Biochemical Pharmacology, 1962, Vol. 11, pp. 1129–1136. Pergamon Press Ltd., Printed in Great Britain.

ENZYMATIC AND ANTIDOTAL STUDIES ON THE NEUROTOXIC EFFECT OF CERTAIN ORGANOPHOSPHATES

R. L. BARON* and J. E. CASIDA

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

(*Received* 25 *June* 1962; *accepted* 16 *August* 1962)

Abstract—The antiesterase activity of tri-*o*-cresyl phosphate (TOCP) and its cyclic phosphate metabolite (SM-1) was examined. Administration of TOCP and SM-1 to hens in ataxia-inducing doses resulted in selective rather than prolonged inhibition of the nerve esterases hydrolyzing butyryl esters of choline, glycerol, and phenols. Nerve esterase inhibition was more persistent with TOCP than with SM-1. Selective inhibition of esterases hydrolyzing butyryl esters was also evident in studies *in vitro* with spinal cord homogenates and SM-1 or di*iso*propyl fluorophosphate (DFP). TOCP and SM-1 did not affect succinate, α-ketoglutarate, or pyruvate oxidation by preparations of nerve and liver from treated birds, nor was the response of the pyruvate oxidation by brain homogenates to thiamine pyrophosphate affected by TOCP. Many compounds tested as possible antidotes for the neurotoxicity induced by TOCP, SM-1, and DFP proved ineffective. Cortisone acetate gave limited improvement in the condition of the birds, thiamine and oxythiamine appeared to be detrimental, and many potential cholinesterase reactivators and other materials were without effect on the neurotoxicity.

CERTAIN organophosphate esters produce a delayed neurological ataxia characterized by central and peripheral demyelination and axon breakdown. This phenomenon has been most intensively investigated in hens. Some compounds such as tri-*o*-cresyl phosphate elicit this delayed response without causing any parasympathomimetic symptoms. Others such as di*iso*propyl fluorophosphate and related fluorophosphorus compounds may give both initial parasympathomimetic effects and delayed ataxia. Theories on the mode of action of the toxicant leading to the delayed paralysis have been recently reviewed.[1, 2] These include prolonged interference with nerve pseudocholinesterase activity, vitamin function, lipid biosynthesis, and respiratory mechanisms. The hypothesis of pseudocholinesterase inhibition[3, 4] has been contested on the basis of the comparative degree and duration of enzyme inhibition by paralytic and non-paralytic organophosphates.[5–9] Administration of various vitamins and other materials to alter the course of the neurotoxic syndrome has given variable results.[10–18] Of the enzymatic systems examined, esterases are most sensitive to inhibition by the paralytic organophosphates (reviewed by Baron[19]). Lipid biosynthesis and nerve respiration have been shown to be affected *in vivo* and *in vitro* by ataxia-inducing materials, but again the significance of the effect is obscure.[20–22] Existing theories for the neurotoxic mechanism are either untenable or as yet unproven.

Recent findings on the metabolism of TOCP indicating an action through esterase-inhibiting metabolites[23–25] prompted a re-examination, utilizing both biochemical and antidotal approaches, of certain of these theories.

* Present address: Division of Pharmacology, Food and Drug Administration, Washington, D.C.

METHODS AND MATERIALS

General

White leghorn chickens between one and two years old and weighing between 1·5 and 2·5 kg were used in all experiments. They were confined in individual cages with ready access to water and complete corn mash ration. The tri-*o*-cresyl phosphate used was of technical grade from Eastman Kodak Co. (Rochester, N.Y.). Di*iso*propyl fluorophosphate was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). The cyclic phosphate metabolite of TOCP, 2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one, was synthesized according to a described procedure.[24] This material, designated for discussion as SM-1, has been identified as the major esterase-inhibiting metabolite of TOCP.[24] TOCP was administered orally at 1·0 ml/kg by a stomach tube either without solvent or dissolved in purified corn oil. Where other doses were used, it is so stated. DFP and SM-1 were administered intraperitoneally as corn-oil solutions at 2·0 and 20 mg/kg, respectively. These solutions were prepared immediately before use to insure against hydrolysis. Atropine was administered to minimize the parasympathomimetic effects of the DFP. Clinical symptoms of ataxia with these organophosphate dosages were evident by the tenth day.

Esterases

Chickens were killed by decapitation and the nerve tissues immediately homogenized with Teflon-glass homogenizers at 4° in bicarbonate buffer. Esterase activity was determined indirectly by CO_2 evolution from bicarbonate buffer (0·357 M $NaHCO_3$ and 0·164 M NaCl) in an atmosphere of 95% N_2 and 5% CO_2 at 37° in 15-ml Warburg flasks. The substrate in 0·40 ml buffer was added to the side arm of the flask and the nerve homogenate to the main compartment. The enzyme level varied from 5 to 160 mg fresh weight nerve tissue per flask depending on the substrate utilized. A 10-min equilibration period at 37° preceded mixing of the substrate and enzyme. The final substrate concentration in all cases was 1×10^{-2} M. Manometric readings were made at 5-min intervals for 30 min. Enzymatic activity was based on the initial rate of CO_2 evolution.

Solutions of acetyl-β-methylcholine chloride, acetylcholine chloride, propionylcholine *p*-toluenesulfonate, butyrylcholine *p*-toluenesulfonate, and O-acetyl and O-propionyl thiamine chloride hydrochlorides were prepared directly in the bicarbonate buffer. Emulsions were used with triacetin, tri-*n*-propionin, tri-*n*-butyrin, phenyl acetate, phenyl butyrate, *o*-nitrophenyl acetate and *o*-nitrophenyl butyrate. These were prepared by adding Triton X-100 (Rohm and Haas, Co. (Philadelphia, Pa.)) in acetone to dissolve the ester, evaporating the solvent, and then adding the buffer with thorough mixing to yield a final concentration of 1·1 mg/ml Triton X-100.

For assay of the inhibitors *in vitro*, freshly prepared aqueous solutions of DFP and SM-1 were added to the nerve homogenates 30 min prior to initiation of the enzymatic assay. All concentrations stated for inhibitors are those during the inhibition reaction and not during the assay of the esterase. The results are expressed as pI_{50} values (negative logarithm of the molar concentration of inhibitor producing 50% inhibition under the stated assay conditions).

Oxidative enzymes

Certain oxidative enzymes of chicken nervous tissue and liver were assayed after poisoning the animals with TOCP and SM-1. Pyruvate and α-ketoglutarate oxidation,

with and without thiamine pyrophosphate chloride (TPP) fortification, and succinic acid oxidation were examined. Homogenates of brain, spinal cord, and liver were made at 10% in 0·25 M sucrose with a Teflon-glass homogenizer. The brain and liver were subjected to differential centrifugation at 600 and 20,000 × *g* for 10 min at 4° to separate a mitochondrial fraction.

Preliminary oxidation studies were made manometrically in 15-ml Warburg flasks containing: in the side arm, 20 μmole substrate as the sodium salt and 0·4 ml of 0·05 M potassium phosphate buffer, pH 7·3; in the main compartment in 2·6 ml total volume, 50 mg wet weight nervous tissue, 6 μmole disodium adenosine-5′-triphosphate (ATP), 5 μmole disodium diphosphopyridine nucleotide (DPN), 3 μmole tetrasodium ethylenediamine tetraacetate, 10 μmole $MgSO_4$, 50 μmole potassium phosphate at pH 7·3, and 300 μmole sucrose. The center well contained 0·2 ml of a 20% KOH solution. No further constituents were involved in the succinate oxidation study. For examining pyruvate oxidation, 2 μmole sodium fumarate were added per flask. Pyruvate and α-ketoglutarate oxidation were examined in the presence and absence of 0·06 μmole TPP per flask. The reactions were followed manometrically for 30 min at 30°.

Further studies were undertaken on pyruvate oxidation in brain homogenates as affected by administration of TOCP *in vivo* and addition of TPP *in vitro*. A 20% brain homogenate was made in 0·25 M sucrose from hens sacrificed 1, 3, 7, 10, 12, and 20 days after TOCP administration. The enzyme reaction at 30° was followed manometrically for 60 min in 15-ml Warburg flasks containing: in the side arm, 0·4 ml 20% brain homogenate; in the main compartment in 2·6 ml total volume, 5 μmole sodium fumarate, 20 μmole sodium pyruvate, 3 μmole ATP, 12 μmole $MgSO_4$, 30 μmole potassium phosphate at pH 7·3, 485 μmole sucrose, and none, 0·1 or 10 μmole TPP. The center well contained 0·2 ml of a 20% KOH solution. The average activities from duplicate analyses on each of four untreated birds expressed as microlitres of oxygen consumed per 80 mg fresh weight of brain per hour, after correction for endogenous enzymatic activity, were as follows: 75 without TPP fortification, 79 with 0·1 μmole TPP added, and 42 on fortification with 10 μmole TPP.

Antidotal studies

Potential antidotes were tested to ascertain their activity in relieving the neurotoxicity induced by TOCP (0·25 to 1·0 ml/kg, oral), SM-1 (20 mg/kg, i.p.) and DFP (2·0 mg/kg, i.p.). The compounds were administered simultaneously with the organophosphate and at intervals thereafter until either symptoms of organophosphate neurotoxicity appeared or toxicity from the antidote was evident. All potential antidotes were tested with TOCP, and certain of the compounds were also tested with SM-1 and DFP. Two animals were used in each preliminary test of an antidote. Where partial relief of the neurotoxic symptoms was evident, five to ten animals were used for confirmatory testing of the antidote. Vitamins, hormones, cholinesterase reactivators, and certain other agents were utilized. In addition to a complete vitamin mixture, varying levels of thiamine, O-acetyl thiamine, oxythiamine, tocopherol, and O-acetyl tocopherol were tested. The cholinesterase reactivators included the following compounds: pyridine-2-aldoxime; pyridine-2-aldoxime methiodide; pyridine-2-aldoxime methyl methansulfonate; pyridine-2-aldoxime benzyl bromide; pyridine-2-aldoxime *o*-methylbenzyl bromide; 1,3-di(pyridinium-4-aldoxime) propane

dibromide; mono*iso*nitrosoacetone; and diacetyl monoxime. In addition, the following materials were also tested: cortisone; cortisone acetate; adrenocorticotropin; 2,3-dimercapto-1-propanol; saponin; pilocarpine hydrochloride; β-diethylaminoethyl diphenylpropylacetate hydrochloride; α[2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene; 2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane; and N-(2-ethylexyl)-bicyclo-[2.2.1]-5-heptene-2,3-dicarboximide. The schedules for antidote dosage, route, and time of administration have been reported.[19]

RESULTS AND DISCUSSION

The specificity of homogenates of chicken nervous tissue in hydrolyzing various esters is shown in Table 1. The results are in general agreement with similar but less

TABLE 1. SPECIFICITY OF HOMOGENATES OF CHICKEN NERVOUS TISSUE IN HYDROLYZING CERTAIN ESTERS AND IN SENSITIVITY TO INHIBITION BY TWO ORGANOPHOSPHATES

Substrate	Brain	Spinal cord	Sciatic nerve	SM-1	DFP
	(μl CO_2/g/hr × 10^{3*})			(pI_{50}, spinal cord†)	
Choline esters					
Acetyl-β-methylcholine	6·26	1·24	0·34	6·1	6·4
Acetylcholine	25·10	3·54		6·3	6·8
Propionylcholine	30·11	5·46	1·23	6·5	7·2
Butyrylcholine	1·75	1·15	0·29	7·4	9·3
Thiamine esters					
O-Acetyl thiamine	15·14	2·51	0·36	6·4	6·8
O-Propionyl thiamine	9·33	1·81		6·6	7·5
Glycerol esters					
Triacetin	6·13	1·86		5·6	6·3
Tri-*n*-propionin	3·81	2·14		7·2	8·5
Tri-*n*-butyrin	2·25	1·71	0·34	7·1	8·4
Aromatic esters					
Phenyl acetate	62·40	17·04		< 4·0	5·5
o-Nitrophenyl acetate	67·60	24·64		< 4·0	< 4·0
Phenyl butyrate	13·60	5·76		7·0	5·8
o-Nitrophenyl butyrate	22·40	8·96		7·3	5·0

* Enzyme levels in mg fresh weight per flask were 32 for brain and 160 for spinal cord and sciatic nerve with the choline, thiamine, and glycerol esters. With the aromatic esters the enzyme levels were 5 for brain and 25 for spinal cord. Each activity value is an average from determinations on 5 to 15 birds.

† The enzyme level in mg fresh weight per flask was 160 for the choline, thiamine, glycerol, and phenol esters. With the *o*-nitrophenol esters, 20 mg of tissue per flask was used. A 30-min preincubation at 37° of tissue homogenate with organophosphate ester was allowed prior to addition of the substrate. Results are based on homogenates from one or two birds with a 10-fold dilution series for the inhibitors.

extensive studies[4,5,7,26] with the exception that the rate of propionylcholine hydrolysis was somewhat higher than previously reported values.[26] The sensitivity to inhibition by DFP and SM-1 for the enzymes in a spinal cord homogenate which hydrolyze various esters is also shown in Table 1. Butyryl ester hydrolysis was generally the most sensitive to inhibition and the acetyl esters hydrolysis the least sensitive. Higher pI_{50} values resulted with DFP than SM-1 except when aromatic butyrates were used as the substrate. These findings confirm and extend previous reports[5, 7] on the sensitivity *in vitro* of pseudocholinesterase of chicken nerve to DFP and certain other organophosphates.

Inhibition results for brain and spinal cord esterases in TOCP- and SM-1-poisoned birds are shown in Table 2. In hens given TOCP, esterases were inhibited to the greatest extent at 5 to 10 days after treatment. Recovery was nearly complete by 20 days except for the esterases hydrolyzing butyrate esters. With SM-1, maximum inhibition occurred within the first 5 days after treatment, with esterase recovery more rapid than that

TABLE 2. PERCENTAGE INHIBITION OF BRAIN AND SPINAL CORD ESTERASES OF CHICKEN AT VARYING INTERVALS AFTER ADMINISTRATION OF TOCP OR SM-1*

		Days after									
		TOCP (1 ml/kg, oral)					SM-1 (20 mg/kg, i.p.)				
Substrate	Tissue†	0·1	5	10	15	20	0·1	5	10	15	20
Choline esters											
Acetyl-β-methylcholine	Br	0	19	31	11	7	16	16	0	4	0
	SC	30	38	43	27	19	21	28	30	13	20
Acetylcholine	Br	12	29	40	10	13	33	24	2	10	0
	SC	16	22	36	12	5	36	12	3	8	0
Propionylcholine	Br	0	29	32	14	5	19	17	3	9	0
	SC	8	40	48	30	19	38	25	31	18	12
Butyrylcholine	Br	7	93	93	64	46	100	43	25	29	8
	SC	32	92	91	72	55	82	49	53	47	39
Thiamine esters											
O-Acetyl thiamine	Br	4	26	32	10	10	15	15	6	11	0
	SC	11	38	48	22	10	35	18	26	20	1
O-Propionyl thiamine	SC	0	29	32	14	5	50	34	19	10	12
Glycerol esters											
Triacetin	Br	9	29	12	0	0	43	17	5	13	0
	SC	13	61	0	14	4	32	28	9	6	9
Tri-*n*-propionin	Br	13	48	61	2	0	72	42	4	11	0
	SC	20	60	63	39	12	76	44	32	29	3
Tri-*n*-butyrin	Br	19	78	100	50	42	72	42	36	25	6
	SC	35	64	67	47	39	66	38	43	30	32
Aromatic esters											
Phenyl acetate	Br	14	27	35	19	0	26	31	18	0	8
	SC	13	14	13	19	13	7	16	19	7	8
o-Nitrophenyl acetate	Br	13	37	37	4	12	18	33	15	38	14
	SC	0	18	16	12	11	14	10	20	18	6
Phenyl butyrate	Br		48	63	6	23	71	56	41	32	26
	SC		43	61	22	30	67	57	43	36	25
o-Nitrophenyl butyrate	Br	34	50	55	29	37	52	50	30	30	30
	SC	27	39	32	20	24	27	37	31	27	19

* Each percentage inhibition figure based on averaging data from two to four birds with single or duplicate analyses on each bird.

† Enzyme levels in mg fresh weight per flask were 32 for brain and 160 for spinal cord with choline, thiamine, and glycerol esters. With the aromatic esters the levels were 5 for brain and 25 for spinal cord.

following TOCP administration. Throughout this experiment the esterases involved in hydrolyzing the butyrate esters were the most sensitive to prolonged inhibition. With the butyrate esters, the choline ester hydrolysis was most sensitive, the glycerol ester hydrolysis next, and the aromatic ester hydrolysis least sensitive to inhibition. The results on butyryl choline and acetyl-β-methylcholine hydrolysis after TOCP administration compare favorably with those of Earl and Thompson,[4, 7] although the degree of inhibition in the present study was somewhat higher.

The biochemical and physiological consequences of inhibition by organophosphates have only been elucidated with the acetylcholine-acetylcholinesterase system. TOCP

effects prolonged inhibition of nerve pseudocholinesterase,[3, 4] but this action does not appear to be directly related to the ataxia and demyelination produced by this compound.[5, 7] Mipafax, DFP[5],[6] and SM-1 (Table 2) produce marked, early inhibition of nerve pseudocholinesterase, but considerable recovery of activity occurs prior to the appearance of ataxia symptoms. In this respect they are similar to certain non-demyelinating compounds such as di*iso*propyl *p*-nitrophenyl phosphate, N,N′,N″,N‴, tetra*iso*propyl pyrophosphoramide and tetra*iso*propyl pyrophosphate.[5] Hydrolysis of tri-*n*-butyrin is also inhibited in the spinal cord of TOCP- treated birds, although to a lesser degree than is the hydrolysis of butyrylcholine.[7] Nerve esterases hydrolyzing the butyrate esters of phenol and *o*-nitrophenol and particularly those hydrolyzing tri-*n*-butyrin were found in the present study to be strongly inhibited at the time of appearance of ataxia symptoms with TOCP but not with SM-1 (Table 2). It therefore appears that *prolonged* inhibition of pseudocholinesterase, or of organophosphate-sensitive esterases hydrolyzing glyceryl and aryl esters, is not a prerequisite for the production of paralysis. Ataxia is produced in hens by tri-*p*-ethylphenyl phosphate, although this compound does not effect inhibition of nerve pseudocholinesterase.[8,9,27] It is, however, active in inhibition of phenyl butyrate hydrolyzing esterases in certain tissues.[8] Although considerable evidence is available to rule out the significance of pseudocholinesterase inhibition in the neurotoxic mechanism, similar evidence is not available on other esterases, such as those hydrolyzing aromatic and glyceryl butyrates or higher acyl derivatives. Selective rather than prolonged inhibition of esterases other than pseudocholinesterase, possibly at localized sites in the nervous system, warrants further investigation as to the part it might play in the neurotoxic mechanism.

Preliminary experiments have been recorded on the rates of decarboxylation or oxidation of certain substrates by homogenates of chicken liver, brain, and spinal cord, and mitochondria of chicken liver and brain.[19] Untreated birds were compared with hens 1, 3, 7, and 12 days after TOCP or SM-1 administration. Succinate, pyruvate, and α-ketoglutarate oxidation rates of brain, spinal cord, and liver homogenates, and brain and liver mitochondria, showed no substantial change from the normal activity. These preliminary findings confirmed previous studies[7, 28] where pyruvate oxidation was found to be unaffected by TOCP administration. An indication was obtained in the preliminary study that fortification *in vitro* with 0·06 μmole TPP caused inhibition of pyruvate oxidation by both nerve homogenates and mitochondrial preparations. This inhibition was not so evident with α-ketoglutarate oxidation. Further studies on brain homogenates oxidizing pyruvate were conducted with varying TPP levels at 1, 3, 7, 10, 12, and 20 days after TOCP administration.[19] Again TOCP had no effect on pyruvate oxidation by brain homogenates. Low TPP levels (0·1 μmole/flask) did not affect the oxidation rates, but high TPP levels (10 μmole/flask) resulted in marked inhibition which was independent of the TOCP administration. The mechanism of TPP inhibition of this enzymatic reaction is not known. It is interesting to note that administration of high levels of thiamine (10 to 100 mg/kg daily for 14 to 21 days starting at the time of TOCP or DFP treatment) resulted in intensifying the neurotoxicity as evidenced by the clinical symptoms.[19] Thiamine and TPP are known to inhibit cholinesterase[29] and certain neurological functions. The activity of high levels of thiamine in enhancement of the detrimental effort of the organophosphate might be related to the inhibitory effect of high levels of thiamine or TPP on pyruvate oxidation, cholinesterase, or other enzymatic systems.

The wide variety of agents tested as possible antidotes for the organophosphate-induced neurotoxicity proved ineffective. Thiamine was tested most rigorously, alone and in combination with other materials. The neurological syndrome associated with a severe thiamine deficiency is similar in certain respects to the clinical and pathological picture in ataxia induced by certain organophosphates.[23, 30, 31] Thiamine has been administered to patients with "ginger paralysis" without beneficial results.[16, 31] No controlled experiments had been reported in hens or other animals susceptible to the organophosphate-induced neurotoxicity where thiamine was administered prior to the onset of symptoms. Thiamine not only failed to give a beneficial response in hens, but at high dosage levels (10 to 100 mg/kg) for extended periods (daily for 14 to 21 days starting at the time of TOCP or DFP administration) actually appeared to be detrimental. The first signs of ataxia appeared slightly earlier and progressed to a greater severity than without thiamine administration. Oxythiamine administered orally or intraperitoneally at levels which appeared grossly to be non-toxic reduced the survival time in birds given TOCP, SM-1, or DFP.

Many agents have been tested on persons accidentally poisoned with cresyl phosphates, but the polyneuritis was already evident before the agents were administered.[16] Pilocarpine was reported to give some beneficial effects.[16] In the present study, however, it did not relieve the ataxia or debilitation in hens.

Glees found that cortisone acetate reduced the severity of ataxia symptoms in hens poisoned by TOCP administered to the comb.[17, 18] Cortisone acetate therapy improved the general physical appearance and food consumption. In the present experiments, cortisone acetate administered at 25 mg per bird in seven doses on alternate days was was the only compound tested that appeared to be beneficial, in that diminished weight loss and improved general appearance were evident. Cortisone, at all levels tested, and other treatment schedules for cortisone acetate showed no such beneficial effects. It is likely that the benefit afforded the hens by cortisone acetate therapy resulted from improved physical condition of the birds rather than from specific relief of the neurological debilitation. That Glees found greater benefit from cortisone acetate than that observed in the present studies may have been due to the difference in TOCP dose or method of administration. The severity of the ataxia symptoms and demyelination vary with dose.[9] Gross relief of the debilitation may be more evident with the less severe neurotoxicity resulting from lower amounts of absorbed organophosphate.

Pyridine-2-aldoxime methiodide failed to relieve the symptoms of ataxia. Since this cholinesterase reactivator does not penetrate readily into the central nervous system, other known or potential reactivators of greater lipoidal solubility were tested. Henschler[15] found that pyridine-2-aldoxime dodeciodide delayed the symptoms of TOCP-induced ataxia in hens. Based on these results, compounds were prepared[19] with the dodecyl group replaced by a benzyl or *o*-methylbenzyl radical. Neither of these two compounds nor any of the other potential cholinesterase reactivators tested was effective in relieving the ataxia. The failure of these aldoximes and related compounds to serve as antidotes may have resulted from inability to reach the localized site of physiologically significant phosphorylated enzyme(s). Another possibility is that the critical phosphorylated enzyme(s) is not susceptible to rapid dephosphorylation or reactivation by the compounds studied. The significant biochemical system, presumably an esterase, involved in initiating the neurotoxicity must be very susceptible

to inhibition or phosphorylation by certain organophosphates. The available evidence is not adequate to define the nature of this system.

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 DA-CML-18-108-61-G-6) and the U.S. Public Health Service, National Institutes of Health (Contract RG-5304). The technical assistance of Judith Engel and Michael Hahn is gratefully acknowledged. The O-propionyl thiamine chloride hydrochloride was prepared and provided by Shirley DeBreuil of American Cyanamid Co., Stamford, Conn.

REFERENCES

1. R. D. O'BRIEN, *Toxic Phosphorus Esters*, Academic Press, New York (1960).
2. D. F. HEATH, *Organophosphorus Poisons*, Pergamon Press, Oxford (1961).
3. C. J. EARL and R. H. S. THOMPSON, *Brit. J. Pharmacol.* **7**, 261 (1952).
4. *Ibid.*, p. 685.
5. A. N. DAVISON, *Biochem. J.*, **54**, 683 (1953).
6. L. AUSTIN and D. R. DAVIES, *Brit. J. Pharmacol.* **9**, 145 (1954).
7. R. H. S. THOMPSON, *Chem. ind. Rev.* 749 (1954).
8. W. N. ALDRIDGE and J. M. BARNES, *Biochem. Pharmacol.* **6**, 177 (1961).
9. J. B. CAVANAGH, D. R. DAVIES, P. HOLLAND and M. LANCASTER, *Brit. J. Pharmacol.* **17**, 21 (1961).
10. C. D. AIRING, W. B. BEAN, E. ROSEMAN, M. ROSENBAUM and T. D. SPIES, *Arch. Neurol. Psychiat. Chic.* **45**, 772 (1941).
11. H. BLOCH and A. HOTTINGER, *Z. Vitaminforsch.* **13**, 9 (1943).
12. H. H. DRAPER, M. F. JAMES and B. C. JOHNSON, *Fed. Proc.* **11**, 204 (1952).
13. D. K. MYERS and H. E. W. MULDER, *Nature, Lond.* **172**, 773 (1953).
14. E. L. HOVE, *J. Nutr.* **51**, 609 (1953).
15. D. HENSCHLER, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **236**, 503 (1959).
16. H. GEOFFROY, A. SLOMIC, M. BENEBADJI and P. PASCAL, *Wld Neurol.* **1**, 294 (1960).
17. P. GLEES and W. G. WHITE, *Nature, Lond.* **187**, 237 (1960).
18. P. GLEES, *Dtsch. med. Wschr.* **86**, 175 (1961).
19. R. L. BARON, *Delayed Neurological Effects of Certain Organophosphate Esters in Chickens* unpub. doctoral Thesis, Univ. of Wisconsin, Madison (1962).
20. G. R. WEBSTER, *Biochem. J.* **57**, 153 (1954).
21. L. AUSTIN, *Brit. J. Pharmacol.* **12**, 356 (1957).
22. G. MANJO and M. L. KARNOVSKY, *J. Neurochem.* **8**, 1 (1961).
23. J. E. CASIDA, M. ETO and R. L. BARON, *Nature, Lond.* **191**, 396 (1961).
24. M. ETO, J. E. CASIDA and T. ETO, *Biochem. Pharmacol.* **11**, 337 (1962).
25. R. L. BARON, D. R. BENNETT and J. E. CASIDA, *Brit. J. Pharmacol.* **18**, 965 (1962).
26. L. C. BLABER and A. W. CUTHBERT, *Biochem. Pharmacol.* **11**, 113 (1962).
27. A. SILVER, *Nature, Lond.* **185**, 247 (1960).
28. C. J. EARL, R. H. S. THOMPSON and G. R. WEBSTER, *Brit. J. Pharmacol.* **8**, 110 (1953).
29. E. GJONE and K. H. SKRAMSTAD, *Acta pharmacol., Kbh.* **11**, 94 (1955).
30. J. M. BARNES and F. A. DENZ, *J. Path. Bact.* **65**, 597 (1953).
31. C. D. AIRING, *Brain* **65**, 34 (1942).