Biochemical Pharmacology, 1962, Vol. 11, pp. 337–352. Pergamon Press Ltd., Printed in Great Britain

HYDROXYLATION AND CYCLIZATION REACTIONS INVOLVED IN THE METABOLISM OF TRI-*O*-CRESYL PHOSPHATE

MORIFUSA ETO,* JOHN E. CASIDA and TADAKO ETO

Department of Entomology, University of Wisconsin, Madison, Wisconsin, U.S.A.

(*Received* 18 *October; accepted* 26 *December* 1961)

Abstract—Tri-*o*-cresyl phosphate is metabolized in rats by hydroxylation and cyclization of the hydroxymethyl derivatives. Indirect evidence was obtained for two intermediates, di-(*o*-cresyl) mono-*o*-hydroxymethylphenyl phosphate and di-(*o*-hydroxymethylphenyl) mono-*o*-cresyl phosphate. These intermediates cyclize spontaneously. The principal cyclic phosphate formed was 2-(*o*-cresyl)-4H-1:3:2-benzodioxaphosphoran-2-one, as ascertained by isolation and synthesis. Evidence is also presented for 2-(*o*-hydroxymethylphenyl)-4H-1:3:2-benzodioxaphosphoran-2-one and 2-(*o*-hydroxybenzyl)-4H-1:3:2-benzodioxaphosphoran-2-one, as either metabolites or products formed on spontaneous cyclization. The primary cyclic metabolite (see above) hydrolyses in mild alkali or reacts with chymotrypsin by cleavage of the cyclic phosphate at the P—O-aryl bond. The deleterious biological activity of tri-*o*-cresyl phosphate appears to result from esterase inhibition by these cyclic phosphate metabolites.

INTRODUCTION

TRI-*o*-CRESYL PHOSPHATE (TOCP) is an undesirable impurity in the tri-cresyl phosphates prepared for a variety of industrial uses.[1] The *ortho*-isomer causes a polyneuritis which progresses to paralysis of the extremities in man and certain other animals,[2–4] and potentiates the toxicity of the insecticide, malathion (O:O-dimethyl S-(1 : 2-dicarbethoxyethyl) phosphorodithioate),[5, 6] and certain ester-containing drugs.[7] Aldridge[8] in 1954 and Myers and co-workers[9] in 1955 reported that TOCP is metabolized to form potent esterase inhibitors. The chemical nature of these metabolites was not elucidated in their studies, nor was the contribution of these metabolites with antiesterase activity to the biological activity of TOCP.

A preliminary report on the present investigations dealing with the relation of the metabolism of TOCP to its biological activity has appeared.[10] The following report considers the experimental evidence for the hydroxylation and cyclization reactions involved in the metabolism of TOCP.

EXPERIMENTAL

Production of metabolites

Male and female rats (from 200 to 400 g) were treated with tri-*o*-cresyl phosphate (Eastman Kodak Co., Rochester, N. Y.) via a stomach tube. One milliliter of tri-*o*-cresyl phosphate per kg was injected into the tube and followed by a corn oil rinse of 1 ml per kg before the tube was withdrawn from the stomach. At varying times after

* Project associate. Permanent address: Department of Agricultural Chemistry, Kyushu University, Fukuoka, Japan.

administration (2 hr optimally), the small intestine and liver were removed and frozen. The frozen tissues were sliced and homogenized in three volumes of acetone with a Lourdes blendor. After filtration of the homogenate, the residue was re-extracted with acetone. The combined filtrates were concentrated with an air jet at room temperature. An equal volume of saturated aqueous sodium chloride was added to the evaporated residue and this mixture was extracted three times with a large volume of benzene; the extract was dried with sodium sulfate, stripped of benzene under reduced pressure and subjected to adsorption column chromatography.

Rat liver microsomes also were used to form the metabolites. The organophosphate substrate in ether was evaporated on the bottom of a 50-ml Erlenmeyer flask. Microsomes, prepared by a described procedure[11] from 2 g of liver, were added in 4 ml of 0·05 M potassium phosphate, pH 7·2, and the flasks were shaken for 2 hr in air at 37 °C. In some studies, reduced diphosphopyridine nucleotide (DPNH) was also added. The final concentration of the organophosphate substrate was always 1×10^{-3}M. Immediately after incubation, 10 vols. of acetone were added to the flasks, the resulting precipitate was filtered, the filtrate assayed for antiesterase activity and prepared for column chromatography with benzene as indicated above.

Chromatography

Adsorption column chromatography. A mixture of 80 g of silicic acid and 40 g of celite was heated at about 150° for 2 hr, cooled, slurried in *n*-hexane and packed to yield a column of 4 × 40 cm. Less than 5 g of benzene-soluble extractives from tissues were added to the column and elution was accomplished with a gradient of 500 ml of benzene to 500 ml of absolute ether. In certain cases this gradient was followed by 200 ml of ether to complete the elution of the compounds under investigation; fractions of 15 ml were collected.

Partition column chromatography. Two partition columns with aqueous methanol coated on celite as the stationary phase were utilized. With the *n*-hexane column the stationary phase was 90% methanol saturated with hexane, and hexane saturated with 90% methanol was used for elution; in a similar manner, carbon tetrachloride and 80% methanol were utilized. These 1·8 × 18 cm columns were prepared with 10 g of dried celite and 10 ml of aqueous methanol.

Paper partition chromatography. Whatman no. 1 paper was used with and without pretreatment with silicone (Dow-Corning 550). Impregnation was accomplished by dipping the paper in 5% silicone in hexane and air-drying. Lack of reproducibility of R_f-values with silicone-impregnated papers, due to variation in the amount of silicone retained, necessitated direct comparison of known with unknown compounds on the same paper strip. Chromogenic reagents were utilized as follows: phosphorus phenolic esters and free phenols were sprayed with 1 N potassium hydroxide in ethanol followed by diazotized sulfanilic acid;[12] mono- and di-aryl phosphates were detected under short wavelength ultraviolet light and by spraying with bromophenol blue or ammonium molybdate-perchloric acid mixture;[12] and free phenols were detected by spraying with 0·08% aqueous 4-aminoantipyrine and overspraying with 0·08% potassium ferricyanide in phosphate buffer, pH 7·7.

Anticholinesterase assay

Cholinesterase activity was assayed potentiometrically, using human plasma, by a slight modification of a described procedure.[13] Human plasma (1·0 ml of 25 per cent) plus 1·0 ml buffer (0·036 M sodium barbiturate, 0·008 M potassium dihydrogen phosphate, 1·20 M potassium chloride, pH 8·0) was incubated with the organophosphate for 30 min at room temperature, 0·20 ml of an 0·66 M solution of acetylcholine chloride were then added and the mixture was incubated at 37° for 50 min, after which time the change in pH was determined. Varying amounts of organophosphate were assayed to determine the level effecting 50 per cent inhibition of cholinesterase. Results are expressed as units of cholinesterase inhibitor or antiesterase agent per mg. One unit is defined as the amount of inhibitor necessary to cause an inhibition of the esterase to the extent of 50 per cent. Thus, an inhibitor effective at the level of 1·0 μg had 1000 units per mg. One drop of the fractions from column chromatography was used to ascertain the position of eluted anticholinesterase agents. In certain cases, paper chromatograms were cut into sections, the sections extracted with 80% methanol and the extracts assayed to determine the approximate R_f-values for anticholinesterase agents. Studies of the stability of metabolites of TOCP were made by incubating the metabolites in barbiturate-phosphate buffer, pH 8·0, for varying periods of time prior to adding the cholinesterase for residual inhibitor assay.

Synthesis of hydroxymethyl-TOCP

Di-*o*-cresyl mono-*o*-hydroxymethylphenyl phosphate (hydroxymethyl-TOCP) was prepared by slow addition at room temperature of the potassium salt of *o*-hydroxybenzyl alcohol to a chloroform solution of di-*o*-cresyl phosphoryl chloride. After 18 hr at room temperature the chloroform solution was washed with water, dilute aqueous sodium bicarbonate and again with water, dried with sodium sulfate, the solvent evaporated and the residue purified by adsorption chromatography. A product obtained in 36% yield, with an infrared spectrum almost identical with that of TOCP, except for a strong associated OH-stretching vibration at 3·00 μ, was considered to be di-*o*-cresyl mono-*o*-hydroxymethylphenyl phosphate. Chromatography of this material on silicone-impregnated paper showed it to be free of *o*-hydroxybenzyl alcohol.

Synthesis of a cyclic phosphate

2-(*o*-Cresyl)-4H-1 : 3 : 2-benzodioxaphosphoran-2-one was prepared by adding triethylamine (16·7 ml) dropwise to a mixture of *o*-hydroxybenzyl alcohol (7·3 g) and *o*-cresyl phosphoryl dichloride (13·3 g) in chloroform with stirring and cooling in an ice-bath. The reaction mixture was kept at 5° for 12 hr, washed with cold water, dilute hydrochloric acid, dilute aqueous sodium bicarbonate, and water; finally the chloroform was dried with sodium sulfate. The crude product (11 g) recovered by evaporation of the solvent was purified by adsorption column chromatography to give 5·8 g of a light brown oil. Distillation *in vacuo* yielded a colorless oil, b.p. 159–161° (0·09–0·1 mm), n_D 24·5° = 1·5584. Anal. calcd. for $C_{14}H_{13}O_4P$: C, 60·87%; H, 4·74%; and P, 11·22%. Found: C, 59·17%; H, 4·67%; and P, 10·86%. Since this compound was subsequently found to be identical to a metabolite of TOCP designated as M-1, the synthetic material was called SM-1.

The infrared spectrum, degradation products and chromatographic characteristics of this cyclic phosphate, as discussed later, were as anticipated for the proposed

structure. Attempts to distil this product prior to chromatographic purification always resulted in decomposition, probably through polymerization. Even after chromatography the pressure during distillation had to be 0·1 mm or below in order to minimize decomposition.

Reaction of a cyclic phosphorus ester with chymotrypsin

The esterase activity of α-chymotrypsin (salt-free from ethanol, Nutritional Biochemicals Corp., Cleveland 28, Ohio) was assayed colorimetrically with *p*-nitrophenyl acetate as the substrate.[14] To the enzyme solution in 5 ml of 0·067 M phosphate buffer, pH 7·0, was added 0·1 ml of a solution of the substrate in isopropanol at 8×10^{-3} M. Absorbance at 400 mμ was measured with a Spectronic 20 spectrophotometer after 20 min at room temperature. The absorbance was proportional to the amount of enzyme from 0·3 to 2·5 mg.

The rate of reaction of the cyclic phosphorus ester with chymotrypsin was studied by mixing 100 mg of chymotrypsin in 2·0 ml of phosphate buffer, pH 7.0, with 0·2 ml of *iso*propanol containing 1% inhibitor. After various incubation times at room temperature, aliquots of 0·05 ml were taken for assaying residual esteratic activity, and of 0·2 ml for analysis of free phenolic groups with 4-aminoantipyrine.

Other methods

4-Aminoantipyrine was used for analysis of free phenolic groups[14–15] in phenolic phosphates. TOCP was hydrolysed, probably to monocresyl phosphate, by incubation with 1 N aqueous potassium hydroxide for 20 min at 100°. The liberated *o*-cresol was determined colorimetrically. The metabolites, either natural or synthetic, were hydrolysed at pH 7·0 to 7·7 prior to color development with aminoantipyrine. The red dye formed could be completely extracted into chloroform if it was derived by coupling with a free phenol, but the color remained in the aqueous phase if the phenolic derivative contained an ionized phosphate grouping.

Infrared spectra were made from 10% chloroform solutions with the Baird model 4-55 or the Beckman model IR-5 infrared spectrophotometer with sodium chloride optics.

Conversion of TOCP to antiesterase metabolites

Tri-*o*-cresyl phosphate (5 g) was subjected to adsorption column chromatography. Anticholinesterase assays on one drop of the eluted fractions failed to detect any inhibitors. The chromatographically pure TOCP was distilled (195° at 0·1 mm) to yield an activity of 1×10^{-2} units/mg (i.e. 100 mg for 50 per cent inhibition of cholinesterase).

The antiesterase activity increased markedly shortly after administration to rats (Fig. 1). The activity of an acetone-soluble fraction of intestine, 2 hr after treatment, was 20,000 times greater than that which would have resulted from the TOCP *per se*, assuming a uniform distribution of TOCP and metabolites in the rat. The material in the liver was considerably less active. Control rats administered only corn oil were devoid of antiesterase activity in similar extracts.

A preliminary study with tissue slices showed that the liver, but not the intestine, was active in converting TOCP to agents with antiesterase activity. Liver microsomes in the presence of DPNH converted the chromatographically purified, distilled TOCP

to derivatives 113,000 times more active than the substrate. Thus, the activity with DPNH-fortification was 1130 units/mg of TOCP and without DPNH-fortification was only 35 units/mg of TOCP; accordingly, the antiesterase metabolites are derived from TOCP *per se* and not from impurities in the technical material.

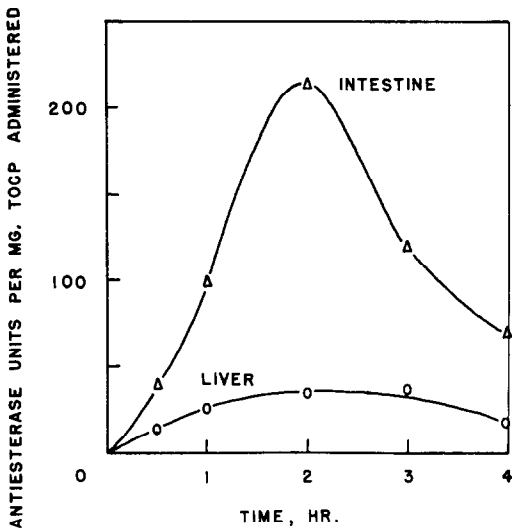


FIG. 1. Antiesterase activity units in rat tissues following oral administration of TOCP (1 ml/kg). Units expressed per mg of TOCP administered, assuming uniform distribution of TOCP and metabolites in the rats; details as in text.

Evidence for three major antiesterase metabolites

Column adsorption chromatography resolved three major metabolites with antiesterase activity, all of which were more polar than TOCP (Fig. 2). These were

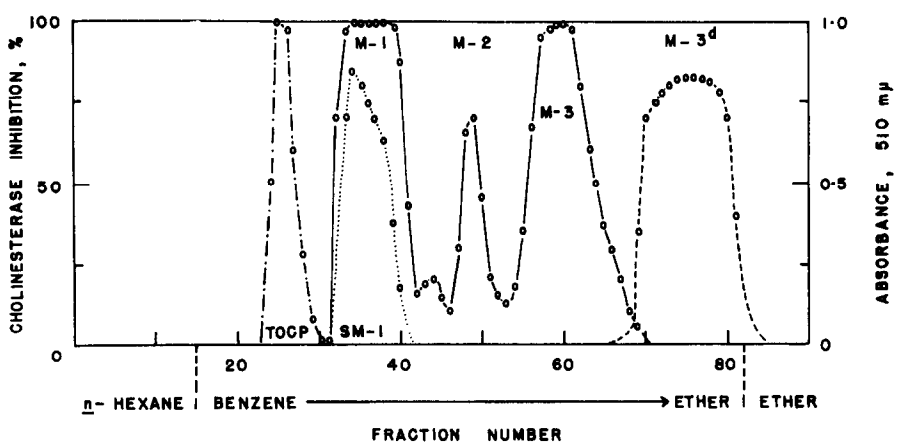


FIG. 2. Separation of derivatives of TOCP by adsorption chromatography on a silicic acid–celite column with a benzene to ether gradient; details as in text. This composite chromatogram based on cholinesterase inhibition by M-1, M-2 and M-3 from intestine, SM-1 from synthesis and M-3^{d} from decomposed M-3 previously separated from liver and intestine; and on absorption at 510 mμ following hydrolysis and reaction with 4-aminoantipyrine for TOCP.

designated, in the order of their elution, as M-1, M-2, and M-3. A fourth minor metabolite, consistently present in the *in vivo*-studies, was eluted just after M-1. Because of its low relative activity, no attempt was made to purify this fourth minor metabolite. The proportion of the total antiesterase activity made up by each of these components varied with the experimental conditions. In the liver and intestine, 2 hr after TOCP administration, the ratio was 100 : 1–1·5 : 4 for M-1 : M-2 : M-3, respectively. With rat liver microsomes and DPNH, three metabolites chromatographing in the same positions were detected and their activity ratio was 100 : 1·6 : 6. A typical experiment illustrating the recovery of these antiesterase metabolites from liver and intestine is presented in Table 1.

TABLE 1. ANTIESTERASE ACTIVITY OF METABOLITES OF TOCP IN RAT LIVER AND INTESTINE 2 HR AFTER THE ORAL ADMINISTRATION OF A TOTAL OF 44·6 ML OF TOCP TO 140 RATS (COMBINED WEIGHT, 44·6 KG)

Tissue and fraction	Total weight (g)	Antiesterase activity (units/mg)
Liver	1695	—
Acetone extract	54	13
Benzene extract	38	17
Col. Adsorp. Chrom		
M-1	1·2	520
M-2	0·17	57
M-3	0·22	109
Intestine	1730	—
Acetone extract	193	47
Benzene extract	191	48
Col. Adsorp. Chrom		
M-1	18·2	318
M-2	2·8	19
M-3	1·7	146

Rats were treated with diphenyl *o*-cresyl phosphate and the liver and intestine extracted and chromatographed on the adsorption column in a manner similar to that employed in the studies with TOCP-treated rats. A single esterase inhibitor was detected and this chromatographed in the approximate position of M-1. Based on this study it appeared likely that the additional metabolites with TOCP might result from attack on the two other methyl groups present in TOCP.

The stability of the three major metabolites of TOCP, and a degradation product of M-3 designated as M-3^{d} which is discussed later, was compared in barbiturate-phosphate buffer of pH 8·0. M-1, M-2 and M-3^{d} were much more labile than M-3, which appeared to be a mixture of components (Fig. 3).

Several column chromatographic systems were tested for their efficiency in further purification of metabolites M-1, M-2 and M-3. Considerable activity was lost with alumina and florisil columns. Partition chromatography with either *n*-hexane or carbon tetrachloride and aqueous methanol proved to be the most effective.

Nature of M-1

M-1 from rat liver and intestine was highly purified by repeated adsorption and partition column chromatography. Purification of the material from the intestines of 490 rats yielded, after the first adsorption chromatogram, 24·5 g with a specific

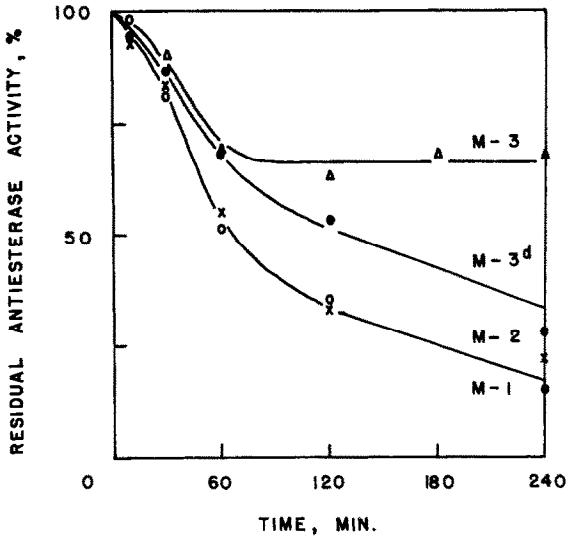


FIG. 3. Stability of TOCP metabolites in pH 8·0 barbiturate–phosphate buffer, based on loss of antiesterase activity; details as in text. Designations: ○——○ for M-1; ×——× for M-2; ●——● for M-3[d]; and △——△ for M-3.

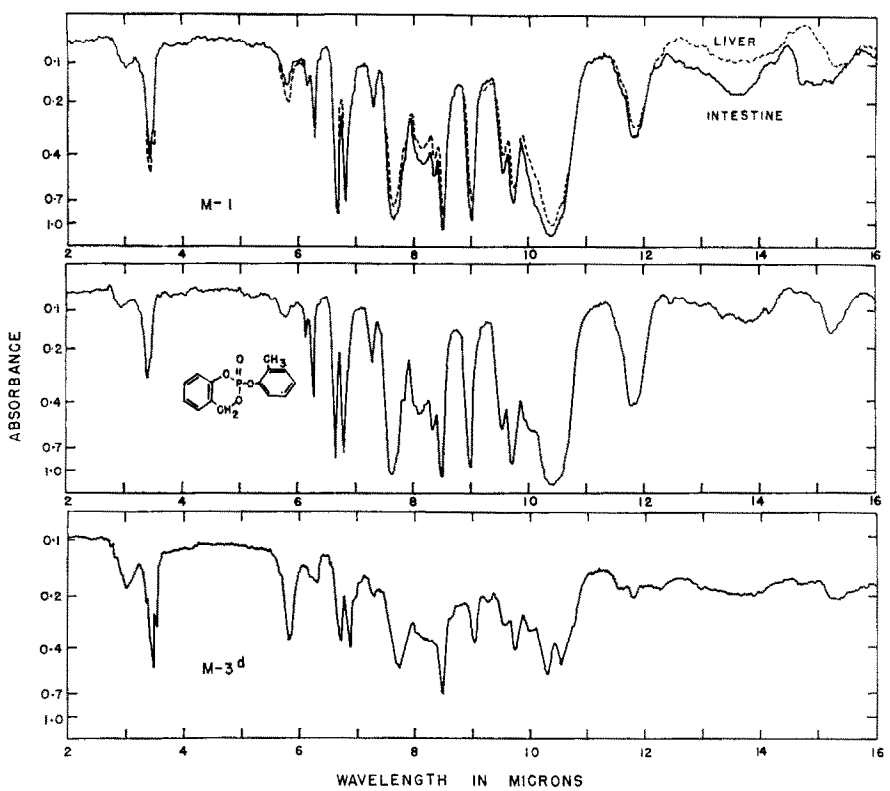


FIG. 4. Infrared spectra of M-1 as purified from liver and intestine, synthetic M-1 as designated by structural formula, and M-3[d] or degraded M-3 from combined liver and intestine. Spectra for M-1 and M-3[d] were prepared at final stage of purification.

activity of 320 units/mg. Two further adsorption chromatograms increased the specific activity to 1100 and 3600 units/mg, respectively, at which time 2·6 g remained. Three partition chromatograms with *n*-hexane–aqueous methanol increased the specific activity to 60,000, 120,000 and 120,000 units/mg, respectively, at which time only 26·6 mg were recovered. An infrared spectrum of this last material is shown in Fig. 4, together with a spectrum obtained with 11 mg of material from liver purified in a similar manner. The specific activity of the purified liver metabolite was 90,000 units/mg; this finding, in conjunction with the infrared spectrum, indicated that the material from liver was less pure than that from the intestine.

M-1 did not appear to have a reactive aldehyde grouping, since it did not lose its anticholinesterase activity on standing with potassium permanganate in acetone solution for 20 hr, and was not extracted from organic solvent with an aqueous solution of sodium bisulfite. A negative result with the ferric chloride-pyridine test[16] indicated no free phenolic hydroxyl. The antiesterase activity was lost on incubation in 1 N HCl, the rate of loss approximating the first-order reaction anticipated from hydrolysis, with half-life values of 20 min at room temperature and 1·5 min at 100°. An ether extract of M-1, following hydrolysis in 1 N hydrochloric acid for 1 hr at room temperature, gave positive spot tests with the following phenolic reagents: Gibbs' reagent, 4-aminoantipyrine, diazotized sulphanilic acid, diazotized nitroaniline and ammoniacal silver nitrate.[12] These spot test reagents were tested with *o*-cresol, *o*-hydroxybenzyl alcohol, salicylaldehyde, salicylic acid, methyl salicylate and tolu-hydroquinol; of these, *o*-hydroxybenzyl alcohol most closely resembled the phenol formed on acid-degradation of M-1. Paper chromatography further indicated that *o*-hydroxybenzyl alcohol is the degradation product (Table 2).

TABLE 2. PAPER CHROMATOGRAPHIC EVIDENCE FOR *o*- HYDROXYBENZYL ALCOHOL AS A PRODUCT OF THE ACID HYDROLYSIS OF M-1. RESULTS AS R_f-VALUES*

Phenols	*n*-Butanol, NH_4OH 5:2	99% Methanol saturated with Skelly C
M-1, acid hydrolysate	0·76	0·77
o-hydroxybenzyl alcohol	0·76	0·76
o-cresol	0·94	0·83
salicylaldhyde	0·70	0·64
methyl salicylate	0·41	—

* Prior to use, the chromatographic paper was washed with the solvent to remove impurities which gave an orange color with diazotized sulfanilic acid and ran at the front to interfere with *o*-cresol analysis. Diazotized sulfanilic acid gave a yellow-orange color with the first three materials and a pale yellow color with methyl salicylate. Salicylaldehyde was detected by yellow fluorescence in ultraviolet light after spraying with ethanolic potassium hydroxide. Known phenols and M-1 hydrolysate were spotted on the same paper and developed for direct comparison.

Exposure of M-1 to ammonia vapors yielded mono-*o*-cresyl phosphoric acid and *o*-hydroxybenzyl alcohol (Table 3); thus, M-1 appeared to be an ester of these two products.

The infrared spectrum of purified M-1 (Fig. 4) showed no free hydroxyl group, but showed a band at 9·7 μ which was not present in triaryl phosphates such as TOCP.

The similarity of this band position with that of P–O–C(alkyl) led to synthesis of a cyclic phosphorus ester derived from *o*-hydroxybenzyl alcohol. The product from the reaction of *o*-cresyl phosphoryl dichloride with *o*-hydroxybenzyl alcohol, 2-(*o*-cresyl)-4H-1 : 3 : 2-benzodioxaphosphoran-2-one, was identical to M-1 in respect to infrared spectrum (Fig. 4), chromatographic characteristics (Fig. 2, Table 4), and antiesterase activity.

TABLE 3. R_f-VALUES FOR HYDROLYSATES OF M-1, M-2 AND M-3[d], AS COMPARED WITH THOSE OF KNOWN COMPOUNDS*

Sample	Mobile phase		Detection			
	n-Butanol, NH_4OH 4:1	*iso*Propanol, NH_4OH 3:1	U.V. absorption†	BPB	Molybdate	Diaz. sulfanilic acid
Known compounds						
Di-*o*-cresyl phosphate	0·79	0·89	+	+	+	–
Mono-*o*-cresyl phosphate	0·13	0·48	+	+	+	–
o-Hydroxybenzyl alcohol	0·80	0·87	–	–	–	Y
Hydrolysates						
M-1	0·13	—	+	+	+	–
	0·80	—	–	–	–	Y
M-2	—	0·19	‡	‡	§	Y§
	—	0·87	‡	‡	–	Y
M-3[d]	—	0·26	+	‡	+	P
	—	0·87	—	‡	—	Y

* Samples were hydrolyzed by exposure to ammonia vapor on the paper for 3 hr before the chromatograms were developed. Y and P are yellow and purple colors with diazotized sulfanilic acid. BPB is bromophenol blue reagent.

† Sensitivity for detection of aryl phosphates was greater than for *o*-hydroxybenzyl alcohol, although the latter could be detected in large amounts.

‡ Amounts available for testing were too small for meaningful results.

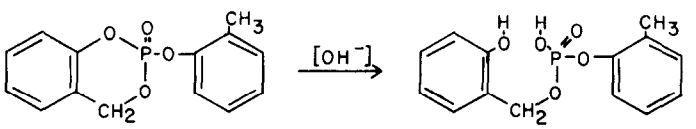
§ Phosphorus content was too small for detection on paper but molybdate test was positive after elution of the R_f 0·19 component and complete digestion with perchloric acid. Partial hydrolysis on the paper to yield a free phenol resulted in the detection of this mono-ester phosphate with the diazotized sulfanilic acid reagent.

TABLE 4. R_f-VALUES FOR CERTAIN DERIVATIVES OF TOCP ON SILICONE-IMPREGNATED PAPER

Mobile phase	TOCP	Hydroxymethyl TOCP	synthetic M-1	M-1	M-2	M-3[d]	*o*-Hydroxybenzyl alc.
Benzene, hexane, methanol, water (3:6:2:7)-lower phase	0·00	0·54	0·07	0·07	0·54	0·64	0·78
Benzene, hexane, methanol, water (4:5:4:5)-lower phase	0·00	0·79	0·41	—	—	—	0·82
Chloroform, ethanol, water (10:10:8)-upper phase	—	—	0·48	0·48*	—	0·90	—
Acetone, acetonitrile, water (12:1:20)	—	—	0·67	0·67*	—	—	—
Acetone, dioxane, water (2:2:5)	—	—	0·85	0·85	—	—	—

* The area corresponding to the yellow-orange spot with diazotized sulfanilic acid was extracted with 80% methanol and found to be the only region on the chromatogram with strong antiesterase activity.

The antiesterase activity of M-1 diminished rapidly on standing in aqueous solutions of pH 8 at room temperature and a free phenolic hydroxyl was released. Most of the colored derivative formed by reaction of this mild hydrolysate after 30 min with 4-aminoantipyrine was not extractable into chloroform, in contrast to the derivative from *o*-hydroxybenzyl alcohol released in 1 N potassium hydroxide. Thus, the product from mild hydrolysis appeared to have a free phenolic hydroxyl and a strong hydrophilic group in the same molecule. These combined observations indicate the following structure for M-1 with the P–O–C(aryl) as the reactive site:



Nature of M-2

M-2 appears to be a further oxidation product of M-1, rather than an intermediate in the formation of M-1. Rat liver microsomes and DPNH effected partial conversion of synthetic M-1 to M-2. Injection into rats of SM-1, 100 mg/kg in corn oil, and assay of the liver and intestine after 1 hr, showed both M-1 and M-2 in ratios similar to those resulting from treating rats with TOCP. In contrast to the TOCP-treated rats, however, the only antiesterase agents evident in SM-1-treated rats were the administered compound and M-2. Partial purification of M-2 formed *in vivo* from synthetic M-1 and TOCP was effected by chromatographing twice on the adsorption column followed by three times on the partition column, using carbon tetrachloride and aqueous methanol. The final specific activity was 24,000 units/mg, with a yield of 6·2 mg, as compared with a specific activity for pure M-1 of 120,000 units/mg. An infrared spectrum of the purified M-2 indicated that it was 5–10 per cent pure, based on the absorbance bands associated with the phosphorus ester groupings (9·74 and 10·30 μ). The presence of the 9·7 μ P–O–C (alkyl) band was indicative of a cyclic phosphate structure, as was its similar hydrolysis rate to M-1 (Fig. 3).

Further support for the cyclic phosphate structure was derived from a consideration of the hydrolysis products. Prior to hydrolysis, M-2 did not contain a free phenolic hydroxyl group, since it failed to form a chloroform-soluble derivative on reaction with 4-aminoantipyrine at pH 7·7. Mild hydrolytic conditions (pH 7·7, 30 min, room temperature) yielded a derivative with a free phenolic group which coupled with 4-aminoantipyrine to form a red dye that was not extracted into chloroform. Hydrolysis in 1 N potassium hydroxide prior to reaction with aminoantipyrine yielded a chloroform-soluble dye.

M-2 was spotted on filter paper, exposed to ammonia vapor and the chromatogram developed with *iso*propanol : ammonium hydroxide (3 : 1); this chromatogram was then sprayed with alkali and diazotized sulfanilic acid. Two yellow spots appeared at R_f 0·19 and 0·87 (Table 3). The higher R_f-value was that of *o*-hydroxybenzyl alcohol. The colored derivative of R_f 0·19 was extracted from the paper with ethyl acetate for comparison with the diazotized sulfanilic acid derivative of *o*-hydroxybenzyl alcohol. These two derivatives were compared by paper chromatography with two systems.

Paper impregnated with 2% sodium carbonate and developed with *n*-butanol saturated with 2% aqueous sodium carbonate (1 : 1) yielded an R_f for both derivatives of 0·03. Non-impregnated paper developed with the upper phase from *n*-butanol : ethanol : water (5 : 1·75 : 5) yielded an R_f of 0·52 for both derivatives. Thus, alkaline hydrolysis of the material with an original R_f in *iso*propanol : ammonium hydroxide of 0·19 appeared to yield *o*-hydroxybenzyl alcohol. This 0·19-region from a chromatogram, prior to hydrolysis by potassium hydroxide, was eluted with 90% methanol and tested for the presence of phosphorus with perchloric acid–molybdate reagent. A positive test indicated that the material of R_f 0·19 was an ester of *o*-hydroxybenzyl alcohol and phosphorus. This relatively low R_f-value, compared with those of mono- and di-*o*-cresyl phosphate (Table 3), suggests a monoester structure. These combined observations indicate that M-2 is the *o*-hydroxymethyl derivative of M-1.

Attempts to prepare this *o*-hydroxymethylphenyl cyclic phosphate by reaction of *o*-hydroxybenzyl alcohol or its potassium salt with phosphorus oxychloride under a variety of conditions failed to yield a product comparable with M-2.

Nature of M-3 and M-3[d]

Isolation of M-3 was not possible, since it decomposed during the purification procedures. This was evidenced by the disappearance of the chromatographic peak associated with M-3 and the appearance of a new peak, M-3[d], eluting from the adsorption column after M-3 (Fig. 2). Decomposition of M-3 to M-3[d] occurred on storage in benzene at — 10° and on the adsorption columns during chromatography. Other compounds with antiesterase activity also were formed, and these chromatographed in the positions of M-2 and possibly M-1. The ratio of the various agents with antiesterase activity, formed on M-3 decomposition, varied with conditions; for example, the products which formed on refluxing M-3 in benzene chromatographed almost entirely in the position of M-2.

M-3[d] was purified by repeated adsorption and partition (carbon tetrachloride-aqueous methanol) column chromatography to yield 4·6 mg of product with a specific activity of 75,000 units/mg of the purity indicated by the infrared spectrum in Fig. 4. Paper chromatography (Table 4) showed this material to be more polar than M-1 or M-2 and to be free of contaminating phenols, as detected by diazotized sulfanilic acid. Reaction of M-3[d] with 4-aminoantipyrine at pH 7·7 prior to hydrolysis yielded a chloroform-soluble red dye, a finding which indicated the presence of a free phenolic hydroxyl group in the molecule. On hydrolysis, by incubation at pH 7·7 for 30 min at room temperature, a further phenolic group was released; this, on reaction with 4-aminoantipyrine, yielded a water-soluble red dye. The latter observation, in combination with the infrared spectrum, suggests a cyclic phosphate structure for M-3[d]. Hydrolysis on paper with ammonia vapor yielded *o*-hydroxybenzyl alcohol and a second phenolic material. This second hydrolysis product gave a purple spot with diazotized sulfanilic acid and contained phosphorus, based on perchloric acid oxidation and the ammonium molybdate reagent. Reaction of this hydrolysis product on a chromatogram with 4-aminoantipyrine gave a red dye which did not shift in position on developing the chromatogram with chloroform. Attempts to hydrolyze this monoester phosphate with alkali or alkaline phosphatase were unsuccessful. These combined observations support the hypothesis that the hydrolysis product is *o*-hydroxybenzyl phosphate and that M-3[d] is the cyclic ester of this phosphate and

o-hydroxybenzyl alcohol. Synthesis of the structure proposed for M-3[d] was not successful.

M-3 appears to be a triaryl phosphate which can spontaneously cyclize to yield three different cyclic phosphates. If the proposed structures for M-2 and M-3[d] are correct, then M-3 must be di-*o*-hydroxymethylphenyl mono-*o*-cresyl phosphate.

Metabolism of hydroxymethyl-TOCP

A theoretical intermediate metabolite in the formation of the cyclic phosphates would be di-*o*-cresyl mono-*o*-hydroxymethylphenyl phosphate or hydroxymethyl-TOCP. Since this material was not detected in rat tissues, it was synthesized in order to study its properties. Paper chromatography (Table 4) showed that the synthetic hydroxymethyl-TOCP contained no more than trace amounts of *o*-hydroxybenzyl alcohol or synthetic M-1. On adsorption chromatography, hydroxymethyl-TOCP eluted just prior to M-1. The antiesterase activity of hydroxymethyl-TOCP was about 90 units/mg, as compared to 120,000 units/mg for M-1. Even if hydroxymethyl-TOCP were present in rat tissues as an intermediate metabolite, its chromatographic position close to M-1 and its low antiesterase activity relative to M-1 would have precluded its detection. A TOCP-metabolite with relatively low antiesterase activity chromatographing on the adsorption column just after M-1 (Fig. 2) could not have been hydroxymethyl-TOCP and its identity remains unknown.

Synthetic hydroxymethyl-TOCP in corn oil was injected at 150 mg/kg into a rat, from which the liver and intestine were removed after 1 hr. The antiesterase activity in the intestine was negligible, but that in the liver was four times the total activity originally administered the rat. A liver extract was chromatographed on the adsorption column. The antiesterase activity was almost entirely attributable to a material chromatographing in the identical position of M-1. Thus, it appears that hydroxymethyl-TOCP is a precursor of M-1 and possibly the other metabolites of TOCP.

Reaction with chymotrypsin

Synthetic M-1 reacted readily with chymotrypsin to inhibit its esteratic activity. By assuming a molecular weight of 22,500 for the α-chymotrypsin used, it was found that 0·68 M equivalents of synthetic M-1 was necessary for 50% esterase inhibition at pH 7·0, with a reaction-time of 30 min. Under the same conditions, di*iso*propyl phosphorofluoridate (DFP) gave a value of 0·50 M equivalents. Free phenolic hydroxyl groups, as detected with 4-aminoantipyrine, were liberated on the reaction of chymotrypsin with synthetic M-1 (Fig. 5), but not on reaction with DFP. Dialysis of the chymotrypsin following reaction with synthetic M-1 failed to remove the major aminoantipyrine-positive component. When the inhibited enzyme was reacted with aminoantipyrine to yield a red pigment and then dialyzed or precipitated by addition of ammonium sulfate (0·8 saturation, 0°), the majority of the pigment remained with the protein. Thus, it appears that the initial reaction involved with the esteratic site of chymotrypsin is a phosphorylation involving opening of the cyclic phosphate structure at the P–O–C(aryl) bond. About 30 per cent of the total phenolic hydroxyl released on reaction with chymotrypsin appeared to be due to free *o*-hydroxybenzyl alcohol, but the mechanism was not examined further in order to determine if the release occurred prior to phosphorylation or was a rapid "aging" type phenomenon

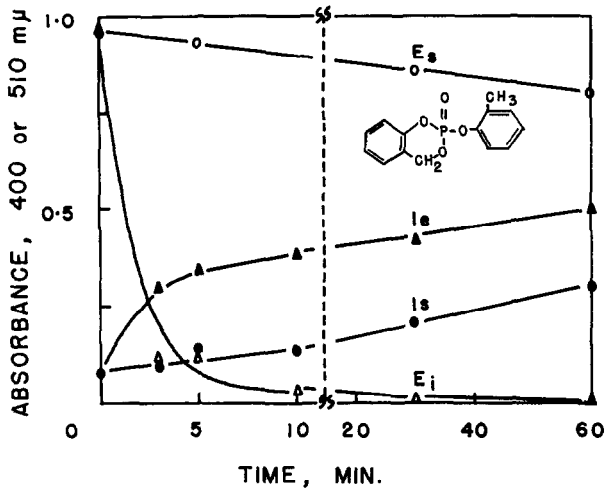


FIG. 5. Reaction of synthetic M-1 with chymotrypsin; details as in text; molar ratio of inhibitor to enzyme 1·6:1·0. Enzymic activity in presence (E_i) and absence (E_s) of inhibitor based on absorbance of *p*-nitrophenate ion at 400 mμ; phenolic hydroxyl release in presence (I_e) and absence (I_s) of enzyme based on absorbance of 4-aminoantipyrine derivatives at 510 mμ.

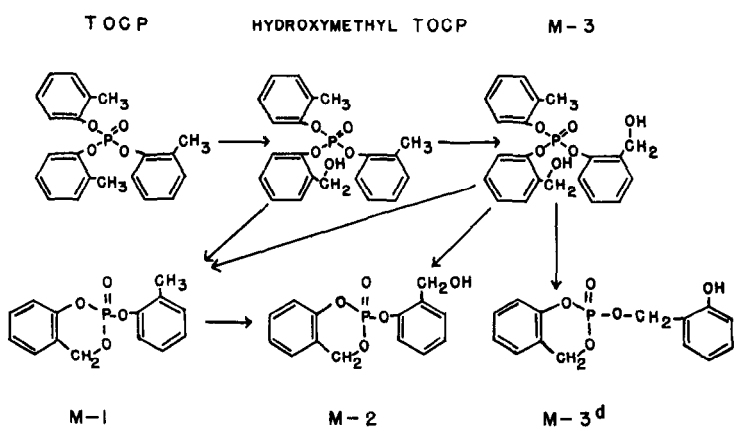


FIG. 6. Proposed pathway for hydroxylation and cyclization reactions in the metabolism of TOCP and degradation of metabolites. Structure for M-1 based on isolation and synthesis, for M-2 and M-3[d] based on infrared spectra and paper chromatography of degradation products; and for M-3 based on derivatives formed on spontaneous degradation. The intermediate, hydroxymethyl-TOCP, was not isolated, but synthetic material was converted to M-1 by rats *in vivo*.

subsequent to phosphorylation. Diphenylphosphoryl chymotrypsin has been shown to undergo a shift from a di-esterified to a mono-esterified phosphoryl enzyme.[17]

DISCUSSION

A proposed metabolic pathway for the hydroxylation and cyclization reactions involved in the metabolism of tri-*o*-cresyl phosphate by rats is presented in Fig. 6. These appear to be the critical reactions in relation to the biological activity of TOCP. The hydroxylated tri-aryl phosphates (hydroxymethyl-TOCP and M-3) were not isolated as metabolites because of their cyclization during purification. The cyclic phosphates were present in only small amounts, a finding which probably is attributable to either their rapid hydrolysis *in vivo*, or to excretion, or both. The reactivity of the *o*-hydroxymethyl group can lead to artifacts during isolation, such as the formation of M-3[d]. This pathway does not consider the hydrolytic reactions nor the fate of the hydrolysis products. Studies with ^{32}P-labeled TOCP have shown that hydrolysis leads to the rapid excretion in the urine of diaryl phosphates, monoaryl phosphates and phosphoric acid.[10] The *in vivo*-distribution and excretion of the cresol from TOCP also has been studied extensively.[18, 19] The *o*-cresol presumably would be conjugated as a sulfate or glucuronide, with a minor portion undergoing ring hydroxylation prior to excretion. Salicyclic acid is probably the main product derived from further metabolism of *o*-hydroxybenzyl alcohol, although small amounts of the unmetabolized compound and ethereal sulfates have also been reported with rabbits.[21]

Several cyclic ribonucleotides with the phosphate in both five- and six-membered rings, either have been isolated from biological material or synthesized.[21-26] Many neutral esters of heterocyclic phosphoric and phosphorothioic acids have been prepared by Lanham.[27] The insecticidal activity of certain of these compounds, such as the experimental insecticide P-chloro-2 : 4-dioxa-5-methyl-P-thiono-3-phosphati*cyclo* (4 : 4 : 0) decane, presumably results from the inhibition of cholinesterase *in vivo*. Although many other cyclic phosphites and phosphates have been prepared, the cyclic phosphate esters of *o*-hydroxybenzyl alcohol have not previously been reported. This also appears to be the first report of spontaneous cyclization of a phosphate ester which results in displacing an alcoholic or phenolic group and yielding a stable derivative. Transitory cyclic phosphates have been considered as intermediates of non-cyclic phosphate esters during certain hydrolytic and phosphorylation reactions (reviewed by O'Brien[28]), but such cyclic phosphate intermediates have not been isolated.

The metabolic pathway of TOCP is of interest with respect to the conversion of this and similar compounds to antiesterase agents and the relation of structure to a delayed neurotoxicity or ataxia. Many *o*-cresyl phosphate esters are metabolized to more potent esterase inhibitors *in vivo* and *in vitro*.[7] The activation mechanism is probably similar to that with TOCP, involving hydroxylation and subsequent cyclization of the *o*-hydroxymethyl derivatives. Activation of *o*-ethylphenyl and *o*-*n*-propylphenyl phosphate esters[7] might similarly involve hydroxylation of the α-alkyl carbon and subsequent cyclization to yield the cyclic phosphates of *o*-hydroxy-α-(methyl or ethyl) benzyl alcohol. Other mechanisms must be involved in the metabolism of such tri-aryl phosphates as tri-*p*-ethylphenyl phosphate,[7, 29] which cannot form six-membered heterocyclic compounds comparable to that formed from TOCP.

TOCP effects a polyneuritis progressing to paralysis of the extremities in man and certain other species. The chicken provides the best laboratory animal for investigating this phenomenon, since rats, mice and many other convenient organisms fail to yield a polyneuritis similar to that in man. Since rats respond differently to TOCP than do chickens, and the metabolite studies were made with rats, it appeared of interest to determine if the chicken also formed the same metabolites of TOCP. Chickens were treated orally at 1·0 ml/kg and the feces collected for the first 3 days following treatment. Extraction of the chicken feces and chromatography of the extract on the adsorption column yielded three metabolites similar in chromatographic characteristics to those formed by rats. The principal antiesterase metabolite, 2-(*o*-cresyl)-4H-1 : 3 : 2-benzodioxaphosphoran-2-one, effected ataxia and demyelination in hens following the injection of 4 to 8 mg/kg.[10] Other triaryl phosphates with one or more *o*-cresyl groups also yield neurotoxicity with hens[29–32] and are metabolized to more potent esterase inhibitors in the hen.[8, 29] Accordingly, the neurotoxicity with *o*-cresyl phosphates would appear to result from metabolites analogous to the cyclic phosphates formed from TOCP. Other types of biological activity associated with TOCP[7, 10] also appear to result from these cyclic phosphate metabolites.

Acknowledgements—Publication approved by the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by grants from the U.S. Army Chemical Research and Development Laboratories (Grant No. DA-CML-18-108-61-G-6) and the U.S. Public Health Service, National Institutes of Health (Contract RG-5304).

REFERENCES

1. P. G. Stecher (Ed.), *The Merck Index of Chemicals and Drugs* (7th ed.) p. 1062. Merck, Rahway, N.J. (1960).
2. M. I. Smith, E. Elvove and W. H. Frazier, *Publ. Hlth Rep., Wash.* **45**, 2509 (1930).
3. M. I. Smith, E. W. Engel and E. F. Stohlman, *Nat. Inst. Hlth. Bull.* **160**, 1 (1932).
4. H. Geoffroy, A Slomic, M. Benebadji and P. Pascal, *World Neurol.* **1**, 294 (1960).
5. S. D. Murphy, R. L. Anderson and K. P. DuBois, *Proc. Soc. Exp. Biol., N.Y.* **100**, 483 (1959).
6. K. P. DuBois, *Advanc. Pest Control Res.* **4**, 117 (1961).
7. J. E. Casida, *Biochem. Pharmacol.* **5**, 332 (1961).
8. W. N. Aldridge, *Biochem. J.* **56**, 185 (1954).
9. D. K. Myers, J. B. J. Rebel, C. Veeger, A. Kemp and E. G. L. Simons, *Nature, London.* **176**, 260 (1955).
10. J. E. Casida, M. Eto and R. L. Baron, *Nature, Lond.* **191**, 1396 (1961).
11. E. Hodgson and J. E. Casida, *Biochem. Pharmacol.* **8**, 179 (1961).
12. R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis* p. 200, 305. Academic Press, New York (1958).
13. J. E. Casida, P. E. Gatterdam, L. W. Getzin, Jr. and R. K. Chapman, *J. Agric. Food Chem.* **4**, 236 (1956).
14. J. E. Casida, K-B. Augustinsson and G. Jonsson, *J. Econ. Entomol.* **53**, 205 (1960).
15. E. Emerson, *J. Org. Chem.* **8**, 417 (1943).
16. S. Soloway and S. H. Wilen, *Analyt. Chem.* **24**, 979 (1952).
17. W. Lee and J. H. Turnbull, *Biochim. et Biophys. Acta* **30**, 655 (1958); *Experientia* **17**, 360 (1961).
18. E. Gross and A. Grosse, *Arch. Exp. Path. Pharmak.* **168**, 473 (1932).
19. M. I. Smith with E. F. Stohlman, *J. Pharmacol.* **51**, 217 (1934).
20. H. G. Bray, W. V. Thorpe and K. White, *Biochem. J.* **46**, 275 (1950).
21. R. T. Williams, *Detoxication Mechanisms* p. 320. John Wiley, New York (1959).
22. H. S. Forrest, H. S. Mason and A. R. Todd, *J. Chem. Soc.* 2530 (1952).
23. R. Markham and J. D. Smith, *Biochem. J.* **52**, 552 (1952).
24. E. W. Sutherland and T. W. Rall, *J. Biol. Chem.* **232**, 1077 (1958).
25. G. M. Tenner, H. G. Khorana, R. Markham and R. H. Pol, *J. Amer. Chem. Soc.* **80**, 6223 (1958).

26. D. LIPKIN, W. H. COOK and R. MARKHAM, *J. Amer. Chem. Soc.* **81**, 6198 (1959).
27. W. M. LANHAM, *Brit. Pat.* 662,125, 762,147, 791,739 and 807,896 (1956–1959). *U.S. Pat.* 2,875,235, 2,894,974, 2,894,016, 2,892,862, 2,892,863, 2,910,499 (1959).
28. R. D. O'BRIEN, *Toxic Phosphorus Esters.* Academic Press, New York (1960).
29. W. N. ALDRIDGE and J. M. BARNES, *Biochem. Pharmacol.* **6**, 177 (1961).
30. C. H. HINE, M. K. DUNLAP, E. G. RICE, M. M. COURSEY, R. M. GROSS and H. H. ANDERSON, *J. Pharmacol.* **116**, 227 (1956).
31. D. HENSCHLER, *Arch. Exp. Path. Pharmak.* **237**, 459 (1959).
32. H. F. BONDY, E. J. FIELD, A. N. WORDEN and J. P. W. HUGHES, *Brit. J. Indust. Med.* **17**, 190 (1960).