Binding of Organophosphates to Cholinesterases

R. D. O'BRIEN
Department of Entomology,
Cornell University,
Ithaca, N. Y.

The interference of tetraethylammonium and tetrapropylammonium with the inhibition of three cholinesterases by eight organophosphates was studied to obtain information on the forces binding organophosphates to cholinesterase. The phosphates differed in the relative contributions of binding to the area of the anionic site and attack upon the esteratic site. Indirect evidence suggested that in housefly head cholinesterase, the separation of the anionic and esteratic sites was between 4.5 and 5.9 A., whereas for human plasma and erythrocytes the separation was less than 4.5 A.

The ORGANOPHOSPHATES attack cholinesterases by phosphorylating their esteratic site through an electrophilic mechanism. Consequently, one can account for the activity of many of these compounds by parameters that measure the positivity of the phosphorus, e.g., Hammett constants of substituents of phenylphosphates (*13*) or susceptibility to alkaline hydrolysis (*2*). However, some organophosphates are unexpectedly better than their close analogs, and this has been attributed to binding to additional areas of the enzyme. Thus, in the substituted diethyl phenyl phosphates, the *m-tert*-butyl and *m*-dimethylamino substituents are so situated that they might bind to the anionic site of cholinesterase (*12*), whose normal function is to bind the quaternary nitrogen group of acetylcholine. In the case of phosphates containing an appropriately placed quaternary nitrogen group, coulombic binding of this group to the anionic site has been postulated (*17*).

In support of the idea of anionic-site binding of organophosphates, it has been shown that several oximes which are potent catalysts for the reactivation of most phosphate-inhibited cholinesterases, are poor reactivators of cholinesterase inhibited by cholinyl methylphosphonofluoridate (*9*). This was suggested to be caused by the cholinyl phosphoryl moiety shielding the esteratic site. It has also been shown that in an organophosphate containing a tertiary nitrogen in a suitable location to bind to the esteratic site, the protonated form was a far more potent inhibitor than the unprotonated (*17*).

The present study is an attempt to obtain more direct evidence of anionic site binding, by examining the way that occupation of the anionic site by quaternary salts interferes with inhibition by various organophosphates. Insect and mammalian cholinesterases are compared, as part of a continuing attempt to discover enzymic differences which can be exploited in the design of selectively toxic compounds.

Experimental Procedure

The enzyme preparations were either: (a) washed "ghosts" of human erythrocytes—1 volume of packed erythrocytes (derived from 2.2 volumes of blood) was washed three times with 0.9% NaCl, laked with 6 volumes of water, acidified with 0.068 volume of 1M acetic acid, and allowed to stand overnight. After decanting, the precipitate was washed three times with equal volumes of 0.1M NaH_2PO_4, once each day. The brown precipitate was centrifuged off, diluted to a creamy consistency with water, and the pH adjusted to 7.0 with 0.1M NaOH, giving 0.25 volume of enzyme. This stock preparation was diluted with 3 volumes of phosphate buffer 7.4, 0.067M before use. (b) Human plasma—diluted with 0.33 volume of the phosphate buffer. (c) Housefly head—16 grams of heads obtained from frozen houseflies were homogenized in 160 ml. of the phosphate buffer, using an ice-cooled Servall

Table I. Interference by Tetraalkylammoniums with Inhibition by Organophosphates

	Plasma Cholinesterase			Erythrocyte Cholinesterase			Housefly Head Cholinesterase		
Inhibitor	*pI₅₀ alone*	*TEA ΔpI₅₀*	*TPA ΔpI₅₀*	*pI₅₀ alone*	*TEA ΔpI₅₀*	*TPA ΔpI₅₀*	*pI₅₀ Alone*	*TEA ΔpI₅₀*	*TPA ΔpI₅₀*
Coroxon	7.5	0.6	0.3	7.1	0.6	0.9	8.9	1.2	1.9
DDVP	6.8	0.1	0.5	5.9[a]	0.9[a]	1.0[a]	8.6	0.2	1.7
DFP	8.7	0.4	0.3	6.5	1.1	1.1	6.4	0.5	2.0
Guthoxon	5.8	0.5	0.4	6.7[a]	0.5[a]	0.3[a]	8.8	1.2	2.0
Paraoxon	7.5	0.7	0.7	7.8	1.0	1.0	7.5	0.6	1.8
Ruelene	4.7	0.8	0.5	4.1	1.1	1.1	7.0	0.8	1.4
Amiton	8.3	0.6	0.5	7.9	1.2	1.2	8.6	1.9	1.8
Quaternarized Amiton	8.4	0.2	0.2	8.5	1.1	0.96	8.7	0.6	0.6
Av.	7.2	0.49	0.43	6.6	0.94	0.95	8.2	0.95	1.65

[a] Extrapolated.

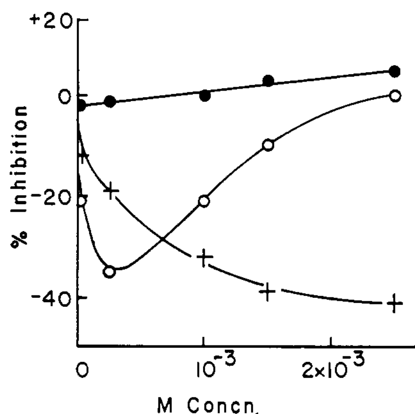


Figure 1. Effect of TEA upon cholinesterases

(● plasma; O housefly head; + erythrocytes)

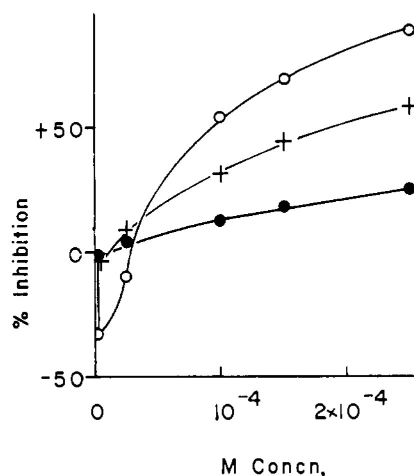


Figure 2. Effect of TPA upon cholinesterases

(● plasma; O housefly head; + erythrocytes)

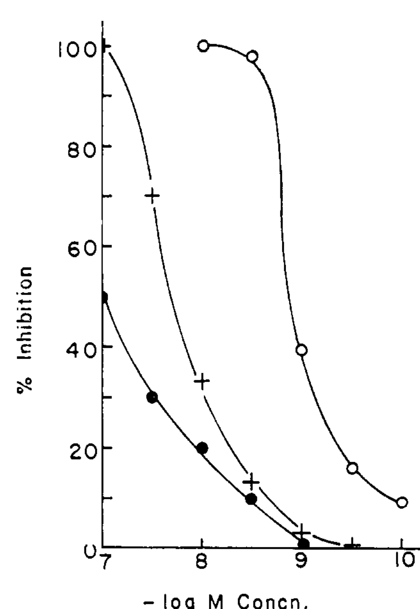


Figure 3. Inhibition of housefly head cholinesterase by Coroxon

(O alone; + with $10^{-2}M$ TEA; ● with $10^{-3}M$ TPA)

Omnimixer for 1 minute, then filtered through glass wool, centrifuged for 5 minutes at 2000 × G, and the supernatant was used.

Stock solutions of organophosphate were prepared at $4 \times 10^{-2}M$ in acetone or ethanol or the phosphate buffer. They were diluted with the buffer to obtain the various concentrations.

In each test tube was placed 0.25 ml. of organophosphate, and a mixture of 0.5 ml. of enzyme and 0.25 ml. of tetraethylammonium bromide (TEA) or tetrapropylammonium bromide (TPA) or water was added at zero time. The concentrations of TEA or TPA in the 0.25 ml. added were 4×10^{-2} and $4 \times 10^{-3}M$, respectively, in the experiments on interference. In the experiments on direct effects, various concentrations were used to give the final concentrations shown in the figures. The tubes were placed in a bath at 37.5° C. Precisely 15 minutes from zero, 9 ml. of prewarmed acetylcholine bromide, 0.1% in Tris buffer pH 8.1, 0.05M, was added. By this means, the TEA and TPA were diluted 10-fold, to reduce their inhibitory effect upon acetylcholine hydrolysis. At 45 minutes from zero, the tubes were transferred to an ice-and-water bath. Residual acetylcholine was then determined by the method of Hestrin (*14*).

The compounds were: Amiton: *O*-,-*O*-diethyl *S*-(diethylaminoethyl) phosphorothiolate; quaternarized Amiton: triethyl analog of Amiton; Coroxon (oxygen analog of Co-Ral): diethyl 3-chloro-4-methyl-7-coumarinyl phosphate; DDVP: diethyl 2,2-dichlorovinyl phosphate; DFP: diisopropyl phosphorofluoridate; Guthoxon(oxygen analog of Guthion):*O,O*-dimethyl *S*-3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-ylmethyl phosphorothiolate; paraoxon: diethyl *p*-nitrophenyl phosphate; Ruelene: methyl 2-chloro-4-*tert*-butylphenyl methylphosphoramidate.

Direct Effects of TEA and TPA on Cholinesterases. Quaternary ammonium salts inhibit cholinesterases (*6, 7*). With the preparations studied here, activation of cholinesterase was found at low concentrations, inhibition at high concentrations of quaternary salts. For TEA, results are shown in Figure 1, using concentrations in the range utilized in the main part of this study. The concentrations given are those prevailing during the assay period, i.e., after addition of acetylcholine. In the studies of interference, the concentrations given are those prevailing during the inhibitory period, before adding acetylcholine, because it is in the inhibitory period that competition between quaternary salt and organophosphate occurs.

Figure 1 shows no inhibitory action by low concentrations of TEA against fly cholinesterase. However, TEA at $2.5 \times 10^{-4}M$ gave maximal activation and thereafter activation lessened, being zero at 2.5×10^{-3}; 50% inhibition was reached at $4.5 \times 10^{-2}M$ (not shown in Figure 1).

For TPA, virtually no activation was found for plasma or erythrocytes, but strong activation of the fly enzyme was found at very low concentrations ($2.5 \times 10^{-6}M$), as shown in Figure 2.

Since the only truly soluble enzyme, i.e., that in plasma, was not activated under any conditions, the activations that were observed were probably due to solubilization, and were not related to the activation by quaternary ammonium salts of plasma hydrolysis of benzoyl choline (*10*).

The above effects were compensated for in the studies with combined organophosphates and quaternary salts, by using a control containing the appropriate amount of quaternary salt.

Interference of TEA and TPA with Cholinesterase Inhibition by Organophosphates. The typical effect observed was that TEA and TPA reduced inhibition, as shown in Figure 3. In two cases, *viz.*, guthoxon and DDVP with erythrocyte cholinesterase, a crossover effect, as shown in Figure 4, was consistently observed. This is attributed to enzymic hydrolysis of the organophosphate at high concentrations, which appears to be inhibited by TEA and TPA.

The reduction of inhibition was expressed as the ΔpI_{50}, or decrease in the pI_{50} caused by TEA or TPA. As this is a logarithmic parameter, a change of one unit signifies a 10-fold reduction in potency. The results are shown in Table I.

Brief experiments showed that $10^{-2}M$ ammonium sulfate or tetramethylammonium had no effect on cholinesterase inhibition by organophosphates.

Discussion

All experiments were performed with relatively crude enzyme preparations. However, for each preparation there is evidence that only one acetylcholine-hydrolyzing enzyme is present. Augustinsson (*3*) found only one cholinesterase in human plasma, in his electrophoretic separations of esterases, and also found (*4*) that human erythrocytes had only acetylcholinesterase, as judged by substrate preference. Dauterman *et al.* (*8*) found no significant change in substrate specificity in the course of a 160-fold purification of housefly head cholinesterase, after the labile aliesterase (which does not hydrolyze acetylcholine) had been destroyed.

It had been anticipated that since Amiton and its quaternary analog have a

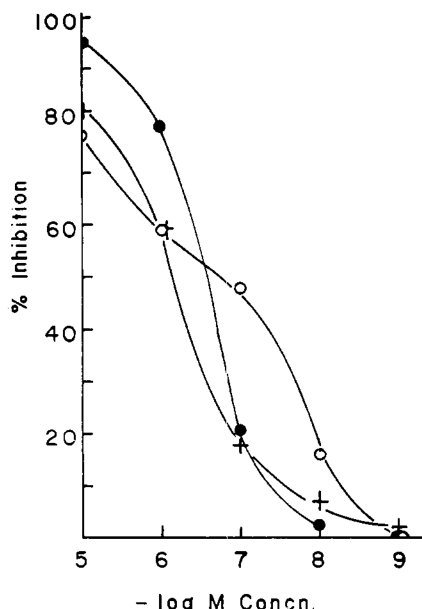


Figure 4. Inhibition of erythrocyte cholinesterase by Guthoxon

(O alone; + with $10^{-2}M$ TEA; ● with $10^{-3}M$)

Table II. Dimensions of Organophosphates (A.)[a]

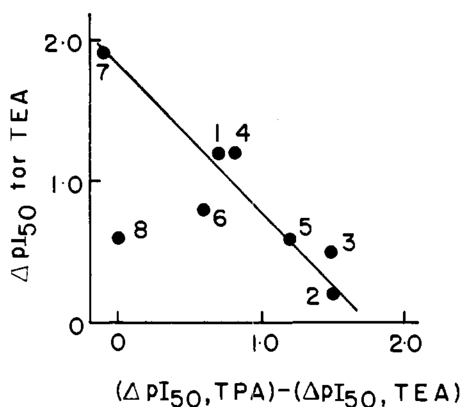
Organophosphates	(ΔpI_{50}, TPA) − (ΔpI_{50}, TEA)	Functional Group	Center of P to Center of Functional Group	Center of P to Farthest Edge of Side Group	Center of P to Farthest Edge of Alkoxy Group
DDVP	1.5	Cl	3.0–5.4	6.0–6.7	4.4
DFP	1.5	F	1.7	5.0	5.4–6.0
Paraoxon	1.2	NO_2	6.7	8.0–8.7	4.7–6.0
Guthoxon	0.8	C=O	2.7–6.0	6.0–10.4	4.4
Coroxon	0.7	Cl	9.4	10.4	4.7–6.0
Ruelene	0.6	$(CH_3)_2C$	6.7	9.4	4.4
Amiton	0.1	N	2.7–5.7	6.7–9.4	4.7–6.0
Quaternary Amiton	0	N	4.7–5.7	8.0–9.4	4.7–6.0

[a] First column is from data on housefly cholinesterase. Other figures are from measurements on Stuart-Briegleb models. Where two figures are shown, they are for fully compressed and extended molecule respectively; in other cases, compression or extension do not effect the particular distance.

Table III. Dimensions of Tetraalkylammoniums (A.)[a]

	Center of N to Edge of Farthest Hydrogens		Center of N to Center of Outermost Carbons	
	Range	Av.	Range	Av.
Tetramethylammonium	2.9	2.9	2.3	2.3
Tetraethylammonium	3.5–4.5	3.0	2.7–3.0	2.85
Tetrapropylammonium	3.9–5.9	4.9	2.3–4.0	3.15

[a] Same as Table II.



igure 5. Effects of quaternary salts on iousefly head cholinesterase

(Numbers refer to compounds listed in Table I)

cationic nitrogen at pH 7, they would owe some of their activity to binding at the anionic site, and would, therefore, suffer more severe interference from TEA and TPA than would the other organophosphates. However, this was not the case; in general, there was a surprisingly uniform response for all the organophosphates, in spite of their different sizes and very different potencies. One can only conclude either that Amiton and quaternary Amiton are not bound to the anionic site, or that many of the compounds owe similar fractions of their potency to binding which is either at the anionic site or near enough to be interfered with TEA or TPA bound to the anionic site.

Comparison of the average interference for plasma as contrasted with erythrocyte and fly head showed less interferences for the plasma enzyme. For instance, the average ratio of TEA potencies (as judged by the antilog of the average ΔpI_{50}'s) for plasma : erythrocyte : fly head was 1:2.8:2.9. The effect of TEA and TPA upon acetylcholine hydrolysis (Figures 1 and 2) were also minimal for the plasma enzyme. These observations support the widely held belief that the anionic site(s) of the plasma enzyme play a lesser role than in erythrocyte and fly head enzyme (*7, 5*). However, the data also oppose the view (*7*) that there is no anionic site in plasma cholinesterase.

Only in the fly head enzyme were profound differences found in the effects of TEA($10^{-2}M$) and TPA($10^{-3}M$). In plasma and erythrocytes, the effects of TEA and TPA were within 0.1 pI_{50} units in 11 out of 16 cases, and the maximum difference was 0.4 unit for DDVP with plasma. Yet with fly head, there were three cases where very large differences occurred: DDVP (1.5 units, i.e., a 32× difference), DFP (32×), and paraoxon (16×). For Ruelene and Coroxon, the differences were intermediate (4.0× and 5.0×), and for the other three, the differences were small (Guthoxon 1.6×, Amiton 1.3×, quaternized Amiton 1×).

In an attempt to account for the special TEA-TPA difference for DDVP, DFP, and paraoxon, measurements on models of organophosphates were made (Table II), and revealed no special features in the dimensions of these three compounds. There was no uniform difference between the anticholinesterase activities of the "special three" against fly head as compared with the others (DDVP is 0.4 unit or 2.5× above the average, DFP 63× below the average, and paraoxon 5× below). The only feature which distinguishes DDVP, DFP, and paraoxon is that their side-chains, i.e., dichlorovinyl-, fluoro- and *p*-nitrophenyl-, are all good electron attracting groups. The sidechains of Coroxon, Guthoxon, and Ruelene are probably poor electron-attracting groups. For Amiton and its quaternary analog, the very weak inductive electrophilic effect of the diethylamino and the strong inductive effect of the quaternary nitrogen would be much diluted by passage through two methylene carbons. For comparison, the hydroxyl of choline is alcoholic, quite unlike that of *p*-nitrophenol. One, therefore, expects the phosphorus of DDVP, DFP, and paraoxon to bear a greater partial positive charge than that of the other five compounds.

The hypothesis is proposed that these three owe a greater share of their effectiveness to the positivity of their phosphorus and thus to their ability to react with the esteratic site. By contrast, the others owe a greater share of their effectiveness to other factors, of which the prime one is probably binding to the enzyme surface by Van der Waals' forces, hydrogen bonding, and the like. Such forces have been shown to be important in binding quaternary ammonium salts to the anionic site (*6, 19*). Measurements of models (Table III) indicated that the maximum radius of TEA is 4.5 A., of TPA 5.9 A. If it is assumed that TEA and TPA were bound only at the anionic site, and that the distance between the anionic and esteratic sites is about 5A. (*7, 16*), only the

TPA would actually overlap the esteratic site. Thus TPA would interfere at both sites, TEA primarily at the anionic site. Consequently, the TEA-TPA difference would be maximal for organophosphates which only attack the esteratic site, and minimal for those which owe much of their potency to an ability to bind to the anionic site while their phosphorus is attacking the esteratic site.

But why do the "special three" compounds not show this TEA-TPA difference when tested against plasma or erythrocyte cholinesterase? On the average, TPA is only a little more effective than TEA for plasma (1.2×) or for erythrocytes (1.5×), but much better than TEA for fly head (5×).

One possibility is that the separation of the esteratic from the anionic site varies in the three enzymes. The maximal TEA-TPA difference would be found when TPA overlaps the esteratic site, but TEA does not. This would only be true if the site separation was between 4.5 A. (the radius of TEA) and 5.9 A. (the radius of TPA). This suggests that for fly head cholinesterase, the separation falls in this range, whereas for the other cholinesterases the separation is greater than 5.9 A. or less than 4.5 A. Now if the separation for plasma or erythrocyte enzyme was greater than 5.9 A., neither TEA nor TPA would overlap the esteratic site, and one would expect that TEA and TPA would be much less effective in blocking, for instance, DFP, which attacks only the esteratic site, than Amiton, which attacks both sites. Since this is not the case (Table I), it must be that in plasma and erythrocyte cholinesterases the separation of the anionic and esteratic sites is less than 4.5 A. Friess and Baldridge (*11*) have proposed a separation of 2.5 A. for electric eel acetylcholinesterase.

A corollary of the hypothesis is that since TEA should cover only the anionic site of housefly cholinesterase, TEA should show maximal blocking of those organophosphates which depend most on anionic site attack. There should, therefore, be a correlation between the TEA-TPA difference and the ΔpI_{50} for TEA. Figure 5 shows that this correlation exists, with the single exception of quaternary Amiton, for which TEA is an unexpectedly poor blocker. This exception is probably due to the fact that quaternary Amiton, the only organophosphate in the series with a quaternary nitrogen, competes more effectively with TEA for the anionic site than do the others. This may be due to the peculiarly favorable disposition of the *N*-alkyl groups of quaternary Amiton, or to the excellence of its quaternary nitrogen in coulombic binding.

The fact that tetramethylammonium ($10^{-2}M$) and ammonium ($10^{-2}M$) do not block organophosphate inhibition suggests at first that none of the organophosphate binding is exactly at the anionic site, and must in fact be 3 A. away from that site, (3 A. is the radius of TMA). But TMA is an extremely poor inhibitor of substrate hydrolysis too (*7*). It may be that quaternary salts are bound primarily by Van der Waals' forces, and very little by coulombic forces, so that TMA has a very low affinity for the anionic site. This suggestion helps account for the proposal in this paper that several un-ionized compounds can be bound to the region of the anionic site.

Conclusions

The conclusion that, in some organophosphates only, an important contribution to anticholinesterase activity is made by binding to the area of the anionic site, leads to interesting possibilities for future organophosphate synthesis. Hitherto, the major factor explored in detail has been the electrophilic nature of the phosphorus, as judged by alkaline hydrolyzability and various substituent constants. More emphasis should be given to exploring the abilities of side groupings to increase the affinity of the whole molecule for the enzyme. When this affinity is especially high, a compound whose phosphorus is a poor electrophile may yet be a good anticholinesterase. To obtain insecticides, the side group must not be ionized because ionic materials cannot penetrate to the vital cholinesterase of insects (*15, 17, 18*). Consequently, the binding forces utilized must be noncoulombic, e.g., Van der Waals or hydrogen bonding. Such forces appear to be important for Guthoxon, less important for Ruelene and Coroxon, and of little importance for DDVP, DFP, or paraoxon.

The other conclusion is that there is an important difference between housefly and mammalian cholinesterase, in the distance between anionic and esteratic sites. This difference could form the basis for the design of selectively toxic anticholinesterases.

Acknowledgment

The author is grateful to L. G. Crawford for technical assistance in preliminary studies and to C. E. Dannelley for the remainder of the technical work. Grateful thanks for supplying the compounds used in this study are due to L. E. Chadwick, G. Schrader, Chemagro Corp., Chipman Chemical Co., and the Dow Chemical Co.

Literature Cited

(1) Adams, D. H., Whittaker, V. P., *Biochim. Biophys. Acta* **4,** 543 (1950).
(2) Aldridge, W. N., Davison, A. N., *Biochem. J.* **51,** 62 (1952).
(3) Augustinsson, K-B., *Acta Chem. Scand.* **13,** 571 (1959).
(4) Augustinsson, K-B., *Acta Physiol. Scand.* **15,** Supp. 52, p. 57 (1948).
(5) Bergmann, F., *Discussions Faraday Soc.*, **20,** 126 (1955).
(6) Bergmann, F., Segal, R., *Biochem. J.* **58,** 692 (1954).
(7) Bergmann, F., Shimoni, A., *Biochim. Biophys. Acta* **8,** 520 (1952).
(8) Dauterman, W. C., Talens, A., Asperen, K. van, *J. Insect. Physiol.* **8,** 1 (1962).
(9) Enander, I., *Acta Chem. Scand.* **12,** 780 (1958).
(10) Erdos, E. G., Foldes, F. F., Zsigmond, E. K., Baart, N., Zwartz, J. A., *Science* **128,** 92 (1958).
(11) Friess, S. L., Baldridge, H. D., *J. Am. Chem. Soc.* **78,** 2482 (1956).
(12) Fukuto, T. R., *Advan. Pest Control Res.* **1,** 147 (1957).
(13) Fukuto, T. R., Metcalf, R. L., J. Agr. Food Chem. **4,** 930 (1956).
(14) Hestrin, S., *J. Biol. Chem.* **180,** 249 (1949).
(15) Kolbezen, M. J., Metcalf, R. L., Fukuto, T. R., J. Agr. Food Chem. **2,** 864 (1954).
(16) Nachmansohn, D., "Chemical and Molecular Basis of Nerve Activity," Academic Press, New York, p. 109, 1959.
(17) O'Brien, R. D., *J. Econ. Entomol.* **52,** 812 (1959).
(18) O'Brien, R. D., Fisher, R. W., *Ibid.* **51,** 169 (1958).
(19) Wilson, I. B., *J. Biol. Chem.* **197,** 215 (1952).

Received for review February 16, 1962. Accepted June 11, 1962. This study was supported in part by Research Grant RG 7804 of the National Institutes of Health, Public Health Service.