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Determination of succinylcholine hydrolytic enzyme activity in human plasma

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ABSTRACT

Succinylcholine is frequently employed in surgical procedures as a pre-anaesthetic However, the lack of existing metabolic activity for this compound in some individuals entails a substantial risk Normally, the risk is assessed indirectly throught the measurement of pseudocholinesterase activity using other substrates, such as acetylcholine, rather than the agent itself Thus, a method was devised to assess directly the hydrolysis of succinylcholine in human plasma samples This method was applied to plasma samples derived from healthy men, healthy women, pregnant women and patients with silent pseudocholinesterasaemia

INTRODUCTION

Succinylcholine has found widespread application as a skeletal muscle relaxant employed for pre-anaesthesia in many surgical procedures. Its utilization is, in part, related to the relative ease with which the anaesthesiologist can control its action. The short duration of action of succinylcholine, in normal patients, is primarily attributed to its rapid hydrolysis by cholinesterase (ChE) in blood plasma and liver [1,2]. However, some patients exhibit a very low activity of cholinesterase owing to genetic factors, hepatic disease or malnutrition [3,4]. Such patients should be screened for their ability to metabolize succinylcholine prior to its use to avoid the possible risk of suffocation resulting from prolonged muscle relaxation [5,6]. The screening of surgical patients typically incorporates a mea-

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surement of the ChE activity in the plasma, using substrates such as acetylcholine, butyrylcholine, or propionylcholine.

In a recent, routine gastrodectomy procedure, one of us (T.H.) noted that the patient exhibited a completely normal recovery time from anaesthesia Subsequent, post-operative determination of the ChE activity in the plasma of this patient, however, indicated a silent pseudocholinesterasaemia condition. Thus, we decided that the simple measurement of ChE activity using alternative substrates was possibly an inadequate predictor of risk in the pre-anaesthetic use of succinylcholine. It seemed that a more rational approach to the assessment of a patient's capability to metabolize this compound would be to incorporate the compound itself in the screening procedure. The ability to degrade succinylcholine may be determined by monitoring the loss of the parent molecule or the appearance of the primary metabolite, choline, upon incubation *in vitro* with a blood sample. The metabolic activity of an individual with respect to succinylcholine *in vivo* could also be derived indirectly by measurement of the remaining substrate in blood at any point in time [7].

Potter *et al.* [8] have recently reported a method for the determination of acetylcholine and choline in tissue samples using liquid chromatography with electrochemical detection (LC–ED). We have reported a modification of this method, incorporating a simplified extraction procedure [9,10]. Fundamentally, this technique measures hydrogen peroxide generated enzymically by either acetylcholine or choline. The reversed-phase separation of the components of interest is followed, on-line, by a post-column reactor. The reactor contains immobilized acetylcholinesterase and choline oxidase, the enzymes that, when exposed to the substrates, lead to the formation of hydrogen peroxide. Since succinylcholine may be considered to be a dimer of acetylcholine, we felt that this approach might be directly applicable to the determination of succinylcholine hydrolytic enzyme activity in blood samples. The activity could be measured either as the disappearance of the succinylcholine or as the appearance of the product, choline. In either case, the enzymic activity would be expressed as the amount of succinylcholine decomposed per minute per millilitre.

EXPERIMENTAL

Subjects

Single 1-ml plasma samples were obtained from a total of 21 subjects. These included nine healthy men (27–33 years of age, 55–85 kg body weight), seven healthy women (21–28 years of age, 43–55 kg body weight), three pregnant women (4–5 months pregnant, 26–28 years of age, 45–55 kg body weight) and two patients with silent pseudocholinesterasaemia (60 and 79 years of age, 50 and 47 kg body weight, respectively).

Succinylcholine hydrolytic enzyme activity

Determination of the degradation of succinylcholine employed the following, individual reaction conditions. The succinylcholine chloride substrate (obtained from Sigma, St. Louis, MO, U.S.A.) was prepared immediately before use at a concentration of 500 pmol/μl in a 0 050 *M* phosphate buffer (pH 7.4). This solution was stored in ice. Incubation began immediately after the addition of 20 μl of plasma to 100 μl of the substrate solution. Incubation was carried out at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of 0.10 *M* perchloric acid. Sample purification was achieved by centrifugation at 20 000 *g* for 5 min and 4°C A 10-μl aliquot of the supernatant was subsequently injected into the LC–ED system for quantitation. Optimization of the incubation conditions and determination of kinetic parameters employed plasma from a single healthy male patient.

LC–ED system

The LC setup incorporated a Beckman 112 pump, operated at a normal flow-rate of 1.5 ml/min The mobile phase was 0.050 *M* phosphate buffer (pH 8.40) containing 0.20 m*M* sodium octyl sulphate (SOS). The choline (retention time 1.26 min) in the sample was separated with an analytical column (ACh Separation Column®, 96.0 mm × 5.0 mm I.D., BAS, Tokyo, Japan). The separated metabolite emerging from the analytical column was directed to an immobilized enzyme column (BAS) containing both acetylcholinesterase and choline oxidase enzymes. On reaction with the choline oxidase, hydrogen peroxide was formed and subsequently measured at a platinum working electrode. The electrode potential, set at +0.6 V *vs.* an Ag/AgCl reference electrode, and the current flowing through the detector were monitored by an LC-3A amperometric detector (BAS, West Lafayette, IN, USA). The current output of the detector was typically set at 100 nA full scale. It is important to note that the acetylcholinesterase immobilized on the LC–ED post-column is distinct from the serum, or pseudo-, cholinesterase mentioned elsewhere in this paper. Acetylcholinesterase (EC 3.1.1.7), commonly called true acetylcholinesterase, has been given the more precise name of acetylcholine acetylhydrolase by the Enzyme Commission of Biochemical Nomenclature [15]. On the other hand, cholinesterase (EC 3.1.1.8), also known as pseudocholinesterase, butyrylcholinesterase, plasma cholinesterase or serum cholinesterase, has been given the name of acylcholine acylhydrolase by the same commission. As shown herein, the former does not exhibit any substantial acitivity towards succinylcholine as a substrate, whereas the latter does.

Pseudocholinesterase activity

Determinations of the pseudocholinesterase activity in serum samples from patients used a slightly modified version of the Garry [11] method. In this procedure, serum was incubated with butyrylthiocholine, the substrate, in a 0.050 *M* phosphate buffer (pH 7.2) to yield 5-thio-2-nitrobenzoic acid (TNB). The product of this reaction was assessed colorimetrically at 470 nm.

RESULTS

Stability of succinylcholine

Although the molecular structure of succinylcholine is similar to that of acetylcholine, we anticipated that it would not be detected by the LC–ED system employed, since the postcolumn reactor in this system uses the highly specific acetylcholinesterase. However, initial injections of succinylcholine solutions did exhibit a detectable LC–ED peak. This peak was identified as choline by its retention time and also by the elimination of the peak on addition of choline oxidase. We thus decided to investigate the non-enzymic degradation of succinylcholine solutions.

Incubation of succinylcholine (0.5 nmol/μl) dissolved in 0.050 *M* phosphate buffers at pH values of 6.0–8.5 were injected into the LC–ED system after incubation at 37°C for 10 min. As can be seen in Fig. 1, the choline measured in these samples was relatively constant at pH values below 7.5. Above this value, the choline concentration rapidly increased.

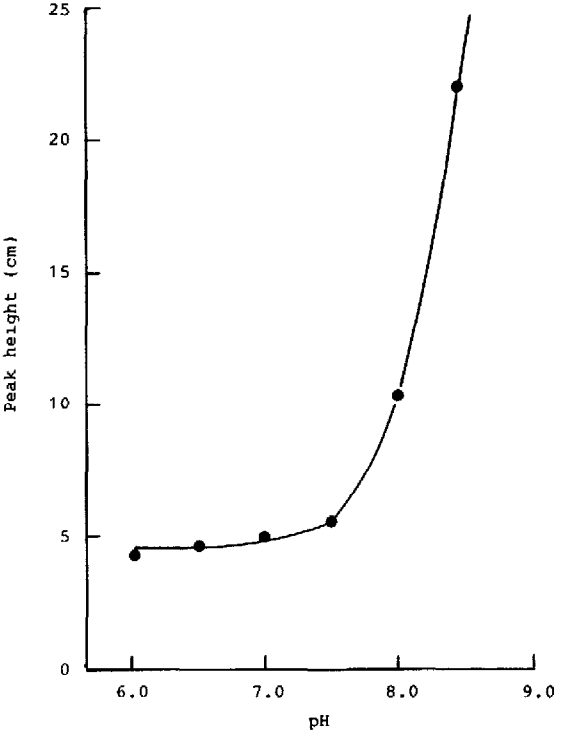

Fig 1 Effect of pH on the stability of succinylcholine Solutions containing 0 5 nmol/μl in 0 050 *M* phosphate buffers were incubated at the indicated pH for 10 min The peak height of choline, the non-enzymic degradation product, was measured.

Since the largest non-enzymic yield of choline from succinylcholine occurred at a pH of 8.5, we further investigated the effect of incubation time on this phenomenon. In these experiments, the same conditions as described above were employed with the pH fixed at 8.5. Aliquots of the incubated samples were then subjected to LC–ED at various times between 0 and 60 min. As seen in Fig. 2, incubation at 0°C indicated no substantial formation of choline. On the other hand, a temperature of 37°C led to increased non-enzymic formation of choline with increasing incubation time Lack of interaction of the succinylcholine with the specific enzyme, acetylcholinesterase, on the LC–ED post-column reactor was demonstrated by separately incubating succinylcholine *in vitro*, with this enzyme being added at an incubation concentration of 1 U/ml, under the same conditions as described above. As seen in Fig. 2, the choline peak, measured at incubation times varying between 0 and 60 min, reflected non-enzymic hydrolysis in exactly the same fashion as the set of experiments in which this enzyme was not incorporated. To further demonstrate that the LC–ED peak measured in these experiments was, indeed, choline, another set of experiments was performed in

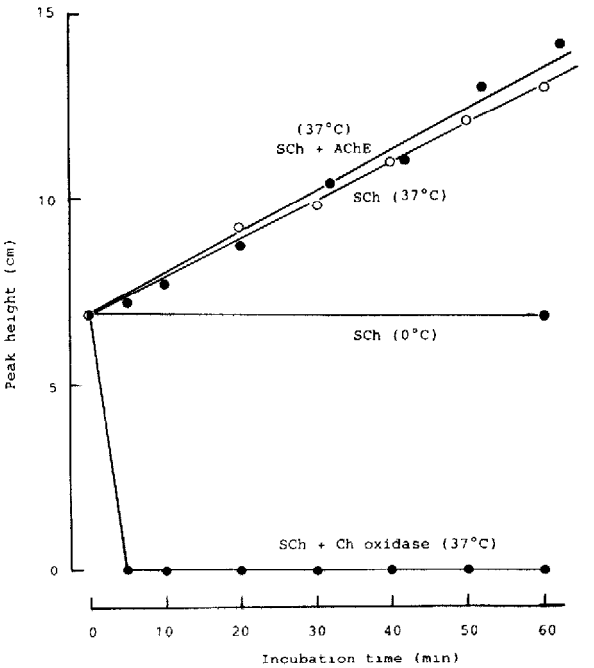

Fig 2 Effect of incubation time on the stability of succinylcholine (SCh) Solutions of succinylcholine in 0.050 *M* phosphate buffer (pH 8 5) were incubated at 0 or 37°C for 60 min Acetylcholinesterase (AChE) and choline oxidase (Ch oxidase) were independently added to some of these incubation mixtures. The peak height for choline, the degradation product of succinylcholine, was measured

which choline oxidase was added to the incubation medium described above. In these experiments, choline oxidase, at an incubation mixture concentration of 2 U/ml, abolished the measured peak, as seen in Fig. 2.

Succinylcholine enzymic hydrolysis by human plasma

The nature of enzymic hydrolysis of succinylcholine in human plasma was investigated from a number of perspectives. First, the linearity of the amount of choline formed as a function of incubation time was examined. In these experiments, the incubation mixture described in Experimental was employed. As seen in Fig. 3, the amount of choline produced was linear for incubation times between 0 and 20 min. The amount of choline formed was independent of the incubation time beyond 40 min. As also shown in Fig. 3, the non-enzymic hydrolysis of succinylcholine after 10 min of incubation accounted for less than 1% of the total measured enzymic activity Consequently, the incubation time of 10 min was selected for all future analyses.

The effect of enzyme concentration on the enzymic hydrolysis of succinylcholine was investigated by varying the amount of plasma sample added to the incubation mixture. As shown in Fig. 4, the reaction was completely linear for 10–40 μl of added plasma, and became independent of the amount of plasma at

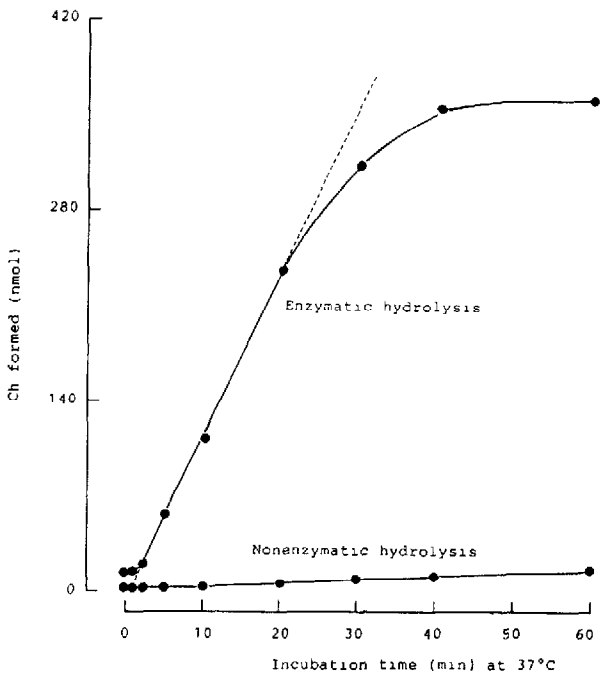

Fig 3. Effect of incubation time on the enzymic and non-enzymic hydrolysis of succinylcholine The volume of plasma added to each incubation mixture was 20 μl Ch = choline

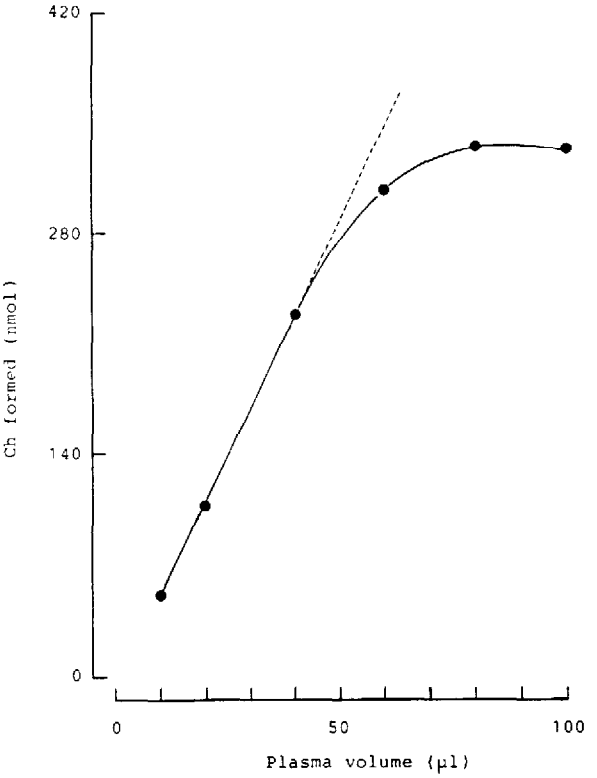

Fig 4 Effect of enzyme concentration on the succinylcholine hydrolytic enzyme activity The enzyme concentration was altered by changing the amount of plasma added to individual incubation mixtures The incubation time was maintained at 10 min for each mixture Ch = choline

values greater than 80 μl. Thus, 20 μl of plasma was selected for all subsequent analyses.

The kinetic parameters for the enzymic hydrolysis of succinylcholine were determined in a set of experiments using various substrate concentrations. The reaction velocity is linear at low values of substrate concentration and eventually reaches a plateau at values greater than 4 10^{-4} M. The Lineweaver–Burk analysis of these data (Fig. 5) yields a Michaelis constant, K_M, of $1.39 \cdot 10^{-4}$ M for succinylcholine and a V_{max} value of 167 nmol/min/ml.

To be useful for routine analysis of the enzymic hydrolysis of succinylcholine by plasma samples, this technique must also obviously demonstrate a linear response for the choline product in a range that reflects the amount of this material expected to be formed in such determinations. As shown in Fig. 6, the LC–ED response for choline is completely linear in the range 12.5–450 pmol of injected compound. An injection volume of 10 μl was used for all these determinations. This range is equivalent to a succinylcholine hydrolysis activity of 7–250 nmol,

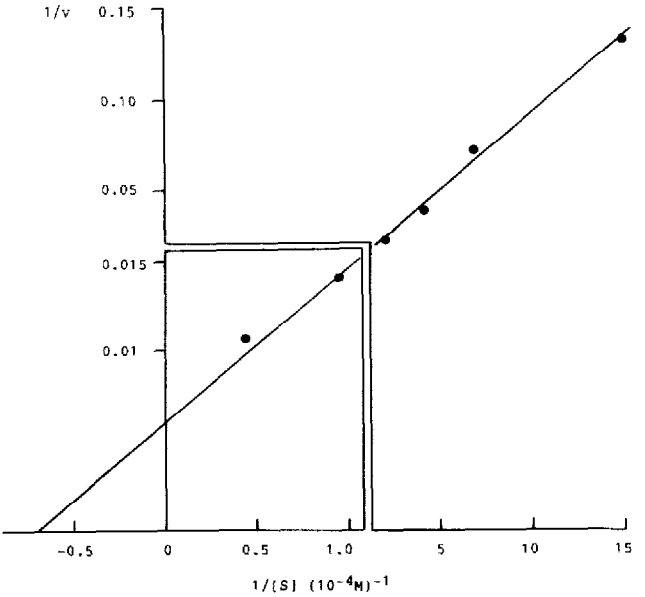

Fig. 5 Determination of Michaelis constant for succinylcholine hydrolytic enzyme activity in human plasma at pH 7 4

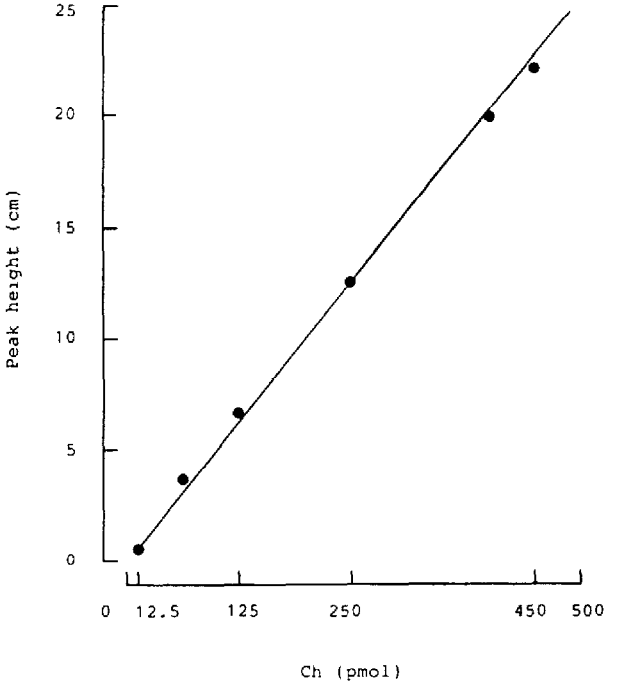

Fig 6 Demonstration of linear response of the LC–ED system for choline (Ch)

min/ml. Thus, the linearity of the response for choline is entirely appropriate for the enzymic determinations described.

Patient analyses

The pseudocholinesterase and succinylcholine hydrolysis activity in blood samples from various human patients were determined. The patients included nine healthy males, seven healthy females, three pregnant women and two patients with silent pseudocholinesterasaemia. The pseudocholinesterase activity, measured by Garry's method, yielded the following results. men (n = 9, 10.5 ± 0.6 μmol/min/ml), women (n = 7, 10.1 ± 0.3 μmol/min/ml), pregnant women (n = 3, 8.9 ± 0.26 μmol/min/ml) and silent pseudocholinesterasaemia patients (n = 2, 0.1 ± 0 2 μmol/min/ml). The succinylcholine hydrolase activity determined for the same patients yielded, respectively, values of 102.7 ± 23.6, 106.8 ± 46.1, 61.8 ± 7.8 and 0 ± 0 nmol/min/ml These results are shown graphically in Fig. 7.

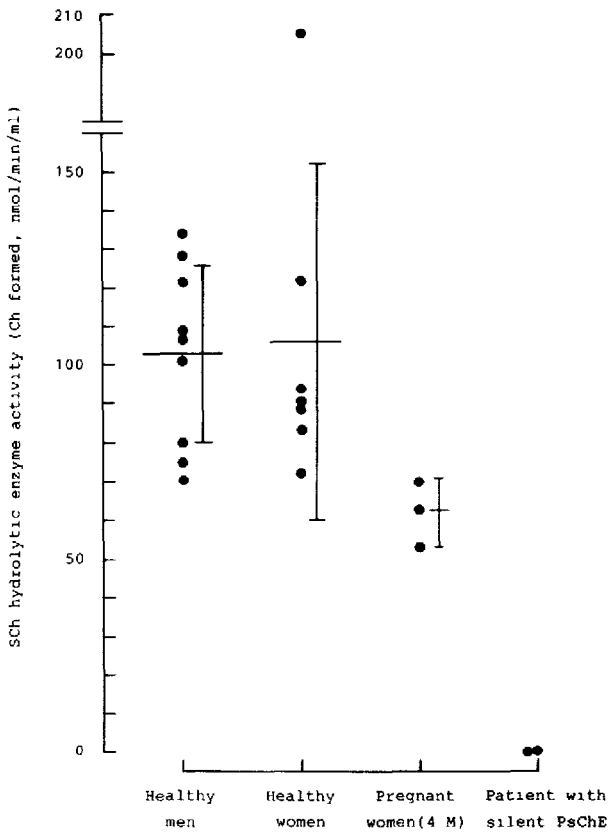

Fig 7 Succinylcholine (SCh) hydrolytic enzyme activity in human plasma from four different groups of patient Ch = choline.

DISCUSSION

The use of succinylcholine as a pre-anaesthetic is typically contraindicated for individuals with low or non-existent levels of plasma pseudocholinesterase. One such patient was treated intravenously with succinylcholine prior to a surgical procedure and surprisingly experienced no problems whatsoever in recovering from this treatment Since the pseudocholinesterase activity of patients is typically determined using acetylthiocholine, propionylthiocholine or butyrylthiocholine as substrate [11–14], we began to wonder if there might be some other mechanism for deactivation of succinylcholine in patients exhibiting this silent pseudocholinesterasaemia. As such, we felt that a more appropriate determination of the metabolic activity towards succinylcholine should employ this substance as the actual substrate. The initial stages of our investigation demonstrated that the LC–ED system employed did not detect succinylcholine. However, it could adequately detect choline, the degradation product from this material. Succinylcholine standard solutions were examined under a variety of pH and temperature conditions. These experiments showed that the non-enzymic degradation of succinylcholine was sufficiently small to provide no major interference with the assessment of enzymic hydrolysis of succinylcholine by blood sample. Examination of succinylcholine solutions in the presence of the specific enzymes, acetylcholinesterase and choline oxidase, allowed us clearly to identify choline as the non-enzymic product of degradation of this compound. These investigations also indicate that the immobilized acetylcholinesterase contained in the LC–ED post-column reactor is not necessary for the determination of succinylcholine hydrolysis enzymic activity. However, the presence of the immobilized acetylcholinesterase enzyme does not in any way interfere with the determination of succinylcholine hydrolysis activity and, therefore, use of commercially available post-column reactors intended for use in the analysis of acetylcholine and choline is entirely appropriate.

In general, succinylcholine is unstable at a pH of 7.5 or greater if one incorporates extended periods of time in the assay. On the other hand, the LC–ED system employed demands an optimal pH of 8 4 for appropriate enzymic activity in the post-column reactor. Fortunately, the residence time of the succinylcholine in the LC–ED system is very short, and decomposition is therefore relatively minimal. Thus, non-enzymic decomposition of succinylcholine in the LC–ED system is negligible, considering the enzymic activities normally achieved by this analytical procedure.

Determination of the succinylcholine enzymic hydrolysis reaction conditions involved consideration of the concentration of the substrates, the sample volume, the incubation time and the pH for optimal activity. The pH of the incubation mixture was fixed at 7.4 to minimize non-enzymic degradation of succinylcholine. The other optimal conditions are mentioned in Results. Since the enzymic activity is determined by measuring the product of hydrolysis, the activity is expressed in

units of choline formed (nmol)/min/ml. The K_M for succinylcholine found in the current experiments, $1.39 \cdot 10^{-4}$ M, is quite comparable with those reported by Szasz [13] for acetylthiocholine and butyrylthiocholine ($0.65 \cdot 10^{-4}$ and $1.2 \cdot 10^{-4}$ M, respectively). It is slightly smaller than those reported for these same alternative substrates in human plasma by Hirata *et al.* [14] ($5.8 \cdot 10^{-4}$ and $6.2 \cdot 10^{-4}$, respectively). Consideration of these facts, the measured V_{max} and other results obtained in this investigation would indicate that the enzyme responsible for hydrolysis of succinylcholine in the current study is most likely pseudocholinesterase [15].

The determination of succinylcholine hydrolytic enzymic activity in patients indicates no significant differences in the normals examined. One should, however, note the slightly lower value obtained for pregnant women. On the other hand, in those patients exhibiting silent pseudocholinesterasaemia, no activity was detected with the present technique.

Even though the current approach to examining possible extenuating circumstances associated with the difficulties to be expected in treatment with succinylcholine of patients at high risk was not established, we feel that the procedure developed is generally applicable and can be readily employed to assess possible problems associated with the use of this compound as a pre-anaesthetic. The use of succinylcholine as the substrate for such investigations provides a much more direct picture for the potential metabolic degradation of this compound by the patients of concern.

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