BBA 65581

THE INHIBITORY EFFECT OF TRIS ON THE ACTIVITY OF CHOLINESTERASES

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(Received November 7th, 1966)

SUMMARY

1. In order to obtain a clearer picture of the effect of Tris on cholinesterases we undertook experiments using acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8).

2. Using the Warburg manometric technique at a temperature of 38° and pH 7.4 we determined the activity of both cholinesterases with various amounts of added Tris. The substrates were acetylcholine and butyrylcholine. Preparations of acetylcholinesterase were obtained from the electric organ of *Torpedo marmorata*. Horse plasma served as the cholinesterase preparation. Kinetic data were analyzed graphically.

3. The inhibition of acetylcholinesterase and cholinesterase by Tris in the concentrations usually used in buffer solutions is appreciable. This should be taken into account when using Tris as a buffer in the study of cholinesterases.

4. The results of our experiments indicate that Tris reacts with the active centres of acetylcholinesterase and cholinesterase. It inhibits both cholinesterases competitively. The inhibition constants for the reaction between Tris and acetylcholinesterase and cholinesterase, respectively, with acetylcholine and butyrylcholine as substrates, are the same: 13–14 mM.

INTRODUCTION

Tris is widely used as a buffer in biochemistry[1]. In studying cholinesterases[2] we used Tris–HCl buffer several times according to the method of GOMORI[3]. The results obtained could only be interpreted on the assumption that Tris influences the activity of cholinesterases. This assumption seemed all the more justifiable in the light of MAHLER's claim[4] that Tris might have an effect on "pseudocholinesterase".

In order to obtain a somewhat clearer picture of the effect of Tris on cholinesterases experiments with acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7) and cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8) were carried out.

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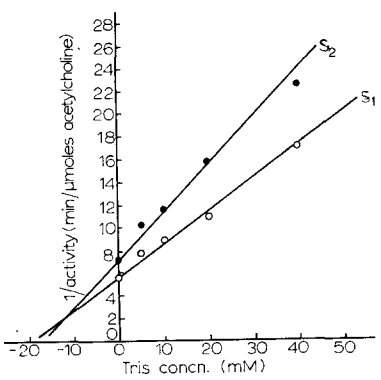
METHODS

The effect of Tris on the activity of cholinesterases was studied by determining the activity of enzyme preparations to which various amounts of Tris were added. The Warburg manometric technique[5] was used at a temerpature of 38° and pH 7.4. The gases in solution were in equilibrium with 5% CO_2 in N_2. The total volume of the reaction mixture in the flask of the Warburg respirometer was 3.0 ml. The amount of cholinesterase preparation added was 0.04 ml. Considering these quantities, the activities of the enzyme preparations and the catalytic-centre activities, we calculated[6] that, in our experiments, the concentration of the active centres of acetylcholinesterase was approx. 0.1 mμM and that of cholinesterase was approx. 0.6 mμM. The substrates were acetylcholine and butyrylcholine. The kinetic data were analysed by the method of DIXON[7].

Preparations of acetylcholinesterase were obtained from the electric organ of the fish *Torpedo marmorata* according to the method of ROTHENBERG AND NACHMANSOHN[8]; after the first stage of preparation, $(NH_4)_2SO_4$ was removed by dialysis against the buffer. When acetylcholine was used as substrate the preparation was conveniently diluted (50–100 times) with the same buffer. The specific activity of such preparations was about 0.01 μmole of acetylcholine hydrolysed per min per mg protein. As cholinesterase preparation, horse plasma was used. The specific activity was about 0.01 μmole of acetylcholine hydrolysed per min per mg protein.

The buffer solution used was the KREBS–HENSELEIT solution[9] without glucose. The basic Tris solution was made by adding Tris to the buffer solution. Prior to each experiment the pH value of the buffer itself and the pH value of the basic Tris solution were adjusted to 7.4 with 0.1 M NaOH or 0.1 M HCl; the solutions were in equilibrium



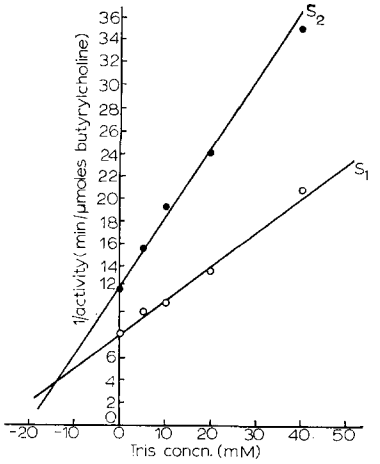


Fig. 1. Inhibition of acetylcholinesterase by Tris, substrate acetylcholine. S_1 = 10 mM acetylcholine, S_2 = 2.5 mM acetylcholine. Temp. 38°, pH 7.4. Each point represents the average of two measurements.

Fig. 2. Inhibition of acetylcholinesterase by Tris, substrate butyrylcholine. S_1 = 10 mM butyrylcholine, S_2 = 2.5 mM butyrylcholine. Temp. 38°, pH 7.4. Each point represents the average of two measurements.

with 5% CO_2 in N_2. After each experiment the pH value of the samples was checked under the same experimental conditions and was found never to have varied from 7.40 by more than ± 0.05.

Chemicals: acetylcholine iodide, butyrylcholine iodide, Tris and all other chemicals, were reagent grade.

RESULTS

Our first experiments were carried out with acetylcholinesterase and cholinesterase preparations, using the manometric technique at 38° (pH 7.4) with acetylcholine as substrate. They showed that Tris in 1 mM and higher concentration considerably inhibits both cholinesterases. The inhibition by 50 mM Tris was greater than 50% under our experimental conditions.

Before continuing the study we estimated, by preincubation, the time required to obtain constant inhibition. The per cent inhibition was independent of the duration

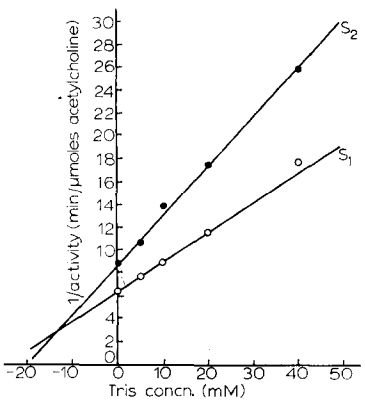


Fig. 3. Inhibition of cholinesterase by Tris, substrate acetylcholine. S_1 = 10 mM acetylcholine, S_2 = 2.5 mM acetylcholine. Temp. 38°, pH 7.4. Each point represents the average of two measurements.

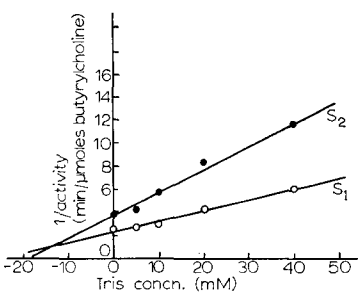


Fig. 4. Inhibition of cholinesterase by Tris, substrate butyrylcholine. S_1 = 10 mM butyrylcholine, S_2 = 2.5 mM butyrylcholine. Temp. 38°, pH 7.4. Each point represents the average of two measurements.

of preincubation between 24 h and 30 min. 30 min sufficed to produce equilibrium. By diluting preincubated enzyme solutions and comparing their activity with that of unincubated enzyme solutions we found that the "dissociation" time of Tris was less than 30 min. Consequently, the time of "association" and "dissociation" of Tris under our experimental conditions is less than 30 min.

Other results are shown in Figs. 1–4. With both cholinesterases and both substrates the inhibition is competitive. The inhibition constants are practically the same: 13–14 mM. In some experiments with cholinesterase, butyrylcholine and Tris the pH value was changed from 7.4 to 8.0. No change in inhibition constant could be detected.

DISCUSSION

The results show that Tris inhibits acetylcholinesterase and cholinesterase. There is little probability that other factors had an influence on the activity changes observed in our experiments: the pH was checked before and after each experiment; the changes in ionic strength due to Tris were relatively small; a possible effect of Tris on the gas equilibrium in the flasks of the respirometer was eliminated by keeping the pH throughout the experiment at 7.00 ± 0.05.

The inhibitory effect on acetylcholinesterase and cholinesterase of Tris in concentrations usually used in buffer solutions is considerable.

MAHLER[4] has mentioned several possible reactions between Tris and some enzyme systems in general. In the case of acetylcholinesterase and cholinesterase we can consider two of them: the reaction of Tris with substrate (carbonyl group) or with the active site of the enzyme.

Our first experiments with acetylcholinesterase and acetylcholine as substrate showed a linear dependence of the reciprocal value of enzyme activity on the concentration of Tris. Experiments with two different concentrations of acetylcholine indicated a competitive inhibition with an inhibition constant of 14 mM.

Approximately the same value was obtained when we repeated the experiments with butyrylcholine as substrate. Since the inhibition constants are the same, apparently Tris reacts with the enzyme and thus with its active sites.

Analogous experiments with cholinesterase have indicated that Tris reacts with the active centres of cholinesterase. The result of this reaction is a competitive inhibition of acetylcholine and butyrylcholine hydrolysis. The corresponding inhibition constants lie between 13 mM and 14 mM.

The same inhibition constants for acetylcholinesterase and cholinesterase, as well as the fact that we could detect no change in the inhibition-constant value for the Tris–cholinesterase–butyrylcholine system when changing the pH value from 7.4 to 8.0 favours a reaction of Tris with the esteratic centres and not with the anionic ones.

In determining the inhibition constants we tacitly supposed an action of Tris molecules on cholinesterases regardless of their state of ionisation. At pH 7.4, 30% of Tris is in its unionised form[1] in equilibrium with its conjugate acid. Presuming an action of the unionised form or of the conjugate acid only the inhibition constants would be 4 mM and 9.5 mM, respectively. Not knowing which of the two forms of Tris is active or if both of them are active the observed inhibition constants of 13 mM represent the apparent inhibition constants.

One interesting point should be mentioned here: when buffer solutions not containing Mg^{2+} and Ca^{2+} were used, an activation of both cholinesterases was observed instead of inhibition. This points to the complexity of the inhibition or activation of cholinesterases in general and to a role for Mg^{2+} and Ca^{2+} in those processes.

ACKNOWLEDGEMENTS

This work was supported by the Boris Kidrič Foundation, Ljubljana, Yugoslavia. The author thanks Miss ERIKA JANŽEKOVIČ for her technical assistance.

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