# Methylene Blue as an Inhibitor of Acetylcholine-Esterase

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Methylene blue is a strong inhibitor of the enzymic hydrolysis of acetylcholine. This was first demonstrated with serum cholinesterase [1-6], later on also found for the acetylcholine-esterase * of leech muscle extract [8] and of erythrocytes, brain, *Helix* blood, and other sources [9]. The action of methylene blue on the serum cholinesterase has been discussed in relation to the positive oxidation-reduction potential of the dye [10, 11].

It is known that compounds with a quaternary ammonium ion are strong inhibitors of both serum cholinesterase and acetylcholine-esterase of various sources. The action of methylene blue is due to the presence of such an ion, for the leuco form of the dye has no inhibiting effect. Regarding the action of the quaternary ammonium bases on acetylcholine-esterase, the present author has recently postulated [9], that such compounds interact with the enzyme (E) by competition with the substrate (S) in the formation of the ES complex. According to the Murray-Haldane hypothesis, an enzyme inhibition by excess of substrate may be explained by assuming the formation of an $ES_2$ complex which is incapable of the breakdown. It actually seems to be the case for the acetylcholine-esterase and its physiological substrate acetylcholine. This view satisfactorily explains the bell-shaped curve obtained by plotting the enzyme activity against the minus logarithm of the molar substrate concentration (pS). The characteristic substrate optimum $(pS_{opt})$ for the acetylcholine-esterase (2.4) has been found to shift to higher substrate concentration in the presence of choline [9] and prostigmine [12]. Both these compounds are quaternary ammonium bases. The shift of optimum becomes increasingly stronger with increasing inhibitor concentration. Prostigmine in $2 \times 10^{-6}$ $M$ solution, for instance, inhibits the enzyme 10 per cent at $3.3 \times 10^{-2}$ $M$ acetylcholine; when

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* The term acetylcholine-esterase is equivalent to the previously used "specific", "true", and "e" cholinesterase [7].

the acetylcholine concentration is 10 times lower, the inhibition by the same concentration of prostigmine is as high as 88 per cent. It has now been postulated that such a shift of optimum acetylcholine concentration is characteristic of the action of quaternary ammonium bases on the acetylcholine-esterase.

It is the purpose of this paper to extend these observations with the inhibitory effect of methylene blue, also to show that the action of this compound is much the same as that of prostigmine on this specific enzyme.

## METHODS

The enzyme activity was measured by the Warburg manometric method described previously [9]. Substrate, enzyme, and inhibitor were dissolved in bicarbonate-Ringer's solution containing 0.12 $M$ NaCl, 0.034 $M$ NaHCO$_3$, and 0.0012 $M$ MgCl$_2$ . 6H$_2$O. The unit, $b_{30}$, an expression of esterase activity, is the amount of CO$_2$ in $\mu l$ evolved during 30 minutes minus the corresponding value for non-enzymic hydrolysis. Measurements were made at 25° C. The substrate solution (1.60 ml) or a mixture of substrate and inhibitor (0.8 + 0.8 ml) was placed in the main compartment of the vessel, and 0.4 ml of the enzyme solution or a mixture of enzyme and methylene blue solutions (0.2 + 0.2 ml) in the side bulb.

The enzyme preparation was obtained by washing cow erythrocytes four times with 0.9 per cent NaCl solution and then haemolysing with distilled water to the same volume as the original blood volume. One part of the haemolysate was diluted with two parts of the bicarbonate solution.

Acetylcholine chloride was used as substrate in all experiments. Methylene blue (mol. wt. 373.89) was a commercial product (Merck & Co.).

## INHIBITION AS FUNCTION OF SUBSTRATE CONCENTRATION

The inhibition of acetylcholine-esterase by methylene blue at various acetylcholine concentrations is shown in Fig. 1. In these experiments the enzyme came in contact with acetylcholine and methylene blue at the same time (*i. e.*, no incubation of enzyme with inhibitor). The final concentration of methylene blue in the reaction mixture in the two series of experiments shown was $1.07 \times 10^{-5}$ $M$ and $3.22 \times 10^{-5}$ $M$ respectively.

In the presence of methylene blue the optimum acetylcholine concentration is changed to a higher concentration. The higher the concentration of methylene blue the higher is the substrate optimum (pS$_{opt}$ lower). In an $1.10 \times 10^{-2}$ $M$ acetylcholine solution when no inhibitor is present, this substrate concentration is higher relative to the substrate optimum. When methylene blue is added in increasing concentrations to such a solution, the new ("apparent") pS$_{opt}$ approaches that corresponding to $1.10 \times 10^{-2}$. When methylene blue is present in still higher concentrations this substrate con-
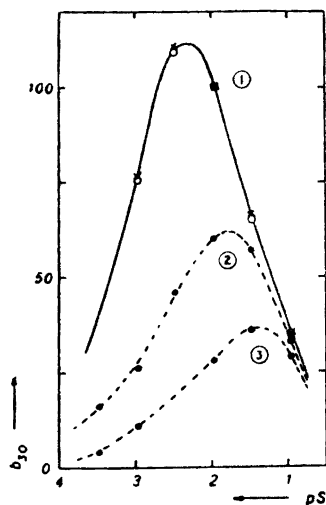
Fig. 1. Activity-pS curves for the enzymic
hydrolysis of acetylcholine by acetylcholine-
esterase (cow erythrocytes) in the presence
of added methylene blue (MB). No incuba-
tion, i. e., MB and acetylcholine in contact
with the enzyme at the same time.

1. Control; haemolysate O——O,
   intact cells X——X; $pS_{opt}2.4$
2. $1.07 \times 10^{-5}M$ MB    $pS_{opt}1.8$
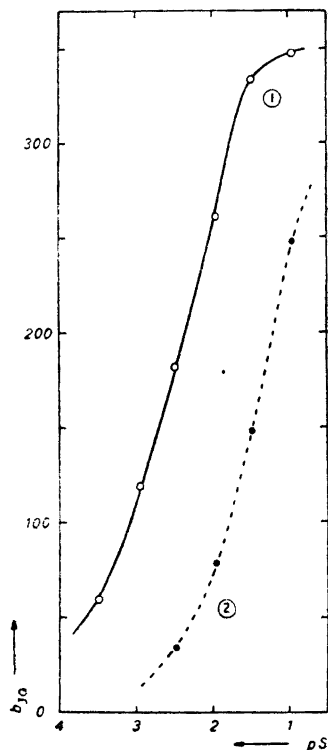3. $3.22 \times 10^{-5}M$ MB    $pS_{opt}1.4$



Fig. 2. Activity-pS curves for the enzymic
hydrolysis of acetylcholine by human serum
cholinesterase in the presence of added meth-
ylene blue (MB).
1. Control
2. $0.27 \times 10^{-5}M$ MB

centration is too low to give optimum enzyme activity (curves 2 and 3).
Starting with a $1.10 \times 10^{-2}$ M substrate solution, therefore, the enzyme activ-
ity increases with increasing substrate concentration when such a high con-
centration of the inhibitor is present, it decreases on the other hand in the
absence of inhibitor or in the presence of low inhibitor concentration.

This change in activity-substrate concentration relationship in the presence
of an inhibitor is important when the degree of inhibition of acetylcholine-
esterase is measured for an inhibitor as methylene blue. The inhibition for a
given methylene blue concentration varies greatly with the substrate concen-
tration. At $3.3 \times 10^{-2}$ M acetylcholine, the enzyme is inhibited 12 per cent
by $1.07 \times 10^{-5}$ M methylene blue; at 10 times lower acetylcholine concentra-
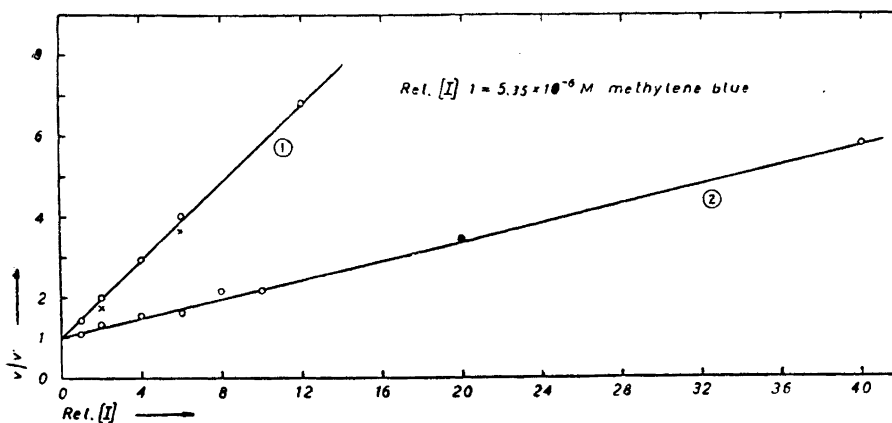
Fig. 3. *Inhibition of acetylcholine-esterase (cow erythrocytes) by methylene blue (MB) as function of inhibitor concentration.* $v$ = *velocity in the absence,* $v'$ = *velocity in the presence of MB, expressed in $\mu l$ $CO_2$ evolved in 30 minutes ($b_{30}$). Acetylcholine concentration $1.10 \times 10^{-2} M$.*

1. *Without incubation; the two values marked with a cross were obtained in the experiments with varying substrate concentrations (Fig. 1)*
2. *Enzyme solution incubated 40 minutes with MB before mixed with the substrate*

tion the inhibition by the same concentration of the dye is 58 per cent. The activity-pS curves are principally similar to those obtained for the electric tissue acetylcholine-esterase in the presence of prostigmine [12].

For comparison the action of methylene blue on the serum cholinesterase is illustrated in Fig. 2. The dye inhibits this esterase also by true competition. The relative degree of inhibition at various acetylcholine concentrations is markedly different from that found for the erythrocyte acetylcholine-esterase. Comparing the inhibitory effect of a compound on these two types of esterases, such differences must be considered. The corresponding value mentioned above for the acetylcholine-esterase and $1.07 \times 10^{-5}$ $M$ methylene blue are for the serum cholinesterase 89 and 98.5 per cent respectively (compared with 12 and 58 respectively).

## INHIBITION AS FUNCTION OF INHIBITOR CONCENTRATION

In the case of competitive inhibition and constant concentrations of enzyme and substrate, a plot of the degree of inhibition (expressed as $v/v'$) against the concentration of the inhibitor I gives a straight line:

*Table 1. Comparison between the inhibitory action of some "anticholinesterases" on acetyl-choline-esterase. Values calculated according to Equation 1. $K_I = 6.3 \times 10^{-4}$. $I_{50} = molar$ inhibitor concentration giving 50 per cent inhibition. In calculating the $K_I$ values (in brackets) for the irreversible inactivators, the slope of the tangents of the curved lines (v/v'; I diagram) has been used [12].*

| Inhibitor | Values obtained in incubation/no incubation experiments | Enzyme | [S] molarity | $K_I$ | $I_{50}$ molarity | Values calc. from |
|---|---|---|---|---|---|---|
| **True competitive inhibitors** | | | | | | |
| Choline chloride | Inc. and no inc. | Erythrocytes (cow) | $3.3 \times 10^{-3}$ | $2.5 \times 10^{-3}$ | $1.6 \times 10^{-2}$ | Ref.   9 |
| »          » | »   »   »   » | »          » | $1.1 \times 10^{-3}$ | $2.4 \times 10^{-3}$ | $6.3 \times 10^{-3}$ | »   9 |
| Prostigmine bromide | »   »   »   » | Electric tissue | $3.3 \times 10^{-3}$ | $1.6 \times 10^{-7}$ | $1.0 \times 10^{-6}$ | »   12 |
| Physostigmine sulphate | »   »   »   » | »          » | $3.3 \times 10^{-3}$ | $6.1 \times 10^{-8}$ | $4.2 \times 10^{-7}$ | »   12 |
| Methylene blue | No incubation | Erythrocytes (cow) | $1.1 \times 10^{-2}$ | $5.7 \times 10^{-7}$ | $1.1 \times 10^{-5}$ | Fig.   2 |
| »          » | 40 min. incub. | »          » | $1.1 \times 10^{-2}$ | $2.3 \times 10^{-6}$ | $4.3 \times 10^{-5}$ | »   2 |
| **Irreversible inactivators** | | | | | | |
| Diisopropyl fluorophosphate (DFP) | Incubation | Electric tissue | $3.3 \times 10^{-3}$ | $(2.1 \times 10^{-7})$ | $1.3 \times 10^{-6}$ | Ref. 12 |
| Tetraethyl pyrophosphate | » | »          » | $3.3 \times 10^{-3}$ | $(1.3 \times 10^{-9})$ | $7.3 \times 10^{-9}$ | »   12 |
| »          »   (TEPP) | » | Erythrocytes(man) | $1.1 \times 10^{-2}$ | $(0.9 \times 10^{-9})$ | $7.0 \times 10^{-9}$ | Unpubl. |

$$\frac{v}{v'} = 1 + [I]\frac{K_S}{K_I([S] + K_S)} \qquad (1)$$

The reaction velocities $v$ and $v'$ represent the enzyme activities in the absence of the inhibitor I and in its presence respectively. $K_S$ and $K_I$ are the dissociation constants of the complexes enzyme-substrate and enzyme-inhibitor respectively. The intercept on the $v/v'$ axis is 1 (one). From known values of [S] and $K_S$ and the slope of the line (i. e., $K_S/K_I$ ([S] + $K_S$)), $K_I$ can be calculated. The present author has applied this method in evaluating the action of choline [9] and other inhibitors [12] on various esterase systems.

Fig. 3 shows the results obtained with methylene blue as an inhibitor of the acetylcholine-esterase of cow erythrocytes. Table 1 compares these results with those obtained for other powerful inhibitors, analysed according to the graphical method described.

The fact that methylene blue gives a straight line according to Equation 1 shows that the dye acts as a competitive inhibitor. The $K_I$ value, $5.7 \times 10^{-7}$, obtained in experiments when inhibitor and acetylcholine are mixed simultaneously with the enzyme (no incubation; curve 1, Fig. 3), is of the same order of magnitude as that of prostigmine ($1.6 \times 10^{-7}$). As pointed out above the $I_{50}$ value (defined in Table 1) greatly varies with the substrate concentration.

This is in contrast to the irreversible inactivators (DFP and TEPP) for which $I_{50}$ is independent on the substrate concentration.

The $I_{50}$ value is higher when the enzyme has been in contact with methylene blue before mixing with substrate (incubation experiments). A similar observation has been made with the irreversible inactivators. In the latter cases the affinity of the enzyme for acetylcholine is much greater than for the inactivators, i. e., acetylcholine protects the enzyme against the action of these compounds when both substrate and inhibitor are coming in contact simultaneously with the enzyme. This is, however, not the case for methylene blue for which the inhibition is a true competitive one. The fact that the incubation of the enzyme solution with the dye leads to a weaker inhibitory action is due to the lowering of that action by the enzyme solution. It is, however, not the enzyme itself which has this effect. This has been proved by destroying the enzyme by heating, after which the enzyme solution still has the power to weaken the inhibitory action of methylene blue. Probably the oxidation-reduction potential is altered in an unfavorable way regarding the inhibitory action, i. e., a part of the dye is converted to the inactive leuco base. This fact is important to consider when methylene blue is used therapeutically in order to depress the activity of acetylcholine-esterase *in vivo*.

## SUMMARY

The kinetics of the inhibition of the acetylcholine-esterase by methylene blue has been studied using cow erythrocytes as enzyme source.

Methylene blue is a true competitive inhibitor and behaves in a similar way found previously for other quaternary ammonium bases. The optimum acetylcholine concentration, characteristic of this esterase, is changed to higher concentration in the presence of the dye. The higher inhibitor concentration the higher is the optimum acetylcholine concentration. Great differences in degree of inhibition are obtained when the action of the dye on the acetyl-choline-esterase is compared with that on the serum cholinesterase.

Evaluating the inhibition as function of methylene blue concentration the dissociation constant $(K_I)$ of the inhibitor-enzyme complex was found to be $5.7 \times 10^{-7}$. A higher value of $K_I$ is obtained when the enzyme solution has been incubated with the inhibitor before mixed with the substrate.

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