# QUATERNARY AMMONIUM SALTS AS INHIBITORS OF ACETYLCHOLINE ESTERASE

## II. pH DEPENDENCE OF THE INHIBITORY EFFECTS, AND THE DISSOCIATION CONSTANT OF THE ANIONIC SITE

by

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In previous papers it was attempted to give a detailed description of the active surface of ACh esterase. The esteratic site was subdivided into a nucleophilic group ($G_1$) and an electrophilic group ($G_2$), in order to account for the reversible inactivation of the enzyme by $H^+$ or $OH'$ ions[1,2]. With the intention to substantiate this subdivision and to determine with which part of the substrate may $G_1$ and $G_2$ combine, we carried out the following experiments.

Based on the assumption that $G_1$ combines with the carbonyl carbon of the ester group and that $G_2$ is responsible for the binding of the ether oxygen* a prediction can be made about the variation with pH of the activity of inhibitors. If we use inhibitors, in which one or the other part of the grouping R–O–C–R′ is missing, the effect of a

$$\begin{matrix} & & \| & & \\ & & O & & \end{matrix}$$

change in hydrogen or hydroxyl ion concentration should disappear. It can thus be foreseen that the inhibitory effect of

1. prostigmine should be dependent on both ions, similar to the change of the rate of hydrolysis of a substrate with pH
2. acetophenone *m*-trimethylammonium iodide should vary only with the concentration of $H^+$ ions
3. choline chloride should be dependent only on the concentration of $OH'$ ions
4. tetraethylammonium bromide should be independent of either ion.

Our experimental results, described in this paper, are in disagreement with the above prediction and reveal a new feature of the influence of a decrease in pH on the active surface of the enzyme. While our investigation was in progress, a paper by WILSON[3] appeared, in which the pH dependence of the inhibitory effect of prostigmine was tested. However, our experiments led to entirely different conclusions from those arrived at by this author.

* This is only one of the possible functions of $G_2$. It could *e.g.* also be involved in attaching the carbonyl oxygen.

*References p. 477.*

## MATERIALS AND METHODS

Acetophenone *m*-trimethylammonium iodide (I) was prepared according to the literature (4.5). However the exhaustive methylation of *m*-aminoacetophenone was carried out in one step as follows: A mixture of *m*-aminoacetophenone (6.8 g) and methyl iodide (43 g; 6 equivalents) in methanol (100 ml) was refluxed for two hours. Upon cooling white crystals were obtained, which were recrystallized from methanol. Colorless prisms of m.p. 233–235°. Yield: 13 g, 85%.

ACh esterase was prepared from the electric tissue of Electrophorus electricus or Torpedo marmorata*. The enzyme preparation used, when diluted 1:1500, hydrolyzed 5 $\mu$moles/ml/hour, if $3.3 \cdot 10^{-3}$ *M* ACh was the substrate. For the short range experiments a dilution 1:400 was applied, which hydrolyzed 0.3 $\mu$moles/ml/min.

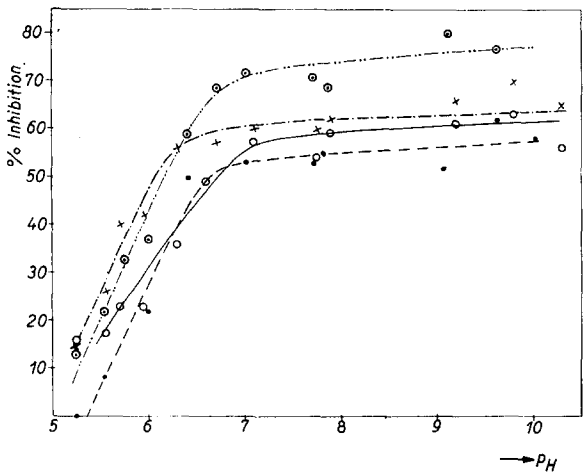



Fig. 1. Inhibition of ACh esterase by quaternary ammonium salts as function of pH
Enzyme 1:400, hydrolyzes 0.3 $\mu$moles ACh/ml/min
Substrate ACh $3.3 \cdot 10^{-3}$ *M*
Buffers 0.1 *M* phosphate for pH below 8.5
0.1 *M* borate for pH above 8.5

Inhibitors:

• – – – • – – – • – – – • prostigmine bromide $8 \cdot 10^{-8}$ *M*
○———○———○ acetophenone *m*-trimethylammonium iodide $8 \cdot 10^{-6}$ *M*
◉–··–·· ◉ ··–··–◉ choline chloride $1 \cdot 10^{-2}$ *M*
×–·–·–×–·–·–×–·–·–× tetraethylammonium bromide $5 \cdot 10^{-3}$ *M*

Hydrolysis of ACh was measured after 4 min for pH $<$ 6 and after 2 min for pH $>$ 6.

The inhibitory activity of the acetophenone derivative[1] was determined as described in a previous paper[6] by the Warburg manometric method. For the determination of the pH dependence of inhibitory effects the colorimetric method of HESTRIN[7] was used. Enzyme and inhibitor were incubated for 20 min, then ACh was added to give a final concentration of $3.3 \cdot 10^{-3}$ *M*. The solutions were made up in such a way that all components of the final mixture besides the substrate occupied a volume of 2.8 ml and the substrate itself 0.2 ml. The addition of such a small volume to the incubated mixture left the enzyme—inhibitor equilibrium practically undisturbed.

The buffer had a final concentration of 0.1 *M*. The final concentrations of the inhibitors were as follows: Prostigmine bromide $8 \cdot 10^{-8}$ *M*; acetophenone *m*-trimethylammonium iodide $8 \cdot 10^{-6}$ *M*; choline chloride $1 \cdot 10^{-2}$ *M*; and tetraethylammonium bromide $5 \cdot 10^{-3}$ *M*. Samples of 1 ml were withdrawn from each reaction mixture after 1 and 2 min for pH $>$ 6, and after 2 and 4 min for pH $<$ 6. Due to the greater sourcer of error involved in the shorter reaction periods we have plotted in Fig. 1 only the values for the longer periods.

* We wish to thank Prof. BACCI of the Marine Biological Station at Naples for the generous gift of these animals.

*References p. 477.*

## RESULTS

### 1. *The inhibitory effect of acetophenone m-trimethylammonium iodide* (I)

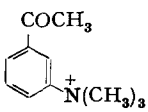
This compound was prepared because of its similarity to 3-acetoxyphenyl *m*-trimethylammonium methosulfate (Nu 2017, II). The latter is rapidly hydrolyzed by ACh esterase, and even its spontaneous hydrolysis in aqueous solution is faster than that of ACh[8]. The acetophenone derivative is of course not subject to hydrolytic fission and should therefore be an effective inhibitor[9]. This prediction is confirmed by our experiments. The values obtained for I are compared in Table I with those for prostigmine. The latter inhibits ACh esterase at about one seventeenth the concentration of the acetophenone derivative, but it is about 150 times more toxic in white mice. The pharmacological properties of the new inhibitor will be reported elsewhere.

TABLE I

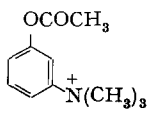
COMPARISON OF PROSTIGMINE BROMIDE AND ACETOPHENONE *m*-TRIMETHYLAMMONIUM IODIDE (I)

| | $C_{50}$ *for ACh esterase** (*ACh as substrate*) | $LD_{50}$ *for white mice* *intraven.* | *intraperition.* |
|---|---|---|---|
| Prostigmine | $4 \cdot 10^{-7}$ *M* | 0.15 mg/kg | 0.6 mg/kg |
| Acetophenone derivative | $7 \cdot 10^{-6}$ *M* | 28 mg/kg | 70 mg/kg |

* For the determination of $C_{50}$ in the Warburg apparatus we used an enzyme concentration which hydrolyzed 5 $\mu$moles/ml/hour. ACh $3.3 \cdot 10^{-3}$ *M*. Buffer 0.2 *M* gelatine — bicarbonate.



Formula I.



Formula II.

### 2. *The pH dependence of the inhibitory effect of quaternary ammonium ions*

In view of the short reaction period in these experiments it can be assumed that addition of the substrate to the incubation mixture does not change the equilibrium enzyme—inhibitor to an appreciable degree. Therefore the degree of inhibition measured represents more or less accurately the position of this equilibrium. Our results are represented in Fig. 1 and demonstrate the fact that all inhibitors used in this investigation show a similar influence of pH changes on their activity: Their effect drops to zero near pH 5. This is especially conspicuous for the tetraethylammonium ion, for which we have demonstrated that VAN DER WAALS' forces between the alkyl chains and the active surface are responsible for the competition inhibitor—substrate[6]. If these forces should change at all with pH, such an effect should certainly be weaker for a $CH_2$ group than for the carbonyl in a ketone or ester. Therefore the fact that all our inhibitors show a similar pH dependence can be explained only by the assumption that they compete with hydrogen ion for the anionic site. We thus come to the conclusion that the anionic site looses its negative charge progressively between pH 7 and 5, owing to combination with $H^+$.

It is apparent in Fig. 1 that choline and tetraethylammonium bromide form a separate group and prostigmine and the acetophenone derivative another one. The latter two compounds show an earlier decline of their inhibitory effect than the first group.

*References p. 477.*

## DISCUSSION

In our first paper[1] it was assumed that the charge of the anionic site does not change over the whole range of pH. However this statement was based on experiments, which were carried out above pH 6 and were extended over sufficiently long periods to obscure the enzyme-inhibitor equilibrium by interference of the competitive substrate. Only short term experiments can be considered as non-competitive. Therefore our results now raise serious doubts on the validity of the value for the constant $K_{EH_2}{}^+$, in connection with the group $G_1$ in the esteratic site, since in these experiments both $G_1$ and the anionic site became progressively inactivated with decreasing pH. The active surface of ACh esterase, at least when ACh serves as substrate, appears to be characterized by *five* equilibrium constants instead of four.

The pH activity curve of ACh esterase, which has been determined by HESTRIN[12] and by WILSON AND BERGMANN[2], shows a rather broad maximum between pH 7.5 and 9. It differs in this respect from the behaviour of unspecific esterases, *e.g.*, serum cholinesterase[10] or liver esterase[11], which possess a much narrower pH optimum. This fact was previously taken as evidence that the active surfaces of "specific" and "unspecific" esterases are different. However in order to compare dissociation constants of the esteratic sites in these two groups of enzymes it is necessary to isolate the effect of pH changes on the esteratic site and on the anionic site in ACh esterase. If two groups in the latter enzyme are inactivated simultaneously by combination with $H^+$, the "true" dissociation constant of $G_1$ is still unknown and must be determined by a new method. We shall report on this problem in a forthcoming paper.

It is surprising that the pH "activity" curves of ACh and TEPP possess a different shape. In view of the fact however that TEPP as a neutral "substrate" is not influenced by changes in the anionic site, this discrepancy now appears in a new light and requires re-investigation.

All our quaternary ammonium compounds show a practically constant inhibitory action throughout the alkaline pH range. This fact was explained by WILSON[3]—for the case of prostigmine—as follows: $K_{EH}$, the dissociation constant associated with $G_2$, belongs not to the free enzyme, but to the enzyme—substrate transition complex. This conclusion is supported by our own results, although other possible explanations have still to be excluded.

## ACKNOWLEDGEMENT

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## SUMMARY

The variation of the effect of a group of inhibitors of ACh esterase with pH has been investigated. These inhibitors all possess a quaternary ammonium group, but are stripped of one or more components of the ester group, present in the substrate. All inhibitors showed a progressive decline of activity with decreasing pH, approaching zero around pH 5.

These observations indicate that the anionic site becomes inactivated between pH 7 and 5 by combination with hydrogen ions. Our results shed new light on the pH activity curve of ACh esterase with ACh as substrate.

*References p. 477.*

## RÉSUMÉ

Les auteurs ont étudié la variation avec le pH d'un groupe d'inhibiteurs de l'ACh-estérase. Ces inhibiteurs contiennent tous un groupe ammonium quaternaire mais sont privés d'un ou plusieurs constituants du groupe ester contenu dans le substrat. L'activité de tous les inhibiteurs diminue, lorsque le pH décroit et tend vers zéro lorsque le pH est 5 environ.

Ces observations indiquent que la partie anionique de l'enzyme est inactivée entre pH 7 et 5 par combinaison avec des ions hydrogène. Nos résultats fournissent un nouvel aspect de la courbe d'activité en fonction du pH de l'ACh-estérase avec ACh comme substrat.

## ZUSAMMENFASSUNG

Die pH-Abhängigkeit der Wirkung einer Gruppe von Hemmstoffen der ACh-Esterase wurde untersucht. All diese Hemmstoffe besitzen eine quaternäre Ammonium-Gruppe, doch fehlen bei ihren ein oder mehrere Bestandteile der Estergruppe, welche im Substrat vorkommt. Die Aktivität aller Hemmstoffe nahm allmählich mit dem pH ab und näherte sich Null bei pH ca 5.

Diese Beobachtungen weisen darauf hin, dass der anionische Teil des Enzyms zwischen pH 7 und 5 durch Verbindung mit Wasserstoff-Ionen seine Aktivität verliert. Unsere Ergebnisse werfen ein neues Licht auf die pH-Aktivitäts-Kurve der ACh-Esterase mit ACh als Substrat.


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