ACTA CHEMICA SCANDINAVICA 15 (1961) 1372–1380

# On the Action of Human Whole Blood and Serum on β-Dimethylaminoethyl *p*-(*n*-amylamino)-Benzoate and Some Homologues

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Blood and serum are shown to hydrolyze β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate and three homologues, *viz.* the *n*-butyl (tetracaine), *n*-hexyl, and *n*-heptyl derivatives. Simple spectrophotometric methods for the determination of the enzymic hydrolysis of β-dimethylaminoethyl *p*-(*n*-alkylamino)-benzoates by serum are described. The velocities of hydrolysis of the four esters are determined in different normal sera. The reaction is shown to be independent of substrate concentration within a concentration range of 1 to 1 000 μg/ml but strongly dependent on pH. Plasma and serum have the same hydrolytic activity towards the esters, whereas blood is only 60 % as active as serum or plasma. The low activity of whole blood depends on the contents of erythrocytes, which do not possess hydrolytic activity towards the present esters. The relative velocities of hydrolysis of the *n*-butyl, *n*-amyl, *n*-hexyl, and *n*-heptyl derivatives are found to be 0.45, 1.00, 0.92, and 0.74, respectively. The absolute values of reaction velocity of the *n*-amyl derivative in different sera range from 5.4 to 11.5 μmole/l · min at 37°C and pH 7.4. Conclusions regarding the relative toxicity of the esters are drawn.

This investigation was undertaken in order to learn about the fate and toxicity of the above-mentioned local anaesthetic esters in blood. Their hydrolysis velocities in blood and serum may give this information, since the hydrolytic products are β-dimethylaminoethanol[1], which is a normal metabolite and *p*-(*n*-alkylamino)-benzoic acids, which are relatively nontoxic. Thus, a high velocity of hydrolysis implies low toxicity. The suggestion that the toxicity of the esters is concerned with the velocity of their enzymic hydrolysis in blood is supported by the work of Foldes [2], which indicates that the toxicity of local anaesthetic agents depends on their plasma concentration, whatever their route of administration. Because of the goal of the investigation no fractionation of the serum to obtain a purified enzyme preparation was performed, nor was the identity of the enzyme with procaine esterase and/or serum choline esterase confirmed [3]. A survey of analytical methods suitable for analysis of substances rela-

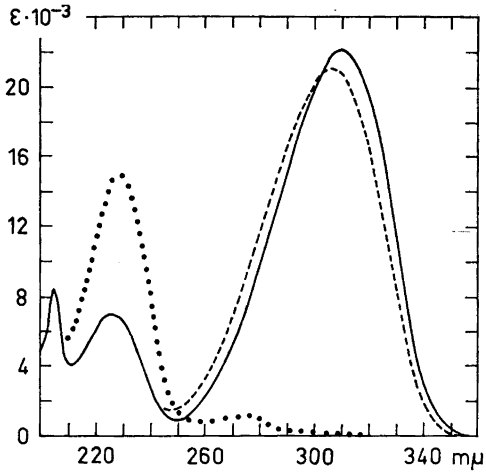


*Fig. 1.* Molecular extinction *vs.* wave length for β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate at three different pH values: . . . pH = 0.2; ——— pH = 6.8; — — — pH = 10.5. Temp. 23°C.

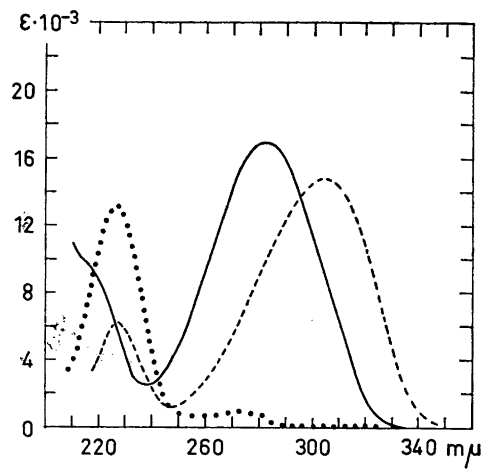


*Fig. 2.* Molecular extinction *vs.* wave length for *p*-(*n*-amylamino)-benzoic acid at three different pH values: . . . pH = 0.2; — — — pH = 3.7; ——— pH = 6.8. Temp. 23°C.

ted to β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate suggested that spectrophotometry would probably be the simplest and most accurate method for the determination of the enzymic hydrolysis of the *n*-butyl (tetracaine), *n*-amyl, *n*-hexyl, and *n*-heptyl derivatives (subsequently to be referred to as compounds 4-R, 5-R, 6-R, and 7-R, respectively). Measurements of the spectrophotometric data of the esters confirmed this suggestion. However, the strongly U.V. light-absorbing proteins of the sera interfered with the absorption measurements of the esters. Two methods were worked out to avoid this interference. One method employed spectrophotometric measurements directly on the incubation mixtures, which consisted of extensively diluted sera and substrates. In this case the possibility of the influence of enzyme-substrate complex formation and U.V. light radiation on the accuracy of the method had to be investigated. The other method involved measurements on deproteinized samples of the incubation mixtures which were then moderately diluted. The two methods were used to elucidate the influence of substrate concentration and pH on the hydrolysis velocities of the esters in human serum, plasma, and blood. In order to obtain information about the normal differences of the hydrolytic activity

*Table 1.* Spectrophotometric data and $pK_a$ values for the esters and their hydrolytic products.

| | Molar ext. × $10^{-3}$ at 310 mμ and pH 7.4 | $pK_a$ for aniline group | $pK_a$ for tert. amine group | $pK_a$ for carboxyl group |
|---|---|---|---|---|
| Tetracaine, 5-R, 6-R, and 7-R | 22.0 | 2.2 | 8.5 | — |
| The corresponding *p*-(*n*-alkylamino)-benzoic acids | 6.1 | 2.5 | — | 4.5 |

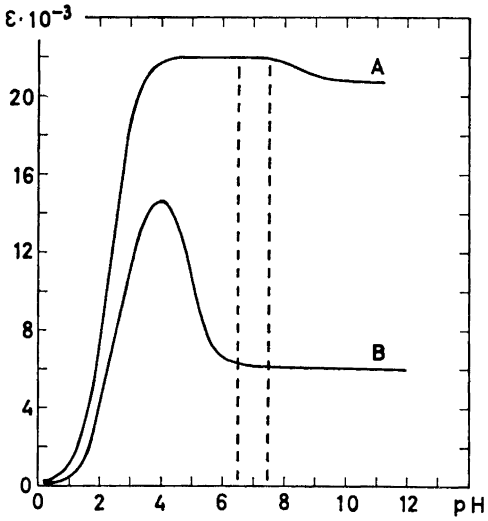


*Fig. 3.* Molecular extinction *vs.* pH at 310 mμ. *A* = β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate. B = *p*-(*n*-amylamino)-benzoic acid. Temp. 23°C.

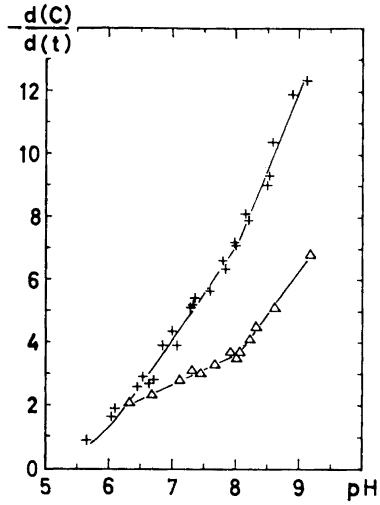


*Fig. 4.* Velocity of hydrolysis of β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate (5-R) *vs.* pH for two sera with different esterase activity. The ordinate refers to μg of the hydrochloride of 5-R hydrolyzed per ml per min at 37°C.

twelve samples of sera from different healthy persons of both sexes were investigated.

To ensure that the enzymic process was a hydrolysis the esters were incubated at 37°C with serum. The U.V. light-absorbing reaction products were isolated (see the experimental part) and identified in three different ways: 1, U.V. absorption spectra were measured at three different pH values and showed to be identical with those obtained with authentic *p*-(*n*-alkylamino)-benzoic acids (Fig. 2); 2, The melting points of the isolated acids coincided with the melting points of the corresponding authentic samples and so did the mixed

*Table 2.* Extinction ratios at 23°C. The figures are valid at pH 5—7.5 for tetracaine, 5-R, 6-R, and 7-R, and at pH 6.5 and upwards for their hydrolytic products.

| | $E_{270}/E_{280}$ | $E_{300}/E_{280}$ | $E_{310}/E_{280}$ | $E_{280}/E_{310}$ |
|---|---|---|---|---|
| Tetracaine, 5-R, 6-R, and 7-R | — | 2.03 | 2.26 | 0.44 |
| The corresponding *p*-(*n*-alkylamino)-benzoic acids | 0.85 | 0.66 | 0.36 | 2.78 |

| | $E_{300}/E_{310}$ | $E_{320}/E_{310}$ | $E_{330}/E_{310}$ |
|---|---|---|---|
| Tetracaine, 5-R, 6-R, and 7-R | 0.89 | 0.88 | 0.52 |
| The corresponding *p*-(*n*-alkylamino)-benzoic acids | 1.87 | — | — |

melting points; 3, The paper-ionophoretic mobilities of the isolated substances were the same as for the authentic samples.

The U.V. absorption spectra measurements of tetracaine [4], 5-R, 6-R, and 7-R showed that the extinction coefficients were identical at different pH values throughout the range tested and thus the $pK_a$ values of the ionizable groups (the aniline and tertiary amine groups). (See Figs. 1 and 2, and Tables 1 and 2.) This is also the case with the U.V. light-absorbing hydrolytic products, the *p*-(*n*-alkylamino)-benzoic acids. (Figs. 2 and 3, and Tables 1 and 2.) β-Dimethylaminoethanol has practically no absorption in the region above 260 mμ. As is evident from Figs. 1, 2, and 3, 310 mμ is a suitable wave length for concentration measurements if the pH value lies between 6.5 and 7.5. Thus, the spectrophotometric data provide a sound basis for the determination of the rates of hydrolysis of the four esters.

However, the difficulty of getting rid of the very strongly U.V. light-absorbing proteins of the sera also had to be overcome. (Too high background extinction will cause great uncertainty in the extinction readings originating from the esters or even make extinction measurements impossible.) This difficulty was overcome in two ways. The first included use of dilute sera (dilution 1:50). The second involved denaturation of the interfering protein material with 7 % perchloric acid, subsequent centrifugation and absorption measurements at 23°C on the appropriately diluted and buffered supernatants.

By using dilute sera it was possible to measure the absorption changes of the incubation mixtures directly in the cuvettes, which in this case were held at 37°C by means of a thermostatically controlled cuvette housing. (All incubations were performed at 37°C.) In this way the rates of hydrolysis were measured at starting concentrations of the esters ranging from 1—10 μg/ml. The second method had to be used at higher ester concentrations. The lowest limit of substrate concentration, 1 μg/ml, is given by the magnitude of the extinction coefficients, the upper limit by the solubility of the hydrolytic products (the *p*-(*n*-alkylamino)-benzoic acids). The solubility of the amyl-, hexyl-, and heptylaminobenzoic acids at 25°C and pH 7.5 are about 1 000, 500, and 330 μg/ml, respectively.

Enzyme-substrate complex formation sometimes interferes with the measurement of the absorption of the substrate. Therefore it is necessary to consider this possibility whenever spectrophotometry is carried out directly on an incubation mixture. Such disturbances were without significance in the first case described above, as the extrapolated "starting" extinctions of the incubation mixtures and the extinctions at the end of the hydrolysis followed the calculated values well within the limits of the accuracy of the method.

It is well known that U.V. radiation denatures proteins. However, any influence of the light of the spectrophotometer on the enzyme activity was not detected, presumably due to the short exposure time and the low intensity of the light beam.

In the second case the values of the "starting" extinctions (extrapolated) and the "end" extinctions did not reach the calculated values as in the first case (for tetracaine, however, they did). The obtained values were too low; 5-R gave 3—7 %, 6-R 7—13 %, and 7-R about 20 % too low values. These findings may be explained to be due to some kind of linking between the dena-

tured protein and the substrates. Corrections for the discrepancies were simply made for every determination by extrapolation of the extinctions to zero time and comparison with the calculated value. Moreover, the obtained correction factors were the same in different experiments with the same substrate, provided that the relation between serum concentration and perchloric acid concentration was identical.

The determinations of the reaction velocities were in most cases performed with serum as the enzyme source. However, to get a correlation between the rates of hydrolysis in serum and whole blood additional determinations on blood were necessary. As the whole blood was heparinized the action of heparin on the reaction velocity was also investigated. The heparinized and nonheparinized blood (from which serum was obtained after coagulation) was taken from the same individuals on the same occasion. Blood from three different persons was investigated to obtain a sure correlation. Serum, plasma (from centrifugation of heparinized blood) and serum + heparin (0.5 mg/ml) hydrolyzed each of the esters with identical velocities. The pH was carefully controlled (*cf.* Fig. 4). Thus heparin did not affect the hydrolysis. In blood the hydrolysis proceeded with velocities which were only 60 % of those obtained with serum or plasma. Temperature, pH, and substrate concentration were identical in the different cases, *viz.* 37°C, 7.4, and 0.25 mM. As 40—45 % of the blood volume is occupied by erythrocytes, it may be concluded that these do not possess hydrolytic activity towards the present esters. This was confirmed through experiments with washed erythrocytes, hemolysate, and erythrocyte cell membranes ("ghosts" which contain the specific acetylcholinesterase of erythrocytes). The reason why whole blood was not chosen as the enzyme source in all determinations was that its high protein content (about 20 % whereas serum has about 7 %) made greater dilution necessary and hence the reaction time much longer. Moreover, direct spectrophotometry at low substrate concentrations was impossible to perform due to the strong absorption and the heterogeneity of blood.

Dilution of serum did not affect the activity of the hydrolytic enzyme(-s) if the dilution was not very great. In these experiments the pH was in each case carefully adjusted to that of the undiluted serum *i.e.*, between 7.6 and 7.8. A fivefold dilution of the serum decreased the reaction velocity to 20 % of the original value as expected, whereas a fiftyfold dilution decreased the reaction velocity to 1.70—1.75 % of the original value (expected value 2 %). The buffer system used in these experiments was 0.05 M trishydroxymethylaminomethane-HCl. The possibility of increasing the enzyme activity to the expected value by using other ionic strengths of the buffer solution or other buffer systems was not investigated.

## RESULTS AND DISCUSSION

The relation between reaction velocity and pH was determined at a substrate concentration of 0.25 mM (the starting value) and a serum to buffer ratio of 1:1. Acetate buffers, 0.1 M, were used at pH 7 and below, 0.05 M "tris" buffers between pH 7 and 8.5 and glycine buffers above pH 8.5. As the buffering capacity of serum is rather great, the pH of the serum-buffer mixtures

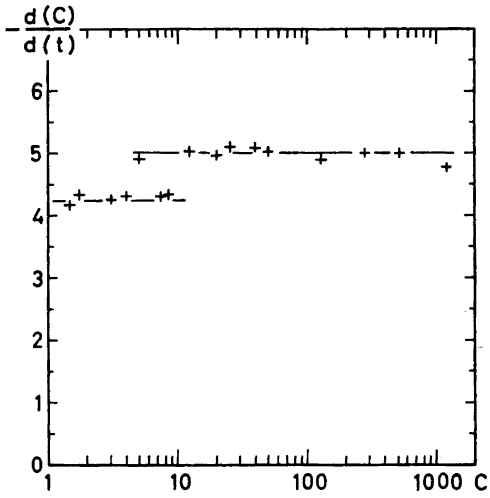


*Fig. 5.* Velocity of hydrolysis of β-dimethylaminoethyl *p*-(*n*-amylamino)-benzoate (5-R) at pH 7.4 and 37°C *vs.* substrate concentration. C = μg of the hydrochloride of 5-R per ml. $-\frac{d(C)}{d(t)}$ = μg of the hydrochloride of 5-R hydrolyzed per ml per min. The two parts of the diagram correspond to experiments at different dilutions of the serum used. The right part was performed at a serum to buffer ratio of 1:1, the left part at a ratio of 1:49. The obtained values have been converted to those assumed for undiluted serum and plotted. See text.

had to be controlled at 37°C (the incubation temperature) before every experiment. The buffer systems used were without influence on the reaction velocity. This was shown for the "tris" buffer (see above) and then for the other buffers by comparison with the "tris" buffers in the pH regions where both "tris", acetate, and glycine buffers, respectively, were utilizable. Two characteristic curves of the reaction velocity-pH dependence for 5-R (two different sera) are shown in Fig. 4. Two other sera were investigated in the same way. Intermediate values of the slopes were obtained. The differences of the slopes are hard to explain, however, similar results were obtained with the other esters. Some considerations may be made. As the reaction apparently follows zero order kinetics (see below), the variation of the ion species of the substrates ($pK_a = 8.5$) cannot be responsible for the great pH dependence of the reaction velocity. It is more adequate to ascribe this dependence to ionization phenomena of groups at the active site of the enzyme (*cf.* Krupka and Laidler [5]). In this connection it is important to stress that nonenzymic hydrolysis is entirely negligible in the pH-range investigated [4], which was shown by experiments performed at 37°C in plain buffer solutions and in sodium arsenite-poisoned sera. More definite explanations of the strong pH dependence may not be given until highly purified and homogeneous enzyme preparations are available for investigation. The practical consequence is quite obvious: pH has to be carefully controlled in order to get a satisfactory comparison of the velocity of hydrolysis between different sera and with different substrate concentrations.

The correlation between reaction velocity and substrate concentration for 5-R is shown in Fig. 5. The left part of the curve was obtained according to the first method described above (*i.e.*, direct spectrophotometry on mixtures with diluted sera), the right part according to the second method. The influence of extensive dilution on the reaction velocity is obvious. The shape of the curves for tetracaine, 6-R, and 7-R was exactly the same as for 5-R. These curves are not plotted in Fig. 5 for the sake of clarity. The reaction follows "zero" order

*Table 3.* Velocity of hydrolysis of tetracaine (4-R), 5-R, 6-R, and 7-R at 37°C and pH 7.4. The figures refer to μg of substrate (hydrochloride) hydrolyzed per ml of undiluted serum per min. The velocity is independent of substrate concentration.

| Serum No. | 4-R | 5-R | 6-R | 7-R |
|---|---|---|---|---|
| 1 | 1.44 | 2.98 | 2.82 | 2.3 |
| 2 | 1.40 | 3.10 | 2.56 | 2.2 |
| 3 | 1.70 | 3.84 | 3.46 | 2.5 |
| 4 | 1.72 | 3.88 | 3.76 | 2.9 |
| 5 | 1.94 | 4.18 | 4.06 | 3.0 |
| 6 | 2.08 | 4.54 | 4.20 | 3.0 |
| 7 | 2.26 | 5.32 | 4.54 | 4.0 |
| 8 | 2.58 | 5.70 | 5.60 | 4.0 |
| 9 | | 2.90 | | |
| 10 | | 3.10 | | |
| 11 | | 5.00 | | |
| 12 | | 5.30 | | |

kinetics throughout the substrate concentration range investigated. This range extends from 1—1 200 μg/ml for tetracaine and 5-R, from 1—500 μg/ml for 6-R, and from 1—20 μg/ml for 7-R. (The figures refer to μg of the hydrochlorides of the substances.) Thus, the Michaelis-Menten constant cannot be calculated from the obtained data. It is possible, however, to establish that the value of the constant is less than $2 \times 10^{-6}$ mole/litre which indicates a great affinity of the substrates for the enzyme.

In Table 3 are summarized the results from experiments with different sera and substrates. It is seen from the table that tetracaine was hydrolyzed at a speed of about 45 % of that of 5-R. The corresponding figures for 6-R and 7-R were 92 % and 74 %. The velocities of hydrolysis in whole blood are 60 % of those in sera (see above) and thus the ranges of these velocities in blood for tetracaine, 5-R, 6-R, and 7-R are 0.8—1.6 μg/ml · min, 1.7—3.5 μg/ml · min, 1.6—3.2 μg/ml · min, and 1.3—2.6 μg/ml · min, respectively.

Due to the high decomposition rates of these esters in the vascular system they do not accumulate and hence they are assumed to be comparatively non-toxic if not given in massive doses. Evidently toxicity also concerns the varying influence on relevant enzyme systems. In this connection it is of interest to consider the strongly decreased activity of the specific acetylcholinesterase caused by the esters. The ester concentrations that cause 50 % inhibition of the activity of this enzyme are $0.32 \times 10^{-3}$ M, $0.18 \times 10^{-3}$ M, $0.95 \times 10^{-4}$ M, and $0.55 \times 10^{-4}$ M for tetracaine, 5-R, 6-R, and 7-R, respectively [6]. If these figures are assumed to be representative for the potencies of the esters with regard to their action and toxicity, some conclusions may be drawn. Tetracaine is hydrolyzed half as rapidly as 5-R and is about half as active as an enzyme inhibitor. Therefore the toxicity may presumably be the same for these two esters. $LD_{50}$ has been determined to 0.683 ± 0.019 g/kg for tetracaine and to 0.672 ± 0.028 g/kg for 5-R (oral administration to rat). In regard to the above assumption 6-R ought to be twice and 7-R four times as toxic as 5-R or tetracaine. Experiments on intravenous administration of tetracaine to homo for the purpose of comparing the toxicity of some local anaesthetics have revealed that doses of 35—60 mg given at a rate of 0.125 mg/kg · min involve no danger [2]. Most probably these figures also apply to 5-R when administered in the same way.

## EXPERIMENTAL

*Materials.* The blood was taken from the antecubital vein of healthy persons of both sexes. Plasma was obtained through centrifugation of heparinized blood and serum from coagulated blood. When stored at 0°C the esterase activities of the sera were unchanged for at least 14 days provided the preparations were performed aseptically. The esters were synthesized in this laboratory according to the methods described in Swed. pat. 5561/59 and 5339/60. The *p-n*-alkylaminobenzoic acids were formed through boiling slightly alkaline sodium *p*-aminobenzoate solutions with *n*-alkylbromide under reflux. The acids were isolated as precipitates since the pH had been adjusted to 3.8 and the temperature to about 0°C. (Fig. 3, curve B). The washed precipitate was recrystallized from ethanol-water. (*Cf.* the above-mentioned patents.) The melting points were 135°—137°C, 122°—124°C, and 107°—109°C for *p-n*-amyl-, *p-n*-hexyl-, and *p-n*-heptylaminobenzoic acid respectively.

The acids were also prepared through enzymic hydrolysis. Mixtures of 50 ml serum and 50 ml 0.05 M "tris"-buffer (pH of the mixtures was 7.4) were shaken with the esters (100 mg) at 37°C for suitable lengths of time. The incubation mixtures were poured into vigorously stirred 100 ml portions of 7 % perchloric acid and the denaturated protein centrifuged off. The pH of the clear supernatants was adjusted to 3.8 with sodium hydroxide. The precipitates were collected after cooling, washed and recrystallized as above. The recoveries were 80 to 90 %. The melting points and mixed melting points coincided with those given above.

Other substances used were commonly available *p.a.* reagents.

Extinction measurements were performed with the Beckman model DU spectrophotometer provided with a cuvette housing whose bottom and walls were attachable to a water thermostat. It was possible to hold the desired temperature in the cell compartment within ± 0.05°C with this arrangement.

A pH-meter (Radiometer, Copenhagen) with glass electrode was used for pH determinations.

Continuous shaking of the incubation mixtures (the second case) was obtained with a "Microid" flask shaker (Griffin and George, London) provided with a suitably designed simple attachment.

Centrifugations were performed with a MSE Minor centrifuge (London).

The incubation temperature was held at 37.0° ± 0.05°C by means of an open water bath in which the reaction flasks were dipped (the second case).

All volumes were measured with calibrated pipettes. Voll pipettes were used for volumes greater than 2 ml and constriction pipettes ("Carlsberg pipettes") for 2 ml and smaller volumes. The error of one measurement of volume therefore was within ± 0.1 %.

Appropriate considerations were taken of the differences in temperature of the fluids to be measured.

*Procedure for the first method.* One part of serum was diluted to 50 parts with 0.05 M trishydroxymethyl aminomethane buffer and the pH was controlled at 37°C. 3 ml of this mixture were transferred to a 10 ml conical flask which was then shaken at 37.0°C for 10 min. 0.1 ml of the desired ester solution (in *aq. dest.*) was now added, the time was noted ("zero time"), and the mixture was quickly transferred into the cuvette (which was held at 37.0°C in the thermostatically controlled cuvette housing) with a suitable, prewarmed pipette. The extinction was read at 310 mμ against a similarly treated blank (water instead of ester solution) at intervals. The correctness of the blank was controlled at 360 mμ. The extinction readings at this wavelength ought to be zero (see Figs. 1 and 2). If they were not, the readings at 310 mμ were corrected according to the values obtained at 360 mμ.

*Procedure for the second method.* One part of buffer was mixed with one part of serum and the pH was controlled at 37.0°C. 3 ml of the mixture were transferred to a 10 ml small-necked conical flask which was stoppered and brought to 37.0°C as above. At zero time 0.1 ml ester solution were added. Aliquots of 0.5 or 0.2 ml (depending on substrate concentration) were withdrawn at intervals and denaturated through pouring into 0.5 ml portions of chilled (0°C) 7 % perchloric acid in small test tubes which were stored in an ice bath. Every withdrawal of a sample from the incubation mixture did not last more

than 20 sec. The times when half the parts of the aliquots had been denaturated were noted. The samples were shaken vigorously for 8 min and centrifugated. Then 0.5 or 0.8 ml of the supernatants were transferred to suitable volumes of 0.3 M phosphate buffer pH 7.5 and measured against a blank prepared in the same way. In this case the accuracy of the blank had to be controlled at 360 mμ for every sample and corrections had to be made if deviations from zero were found (see above).

Example: At a substrate concentration of 76.2 μg/ml of 5-R, 0.5 ml aliquots of the incubation mixture were denaturated with 0.5 ml of 7 % perchloric acid. Thereafter 0.5 ml of the supernatants of the denaturated samples were buffered with 3 ml phosphate buffer each. A dilution of 1:14 was thus obtained. The plot of extinction *versus* time gave a fall in extinction of 0.261 in 36 min. The calculated extinction at the beginning of the experiment was 0.379, the obtained value 0.365. Thus the correction factor was 0.379: 0.365 = 1.04. The molecular weight of 5-R (hydrochloride) is 314.8 and the molar extinction $22.0 \times 10^3$. The molar extinction of the hydrolysis product is 6.1 and hence the "molar extinction change" due to hydrolysis $15.9 \times 10^3$ at 310 mμ. The velocity of hydrolysis was $14 \times 0.261 \times \frac{1}{36} \times \frac{1}{15.9} \times 1.04 \times 314.9 = 2.09$ μg/ml · min. (The molecular weights of the hydrochlorides of tetracaine, 6-R, and 7-R are 300.8, 328.9, and 342.9, respectively.) In the undiluted serum the reaction velocity at the same pH ought to be $2.09 \cdot \frac{3.10}{1.50} = 4.32$.

*Estimation of errors.* An estimation of the maximal error of a single extinction determination involves the following points:

| | |
|---|---|
| 1: Dilution errors | ± 1 % |
| 2: Accuracy of spectrophotometry | ± 2 % |
| 3: Blank error | ± 1 % |
| 4: Time error (depends on time for complete hydrolysis) | ± 0.5 – ± 3 % |
| 5: Error due to uncertainty in pH determination (See Fig. 4) | ± 2 % |

A value of reaction velocity was obtained from the slope of the straight line: Extinction = f(time). This line was drawn by means of at least six different points and every value of reaction velocity was the average of two determinations. Hence, the error of the reported reaction velocities most probably is considerably below ± 7 %.

*Determination of acetylcholinesterase activity.* In these experiments the specific acetylcholinesterase of human erythrocyte cell walls ("ghosts") was used as enzyme and acetylcholine chloride as substrate. The esterase activity was determined with common Warburg technique [7]. The inhibitor concentrations that effected 50 % inhibition of the enzyme activity were determined at a substrate concentration of $3.30 \times 10^{-3}$ M and at an enzyme concentration such that 44 % of the substrate was hydrolyzed in 30 min. Further details will be published elsewhere.

*Acknowledgement.* The author gratefully acknowledges the assistance of Miss B. Olsson and Mr. B. Bunke.

## REFERENCES

1. Pepen, G., Freedman, D. X. and Giarman, N. J. *J. Pharmacol. Exptl. Therap.* **129** (1960) 291.
2. Foldes, F. F., Molloy, R., McNall, P. G. and Koukal, L. R. *J. Am. Med. Assoc.* **172** (1960) 89/1493.
3. Kalow, W. *J. Pharmacol. Exptl. Therap.* **104** (1952) 122.
4. Karlén, B. and Ågren, A. *Acta Chem. Scand.* **14** (1960) 197.
5. Krupka, R. M. and Laidler, K. J. *Trans. Faraday Soc.* **56** (1960) 1477.
6. Persson, K. Ö. U. *Unpublished paper.*
7. Augustinsson, K. B. *Acta Physiol. Scand.* Suppl. 52 (1948).

Received February 6, 1961.