MOLECULAR PHARMACOLOGY, 7, 129–135

The Influence of Tetraethylammonium Ion on the Reaction between Acetylcholinesterase and Selected Inhibitors

F. IVERSON[1]

Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607

(Received December 22, 1970)

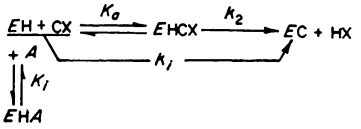
SUMMARY

The values of the affinity constant (K_a) and the first-order inhibition rate constant (k_2) of selected inhibitors of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) were determined in the presence and absence of tetraethylammonium ion (TEA). Although TEA is believed to act as a purely competitive inhibitor toward cationic compounds, the present study reveals that this is not a general case. TEA did reduce the rate of inhibition by the organophosphate diisopropylphosphorylthiocholine in a purely competitive manner, but its effect on the rates of inhibition by two cationic carbamates was more complex. TEA at 0.5 mM reduced the apparent value of K_a, but increased the apparent k_2 value of neostigmine. At higher TEA concentrations anomalous curving was observed in the inhibition plots of dimethylcarbamylcholine. These results indicate that the inhibitors are bound simultaneously with TEA to the enzyme, and this suggests that TEA binds to an allosteric site.

Similar studies on inhibitors that contained the smaller fluoride leaving group revealed that TEA acted as a purely competitive inhibitor toward diisopropyl phosphorofluoridate. On the other hand, it is estimated that TEA may increase the first-order sulfonation rate by methanesulfonyl fluoride from 72.5 min^{-1} to 2376 min^{-1}.

INTRODUCTION

Tetraethylammonium ion is believed to bind to the anionic portion of the active site of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), thereby preventing the binding of substrates and inhibitors containing cationic leaving groups (1, 2). This purely competitive effect is described by the following scheme.



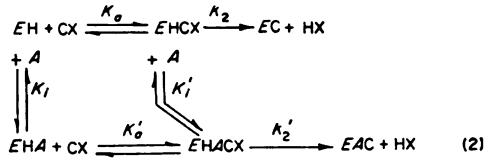
(1)

Here EH is the enzyme, CX is a carbamate, organophosphate, or sulfonate inhibitor, and A is tetraethylammonium ion. Formation of a reversible complex between enzyme and

This is a contribution from the Biochemistry Department, School of Agriculture and Life Sciences and School of Physical Sciences and Applied Mathematics, and is Paper No. 3108 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina. These studies were supported in part by Research Grants AM-12355 from the National Institute of Arthritis and Metabolic Diseases and ES-00044 from the National Institute of Environmental Sciences, National Institutes of Health.

[1] Present address, Pharmacology Division, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada.

inhibitor, and between enzyme and TEA,[2] is controlled by the affinity constants, K_a and K_i, respectively. Carbamylation (phosphorylation, sulfonation) proceeds by the irreversible k_2 step with release of the leaving group HX. When $i \ll K_a$, the over-all inhibition rate is given by the bimolecular rate constant k_i. Other evidence (3, 4) indicates that if the inhibitor contains a small fluoride leaving group, TEA may bind to the vacant anionic site and accelerate the inhibition reaction occurring at the esteratic site. The following scheme depicts the influence of TEA on this inhibition reaction.



(2)

In addition to K_a, k_2, and K_i, Eq. 2 contains three other constants: K_i', is the binding constant for TEA and the enzyme-inhibitor complex, while K_a', and k_2', are the reaction constants for the inhibitor and TEA-enzyme complex, respectively.

Equation 1 indicates that TEA will affect only the K_a value of cationic inhibitors. There are at least three reports (5–7) suggesting that acetylcholinesterase may possess additional (allosteric) binding sites. If this does occur, TEA and a cationic inhibitor may bind to the enzyme simultaneously. The inhibition scheme may then resemble that of Eq. 2, in that the apparent k_2 value as well as the apparent K_a value may be changed in the presence of TEA. The present study was initiated to determine whether TEA would affect the K_a and k_2 values of cationic inhibitors.

The influence of TEA on inhibition by compounds with small fluoride leaving groups was also studied, since all previous work has related the acceleration effect of TEA to the over-all inhibition constant k_i, which is actually k_2/K_a (8).

Not all the inhibitors of interest were suitable for kinetic analysis. The magnitudes of K_a and k_2 could not be determined for dimethylcarbamyl fluoride. These constants were obtained for methanesulfonyl fluoride, but only in the absence of TEA. It was found that TEA produced purely competitive inhibition toward inactivation of acetylcholinesterase by the cationic organophosphate diisopropylphosphorylthiocholine. However, a purely competitive effect was also noted with DFP, which has a fluoride leaving group.

TEA did not produce purely competitive inhibition with the cationic carbamates neostigmine and dimethylcarbamylcholine. At 0.5 mM, TEA produced a decrease in affinity, but an increase in k_2, with neostigmine. At higher TEA concentrations, anomalous curving was observed in the dimethylcarbamylcholine inhibition plots.

MATERIALS AND METHODS

Inhibition procedure. K_a and k_2 values were determined by the procedure reported by Main and Iverson (9). Inhibition occurs in the absence of substrate in an all-glass inhibition reaction vessel incorporating two side arms, a mixing plenum, and a substrate chamber. A removable plunger isolates the mixing and substrate chambers. A mixture of TEA and inhibitor (0.5 ml) is placed in one side arm, and 0.5 ml of enzyme solution is pipetted into the other side arm. The plunger is inserted, and the vessel is placed in a water bath at 25°. After temperature equilibration, 10 ml of substrate (30 mM acetylcholine chloride) at pH 7.0 are pipetted into the substrate chamber. The inhibition reaction is started by tipping the vessel and shaking it quickly to mix the contents. Inhibition occurs at pH 7.0 and 25°; the ionic strength is 0.02 M. The inhibition reaction is stopped by pulling on the plunger, allowing substrate to flood the side arms and dilute the inhibitor as well as complex the remaining free enzyme. Elapsed time is measured on a foot-operated timer calibrated to 0.1 sec. The contents of the vessel are then dumped into a pH-stat (Radiometer, Copenhagen) vessel containing 20 ml of 30 mM acetylcholine chloride, pH 7.0, at 25°. The total assay volume is 31 ml. The residual activity

[2] The abbreviations used are: TEA, tetraethylammonium chloride; DFP, diisopropyl phosphorofluoridate.

is assayed approximately 10 sec after termination of the inhibition reaction. The activity of the enzyme remaining uninhibited is recorded as a progress curve. In most instances these recordings are linear, but some potent organophosphate inhibitors may continue to inactivate the enzyme even after dilution and in the presence of high substrate concentrations. In addition, carbamate-inhibited enzyme undergoes regeneration during the assay, producing nonlinear recordings. The effects of these interfering reactions may be eliminated by drawing tangents to the progress curves and extrapolating to zero time to provide a true initial velocity (10).

Enzyme. Acetylcholinesterase, purified from *Electrophorus electricus* tissue, was obtained from Worthington Biochemical Corporation. Stock solutions were prepared in 10 mM sodium phosphate buffer, pH 7.0. No other salts were added. One milliliter of the working solution hydrolyzed 5.4 μmoles of 30 mM acetylcholine in 1 min at pH 7.0 and 25°.

It has been reported that preparations of acetylcholinesterase are not homogeneous but contain multiple molecular forms of the enzyme. Previous inhibition studies (11, 12) have shown that in the preparation used in the present study, approximately 98% of the activity is accounted for by one major form of the enzyme.

Substrate. Acetylcholine chloride was obtained from Sigma Chemical Company. The residual enzyme was assayed in 30 mM acetylcholine. The high substrate concentration was used to reduce inhibition by organophosphate during the assay, and to bind quickly the free enzyme remaining after inhibition.

Inhibitors. Tetraethylammonium chloride was obtained from Sigma Chemical Company; methanesulfonyl fluoride, from Eastman Kodak; and DFP, from Mann Research Laboratories. Neostigmine(3-trimethylaminophenyl *N*,*N*-dimethyl carbamate) was a gift from Hoffmann–La Roche, Inc. Diisopropylphosphorylthiocholine iodide [methiodide of *O*,*O*-diisopropyl *S*-(2-dimethylamino)ethyl phosphorothiolate] was prepared by Dr. W. C. Dauterman. Volhard titration yielded a molecular weight of 413 (calculated, 411.3); m.p. 154° (recorded, 153–154°). Dimethylcarbamylcholine iodide was prepared according to Wilson *et al.* (13). Volhard titration gave a molecular weight of 300 (calculated, 302.2); m.p. 213–214° (recorded, 212°).

With the exception of neostigmine, all inhibitors were diluted directly into distilled water. No alcohol was used. The maximum final concentration of ethanol in the neostigmine determinations was 0.5%. It was found that relatively low concentrations of TEA had to be used (0.5, 1.0, and 2.0 mM) to approach the K_a values of the inhibitors acting in the presence of TEA.

RESULTS

Values of K_a, k_2, and k_i were determined graphically as described by Main and Iverson (9), using the equation

$$\frac{i}{\rho} = \frac{i}{k_2} + \frac{K_a}{k_2} \tag{3}$$

where $\rho = 2.3\ (\Delta \log v/\Delta t)$ and v is the observed velocity of the uninhibited enzyme. Equation 3 is a linear transformation of

$$\rho = \frac{ik_2}{i + K_a} \tag{4}$$

When $i \ll K_a$, Eq. 4 reduces to

$$\rho = \frac{ik_2}{K_a} \tag{5}$$

which can be written as $\rho = ik_i$, where k_i is the bimolecular rate constant (see Eq. 1).

The rates of inactivation of acetylcholinesterase by selected inhibitor concentrations were fitted by regression analysis, yielding $\rho/2.3$. The abscissa intercept of a plot of i/ρ against i yields $-K_a$, the slope is $1/k_2$, and the ordinate intercept is $1/k_i$.

Influence of TEA on inhibition by cationic inhibitors. The plots of i/ρ against i for the cationic organophosphate diisopropylphosphorylthiocholine are shown in Fig. 1. The values for the inhibition constants are given in Table 1. As expected, TEA affects the value of K_a and not that of k_2.

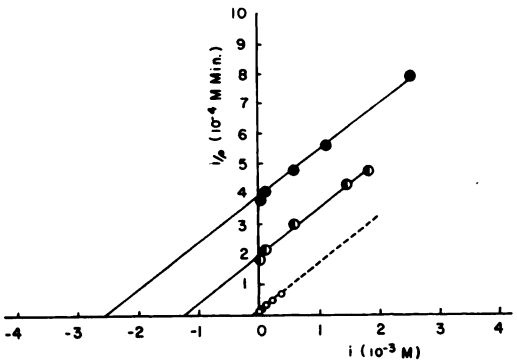


FIG. 1. *Effect of TEA on inhibition of acetylcholinesterase by diisopropylphosphorylthiocholine*

○, no TEA present; ●, 1 mM TEA present; ◐, 0.5 mM TEA present. The slopes of the plots of i/ρ against i are constant, indicating that k_2 does not change and that TEA acts in a purely competitive manner toward inhibition by the organophosphate. Inhibition occurred at 25° and pH 7.0.

The following equation describing inhibition is obtained from Eq. 1.

$$\frac{i}{\rho} = \frac{i}{k_2} + \frac{K_a}{k_2}\left(1 + \frac{A}{K_i}\right) \qquad (6)$$

It yields a value of 32 μM for K_i, the TEA binding constant. This K_i value is considerably lower than estimates obtained from the interference of TEA with acetylcholine hydrolysis, i.e., 250 μM (4). This may be due in part to the relatively low ionic strength (0.02 M) used in the present study, compared to the ionic strength of 0.1 M used by other workers (4). As the ionic strength decreases, it is expected that Coulombic interaction of TEA with an anionic site will increase, producing greater affinity.

The inhibition constants for the cationic carbamate dimethylcarbamylcholine are listed in Table 1. The plots of i/ρ against i

TABLE 1

Effect of TEA on apparent values of inhibition constants K_a, k_2, and k_i

K_i is the apparent TEA binding constant determined from the k_i values, assuming purely competitive inhibition (see Eq. 6).

Inhibitor	TEA concentration	K_a	k_2	k_i	K_i
	mM	M	min^{-1}	$M^{-1}\ min^{-1}$	M
Diisopropylphosphorylthiocholine	0	7.8×10^{-5}	6.4	8.3×10^{4}	
	0.5	1.3×10^{-3}	6.4	5.1×10^{3}	3.2×10^{-5}
	1.0	2.5×10^{-3}	6.3	2.5×10^{3}	3.2×10^{-5}
Diisopropyl phosphorofluoridate	0	1.6×10^{-3}	21.6	1.3×10^{4}	
	0.5	1.3×10^{-2}	22.6	1.7×10^{3}	7.0×10^{-5}
	1.0	2.4×10^{-2}	21.2	8.9×10^{2}	7.0×10^{-5}
Dimethylcarbamylcholine	0	3.4×10^{-3}	3.9	1.1×10^{3}	
	0.5	2.3×10^{-2}	6.4	2.8×10^{3}	1.7×10^{-4}
	1.0	1.6×10^{-2}[b]	4.6[b]	2.8×10^{2}[b]	1.7×10^{-4}[a]
	2.0	1.8×10^{-2}[b]	3.6[b]	2.0×10^{2}[b]	2.1×10^{-4}[a]
Neostigmine	0	6.2×10^{-5}[c]	110.4[c]	1.7×10^{6}[c]	
	0.5	1.9×10^{-4}	151.7	7.8×10^{5}	4.0×10^{-4}
	1.0			3.9×10^{5}	2.9×10^{-4}
Methanesulfonyl fluoride	0	3.0×10^{-1}	72.5	2.4×10^{2}	
Dimethylcarbamyl fluoride	0			2.0×10^{3}	
	1.0			1.2×10^{4}	

[a] Determined from the i/ρ values at the lowest carbamate concentration (see Fig. 2).

[b] Determined from the linear portion of the curves (see Fig. 4).

[c] Taken from Iverson and Main (11).

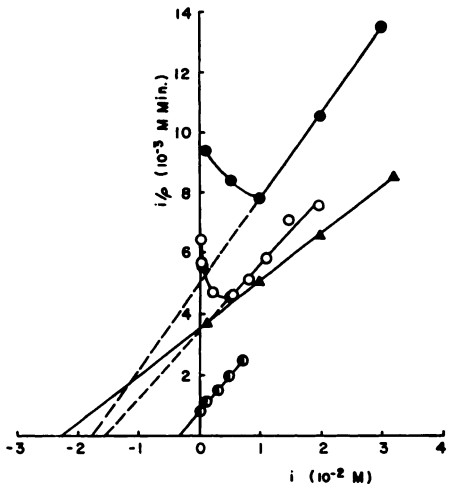


FIG. 2. *Effect of TEA on inhibition of acetylcholinesterase by dimethylcarbamylcholine*

◐, no TEA added; ●, 2 mM TEA present; ○, 1 mM TEA present; ▲, 0.5 mM TEA present. Inhibition occurred at 25° and pH 7.0. The fitted dashed lines were used to determine the K_a and k_2 values with 1 mM and 2 mM TEA present. The K_i values for TEA at these concentrations were determined from the i/ρ values of the extrapolated lines and also from the i/ρ values of the lowest carbamate concentration. The K_i values obtained from the latter method are given in Table 1; at 1 mM TEA i/ρ = 6.42 mM min^{-1} for 0.22 mM carbamate, and with 2 mM TEA i/ρ = 9.40 mM min^{-1} for 1 mM carbamate.

are shown in Fig. 2. In the presence of 0.5 mM TEA, the apparent affinity decreases but the apparent value of k_2 increases from 3.9 to 6.4 min^{-1}. In the presence of 1 mM and 2 mM TEA, the i/ρ plots are nonlinear, but approximate linearity at the higher inhibitor concentrations. The K_a and k_2 values were obtained by fitting lines to the linear portion of the curve. The k_2 values so obtained were close to that found in the absence of TEA, giving the appearance of purely competitive inhibition. Using these extrapolated k_i values and assuming purely competitive inhibition, the K_i for TEA varies from 0.17 mM at 0.5 mM TEA to 0.45 mM at 1.0 mM TEA. However, if the i/ρ values at the lowest actual carbamate concentrations are used as a measure of k_i, i.e., $\rho = ik_i$ (see Eq. 5), then K_i becomes relatively constant at 0.17 mM, 0.17 mM, and 0.21 mM with 0.5, 1.0, and 2.0 mM TEA, respectively. These results show that at selected inhibitor concentrations, TEA appears to be a purely competitive inhibitor. Nevertheless, it is apparent that this is not the case over a range of inhibitor concentrations, since the i/ρ plots were not linear.

The nonlinear nature of the dimethylcarbamylcholine inhibition plots was confirmed and found to be reproducible. Analysis of the carbamate by thin-layer chromatography, infrared spectrometry, and titration of iodide did not indicate the presence of a second inhibitor. Because of the anomalous kinetic results, attempts were made to study the effect of TEA on another cationic carbamate, neostigmine. With 0.5 mM TEA, the K_a value could not be measured directly, but reasonable estimates of K_a and k_2 could be made. As with dimethylcarbamylcholine, affinity was decreased and the apparent k_2 was increased, but the i/ρ plot did not curve. Only k_i could be determined in the presence of 1 mM TEA. The values of the inhibition constants are listed in Table 1. The apparent K_i values for TEA as determined from the k_i values by Eq. 6 were 0.40 mM at 0.5 mM TEA and 0.29 mM at 0.1 mM TEA.

Influence of TEA on inhibition by compounds with a fluoride leaving group. Attempts were made to determine the inhibition constants for dimethylcarbamyl fluoride, but although values of 70 min^{-1}

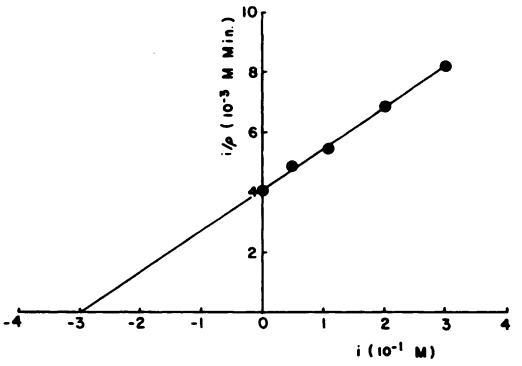


FIG. 3. *Plot of i/ρ against i for inhibition of acetylcholinesterase by methanesulfonyl fluoride*

k_2 is 72.5 min^{-1}; K_a is 0.3 M. Inhibition occurred at 25° and pH 7.0.

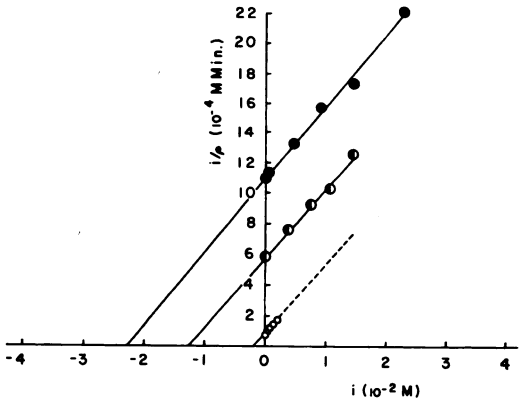


FIG. 4. *Effect of TEA on inhibition of acetylcholinesterase by DFP*

○, no TEA added; ●, 1 mM TEA present; ◐, 0.5 mM TEA present. The inhibition plots are parallel, indicating that TEA acts as a purely competitive inhibitor. Inhibition occurred at 25° and pH 7.0.

were determined at inhibitor concentrations up to 30 mM, the K_a value was too high to be measured. In the presence of 0.5 mM TEA, values as high as 72 min^{-1} were measured, but again K_a was beyond the limits of the method. The addition of TEA resulted in a 6-fold acceleration in the k_i value, an effect previously noted by Metzger and Wilson (4).

K_a and k_2 values of 0.3 M and 72.5 min^{-1}, respectively, were obtained for methanesulfonyl fluoride. The i/ρ plot is shown in Fig. 3. Since the values of the inhibition constants were already at the limits of resolution, determinations in the presence of TEA were not attempted. Kitz and Wilson (3) have reported that TEA accelerates the inhibition rate a maximum of 33-fold.

Inhibition constants were obtained for DFP in the presence and absence of TEA and are listed in Table 1. The i/ρ plots are shown in Fig. 4. Although the apparent K_a varies, k_2 remains constant, indicating purely competitive inhibition. This result was unexpected in view of the possibility that TEA and the inhibitor may bind simultaneously to the enzyme. The value of K_i for TEA obtained from these data is 70 μM.

DISCUSSION

The results reveal that TEA may or may not act as a purely competitive inhibitor of the reaction between acetylcholinesterase and cationic inhibitors. The mechanism depicted in Eq. 1 predicts that the K_i values for TEA should be independent of the inhibitors used, since interaction will occur only with the free enzyme. While the K_i values were in fact relatively constant over the range of TEA concentrations employed with any one compound, they varied more than 10-fold between inhibitors, from 32 μM for diisopropylphosphorylthiocholine to 400 μM for neostigmine. Considering first the purely competitive cases of DFP and its thiocholine analogue, it is apparent that the relatively large isopropyl function is almost as significant as a cationic leaving group in interfering with the binding of TEA. This is consistent with earlier work of Wilson (14), who observed that in the diisopropylphosphorylated enzyme the alkyl functions strongly interfered with the approach of quaternary ammonium ions to the anionic site.

While the effect of TEA on these organophosphate inhibitors may be interpreted on the basis of purely competitive inhibition, the results show that TEA does not act in this manner toward the two charged carbamates, neostigmine and dimethylcarbamylcholine. These results at first suggest that a distinction may be made between the organophosphate and carbamate inhibitors on the basis of the effects of TEA on k_2. However, the number of compounds used here is limited, and a recent investigation by Chiu and Dauterman (15) has shown that 20 mM TEA reduces the apparent k_2 of the cationic organophosphate Tetram [*O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate] from 126 min^{-1} to 8.0 min^{-1}. It seems likely, then, that k_2 effects will occur with both classes of inhibitors.

If TEA does not act as a purely competitive inhibitor, the ion and inhibitor must be bound simultaneously to the enzyme. The present evidence (16, 17) indicates that the anionic site is some 2.5–5 A from the esteratic site, making it unlikely that any inhibitor

with a cationic leaving group could bind and inhibit the enzyme while TEA is bound at the anionic site. An alternative explanation is that TEA may bind to a site distinct from the inhibitor-binding site. This site may resemble the "peripheral anionic site" that Changeux (5) has suggested to explain the anomalous kinetics he observed for the Flaxedil - acetylcholine - acetylcholinesterase system. However, unless complete specificity exists for TEA at this allosteric site, there is no reason to believe that TEA cannot bind to the anionic site as well, or that the charged inhibitor itself could not bind at a site other than the "active site" of the enzyme.

With this number of possibilities existing, the schemes describing inhibition expand to a point where, at present, it is not possible to evaluate the inhibition mechanism. Nevertheless, the inhibition plots for dimethylcarbamylcholine (Fig. 2) show that as the TEA concentration increases, the plot of i/ρ becomes nonlinear. This suggests that at the higher TEA concentrations, an enzyme-TEA-dimethylcarbamylcholine complex may exist in which TEA and the carbamate are bound to different sites on the enzyme. Since TEA and the organophosphate compounds are not bound simultaneously to the enzyme, it seems possible that the bulky isopropyl groups on these inhibitors may occupy the allosteric site and prevent the binding of TEA.

Although the results presented above do not permit determination of individual reaction constants, a first-order sulfonation rate in the presence of TEA may be estimated by incorporating data from a study by Kitz and Wilson (3). These authors found that TEA accelerated the inhibition rate of methanesulfonyl fluoride a maximum of 33-fold. Since this inhibitor has a small leaving group as well as a small alkyl function, it is possible that TEA may not affect K_a. The full acceleration may then be attributed to the k_2' (Eq. 2) rate when the TEA binding sites are saturated. This means that the k_2 value of 72 min^{-1} would be accelerated to a k_2' value of 2376 min^{-1}, which is more than 20 times greater than the k_2 obtained for the potent cationic inhibitor neostigmine (11). The existence of a sulfonation rate of this magnitude suggests that some of the more active acetylcholinesterase inhibitors, i.e., the organophosphonates, may possess k_2 values that would approach or even exceed this estimated value.

REFERENCES

1. R. M. Krupka, *Biochemistry* **4**, 429 (1965).
2. R. Kitz and I. B. Wilson, *J. Biol. Chem.* **237**, 3245 (1962).
3. R. Kitz and I. B. Wilson, *J. Biol. Chem.* **238**, 745 (1963).
4. H. P. Metzger and I. B. Wilson, *J. Biol. Chem.* **238**, 3432 (1963).
5. J. P. Changeux, *Mol. Pharmacol.* **2**, 369 (1966).
6. W. N. Aldridge and E. Reiner, *Biochem. J.* **114**, 1860 (1969).
7. R. D. O'Brien, *Biochem. J.* **113**, 713 (1969).
8. A. R. Main, *Science* **144**, 992 (1964).
9. A. R. Main and F. Iverson, *Biochem. J.* **100**, 525 (1966).
10. A. R. Main and W. C. Dauterman, *Nature* **198**, 551 (1963).
11. F. Iverson and A. R. Main, *Biochemistry* **8**, 1889 (1969).
12. A. R. Main, *J. Biol. Chem.* **244**, 829 (1969).
13. I. B. Wilson, M. A. Hatch and S. Ginsberg, *J. Biol. Chem.* **235**, 2312 (1960).
14. I. B. Wilson, *J. Biol. Chem.* **199**, 113 (1952).
15. Y. C. Chiu and W. C. Dauterman, *Biochem. Pharmacol.* In press.
16. S. L. Friess and H. D. Baldridge, *J. Amer. Chem. Soc.* **78**, 2482 (1956).
17. I. B. Wilson, *in* "The Enzymes" (P. D. Boyer, H. Lardy and K. Myrbäck, eds.), Vol. 4, Ed. 2, p. 501. Academic Press, New York, 1960.