Hydrolysis of Succinyldicholine and Succinylmonocholine in Human Serum

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SUMMARY

The enzymatic hydrolysis of the muscle relaxant suxamethonium (succinyldicholine) and the first product of its degradation, succinylmonocholine, in sera of different pseudocholinesterase phenotypes have been investigated. Kinetic data were obtained, using ^{14}C-labeled substrates, thus making the method very sensitive and accurate at concentrations of pharmacologic interest. Radioactivity of substrates and split products were estimated after separation by high voltage electrophoresis on paper. The results give much evidence that in human serum both esters are hydrolyzed by pseudocholinesterase.

INTRODUCTION

Since Glick in 1941 (1) observed the enzymatic hydrolysis of succinyldicholine in horse serum, and Bovet-Nitti (2) showed the powerful blocking properties of this drug, some investigators have studied by various methods the fate in the body and the kinetics for both the drug and its degradation product succinylmonocholine (3–8). The methods used did not allow exact measurements below 10^{-4} *M* succinyldicholine. On the other hand, the concentrations expected during anesthesia are lower. For this and other reasons we tried to find a method to measure the hydrolysis of succinyldicholine at concentrations down to 10^{-6} *M*. Using a modified method of Neubert *et al.* (9), ^{14}C-methyl-labeled succinyldicholine (specific activity, 5–10 mC/mmole) can be separated from its split products choline and succinylmonocholine by high voltage electrophoresis. The limit for the detection of succinyldicholine by this method can be brought to about 10^{-5} μmoles. Thus there arises the possibility of studying the pharmacologically interesting kinetics at such low concentrations. This method is described, and that of another more semiquantitative assay as well as some kinetic data on the hydrolysis of the two succinylcholine esters in sera of different pseudocholinesterase phenotypes.

METHODS

Direct Method

Incubation assay. Human serum or purified human pseudocholinesterase is incubated with succinyldi(choline-methyl-^{14}C-iodide) (from The Radiochemical Centre, Amersham; specific activity, 8.6 mC/mmole) in 6.7×10^{-2} *M* phosphate buffer pH 7.4. In a second incubation, serum or purified enzyme is omitted to get data on the spontaneous hydrolysis of the substrate under the experimental conditions. After incubation, the protein is precipitated with concentrated sulfuric acid. Subsequent to centrifugation, a sample of the supernatant solution (0.02–0.04 ml) is taken for electrophoretic separation of substrate and reaction products. Acid precipitation of the protein is not necessary if the sample is subjected to electrophoresis in a well-defined short time of about half a minute and if the enzyme concentration is low and

the electrophoresis chamber cooled to below 0°.

High voltage electrophoresis. Strips of Whatman paper No. 3 MM (4 × 42 cm) are soaked with 0.05 *M* citrate buffer pH 5.15 and passed between two rubber rollers to remove excess of buffer solution and to get an equally distributed humidity on the paper. Then the strips are rolled gently on a glass plate, which is set into the electrophoresis chamber, cooled at −8° (Pherograph Original Frankfurt, Hormuth & Vetter Ind.). Samples of 0.02–0.04 ml are applied within 3–8 min at a line a few centimeters from the anodic end of the paper. The anodic as well as the cathodic ends of the papers are connected with strips (2.5 × 35 cm) of MN paper for electrophoresis (Machery & Nagel, Düren, Germany), soaked with 0.05 *M* citrate buffer. Platinum block electrodes fixed to polyvinylchloride rods are pressed on these thick paper strips with a heavy glass plate. Then, electrophoresis is started at 40 V/cm and 7–9 mamp/strip. The whole procedure before starting electrophoresis must be done quickly, otherwise the buffered papers will freeze on the cooling plate. The same will happen if the current is too low (<6 mamp). After 25 min, choline is separated from succinylmonocholine, after 40 min succinyldicholine is separated from succinylmonocholine, and after 90 min choline is separated from succinyldicholine.[1]

Evaluation of the electropherogram. After electrophoresis, the papers are dried at room temperature and the radioactive spots are identified by means of a strip scanner (see Fig. 1). For a quantitative estimation of the split products, the electropherogram is cut into small pieces of 2 × 1 or 4 × 1 cm, which are subsequently counted by the liquid scintillation spectrometer (Packard Tri-Carb 3000) in 12 ml of toluene–PPO[2]–POPOP scintillator (17% gain, window 70–1000/350–1000 with background substraction).

The counts of succinylmonocholine or choline, respectively, multiplied by two and divided by total counts give the proportion of converted succinyldicholine (no hydrolysis of succinylmonocholine predicted). The peak of succinyldicholine sometimes interferes with the peak of succinylmonocholine when block electrodes are used. The choline peak has almost no tailing. Therefore, one gets better results relating the counts of the choline peak to total counts. Using block electrodes, no such difficulties arise when only succinylmonocholine is separated from choline.

Preparation of Succinylmonocholine

Succinyldi(choline - methyl-^{14}C - iodide), 5.0×10^{-3} *M* was incubated 30 min in 6.7×10^{-2} *M* phosphate buffer pH 7.4 with serum (phenotype U, diluted 1:2) at 37°. The protein was precipitated with concentrated sulfuric acid. After centrifugation, the supernatant solution was subjected to electrophoresis as described above, and the succinylmonocholine, identified by means of radio paper chromatography, was eluted with H_2O. The eluate was concentrated by freeze drying. Later experiments were done with succinylmono(choline-methyl-^{14}C-iodide) (specific activity: 1 mC/mmole) from the Farbwerke Hoechst. Nonradioactive succinylmonocholine chloride was obtained from Lentia (Linz, Austria).

Indirect Method

The principle of this method is based on the fact that succinyldicholine, in close relation to its concentration, inhibits the enzymatic activity of pseudocholinesterase on benzoylcholine. From a calibration curve giving the percent inhibition of the enzymatic hydrolysis of benzoylcholine in relation to the concentration of added succinyldicholine, the decrease of succinyldicholine can be calculated. With this method it is also possible to determine easily, although not very accurately, the concentration of succinyldicholine in fluids which do not contain other inhibitors or substrates of

[1] Often the separation of choline and succinyldicholine is unsatisfactory if block electrodes are used. Separation should be done by means of electrode vessels connected with the strips by paper or other suitable bridges.

[2] PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(5-phenyloxazolyl)benzene.

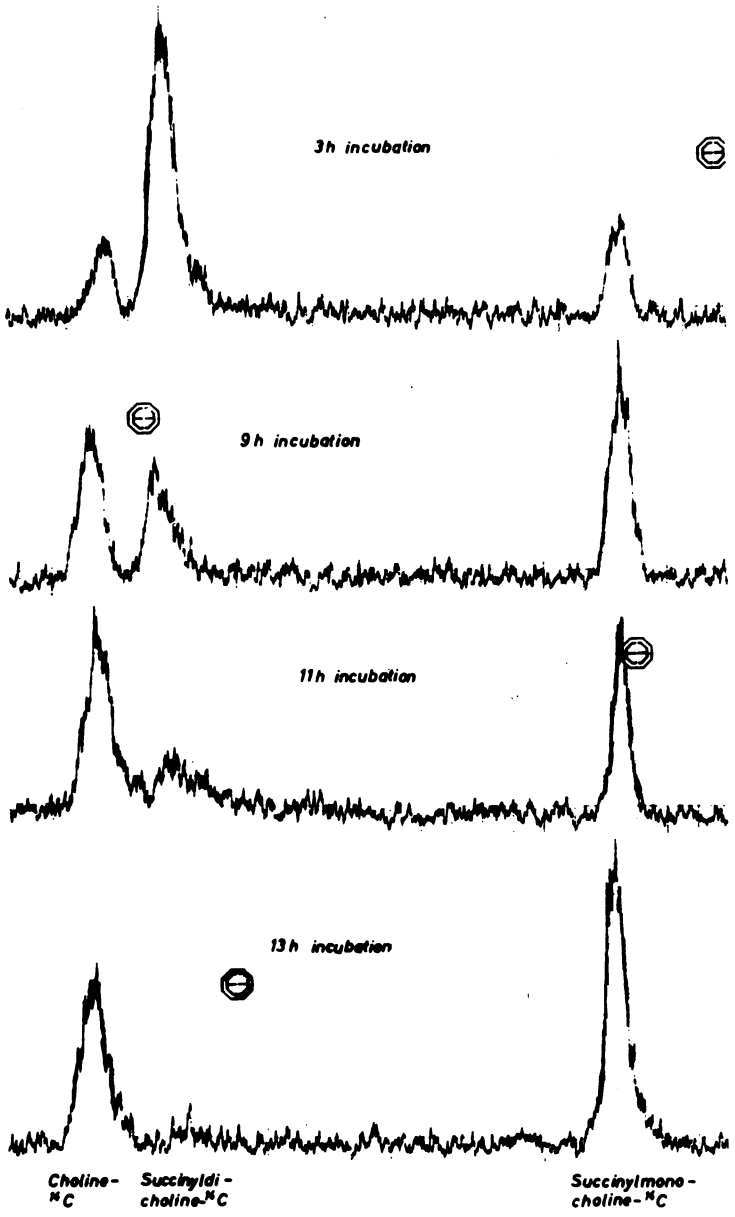

FIG. 1. *Radiopaper electropherogram of succinyldicholine-^{14}C and split products after different incubation times with serum of phenotype U (10)*

Ordinate: counts per minute.

pseudocholinesterase. However, this method is a semiquantitative one.

Calibration curve. The calibration curve is estimated using the standard spectrophotometric test for pseudocholinesterase with benzoylcholine as substrate according to Kalow (11). The percent inhibition is determined by use of the equation:

$$\text{Inhibition (\%)} = 100\left(1 - \frac{\text{reaction with succinyldicholine}}{\text{reaction without succinyldicholine}}\right)$$

A calibration curve determined in this way is given in Fig. 2.

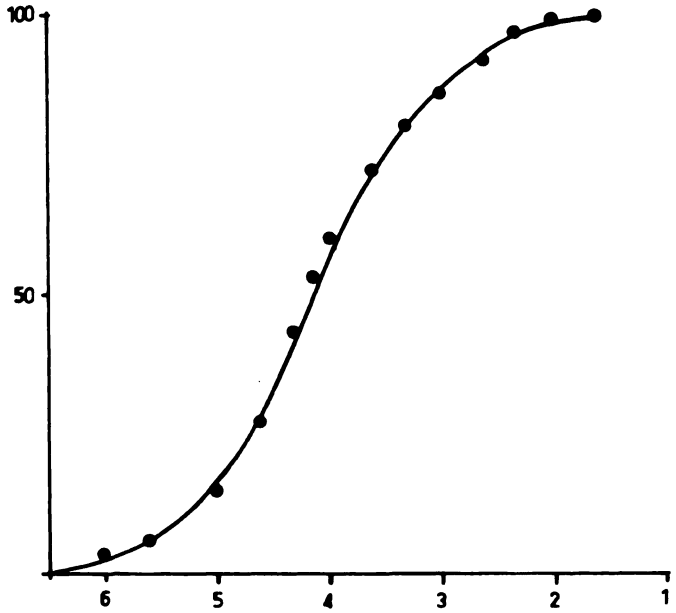

FIG. 2. *Calibration curve for indirect determination of succinyldicholine*

Abscissa: log concentration of succinyldicholine. Ordinate: percent inhibition of the reaction of pseudocholinesterase with benzoylcholine ($5 \times 10^{-6} M$).

Assay for measuring the activity of pseudocholinesterase on succinyldicholine. Procedure: Use 0.5 ml succinyldicholine chloride (Fluka, Buchs, Switzerland) in *M*/15 phosphate buffer pH 7.4 + 1.0 ml serum 1:100 or purified pseudocholinesterase in buffer. Incubate at 26° for the desired time interval. Then add 0.5 ml of benzoylcholine $2 \times 10^{-4} M$ and measure the enzymatic activity with benzoylcholine as substrate as described.

Comparing this activity with that of the reaction without succinyldicholine, the percent inhibition can be calculated and the calibration curve will show the final concentration of succinyldicholine. This concentration, corrected for dilution, can be compared with the initial concentration of succinyldicholine, thus giving a standard for the enzymatic activity with succinyldicholine after correction for spontaneous hydrolysis.

The method can be modified for various purposes. The reaction of pseudocholinesterase on succinyldicholine can be stopped by acid denaturation of the protein. After centrifugation and adjustment of the supernatant to pH 7.4, 0.5 ml of the supernatant is added to 0.5 ml $2 \times 10^{-4} M$ benzoylcholine and 1 ml of a standard enzyme solution. Using always the same purified pseudocholinesterase sample for this variation of the assay, only one calibration curve is necessary. Otherwise for each new enzyme source a special calibration curve ought to be established; this is very time-consuming in routine work. For the determination of unknown concentrations of succinyldicholine in fluids, the latter are ultrafiltered, 0.5 ml of the filtrate is added to the reaction mixture, and the concentration is calculated as described above. This indirect method is not very accurate and depends on the total absence of any inhibitors other than succinyldicholine. However, the method may be useful if no special laboratory equipment is available for more precise measurements, such as for titrimetric, manometric, or radioactivity measurements.

Purification of Pseudocholinesterase

Purified normal pseudocholinesterase has been prepared by ammonium sulfate fractionation at different pH, adsorption on

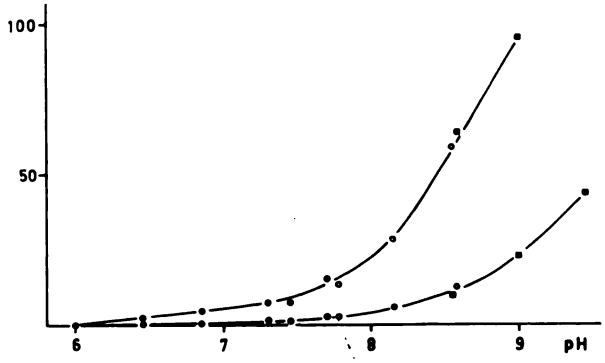

FIG. 3. *pH dependence of spontaneous hydrolysis of succinyldicholine-^{14}C (10^{-5} M, upper curve) and succinylmonocholine-^{14}C (5×10^{-5} M) at 37°*

Succinyldicholine-^{14}C was incubated for 1 hr; succinylmonocholine-^{14}C for 3 hr. Ordinate: Percentage relation of choline-^{14}C to substrate. ●, 0.1 *M* phosphate buffer; ○, 0.1 *M* Tris-HCl buffer; ■, 0.1 *M* glycine buffer.

Al-C_γ-gel followed by adsorption on hydroxylapatite and stepwise as well as gradient elution with increasing phosphate concentrations or chromatography on DEAE-cellulose and gel filtration on Sephadex G-200. Specific activities of about 1000–4000 times that of normal serum are thus easily obtained. For about 1200-fold purification see Goedde *et al.* (12); details for further purification will be published elsewhere.

Preparation of Antiserum against Human Pseudocholinesterase

The antiserum was obtained from rabbits immunized with 500-fold purified human pseudocholinesterase (phenotype U), as described (12, 13).

RESULTS

Spontaneous Hydrolysis of Succinyldicholine and Succinylmonocholine

The spontaneous hydrolysis of both esters at pH values ranging from 6.0 to 9.5 in buffer solutions without protein is shown in Fig. 3. The hydrolysis of the dicholine ester is always much higher than that of the monocholine ester; for instance at pH 7.4 in 0.1 *M* phosphate buffer 6% of succinyldicholine and only 0.5% of succinylmonocholine are hydrolyzed within 1 hr. The hydrolysis of succinyldicholine rises quickly above pH 8, that of succinylmonocholine above pH 8.5.

pH optima of the Enzymatic Hydrolysis of Succinyldicholine and Succinylmonocholine in Human Sera

The pH optimum for the enzymatic breakdown of the dicholine ester in serum (phenotype U) is very similar to that of other choline esters. The data illustrated in Fig. 4 give maximum values between pH 8.0 and 9.0. It was not possible with this

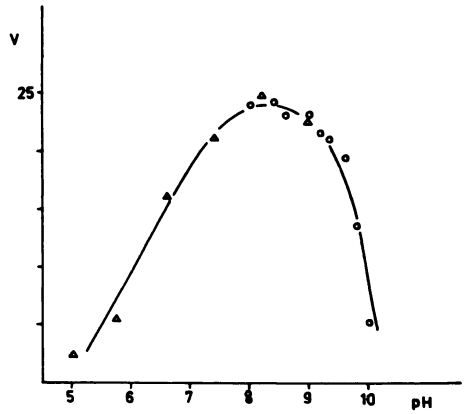

FIG. 4. *pH optimum for enzymatic hydrolysis of succinyldicholine-^{14}C in ierum (phenotype U; 1:50)*

Substrate concentration: 5×10^{-5} *M*; ordinate: *V* = μmoles of succinylmonocholine-^{14}C per liter for 15 min at 37°. △, *M*/15 phosphate buffer; ○, *M*/15 glycine buffer.

method to get data of the corresponding pH optimum in serum of the pseudocholinesterase phenotype A because this enzyme variant is active only at high substrate concentrations, such as 10^{-2} *M* succinyldicholine. The enzymatic hydrolysis in these sera is too low to be determined with confidence by this method (see below). Figure 5 shows the pH optima for the enzymatic breakdown of succinylmonocholine in sera of the pseudocholinesterase phenotype U and A. Both optima were found between pH 5.0 and 6.0.

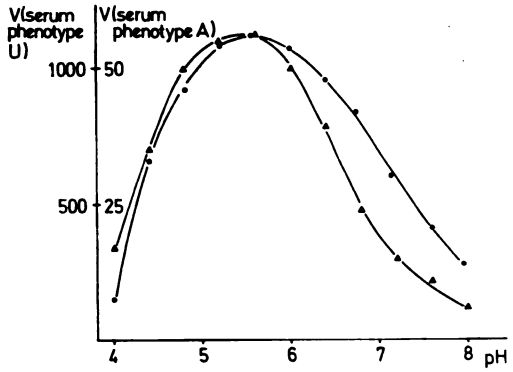

FIG. 5. *pH optima for enzymatic hydrolysis of succinylmonocholine-^{14}C in sera of phenotype U and A*

Substrate concentration: 5×10^{-3} *M*; ordinate: *V* = μmoles of ^{14}C-choline per liter in 3 hr at 37°. ●, Serum (phenotype U; 1:10) in 0.1 *M* citrate phosphate-buffer; ▲, serum (phenotype A; 1:10) in 0.1 *M* citrate-phosphate-buffer.

Saturation Curves and K_m Determinations for the Enzymatic Hydrolysis of Succinyldicholine and Succinylmonocholine in Sera of Pseudocholinesterase Phenotypes U and A

The K_m for succinyldicholine and normal pseudocholinesterase was determined both with serum (phenotype U) and about 1000-fold purified pseudocholinesterase (phenotype U). All data were corrected for spontaneous hydrolysis. The serum was used in a final dilution of 1:400. The data from serum and purified esterase were plotted according to Lineweaver and Burk as shown in Fig. 6. The resulting K_m values are 3.8×10^{-5} *M* with serum (phenotype U) and 4.2×10^{-5} *M* with purified enzyme of the same phenotype. As will be discussed below, it was not possible to get the corresponding data with serum of phenotype A when this method was applied.

The enzymatic hydrolysis of succinylmonocholine at various concentrations of substrate was estimated at pH 5.7. The saturation curves and plots of the data according to Lineweaver and Burk are given in Figs. 7A and 7B. The serum dilution for all measurements with sera of phenotype U (Fig. 7A) and phenotype A (Fig. 7B) was 1:10. The data from the straight part of the saturation curves were plotted in a diagram $1/v$ versus $1/S$. From this plot the values of the Michaelis constant and of V_{max} can

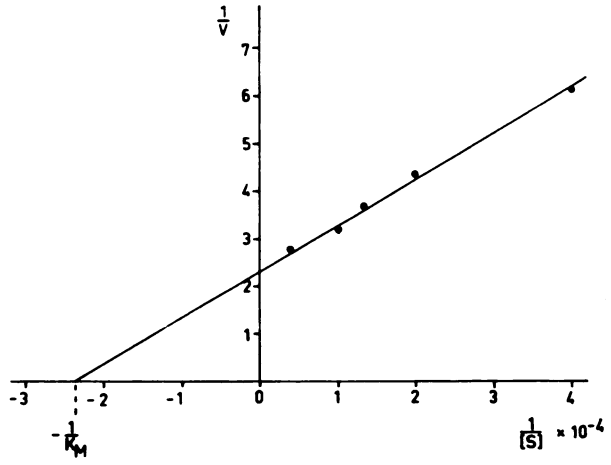

FIG. 6. *K_m determination for succinyldicholine-^{14}C and 1000-fold purified pseudocholinesterase (phenotype U)*
Ordinate: $1/V \times 10^4$; *V* = μmoles of succinylmonocholine-^{14}C per liter in 15 min at 37°. $K_m = 4.2 \times 10^{-5}$.

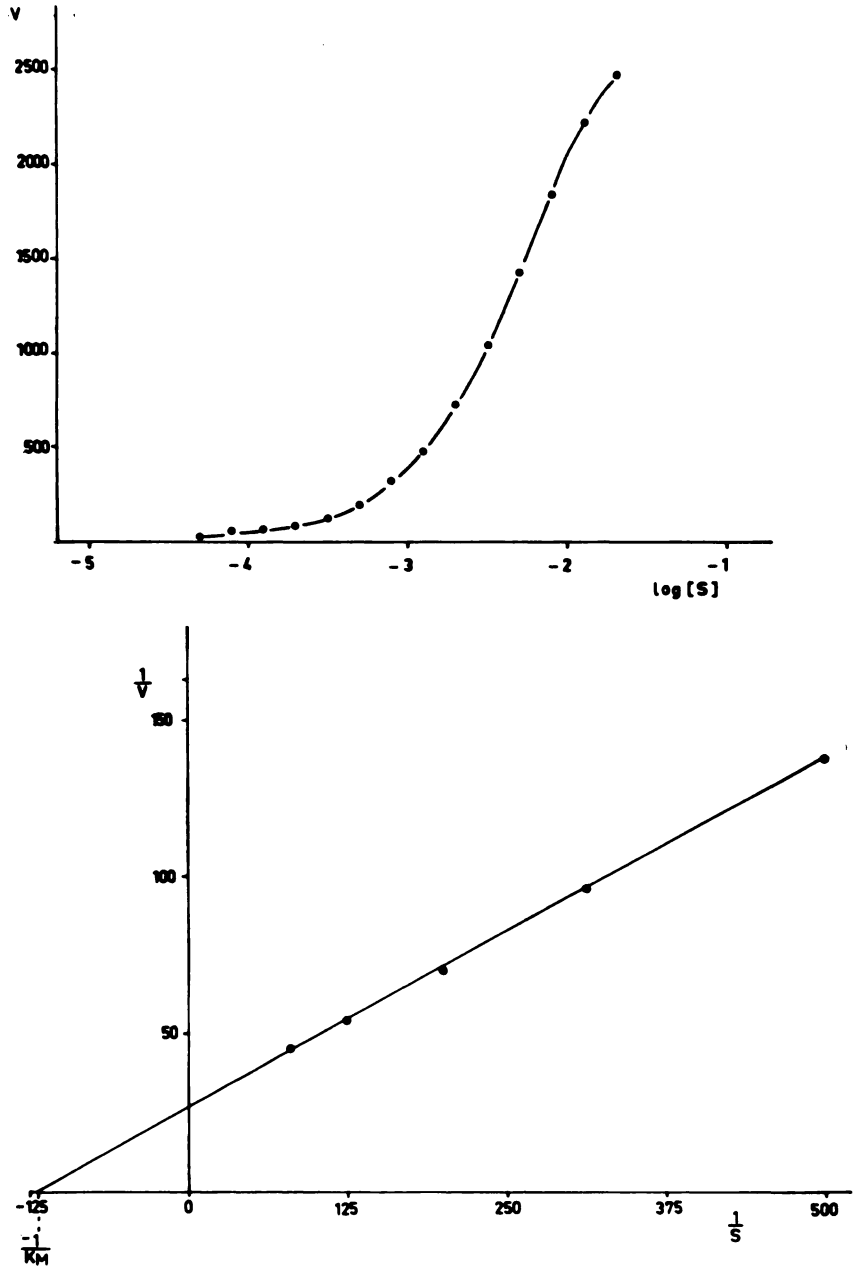

FIG. 7A. *Saturation curve and K_m for the enzymatic hydrolysis of succinylmonocholine-^{14}C in serum (1:10) of phenotype U*

pH 5.7 in 0.1 *M* citrate buffer. Ordinate: *V* and $1/V \times 10^5$, respectively; *V* = μmoles of choline-^{14}C per liter in 3 hr at 37°. $K_m = 8.4 \times 10^{-3}$.

be calculated. For serum of phenotype U V_{max} would be some 3700 μmoles per liter in 3 hr and the K_m value 8.4×10^{-3}. The corresponding values for serum of phenotype A were found to be about 2000 for V_{max} and 1.7×10^{-1} for K_m. This is a distinct kinetic difference between the reactions of succinylmonocholine with sera of

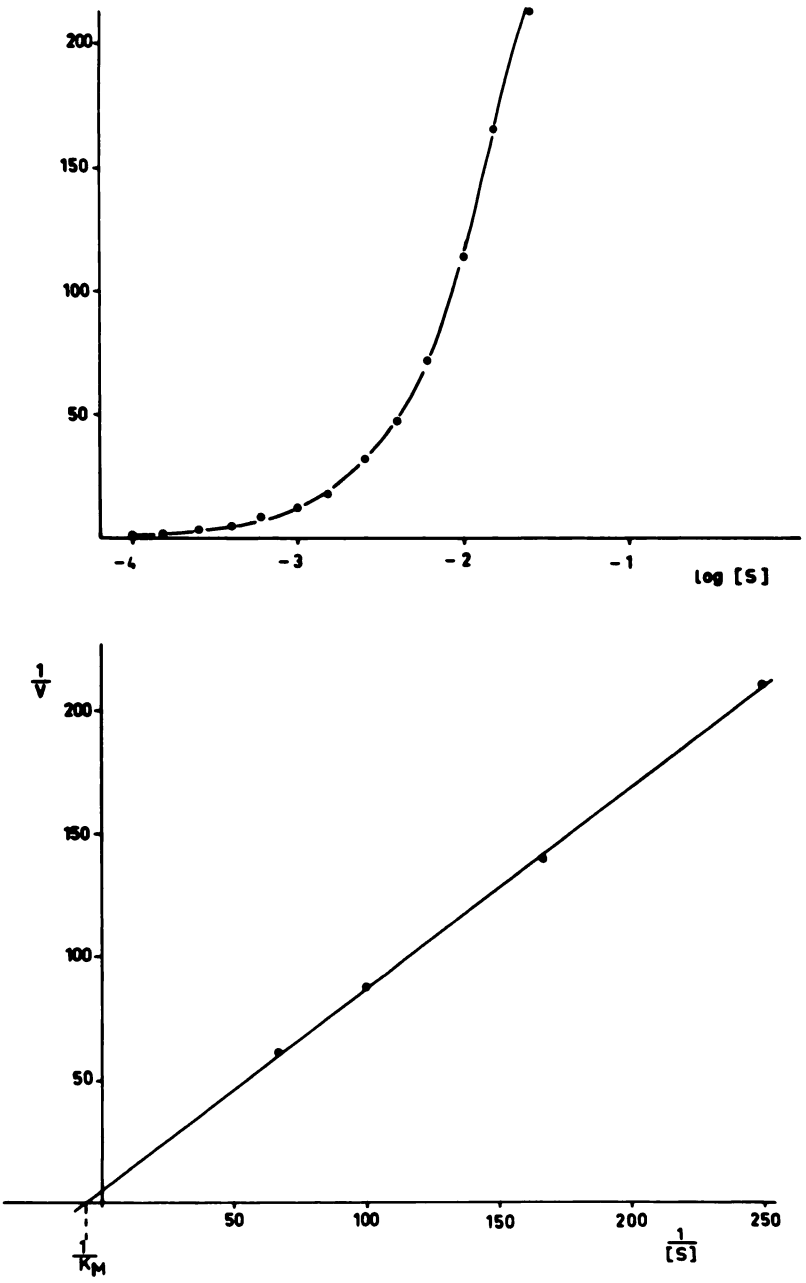

FIG. 7B. *Corresponding data for serum of phenotype A*
Ordinate: V and $1/V \times 10^4$: see Fig. 7A. $K_m = 1.7 \times 10^{-1}$.

these two homozygous phenotypes of pseudocholinesterase polymorphism.

In order to get more evidence that the serum activity on succinylmonocholine is controlled by the alleles at the E_1 locus of pseudocholinesterase, 15 "silent gene" sera were tested at pH 5.7 and 5×10^{-3} M succinylmonocholine under the same experimental conditions. With most of these sera the breakdown of the substrate was found

to be not distinctly different from spontaneous hydrolysis over the same time of incubation.

An activity assay on succinylmonocholine with 9000-fold purified pseudocholinesterase of phenotype U showed high catalytic activity of the enzyme for succinylmonocholine. From Fig. 8 it can be seen that antiserum against normal purified pseudocholinesterase is able to block the reaction of serum with succinylmonocholine.

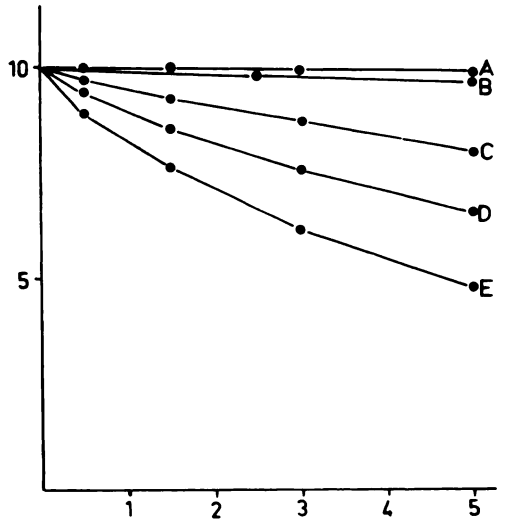

FIG. 8. *Succinylmonocholine-^{14}C (10^{-4} M in M/15 phosphate buffer pH 7.4 at 37°)*

Incubated with: *A*: albumin/globulin (1.2 mg/ml)-*B*: serum (U) + antiserum against pseudocholinesterase; *C*: 1000-fold purified pseudocholinesterase (phenotype U); *D*: serum (phenotype U) 1:10; *E*: serum (phenotype U) 1:5. Ordinate: moles $\times 10^{-5}$ of succinylmonocholine-^{14}C per liter; abscissa: time of incubation in hours.

Inhibition of Pseudocholinesterase (Phenotype U and A) by Succinyldicholine and Succinylmonocholine

Figures 9A and 9B show the data for the inhibition of the reaction of benzoylcholine and pseudocholinesterase (phenotype U) by succinyldicholine. They correspond to an inhibitor constant of 2.2×10^{-5} *M* and a pI_{50} at 5×10^{-5} *M* benzoylcholine of 4.1. As can be seen from the figure, the inhibition is competitive.

Figures 10A and 10B show the corresponding data for succinylmonocholine as inhibitor. The inhibitor constant (K_I) was 4.4×10^{-3}. The inhibition is competitive.

From Figs. 11A and 11B the K_I for the inhibition of pseudocholinesterase (phenotype A) by succinyldicholine can be calculated as 3.1×10^{-3}. The pI_{50} at 5×10^{-5} *M* benzoylcholine was found to be 2.1. The inhibition is competitive. The ratio of I_{50} (phenotype A):I_{50} (phenotype U) at 5×10^{-5} *M* benzoylcholine was 98. The I_{50} at 2.5×10^{-5} *M* benzoylcholine of succinylmonocholine was 1.3×10^{-2} *M* with serum (phenotype U) and 10^{-1} *M* with serum (phenotype A). In this case the ratio of I_{50} (phenotype A):I_{50} (phenotype U) is 7.6.

Comparison of the Pseudocholinesterase Activity of Normal and Atypical Sera with Succinyldicholine and Benzoylcholine as Substrates

Figure 12 shows the relation of the enzymatic activity on two different substrates. As is to be expected, the data for different pseudocholinesterase phenotypes are distributed along different straight lines, demonstrating the different affinities of benzoylcholine and succinyldicholine to the usual and dibucaine-resistant enzymes.

DISCUSSION

If one accepts that the 4 alleles E_1^u, E_1^a, E_1^f, and E_1^s, known to control the polymorphism of human pseudocholinesterase, modify the pseudocholinesterase protein only, it can be tested in a simple way whether or not the catalytic activity of serum on different esters derives from different enzymes. Thus the striking difference of kinetic data for the enzymatic hydrolysis of succinyldicholine in sera of the homozygotes for the alleles E_1^u and E_1^a, respectively, and the fact that serum of homozygotes for the "silent gene" does not catalyze the hydrolysis of this ester give much evidence that in human serum only the pseudocholinesterase protein catalyzes hydrolysis of succinyldicholine, and this is the same enzyme that catalyzes the hydrolysis of benzoylcholine.

On the same assumption the data reported in this paper show that succinyl-

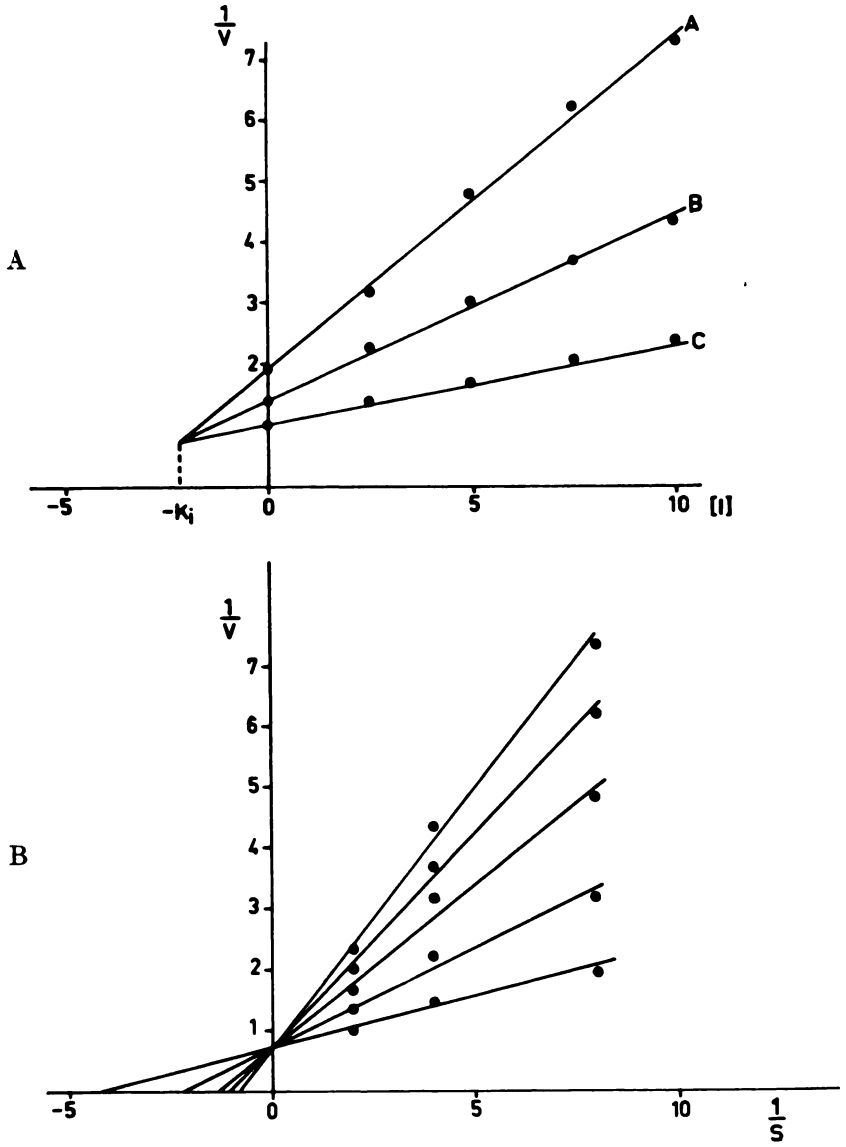

FIG. 9. *K_I for the reaction of benzoylcholine and pseudocholinesterase (serum 1:150; phenotype U) inhibited by succinyldicholine in M/15 phosphate buffer pH 7.4 at 26°*

Ordinate: $1/V \times 10^3$; $V = \Delta E_{240} \times 1000/3$ min; abscissa: $[I] \times 10^{-5}$ and $1/S \times 10^{-4}$. $K_I = 2.2 \times 10^{-5}$.

monocholine is hydrolyzed only by the pseudocholinesterase protein, too, because otherwise sera from homozygotes for the "silent gene" should contain enzymatic activity, which they in fact do not. Recent experiments with monospecific antiserum against pseudocholinesterase of phenotype U have shown that in human serum only pseudocholinesterase catalyzes the hydrolysis of succinylmonocholine. From the biochemical point of view it seems to be interesting that the catalytic activity of pseudocholinesterase for the monocholine ester does not have its pH optimum in alkaline solutions, as do the activities for other known substrates of pseudocholinesterase. More data on this subject with purified enzymes possibly will give more

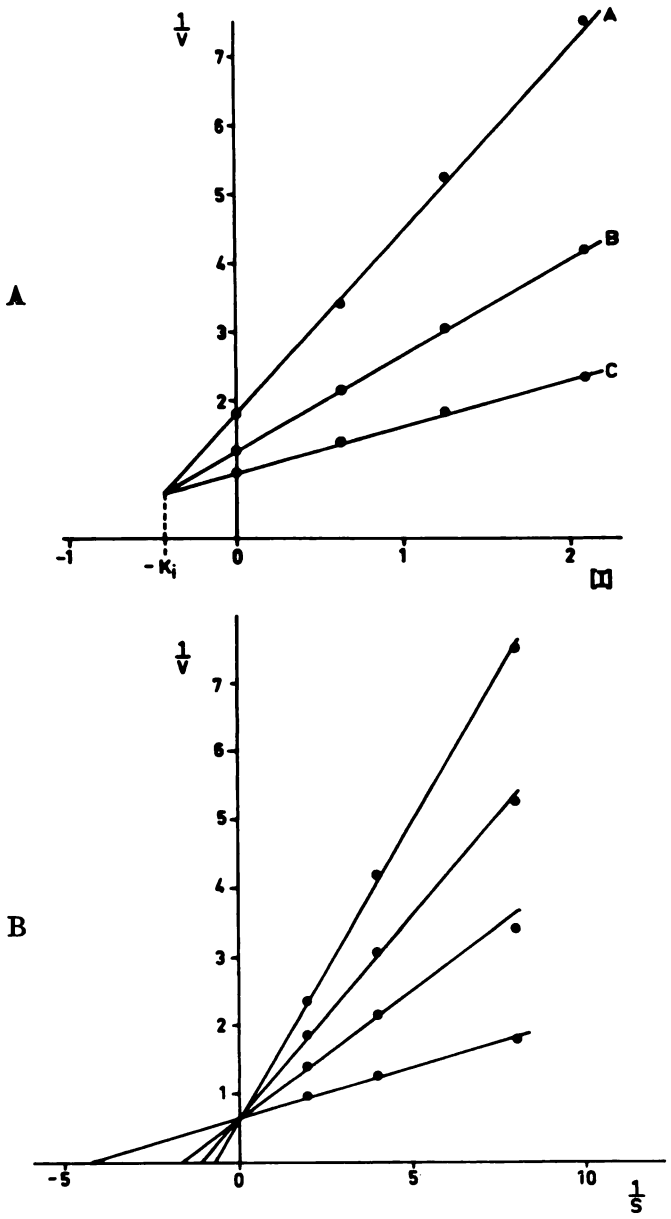

FIG. 10. *K_I for the reaction of benzoylcholine and pseudocholinesterase (serum 1:200; phenotype U) inhibited by succinylmonocholine in M/15 phosphate buffer pH 7.4 at 26°*

Ordinate: see Fig. 9; abscissa: $[I] \times 10^{-3}$ and $1/S \times 10^{-4}$. $K_I = 4.4 \times 10^{-3}$.

light on the nature of the two active sites discussed for the pseudocholinesterase protein. Because of the negligible spontaneous hydrolysis of succinylmonocholine at pH 5.7 and the quantitative electrophoretic separation of the ester from choline, the assay described becomes very sensitive for small enzyme activities. Thus it is suitable to test sera from homozygotes for the so-called "silent gene" which in some cases were found to have very small activities when tested with sensitive methods.

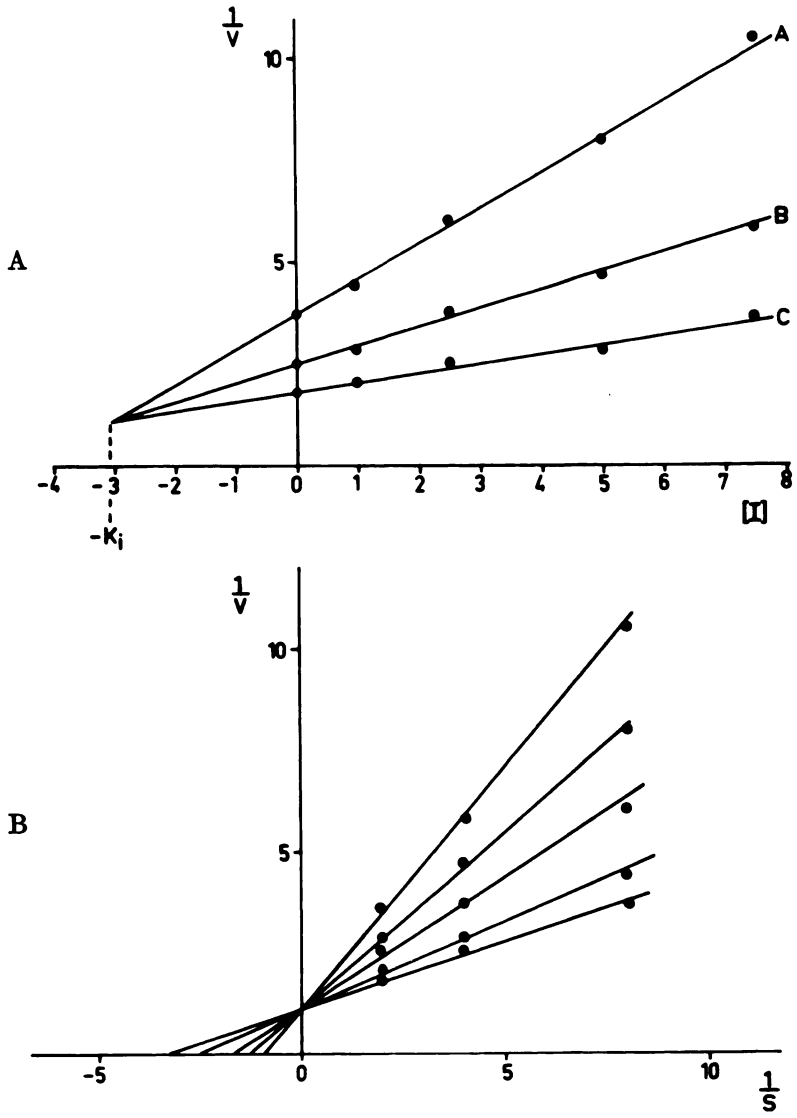

FIG. 11. *K_I for the reaction of benzoylcholine and pseudocholinesterase (serum 1:75; phenotype A) inhibited by succinyldicholine in M/15 phosphate buffer pH 7.4 at 26°*

Ordinate: see Fig. 9; abscissa: $[I] \times 10^{-3}$ and $1/S \times 10^{-4}$. $K_I = 3.1 \times 10^{-3}$.

However, the method described has some limitations. For instance, it is not possible to estimate small enzymatic activities at alkaline pH because the spontaneous hydrolysis will be higher than enzymatic hydrolysis and an unreasonable relation of both reactions will make the test inaccurate. This mainly will be a trouble at high substrate concentrations, as can be easily understood from the different types of reaction involved. Therefore, it was not possible to obtain the pH-optimum curve for the enzymatic hydrolysis of succinyldicholine by sera of phenotype A.

According to the results of *in vitro* and *in vivo* experiments of Kalow and others (for references, see 14–16) as well as those of this paper, the prolonged apnea of some patients after injection of succinyldicholine can be explained by a lack of

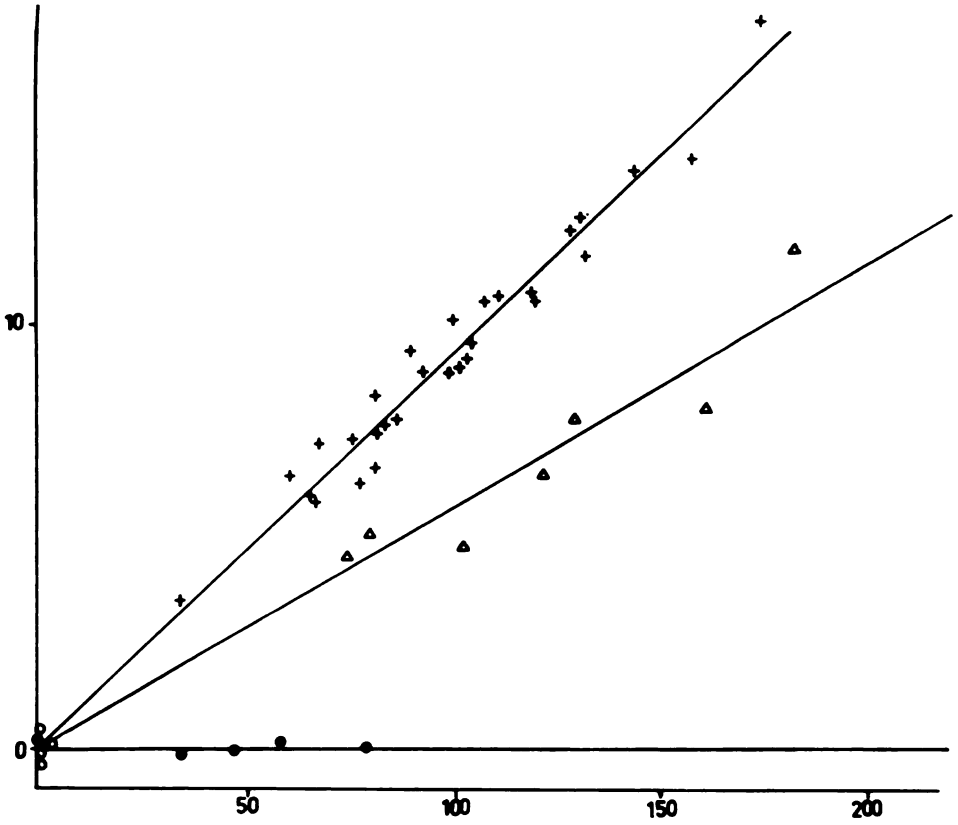

FIG. 12. *Relation of the hydrolysis of benzoylcholine and succinyldicholine-^{14}C in human sera of different phenotype*

The hydrolysis of succinyldicholine-^{14}C was measured by the direct method; that of benzoylcholine was tested according to Kalow

Ordinate: reaction velocity with succinyldicholine-^{14}C [μmoles/8 min; 5×10^{-5} M; 37°; $M/15$ phosphate buffer, pH 7.4; serum 1:50]; abscissa: reaction velocity with benzoylcholine [$\Delta E \times 1000/3$ min; 5×10^{-6} M; 26°; $M/15$ phosphate buffer pH 7.4; serum 1:200]; +, phenotype U and US; ▲, phenotype UA; ●, phenotype A and AS; O, phenotype S. Some of the sera of the rare phenotypes were stored for a long time. It is known that pseudocholinesterase activity in sera rises on longer storage; this may be the reason for the high activities of some atypical sera.

enzymatic hydrolysis of the muscle relaxant in serum. Thus one would expect that the injection of normal pseudocholinesterase should normalize the response to succinyldicholine in those patients. Early attempts to shorten the apnea by injection of cholase (concentrated human pseudocholinesterase) or by infusion of fresh plasma did not show an abbreviation of relaxation (17, 18). It has been shown by Goedde *et al.* (19) that the duration of apnea can be normalized by injection of highly purified pseudocholinesterase corresponding to the whole plasma activity, calculated from normal enzyme level and individual plasma volume. Thus, an effective therapy seems to be a question of dosage.

ACKNOWLEDGMENT

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