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NEUROTOXIC SYNDROME PRODUCED IN CHICKENS BY A CYCLIC PHOSPHATE METABOLITE OF TRI-o-CRESYL PHOSPHATE—A CLINICAL AND PATHOLOGICAL STUDY

BY

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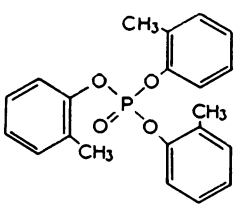
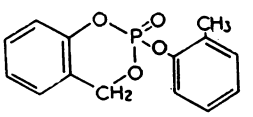
A clinical and neuropathological comparison was made between chickens poisoned with tri-*o*-cresyl phosphate and those treated with the major cyclic phosphate metabolite of tri-*o*-cresyl phosphate. Ataxia was evident with both materials, and the degree of peripheral neuropathy increased with higher doses of these agents. Morphological changes were evident with the onset of symptoms and increased in number as the neurological signs progressed. Demyelination of the spinal cord following administration of the metabolite occurred at doses considerably below those necessary to effect peripheral nerve degeneration. Axon and myelin damage was prominent with both agents. The relationship between these effects and those produced by certain other organophosphates such as dyflos (di*iso*propyl fluorophosphonate) is discussed.

The clinical and neuropathological changes produced by tri-*o*-cresyl phosphate have been studied in man and experimental animals, especially the chicken. Wallerian degeneration has been reported in specific long tracts of the spinal cord and brain stem and in peripheral nerves (Barnes & Denz, 1953 ; Cavanagh, 1954). Many aryl phosphates and organofluoro phosphates, particularly di*iso*propyl fluorophosphonate (dyflos), also produce similar effects (Barnes & Denz, 1953 ; Fenton, 1955 ; Bondy, Field, Worden & Hughes, 1960 ; Davies, Holland & Rumens, 1960 ; Lancaster, 1960).



Tri-*o*-cresyl phosphate



Cyclic phosphate metabolite of tri-*o*-cresyl phosphate

Recent studies on the metabolism of tri-*o*-cresyl phosphate have shown that a major pathway involves methyl hydroxylation and subsequent cyclization to yield potent esterase inhibitors (Casida, Eto & Baron, 1961 ; Eto, Casida & Eto, 1962). The structural relationship of tri-*o*-cresyl phosphate and its main cyclic phosphate metabolite is shown above.

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It appeared of interest to see if this cyclic phosphate metabolite would produce clinical and pathological changes similar to those resulting from tri-*o*-cresyl phosphate and the organofluoro phosphates.

METHODS

Treatment and clinical observations

White leghorn chickens (12 to 18 months of age) averaging 2.0 kg in weight were used throughout the experiment. Tri-*o*-cresyl phosphate (Distillation Products, Eastman Kodak Co., Rochester, N.Y.) was administered in one dose of 1.0 ml./kg orally. 2-(*o*-Cresyl)4H-1,3,2-benzodioxaphosphoran-2-one, or the cyclic phosphate metabolite of tri-*o*-cresyl phosphate, was prepared by reaction of *o*-cresyl phosphoryl dichloride with *o*-hydroxybenzyl alcohol and purified by chromatography and distillation (Eto *et al.*, 1962). This metabolite was administered in corn oil as a single dose varying from 8 to 400 mg/kg. Those animals receiving intraperitoneal injections of 100 mg/kg of the metabolite were protected from parasympathomimetic effects by intraperitoneal administration of 1 mg/kg atropine simultaneously with the organophosphate and again 2 hr later. Chickens 1 to 24 were treated with the metabolite while 25 to 38 received tri-*o*-cresyl phosphate as indicated in Table 1. The chickens were observed daily for the onset and severity of neurological signs. Body weights were taken initially, on the 7th and 14th days following treatments, and at the time of sacrifice.

Histology

General. Following anaesthesia with pentobarbitone sodium, 45 mg/kg, the chickens were perfused through the left ventricle with 100 to 200 ml. of 10% formalin. Tissues from the thoracic and abdominal cavities were taken from chickens 1, 4 and 10 treated with the metabolite, and 25, 28, 31 and 35 treated with tri-*o*-cresyl phosphate. They were fixed in 10% formalin for 48 hr, embedded in paraffin and stained with haematoxylin and eosin. Longitudinal sections were made of the biceps flexor cruris muscle from most of the chickens and stained in a similar manner.

Central nervous system. In the majority of the chickens the entire brain and portions of the cervical and lumbo-sacral cord were dissected out and fixed in 10% formalin for 48 hr. Two normal chickens were used as controls. The following histological examinations were made: the Swank-Davenport modification of the Marchi stain was used on sagittal sections of the cerebrum and midbrain, sagittal cuts of the brain stem and cerebellum at the level of the restiform body, and cross-sections of the cervical and lumbo-sacral cord; paraffin-embedded tissue from areas adjacent to these was routinely stained with haematoxylin and eosin; special stains (Bodian, Klüver and Nissl) were used only on paraffin-embedded tissue from chickens just prior to the anticipated time of initial symptoms or after their appearance. They were used primarily to study axon, myelin and horn cell changes. Several longitudinal sections of the dorso-lateral area of the cervical cord were also made and stained in a similar manner.

Peripheral nervous system. Either one or both sciatic nerves from the level of the greater trochanter to the knee were dissected out in most of the chickens. In about one-fourth of the birds, the anterior tibial nerve was removed as well. The lumbo-sacral plexus, dorsal ganglion and ventral and dorsal roots were not included. Forty-one longitudinal sections (average length, 5.7 mm) were taken from the mid-portion of these sciatic nerves and stained with haematoxylin and eosin. Nineteen anterior tibial nerve sections (average length, 6.4 mm) were similarly examined. The Marchi stain was utilized on 37 longitudinal sections of the sciatic nerve (average length, 3.4 mm) and 9 sections of the anterior tibial nerve (average length, 2.3 mm). Bodian, Klüver and Sudan IV stains were used to study the axon and myelin breakdown. Similar sections and stains were made from the sciatic nerves of 2 normal chickens.

RESULTS

Clinical observations

The initial neurological manifestations appeared 9 to 11 days after the administration of either tri-*o*-cresyl phosphate or the metabolite. The progressive nature of the clinical signs is indicated in Table 1. The initial symptom with both compounds

TABLE 1

CLINICAL AND PATHOLOGICAL RESPONSE OF CHICKENS TO TRI-O-CRESYL PHOSPHATE AND ITS CYCLIC PHOSPHATE METABOLITE

Numerical designations are as follows: for clinical effects, 0—normal, 1 and 2—severity of ataxia, 3 and 4—severity of ataxia and peripheral neuropathy; for pathological effects, peripheral nerve, 0—no degenerated fibres, ?—equivocal changes, 1–3—number of degenerated fibres; spinal cord, 0—no Marchi positive material, 1–3—intensity of Marchi positive material. P.N.—peripheral nerve. S.C.—spinal cord. Birds 21–23 were given protective doses of atropine

Compound and dose		Chicken no.	Days killed after		Clinical	Pathological	
			Treatment	Symptoms		P.N.	S.C.
Metabolite (mg/kg intraperitoneally)	20	1–3	3	—	0,0,0	0,0,0	0,0,0
	20	4–6	8	—	0,0,0	0,0,0	0,0,0
	20	7–9	11	1	1,1,1	?,0,0	1,2,1
	20	10–12	15	4	1,1,1	?,0,?	1,2,2
	20	13–15	20	11	2,2,2	1,0,0	3,3,3
	8	16	29	19	2	0	3
	16	17	29	19	2	0	3
	16	18	50	41	2	0	3
	33	19	29	19	2	0	3
	60	20	18	8	3	0	3
	100	21	19	9	1	?	—
	100	22	20	7	3	3	—
	100	23	28	18	2	2	—
(orally)	400	24	21	12	3	3	—
Tri-*o*-cresyl phosphate (ml./kg orally)	1	25–27	3	—	0,0,0	0,0,0	0,0,0
	1	28–30	8	—	0,0,0	0,0,0	0,0,0
	1	31–33	11	1	1,1,1	1,?,?	1,1,2
	1	34–35	15	5	3,3	3,3	–,2
	1	36–38	20	10	4,4,4	3,3,3	3,3,3

was an unsteadiness of gait, the birds swaying while walking and occasionally falling. They spent more time sitting than normal birds. Several days after the onset of symptoms they were able to walk only a few steps before losing their balance. The first major difference between the two materials appeared by the 5th day following the onset of symptoms. The metabolite-poisoned chickens showed only a slight progression in the ataxic symptoms, while with tri-*o*-cresyl phosphate the chickens developed weakness in the legs, especially in the extensor group of muscles. The degree of disability in the metabolite-poisoned birds reached its maximum around 11 days after symptoms and remained stationary in the chickens observed for 19 and 41 days with symptoms. Metabolite-treated birds showed definite weakness of the legs only with doses of 60 mg/kg and above. The paresis in the tri-*o*-cresyl-phosphate-poisoned chickens was progressive, affecting mainly the legs and to a lesser extent the wings. These birds had difficulty in rising to a standing position, and since the legs were held in flexion the chickens moved about on their knees. By 10 days there was little spontaneous movement in the lower extremities. The limbs were flaccid and response to pin-prick was feeble, respiration became laboured and the comb cyanotic. Weight loss was much greater in the

tri-*o*-cresyl-phosphate-treated chickens than in the metabolite-poisoned or control birds (Fig. 1). With higher doses of metabolite (chickens 21–24 of Table 1), the weight pattern was similar to the 20 mg/kg level shown in Fig. 1. Weight loss in metabolite-treated chickens was marked only after the appearance of muscle weakness, in contrast to the tri-*o*-cresyl-phosphate-poisoned birds where weight loss appeared several days before the appearance of muscle weakness.

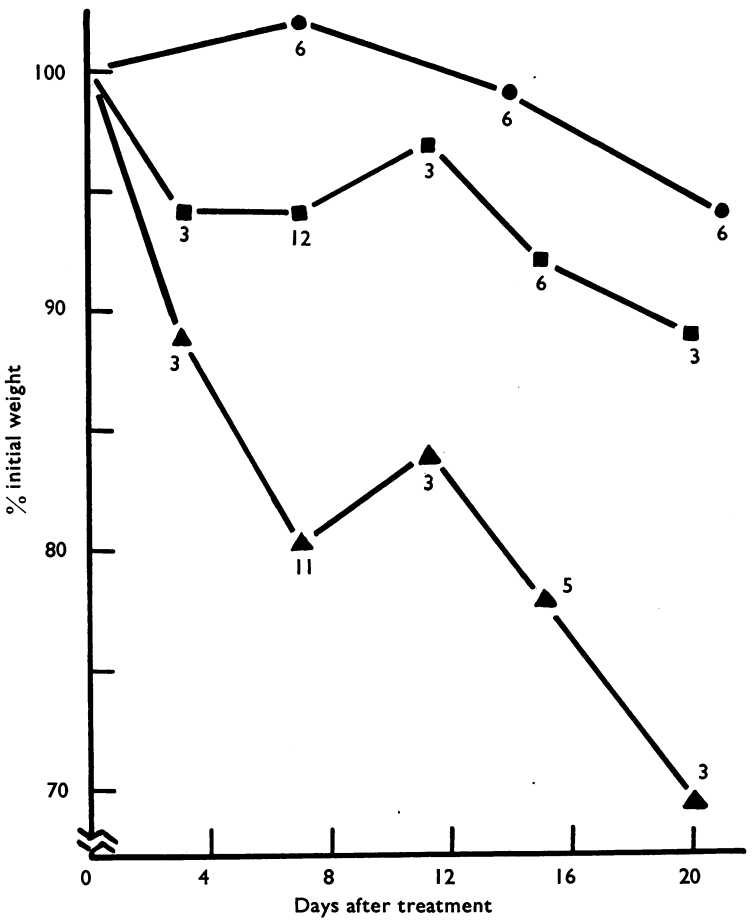


Fig. 1. Effect of tri-*o*-cresyl phosphate (▲—▲) (1 ml./kg orally) and its cyclic phosphate metabolite (■—■) (20 mg/kg injected intraperitoneally) on weight of chickens compared with that of control, untreated chickens (●—●). Numbers indicate number of birds observed.

Pathology

General

Gross examination showed that the thoracic and abdominal organs in all the chickens were essentially normal. Microscopic sections examined from the myocardium, lung, pancreas, small intestine, spleen, kidney and adrenal were within normal limits. These were taken from 3 metabolite-poisoned birds sacrificed at

3, 8 and 15 days and 4 tri-*o*-cresyl-phosphate-poisoned chickens killed at 3, 8, 11 and 15 days from treatment. Slight fatty infiltration of the liver was found in one chicken from each group. The biceps cruris flexor muscle was normal grossly and microscopically except in the severely poisoned tri-*o*-cresyl phosphate birds, where atrophy was noted.

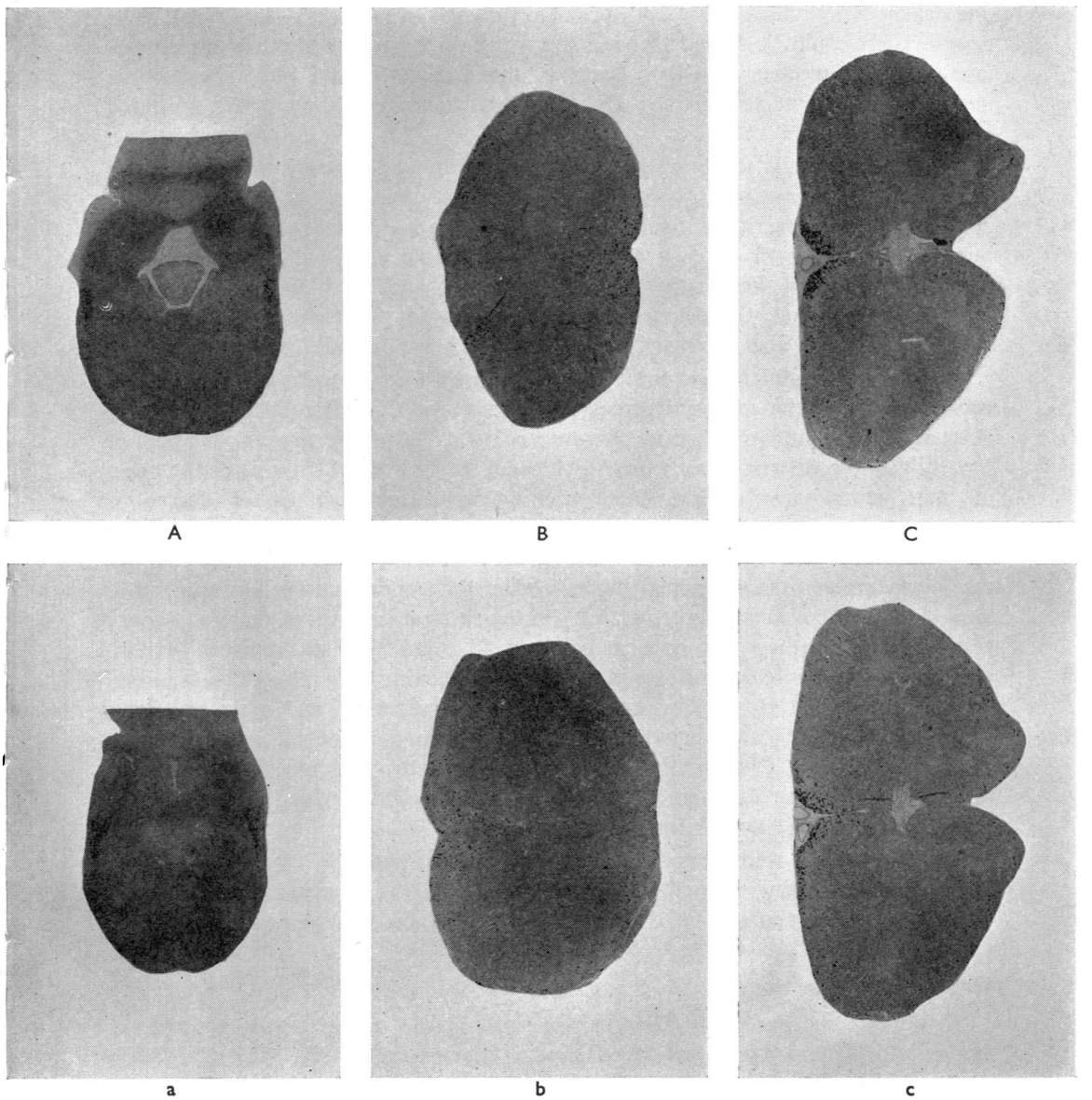


Fig. 2. Brain stem (A,a, × 11), cervical cord (B,b, × 30) and lumbo-sacral cord (C,c, × 30) in chickens 10 to 11 days after symptoms resulting from tri-*o*-cresyl phosphate (1 ml./kg, orally) (A,B,C) and its cyclic phosphate metabolite (20 mg/kg, intraperitoneally) (a,b,c). Marchi stain.

Central nervous system

Cerebrum and midbrain. Degeneration was not evident in the tracts of the cerebrum and midbrain with either compound. Cortical and subcortical neurones appeared normal.

Brain stem. Positive Marchi material was noted in the spinocerebellar tracts in equal amounts with both compounds. This was present in chickens sacrificed after one day of symptoms and did not appear to progress in intensity (Fig. 2A, a). The degeneration could be traced through the restiform body into the ipsi- or contralateral cerebellar cortex. Other tracts of the brain stem did not appear to be involved, and the nuclear groups of the brain stem and cerebellar cortex appeared normal.

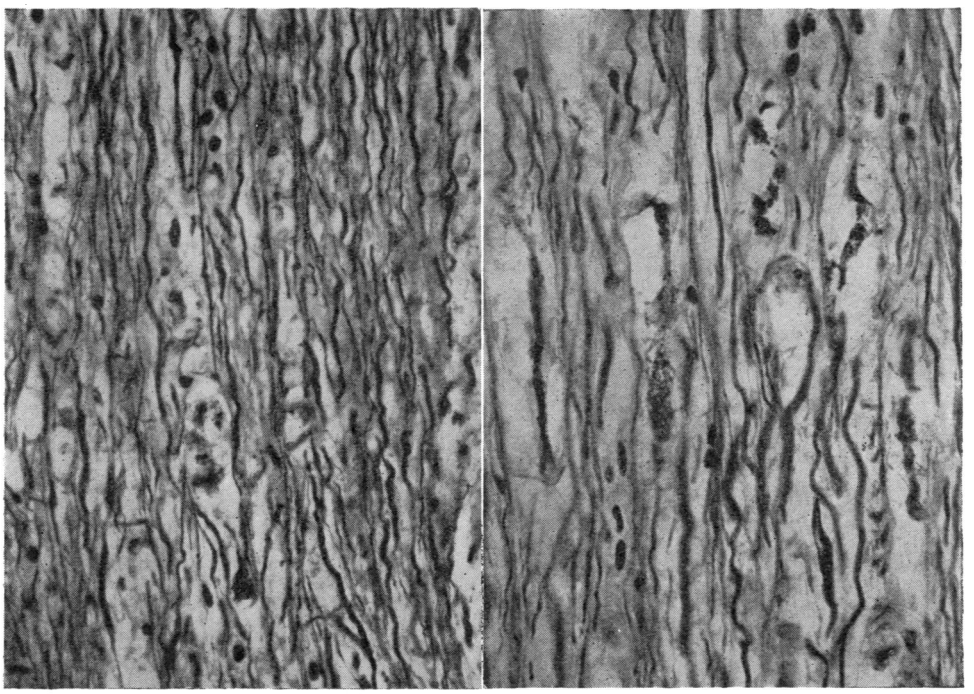
Spinal cord. As in the brain stem, the topography and severity of change in the spinal cord were most evident with the Marchi stain. Because of the inherent difficulties in judging severity of myelin degeneration with the Marchi stain when limited numbers of sections from each chicken were available, it was difficult to make a correlation between the two compounds or birds treated with the same material. However, in general, the degree of damage appeared to be the same with both compounds. This also increased as the symptoms progressed.

Swelling and granular degeneration of the axon with associated myelin breakdown was apparent in the metabolite-treated chickens one day after initial symptoms. Soon after this the axon became fragmented (Fig. 3a). The greatest amount of Marchi-positive material was found in chickens killed 8 to 41 days after the onset of their neurological signs. There was no appreciable change in degree of involvement between 19 and 41 days after the initial symptoms. The spinal cord tracts affected were a ventral descending tract located adjacent to the ventral median fissure and the ascending spinocerebellar tract in the superficial dorso-lateral area of the lumbo-sacral cord (Fig. 2c). In the cervical cord the dorsal and ventral spinocerebellar pathways were most involved, followed by a scattering of Marchi-positive material in the ventral and posterior funinculus (Fig. 2b). The neuronal groups of the cord grey matter examined with haematoxylin and eosin and Nissl stains did not show any definite alterations even in the chicken sacrificed after 41 days of illness. Chromatolysis was not seen. Astro- and micro-glial cell proliferation within the degenerated tracts was seen in chicken no. 18, which was observed the longest time before sacrifice.

Similar changes were found with tri-*o*-cresyl phosphate in regard to severity and location of positive Marchi material (Figs. 2B and C). Again axon and myelin breakdown were present (Fig. 3b). The neurons of the ventral and dorsal horns appeared normal.

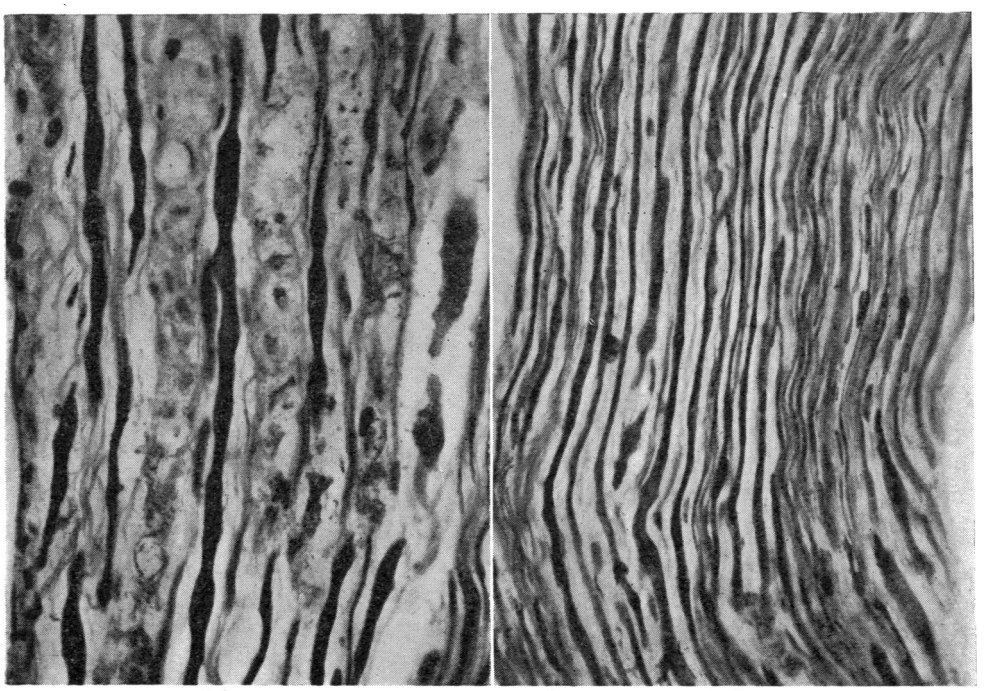
Peripheral nervous system

No definite morphological changes appeared in the sciatic nerves of chickens treated with the metabolite and killed prior to the onset of ataxia. This conclusion is based upon examination of 7 sections from the sciatic nerves with haematoxylin and eosin stain and 6 sections with the Marchi stain. Of the 18 symptomatic chickens, 3 sections of the sciatic nerve showed equivocal changes (chickens 7, 12 and 21), while 1 (chicken 13) showed definite though infrequent granular and



a b

Fig. 3. Axon degeneration in dorso-lateral tracts of cervical cord in metabolite (20 mg/kg, intraperitoneally) (a) and tri-*o*-cresyl phosphate (1 ml/kg, orally) (b) treated chickens. Bodian × 550.



a b

Fig. 4. (a) Diseased sciatic nerve from tri-*o*-cresyl-phosphate-poisoned chicken (1 ml./kg, orally, 5 days of symptoms). (b) Normal sciatic nerve from metabolite-treated bird (20 mg/kg, intraperitoneally, 5 days of symptoms). Bodian × 550.

fragmentary degeneration of the axon along with rupture of the myelin into globules. Extensive positive Marchi material appeared in the sciatic nerves of chickens 22 to 24 which received high metabolite doses. Also one section of an anterior tibial nerve (chicken 10) showed equivocal changes. However, 11 sciatic and 9 anterior tibial nerve sections stained with haematoxylin and eosin and 13 sciatic and 2 anterior tibial nerve sections stained with the Marchi preparation were normal.

In the tri-*o*-cresyl-phosphate-treated birds without symptoms, 5 sciatic and 2 anterior tibial nerve sections stained with haematoxylin and eosin and 6 sciatic and 1 anterior tibial nerve preparations examined with the Marchi stain failed to show any definite changes. However, in chickens with symptoms, 9 sciatic nerve sections showed definite alterations, 5 were equivocal, and 1 was negative with haematoxylin and eosin. With this stain, 4 anterior tibial nerve preparations were positive, while one was negative. In all, 15 sciatic and 5 anterior tibial nerve specimens from symptomatic birds were examined with haematoxylin and eosin. The histological changes observed in the peripheral nerves of the tri-*o*-cresyl-phosphate-poisoned birds were similar to those reported by Cavanagh (1954). These consisted mainly of swelling and granular and fragmentary degeneration of the axon together with myelin breakdown. Positive Marchi material in the form of droplets or globules was seen only in nerve sections taken from chickens after 5 or more days of symptoms. Fig. 4a and b shows a comparison between a sciatic nerve section taken from a tri-*o*-cresyl-phosphate-poisoned bird (5 days of symptoms) which shows the aforementioned changes and a comparable specimen from a metabolite-treated chicken (5 days of symptoms) which is essentially normal.

DISCUSSION

Chickens treated with tri-*o*-cresyl phosphate or the metabolite showed a weight loss which appeared to be dose dependent. With lower doses of tri-*o*-cresyl phosphate milder symptoms were evident, the weight loss was less marked and the survival period prolonged. A similar dose-dependent pattern appeared with metabolite-treated birds except that marked weight loss did not result until after the appearance of marked muscle weakness. Cavanagh (unpublished observations, cited by Cavanagh, Davies, Holland & Lancaster, 1961) has noted that decreasing the tri-*o*-cresyl phosphate dose reduced the extent of damage to the peripheral nerves to a greater degree than it did to the long tracts of the spinal cord. The dose differential in affecting the peripheral nerves in contrast to the spinal cord appeared to be even greater for the metabolite. The weight loss might therefore be related in some manner to the degree of peripheral nerve involvement.

The onset of neurological signs appeared at approximately the same time with tri-*o*-cresyl phosphate and its metabolite, and involved a similar degree of degeneration in the same tracts of the spinal cord and brain stem. Soon after the onset of ataxia the tri-*o*-cresyl-phosphate-treated chickens developed a rapidly progressive weakness, mainly of the lower extremities, while birds treated with the metabolite at doses below 60 mg/kg showed no or minor signs of a neuropathy. This was substantiated pathologically by haematoxylin and eosin staining of sections from sciatic and anterior tibial nerves. After the onset of symptoms, most of the sections from chickens treated with tri-*o*-cresyl phosphate showed a significant number of

degenerating fibres, while in the metabolite-poisoned birds at doses below 60 mg/kg only 3 showed equivocal changes and one positive alterations of the 24 sections examined. Metabolite doses of 100 mg/kg or greater resulted in increased severity of symptoms and positive Marchi material in sciatic nerve sections. Axon and myelin breakdown was noted with both compounds.

The tri-*o*-cresyl phosphate metabolite at 20 mg/kg acted predominantly on the spinal cord while tri-*o*-cresyl phosphate at 1.0 ml./kg affected both the spinal cord and peripheral nerves. Metabolite doses above 60 mg/kg, requiring oxime and/or atropine for survival from initial cholinergic effects, resulted in both spinal cord and peripheral nerve damage. Similar results to those encountered with the tri-*o*-cresyl phosphate metabolite have been reported with dyflos (Lancaster, 1960) and tri-*o*-cresyl phosphate (unpublished results cited by Cavanagh *et al.*, 1961) where the extent of damage to peripheral nerves in contrast to the tracts of the spinal cord decreased with reduced doses of the toxicant. The dose differential for the metabolite to effect damage in both sites must be rather large. Spinal cord degeneration occurred predominantly over a large range (8 to 400 mg/kg verified both clinically and pathologically and as low as 4 mg/kg based on clinical symptoms), while peripheral degeneration occurs only at doses of 100 mg/kg or above. By contrast it has been recently shown that tri-*p*-ethylphenyl phosphate produces mainly a peripheral neuropathy over a large dose range (Cavanagh *et al.*, 1961). This does not necessarily mean that the tri-*o*-cresyl phosphate metabolite and tri-*p*-ethylphenyl phosphate act on different biochemical systems, but may be related only to selectivity in localization of the agent or reaction at the active site within the nervous system.

The nature of the biochemical lesion effected by certain organophosphate esters to produce ataxia in chickens is not known. Many organofluoro phosphorus compounds that are potent esterase inhibitors will produce this effect at intramuscular doses as low as 0.25 mg/kg (Davies *et al.*, 1960). The tri-*o*-cresyl phosphate metabolite is a potent esterase inhibitor and will cause ataxia with intraperitoneal doses of 4 to 8 mg/kg, while certain anti-esterase analogues of this cyclic phosphate are effective below 1 mg/kg (Baron, Casida & Eto, unpublished observations). The clinical and pathological findings are similar with the organofluoro phosphates and the tri-*o*-cresyl phosphate metabolite. Other tri-aryl phosphates producing ataxia are enzymatically oxidized *in vitro* and *in vivo* to potent esterase inhibitors (Casida, 1961 ; Aldridge & Barnes, 1961). One common characteristic of the ataxia-producing agents, with the possible exception of tri-*p*-ethylphenyl phosphate, is their high degree of reactivity with esterases either *per se* or as metabolites. The selectivity of these agents in inhibition of different esterases varies considerably, and many esterase inhibitors will not yield ataxia. The critical reaction producing ataxia probably occurs within the nerve to yield a phosphorylated esteratic site on some protein with the release of fluoride or a substituted-phenolate ion. Davies *et al.* (1960) have proposed that the ion released produces the lesion. It appears more probable that the damage to nerve fibre results from changes in metabolism initiated by phosphorylation at a critical esteratic site of an as yet undefined protein. Pseudocholinesterase has been proposed as the site of disruption (Earl & Thompson, 1952a and b), but considerable evidence against this hypothesis has been presented

(Davison, 1953 ; Aldridge & Barnes, 1961 ; Cavanagh & Holland, 1961 ; Cavanagh *et al.*, 1961). Because of their high degree of reactivity and selectivity in producing ataxia in chickens, the cyclic phosphorus esters of *o*-hydroxybenzyl alcohol should prove useful in further pathological and biochemical investigations of this intriguing phenomenon.

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