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Cholinesterase Activity Determined with an Electrometric Method

L.-E. TAMMELIN and B. STRINDBERG

Research Institute of National Defence, Department I, Sundbyberg 4, Sweden

The organo-phosphorus compounds which are used as insecticides or which are potential war gases are all potent cholinesterase inhibitors. Their toxic properties depend more or less on their cholinesterase inhibiting action. Work on these types of compounds requires a rapid and convenient method for determinations of esterase activity. Determinations according to the Warburg manometric technique are very accurate but require specially trained personel. It was felt, therefore, that an investigation of the method reported by Michel [1] would be of interest, the apparatus being a pH-meter.

The activity of a cholinesterase preparation is determined from the pH-change in a buffer solution to which acetylcholine and enzyme have been added. The principles of this method have been published by Michel [1] and our apparatus has been described by Tammelin and Löw [2] in a paper on the calibration of the electrometric method. In the papers mentioned above it has been shown that it is possible to obtain pH as a linear function of time. The purpose of the present paper is to give a detailed description of an electrometric method for esterase determinations, to show some simplifications of Michel's method, and to demonstrate the usefulness of the method for cholinesterase determinations.

Michel performed his measurements in the pH region between 8 and 6, in which range the activity of the cholinesterase varies with the pH level. The composition of the buffer solution counteracts this effect, but some deviation from the straight line remains owing to the pH change. In correcting this error, Michel used a correction factor arbitrarily set at 1.00 at pH 7.00. Since our apparatus permits reproducible registrations in a much smaller range of the pH scale, *i.e.* from 8 to 7.7, this correction can be disregarded, and moreover each test can be carried out in twenty minutes, thus saving

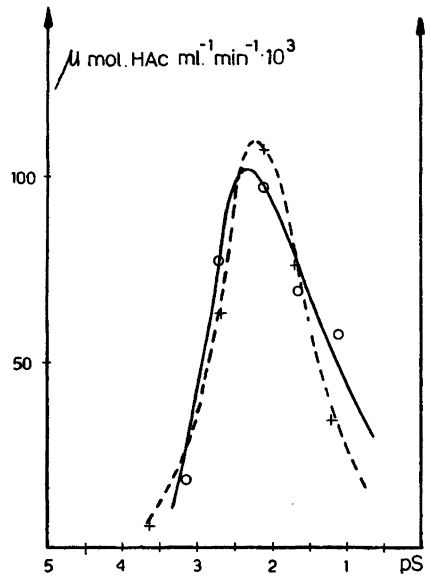


Fig. 1. Enzyme activity as function of pS. Electrometric method denoted by continuous line; manometric method by broken line.

time. The correction for non-enzymatic hydrolysis generally has only a negligible influence on the final value for the enzymatic activity.

The activity of an enzyme preparation was expressed in △ pH per unit of time by Michel, and the values from the manometric technique are originally expressed as b_{30} values according to Augustinsson [3], *i.e.* μl carbon dioxide evolved in 30 minutes from a bicarbonate solution. Both these ways of expressing the activity relate to secondary effects of the hydrolysis of acetylcholine and are incommensurable as absolute values. *A direct and commensurable measure of the activity is the velocity of the development of acetic acid in the solutions expressed in micromoles of acetic acid per ml per minute.* The amount of carbon dioxide obtained with the manometric technique can readily be converted to this measure, but the △ pH values are not directly convertible. If the electrometric apparatus is calibrated according to Tammelin and Löw [2] infusing a solution of acetic acid into the mixture of enzyme and buffer solution, the amount of acetic acid can be set against the slope of the straight line obtained on the recorder paper. The slope of the line is originally determined in cm/cm.

Apparatus and solutions. The apparatus is identical with that described by Tammelin and Löw [2] except for the use of a Micromax recorder. The recorder is connected to the valve potentiometer by a bridge making the total scale of the recorder paper correspond to about two pH units.

The reaction mixture in which the pH determinations were made had the following composition: 3.00 ml Michel's buffer solution, 2.34 ml redistilled water, 0.66 ml enzyme

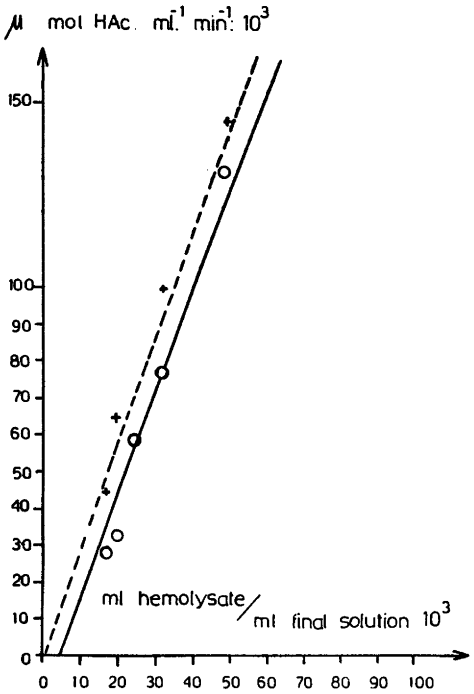


Fig. 2. Enzyme activity as function of hemolysate concentration. Electrometric method denoted by continuous line; manometric method by broken line.

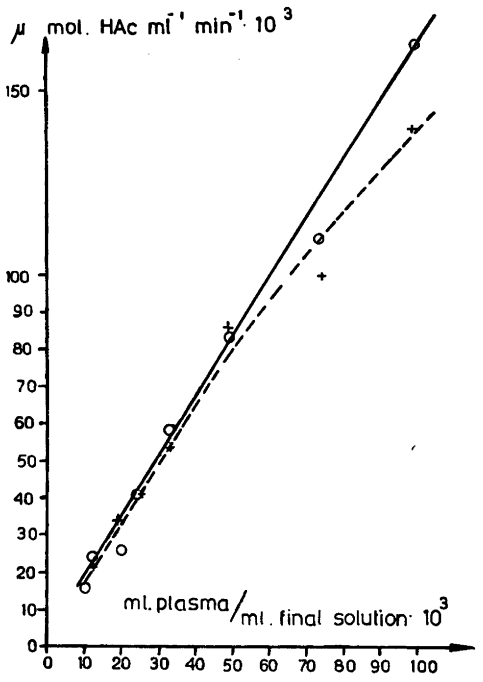


Fig. 3. Enzyme activity as function of plasma concentration. Electrometric method denoted by continuous line; manometric method by broken line.

solution, and 0.60 ml acetylcholine solution. For inhibitor tests the enzyme was suspended in the 2.34 ml of water and 0.66 ml of the inhibitor solution added.

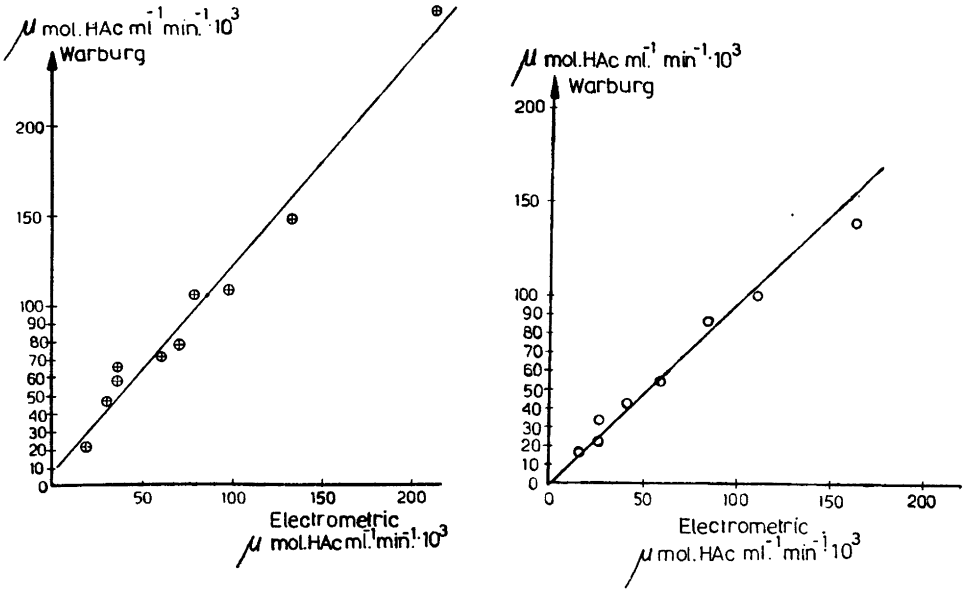
Michel's buffer solution has the following composition: 0.02 *M* sodium barbital (4.1236 g); 0.004 *M* KH_2PO_4 (0.5446 g); 0.60 *M* KCl (44.730 g); 28.0 ml 0.1 *M* HCl solution and water to 1 l.

The reaction mixture in the Warburg vessels has the following composition: 1.60 ml acetylcholine solution (0.100 g per 40 ml Ringer's solution) + 0.40 ml enzyme solution.

Or 1.60 ml acetylcholine solution + 0.20 ml enzyme solution + 0.20 ml inhibitor solution.

The enzyme solutions were prepared from heparinized centrifuged human blood. The supernatant plasma was used directly for the investigations. The red cells were washed four times with saline and then hemolyzed by addition of redistilled water to blood volume. The enzyme solutions were prepared from this stock solution immediately before use.

The substrate solution was made immediately before the experiments. In the electrometric method, 0.110 g acetylcholine iodide was dissolved in 5.00 ml redistilled water. In the Warburg manometric method, 0.100 g was dissolved in 40 ml Ringer's solution.



Figs. 4 and 5. The relation between manometric and electrometric values for the cholinesterase activity. Fig. 4 shows the erythrocyte values and Fig. 5 the plasma values.

Enzyme concentrations and acetylcholine concentrations are varied in the following, but the added volume is in each case consistent with the above composition. For the calibration we used a solution of 0.0504 M acetic acid.

Calibration and formulas. The purpose of the calibration was to elucidate the relation between the slope of the straight line obtained on the recorder paper expressed in cm/cm and the change in the concentration of acetic acid in the test solution. Therefore we immersed the electrode in a solution consisting of 3.00 ml Michel's buffer solution, 2.94 ml water and 0.66 ml plasma solution diluted to three times of the blood volume, or 0.66 ml erythrocyte hemolysate diluted to three times of the blood volume. The acetic acid solution was then infused by means of an Agla Micrometer syringe driven by a synchronous motor. The infusion time for 0.20 ml 0.0504 *M* acetic acid and the slopes of the lines obtained in the recorder were determined in respect of plasma and erythrocytes. Seventeen determinations were made on plasma solution and ten on erythrocyte solution. The mean value of the time readings is 12.7 ± 0.2 minutes for the infusion of 0.20 ml of the acetic acid, and for the corresponding "activity" in cm/cm, the plasma value is 0.38 ± 0.04 and the erythrocyte value 0.39 ± 0.03 cm/cm. There is no significant difference between these values, and the result of the determination is that a velocity of 0.20 ml 0.0504 *M* acetic acid solution in 12.7 minutes gives an "activity" which corresponds to 0.39 ± 0.04 cm/cm.

From this it follows that an activity measured in cm/cm, multiplied by the factor 0.31, gives the activity measured in micromoles of acetic acid per ml per minute. This conversion factor is limited to our special apparatus. However, one centimeter corresponds to 0.059 pH units and on the time axis to 0.98 minutes. The conversion factor

from pH per minute to micromoles of acetic acid per ml per minute is 5.15, and this factor is characteristic of the volume and composition of our reaction mixture. It may be used whenever an electrometric method is employed where time and pH will be determined under the conditions mentioned above. The conversion factors are subject to fairly substantial errors because of the errors in the determinations of infusing time and the obtained corresponding "activities". The errors are sufficiently small, however, to justify use of the factors, as is confirmed by a comparison of the electrometric and the manometric results (see Figs. 2 and 3). The conversion of b_{30} values to micromoles of acetic acid per ml per minute is done by using the factor $7.44.10^{-4}$.

EXPERIMENTAL

In order to find suitable conditions for the determination of the cholinesterase activity, the substrate concentration and the enzyme concentration were varied. According to Augustinsson [3], cholinesterase from erythrocytes shows optimum activity at a certain substrate concentration, since high concentrations give a complex of the enzyme with two molecules of the substrate. This complex cannot break down and yield acid and choline. The concentration of substrate which gives maximum activity is demonstrated by diagrams where the activity is plotted against the negative logarithm of the molar concentration of the substrate (pS). For comparison between electrometric and manometric methods, solutions equimolar in respect of substrate were used in the parallel experiments. The enzyme concentration was 0.033 ml erythrocyte hemolysate per ml of the final solution in the two methods.

Fig. 1 shows that a pS of 2.25 gives optimum activity. A concentration of $7.32.10^{-3}$ M will be obtained if solutions are prepared as recommended under Apparatus and solutions. The difference between the obtained value and the concentration used is negligible.

In order to show the relative activities at various enzyme concentrations, experiments were conducted with different dilutions of the hemolysate and plasma. The results are summarized in Figs. 2 and 3.

The manometric method results in somewhat higher activities in determinations of the specific cholinesterase from erythrocytes, but in the determinations of the non-specific cholinesterase from plasma the methods show the same activity until the manometric technique loses precision because of the high reaction velocity. The slope of the curves in Figs. 2 and 3 is the same for each method and hence the sensitivity of the methods is equal in spite of the higher values for erythrocytes in the manometric technique.

Values obtained by the manometric and the electrometric methods are plotted against each other, in Fig. 4 for erythrocytes and in Fig. 5 for plasma.

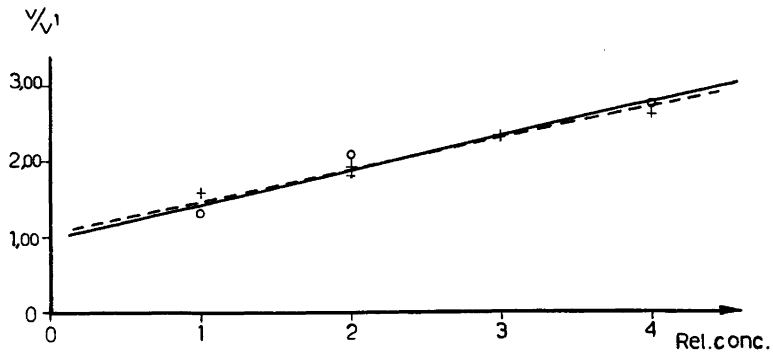


Fig. 6. Inhibition of erythrocyte cholinesterase as a function of the inhibitor concentration. v *is the reaction velocity without inhibitor,* v′ *reaction velocity in the presence of various inhibitor concentrations. Electrometric method denoted by continuous line, manometric method by broken line.*

The plotted values were obtained from the preceding experiments. Diagrams obtained in this way are useful when values of one kind are to be converted to the other.

The molar concentration of an inhibitor reducing the activity of an enzyme preparation by fifty per cent is called I_{50} and is a suitable constant to characterize an inhibitor [3]. In order to compare the two methods, a determination of the value of I_{50} of a preparation containing bis-dimethylamido-phosphoryl fluoride was done manometrically and electrometrically. Only relative values for the activity and the value in cm/cm were used in the electrometric method. These values are very accurate, since no conversion factor is necessary. The results are shown in Fig. 6.

DISCUSSION

The determination of the activity-pS curve shows that the same pS value gives optimum activity in the electrometric and the manometric method. Evidently, the pS value that corresponds to optimum activity is characteristic of the enzyme itself and independent of the buffer solution used. Essential for our comparison of the two methods is Strindberg's [4] demonstration, that potassium of the same concentration as in Michel's buffer may be substituted for the magnesium in the Ringer's solution without effect on the enzymatic activity.

A method for determining cholinesterase activity has to show a linear relation between activity and enzyme concentration within the range of measurements. The Warburg manometric technique shows a good relation,

and Figs. 2 and 3 demonstrate that the electrometric method also is satisfactory in this respect. At very low enzyme concentrations, both methods yield somewhat erroneous results. High concentrations cause a high reaction velocity, and in the Warburg technique the capacity of the manometer limits the measurable enzyme activity. The rapid movement of the manometer fluid makes accurate determinations impossible. The continuous registration in our electrometric method eliminates these disadvantages, and the method is therefore applicable to higher activities than the manometric technique. However, erythrocytes gave a deviation from the straight line at high enzyme concentrations in both methods, perhaps due to a high protein content. Plasma did not show this deviation when the electrometric method was used, but manometric values showed a deviation, presumably for reasons mentioned above.

The main purpose of this investigation was to find a rapid and convenient method for determinations of I_{50} values and studies of the blood of intoxicated animals. Since the results were satisfactory, an apparatus is under construction for simultaneous determinations of six enzyme preparations, thus reducing the time necessary for six determinations to about forty minutes.

SUMMARY

An electrometric method for the determination of cholinesterase activity is described. Values obtained with the electrometric method are compared with manometric values in determinations of enzyme activity as functions of substrate concentrations, erythrocyte concentrations, and plasma concentrations. The accuracy of the methods is discussed.

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