40	19	IV	4	1	1	1

TABLE 2
CLINICAL AND PATHOLOGICAL EVALUATION OF HENS TREATED WITH MERPHOS
Ip=Intraperitoneal; * and † as in Table I

Toxic agent and route	Dose (mg/kg)	No. of treatments	Time: Maintained (days)	Time: To onset of symptoms (days)	Time: Final* condition	Pathology†: Sciatic nerve	Pathology†: Cord: Cervical	Pathology†: Cord: Lumbar	Pathology†: Cord: Lumbosacral
Merphos, i.p.	100	5	5	—	—	—	—	—	—
		10	10	—	—	0	0	0	—
		10	10	—	—	—	2	1	1
		10	10	—	—	—	3	2	2
		10	15	—	—	3	—	0	2
		10	30	29	I	—	0	1	—
		10	50	—	—	2	4	4	3
		10	50	29	>I	—	4	4	3
		10	50	—	—	2	4	3	2
		15	16	—	—	—	3	—	2
		15	16	—	—	4	2	2	2
		15	21	17	<I	3	3	5	2
		15	21	17	<I	3	3	3	2
Merphos, oral	100	10	50	—	—	—	1	0	0
		10	50	—	—	2	4	3	2
	150	10	50	—	—	2	2	2	1
		10	50	—	—	3	2	0	2
	200	10	50	—	—	3	2	2	2
		10	50	—	—	2	3	2	2
	300	10	60	—	—	—	—	0	1
		10	60	—	—	—	2	1	1

treated orally with DEF at 50 to 100 mg/kg showed signs of peripheral weakness within the test interval of 44 to 60 days. The one hen surviving administration of 150 mg/kg for 5 days developed severe peripheral weakness. Administration of high oral doses of Merphos (up to 300 mg/kg daily for 10 days), in contrast to DEF, was well tolerated. Animals held for 50 to 60 days appeared normal at that time.

The clinical signs of neurological disruption in hens, consistently seen after multiple intraperitoneal injections of DEF and sporadically seen after Merphos, were typical of those induced by tri-*o*-cresyl phosphate, an antiesterase metabolite of tri-*o*-cresyl phosphate, dyflos and other fluorophosphates. The clinical signs, in general, were irreversible and dependent on the dose administered and the length of time the animals were maintained. In certain instances when the paralysis did not progress to the point of death and the birds were maintained for extended periods, slight regression of signs was evident. However, no animal showing a definite peripheral weakness was known to recover fully within the time intervals tested.

Those paralysed hens progressing to terminal stages of ataxia lost the most weight, and malnutrition was evident although food and water were readily available. Where minor signs of weakness were noted, the animals maintained their weight and a healthy appearance.

Pathology

Results of the microscopic examination of sections from the spinal cord and sciatic nerve using the Marchi stain (Swank-Davenport modification), are summarized for those animals treated with DEF and Merphos in Tables 1 and 2, respectively. Evaluation of these histological findings, made with the aid of an arbitrary grading system, indicates a normal tissue (−), the probable artifact (0), and the extent of Marchi-positive staining (1 to 5). No attempt was made to limit the examination of the spinal cord to an exact location. Rather, representative cross-sections of the cervical, lumbar and sacral areas were used in this study. Thus an evaluation of these randomly selected areas of the spinal cord precludes a detailed quantitative comparison of the microscopic findings of one hen with another.

Results of the histological examination in most instances qualitatively resembled those shown after treatment with tri-*o*-cresyl phosphate or other organophosphate esters. With hens treated intraperitoneally with DEF (Fig. 1) and Merphos, the regions of the spinal cord most evidently affected were ventral tracts located adjacent to the ventro-median fissure, dorso-lateral tracts in the sacral area and dorsal and ventral tracts in the cervical area. The appearance of Marchi-positive staining generally coincided with the onset of clinical signs and increased in proportion to the extent of physical involvement, although in several instances a number of positive-staining areas were seen in hens without apparent muscular weakness. The appearance of Marchi-positive staining in the spinal cord before the appearance of clinical symptoms was evident with at least two and possibly three hens. As the general pattern of neurological involvement with Merphos was not clear, a full evaluation

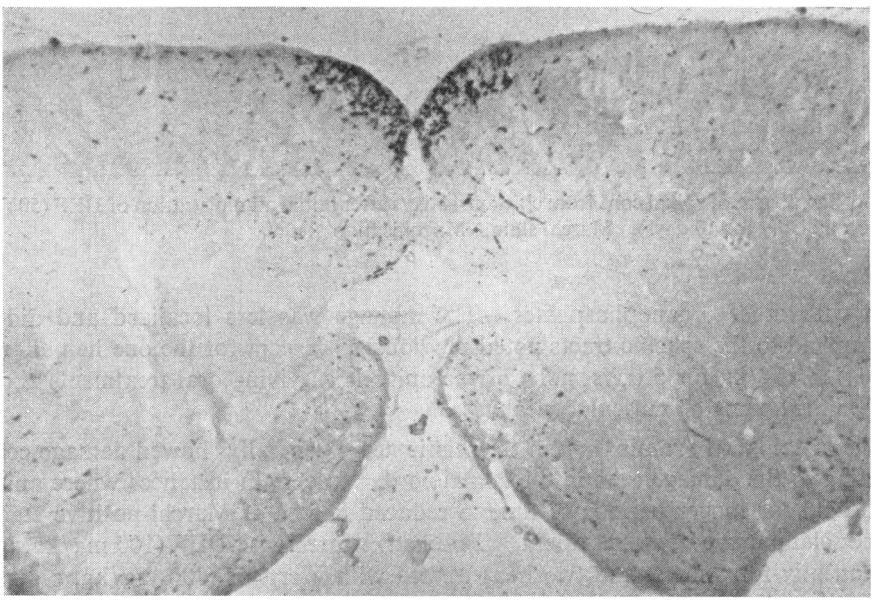

Fig. 1. Lumbo-sacral area of spinal cord from chicken 29 days after onset of signs of ataxia resulting from DEF (50 mg/kg, intraperitoneally, daily for 10 days). Marchi stain. Magnification × 6·7.

of this occurrence with this compound cannot be made. On the other hand, the response of hens to intraperitoneal injections of DEF consistently followed a uniform clinical pattern (as seen with other neurologically active organophosphates) and the histological findings were more readily evaluated.

An unusual result seen in the eight hens receiving Merphos administered orally for 10 days at dosages up to 300 mg/kg was the lack of clinical signs of muscular weakness coinciding with Marchi-positive stained sections of the spinal cord. Of the ten animals surviving oral administration of DEF, two showed questionable positive staining in the spinal cord areas examined, while eight showed moderate to severe staining. The pattern of disruption in these instances (Fig. 2) was not typical of that

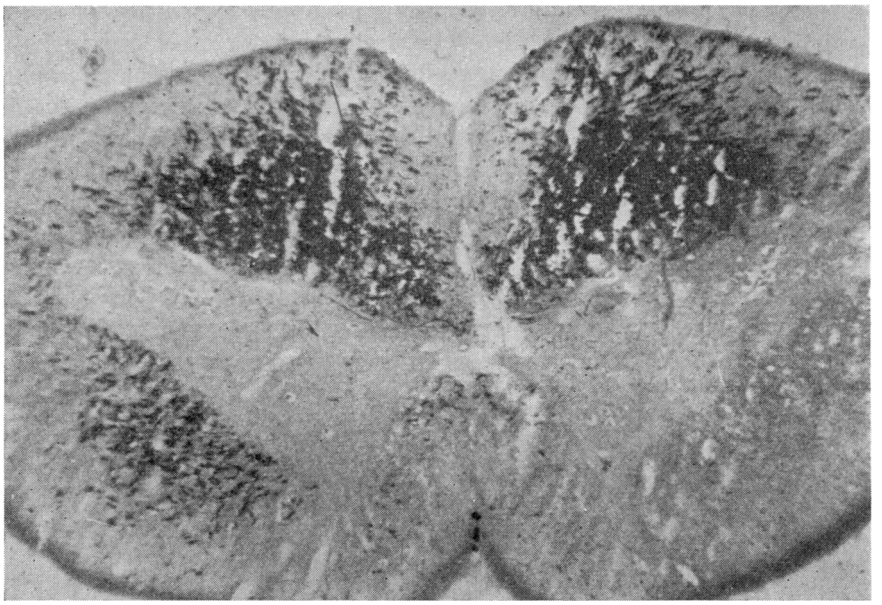

Fig. 2. Sacral area of spinal cord from chicken 44 days after initial administration of DEF (50 mg/kg orally daily for 10 days). Marchi stain. Magnification × 6·7.

seen with other organophosphates. The damage was less localized and did not correspond to the specific tracts noted previously. Except for the one hen administered 150 mg/kg for 5 days, none of the animals surviving oral treatments showed any clinical signs of muscular weakness.

The histological examination of the sciatic nerve generally showed damage corresponding to the damage seen in the spinal cord. In certain instances where animals were held for longer periods of time, a reduced degree of Marchi-positive staining in the sciatic nerve became evident. Two hens treated with DEF (100 mg/kg, intraperitoneally for 5 days) and two hens treated with Merphos (300 mg/kg, orally for 10 days), killed 19 to 39 days later than the other animals treated in a similar manner, showed no indications of Marchi-positive staining in the sciatic nerve,

TABLE 3

INHIBITION (%) OF BRAIN ESTERASES AT VARIOUS INTERVALS AFTER ADMINISTRATION OF DEF

*Activity expressed as μl. of carbon dioxide per g (wet weight)/hr × 10^3. For glyceryl and choline ester hydrolysis 32 mg fresh weight of tissue per flask were used. For aromatic esters 5 mg of tissue were used. Averages of duplicated analyses of four hens were used for all figures. The hens of column 1 received the drug for 5 days, the other hens for 10 days

		Time (days) after first dose of drug				
Substrate	Normal*	5	10	15	22	25
Choline acetate	27·66	68	56	56	34	35
Triacetin	7·03	54	42	46	15	31
Phenyl acetate	54·48	46	41	39	33	29
Choline propionate	33·02	67	55	55	34	34
Tripropionin	5·27	74	78	66	53	52
Choline butyrate	2·50	95	100	87	75	70
Tributyrin	2·86	88	92	69	67	65
Phenyl butyrate	14·64	98	100	99	83	81

TABLE 4

INHIBITION OF SPINAL CORD ESTERASES AT VARIOUS INTERVALS AFTER ADMINISTRATION OF DEF

*Activity expressed as μl. of carbon dioxide per g (wet weight)/hr × 10^3. For glyceryl and choline ester hydrolysis 160 mg fresh weight of tissue per flask were used. For aromatic esters 25 mg of tissue were used. Averages of duplicated analyses of four hens were used for all figures. The hens of the first column received the drug for 5 days, all other hens for 10 days

		Time (days) after first dose of drugs				
Substrate	Normal*	5	10	15	22	25
Choline acetate	3·77	51	42	48	27	28
Triacetin	1·98	31	19	25	2	18
Phenyl acetate	17·57	27	12	20	13	28
Choline propionate	4·97	65	56	50	33	32
Tripropionin	2·78	72	74	71	63	56
Choline butyrate	1·22	93	88	83	70	64
Tributyrin	2·00	81	78	77	46	62
Phenyl butyrate	6·70	88	83	75	77	67

whereas the animals sacrificed at an earlier time showed positively-stained regions in the sciatic nerve.

Esterase analyses

Results of the manometric assay of esterases of the brain and spinal cord from hens treated intraperitoneally with DEF at 100 mg/kg daily for periods of either 5 or 10 days are summarized in Tables 3 and 4. Values expressed as percentage inhibitions of control values, indicated a greater initial inhibition and a greater delay in recovery of both brain and spinal cord esterase activity on butyrate esters of choline, glycerol and phenol than on the corresponding acetate or propionate esters. The hydrolysis of all acetate esters and of choline propionate by brain and spinal cord was found to be maximally inhibited within 5 days. At the 25th day esterase activity had returned to between 65 and 80% of control levels. Inhibition of the hydrolysis of butyrate esters and of tripropionin by brain and spinal cord was also maximal within 5 days. However, the hydrolytic activity towards these esters at 25 days was

only 20 to 50% of the control. The rate of recovery of esteratic activity on all substrates appeared to be of the same relative order. The apparent delay in recovery of butyrate ester hydrolysis as compared with hydrolysis of other substrates appeared to be due to the greater extent of initial inhibition rather than to differences in rate of recovery. Thus the inhibition of the acetate ester hydrolysis ranged from 46 to 68% of normal at 5 days and from 29 to 35% of normal at 25 days; inhibition of butyrate ester hydrolysis ranged from 88 to 98% at 5 days and from 65 to 81% at 25 days.

DISCUSSION

The histological examination of the spinal cord and sciatic nerve was made using the Marchi stain for myelin disruption. Cavanagh (1954) has pointed out the disadvantages of relying too heavily on results seen with this staining technique. However, our experiences with nervous tissue from both normal animals (serving as positive and negative controls) and from those treated with tri-*o*-cresyl phosphate have indicated the qualitative and semi-quantitative effectiveness of this procedure for the routine screening and examination for myelin disruption.

Intraperitoneal treatment with DEF and Merphos resulted in a time/dose relationship such that, with lower doses and correspondingly shorter intervals before killing the hen, the extent of clinical involvement was less. As with other organophosphates, weight loss was evident only after the onset of muscular weakness. Following oral administration (except for one animal treated with DEF at 150 mg/kg), the hens maintained normal weight with no signs of clinical weakness. The loss of body weight has been thought to be directly related to the degree of damage to the peripheral rather than to the central nervous system. Were the weight loss related to the degree of peripheral involvement, a histological examination of the sciatic nerve in those birds showing no weight loss would be expected to show no lesions. While the weights of orally-treated hens were normal, the corresponding histological results showed more degeneration, indicating that a loss in weight may not be related to peripheral nerve involvement. On the other hand, isolated effects of these compounds on specific peripheral nerves (possibly such as those controlling certain areas of the digestive system) may be involved in producing unusual results. Because peripheral nerves other than the sciatic were not examined, this question cannot be answered.

Neurological signs of weakness appeared at approximately the same time in all animals treated intraperitoneally with DEF. The onset of symptoms in the only orally-treated hen exhibiting muscular weakness was somewhat delayed. In contrast to prior work which showed that the histological lesions occur at the time clinical ataxia was noted, this study indicates the occurrence of lesions in the spinal cord before the onset of muscle weakness. Histological lesions were observed first in the spinal cord and were quickly followed by lesions in the sciatic nerve. This pattern of lesion development contrasts with patterns seen in hens given dyflos, tri-*p*-ethylphenyl phosphate and a metabolite of tri-*o*-cresyl phosphate, but it is similar to the effects of tri-*o*-cresyl phosphate itself. Tri-*p*-ethylphenyl phosphate has been reported to affect primarily the peripheral nervous system, while dyflos and the metabolite of

tri-*o*-cresyl phosphate exhibited their effects on the central nervous system. Tri-*o*-cresyl phosphate has been shown to affect both the peripheral and central nervous system in a manner similar to that seen with DEF.

The absence of any muscular weakness coinciding with the extensive Marchi-positive nerve damage in hens treated orally with both DEF and Merphos is intriguing. Hens treated with Merphos at daily doses up to 300 mg/kg for 10 days showed no signs of muscular weakness. However, as the hens treated intraperitoneally with Merphos responded only sporadically with muscular weakness, too much emphasis should not be placed on the results with this compound. Animals treated orally with DEF at daily doses of 50 mg/kg for 15 days also showed no signs of muscular weakness after periods of observation of more than a month. On the other hand, intraperitoneal administration of 50 mg/kg for 5 days resulted in definite ataxia within 11 days. As the microscopic lesions in the orally-treated animals, in contrast to those treated by intraperitoneal administration, appeared to be much more severe and yet did not coincide with signs of peripheral weakness, it is believed that metabolism and/or nerve sites affected by the organophosphates differ with the route of administration. Detoxication and metabolic transformation either within or related to the gastro-intestinal tract are presumably responsible for these unusual results. The difference in metabolism appears to be such that, by intraperitoneal administration of DEF or Merphos, the original compound, or the metabolite(s) produced, selectively affect those areas of the nervous system controlling muscular stability. With oral administration the metabolite(s) produced also attack the central and peripheral nervous system, as indicated by the severe Marchi-positive staining. However, the treatment results in clinically normal animals. Until the metabolic products of DEF and Merphos are defined, it will be difficult to interpret the different effects of oral and intraperitoneal administration of these compounds to hens.

The nature of the biochemical lesion which produces ataxia in response to organophosphorus esters in hens is not known. Although considerable variation exists between individual inhibitors and their selectivity, several proposals have been put forth for the possible interaction of these compounds with the nervous system. The suggestion that disruption of the pseudocholinesterase present in the nervous system, or that the fluoride ion, liberated upon hydrolysis of certain fluorophosphates, produced the nervous system lesions, has met with some resistance. The most recent proposal of selective phosphorylation and prolonged inhibition of an esteratic site of an, as yet undefined, protein (possibly a nonspecific aliphatic esterase hydrolysing butyrate or esters of a four-carbon chain length of or longer) has yet to be fully evaluated.

Enzymatic evaluation of the effects of oral intoxication by DEF, which induces no muscular weakness but does induce histological changes, has not been made. This, and investigation of effects of other antiesterase agents on the susceptibility of butyrate-hydrolysing enzymes with respect to clinical and histological damage, is currently in progress.

Previous work on the delayed muscular weakness induced by certain organophosphorus esters has correlated the extent of weakness to the degree of spinal cord or peripheral nerve involvement. In this study, a correlation between nervous system

disruption and muscular weakness was evident when DEF (and occasionally Merphos) was administered intraperitoneally. However, as the oral route of administration produced severe neurological disruption with no corresponding muscular weakness, evaluation of the potential neurological hazard of antiesterase phosphates (and possibly other neurologically active compounds) becomes more difficult. Evaluation of the effects of those compounds not eliciting a clinical response in test animals may necessitate an extensive histological examination of the nervous system. For the study of the neurotoxic hazards of pesticides a combination of histological and clinical evaluation may be more important than a clinical evaluation alone.

REFERENCES

BARNES, J. M. & DENZ, F. H. (1953). Experimental demyelination with organophosphorous compounds. *J. Path. Bact.*, **65**, 597–605.

BARON, R. L. (1962). *Delayed Neurological Effect of Certain Organophosphate Esters in Chickens.* Doctoral thesis, Madison, Wisconsin.

BARON, R. L. & CASIDA, J. E. (1962). Enzymatic and antidotal studies on the neurotoxic effect of certain organophosphates. *Biochem. Pharmacol.*, **11**, 1129–1136.

CASIDA, J. E., BARON, R. L., ETO, M. & ENGLE, J. L. (1963). Potentiation and neurotoxicity induced by certain organophosphates. *Biochem. Pharmacol.*, **12**, 73–83.

CAVANAGH, J. B. (1954). The effects of tri-*ortho*-cresyl phosphate on the nervous system ; experimental study in hens. *J. Neurol. Neurosurg. Psychiat.*, **17**, 163–172.

DAVIES, D. R. (1963). Neurotoxicity of organophosphorus compounds. *Hbh. Exp. Pharmak.*, ed. KOELLE, G. B., **15**, 860–882.

HEATH, D. F. (1961). *Organophosphorus Poisons.* Oxford : Pergamon Press.

LANCASTER, M. C. (1960). A note on the demyelination produced in hens by dialkylfluoridates. *Brit. J. Pharmacol.*, **15**, 279–281.

O'BRIEN, R. D. (1960). *Toxic Phosphorus Esters*, New York: Academic Press.