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The Hydrolysis of Succinyldicholine by Cholinesterase

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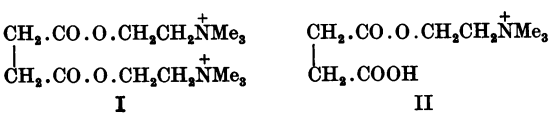
The dicholine ester of succinic acid (I) has recently found clinical application as a powerful curare-like drug with a short-lasting action and relative freedom from undesirable reactions (Bovet, Bovet-Nitti, Guarino, Longo & Marotta, 1949). Unlike natural curare, its action is potentiated, not antagonized, by eserine, and since it is also known to be hydrolysed *in vitro* by plasma cholinesterase (Glick, 1941; Bovet-Nitti, 1949), its transient action is probably due to destruction by cholinesterase *in vivo*. As the half ester (II) is without



I II

curare-like action, inactivation could conceivably be brought about either by simultaneous hydrolysis of both links of (I), giving a mixture of choline and succinic acid, or by hydrolysis to (II) as the intermediate or final product. The mechanism of the destruction of succinyldicholine by cholinesterase is thus of interest both from the pharmacological and enzymological points of view. We have studied the problem *in vitro* by means of chromatographic separation of the constituents of the reaction mixture at different stages of the hydrolysis. In earlier work (Whittaker, 1951) the solvent system used effected the separation of the two esters of succinic acid but not that of succinylmonocholine and choline, and these could only be distinguished by means of a reagent giving a coloration with carboxylic esters. A solvent mixture has now been found which gives good separation of all four of the possible constituents of the reaction mixture, namely succinyldicholine, succinylmonocholine, choline and succinic acid, and the chromatographic studies have been supplemented by a kinetic analysis, using the Warburg technique.

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METHODS

Source of enzyme. Horse serum cholinesterase was prepared by the method of Strelitz (1944) from commercial horse serum. The preparation corresponded to that obtained at stage 3 of her procedure and had initially a specific activity of 33 120 μl./ml. enzyme solution/hr., measured manometrically at pH 7·4 and 38° with acetylcholine (30 mM) as the substrate. The enzyme was stored at 0° with $CHCl_3$ as a preservative; its activity was unchanged by incubation without added substrate for several hours at 38°, but slowly declined during the course of the work to 23 600 units. Initial velocities are expressed as μmoles acid liberated/3 ml./hr. in presence of 23 600 units of enzyme activity.

Substrates. The choline esters and choline were employed as the perchlorates.

Paper chromatography. This was carried out as described in a previous paper (Whittaker & Wijesundera, 1952). Immersion in I_2 vapour (Brante, 1949; Marini-Bettolo-Marconi & Guarino, 1950) proved a useful general method, giving yellowish or brown spots with choline and its esters and succinic acid. The carboxylic ester reagent (Whittaker & Wijesundera, 1952) was used to detect the succinylcholine esters, and bromothymol blue, used as described by Brown (1950), detected succinylmonocholine and succinic acid as yellow spots. Whatman no. 4 filter paper was used throughout.

Table 1. *R_F values of choline and succinylcholines in various solvents*

(Compositions are by vol.; aliphatic alcohols *n* unless stated otherwise; upward irrigation in all cases.)

Solvent system	Succinyl-dicholine*	Succinyl-monocholine*	Choline*
Phenol-water	1·0	1·0	1·0
Acetone-acetic acid-water (8:1:1)	0·9	0·93	0·5
*cyclo*Hexanol-ethanol-water (4:2:1)	0·19†	0·41†	0·33†
Dioxan-water (9:1)	0·44†	0·53†	0·63†
Butanol-acetic acid-water (4:5:1)	0·24	0·71	0·71
Propanol-formic acid-water (8:1:1)	0·3	0·62	0·6
Propanol-formic acid-water (80:5:15)	0·46	0·49	0·43
*iso*Propanol-formic acid-water (8:1:1)	0·6	0·81	0·82
Butanol-propanol-water (4:2:1)	0·12	0·04	0·31
Propanol-water-conc. aqueous ammonia (sp.gr. 0·880) (8:1:1)	0·33	0·16	0·42
Propanol-benzyl alcohol-water (5:2:2)‡	0·30	0·17	0·45
Ethyl acetate-acetic acid-water (7:2:1)	0	0	0
Ethyl acetate-pyridine-water (7:2:1)	0	0	0
*cyclo*Hexanone-ethanol-water (4:2:1)	0	0	0

Collidine, dioxan-propanol-water (9:2:2), chloroform-acetic acid (8:2) sat. with water, acetone-water (5:1), benzy alcohol-ethanol-water (9:1:1), benzyl alcohol-water were all unsatisfactory.

* Perchlorates.

† Tailing.

‡ Higher water content in the propanol-benzyl alcohol-water mixture increased R_F values and reduced separation; increased benzyl alcohol content gave ill-defined spots.

Several solvent mixtures of the large number tried gave good separation between the two choline esters, but only one neutral mixture (*n*-propanol-benzyl alcohol-water, 5:2:2 by vol.) effectively separated all four possible components of the reaction mixture. Acid and alkaline solvents were avoided owing to the risk of hydrolysis on the paper. The results are summarized in Table 1.

Manometric technique. The hydrolysis of the succinylcholines was followed manometrically at 38° and pH 7·4 using an adaptation (Ammon, 1933) of the Warburg technique in which acid formed during hydrolysis liberates CO_2 from a $NaHCO_3/CO_2$ buffer. All solutions contained 0·2% (w/v) $NaHCO_3$, the total volume being 3 ml. The enzyme solution (usually 1 ml.) was contained in the side bulbs of Warburg flasks and the esters (neutralized where necessary) were in the main compartment. After attachment to the manometers, the flasks were gassed with 5% (v/v) CO_2 in N_2 (20 cm. water pressure for 2·5 min.). After equilibration in the thermostat for 20 min. the enzyme was tipped in and the first reading taken 3 min. later.

RESULTS

Hydrolysis of succinylcholines by cholinesterase

Fig. 1 shows the hydrolysis of succinylmonocholine and succinyldicholine by horse serum cholinesterase as a function of substrate concentration. Succinyldicholine shows some inhibition by excess substrate at higher concentrations. At the optimum, it is split at 3·4% of the maximum rate for acetylcholine. This is in good agreement with the figure of 4% as found by Glick (1941).

Succinylmonocholine is also hydrolysed by horse serum cholinesterase, though only very slowly (10% of the rate found with succinyldicholine). Its affinity for the enzyme is also less than that of the diester. As hydrolysis of the latter continues beyond 50% (Fig. 2), there are thus two possible mechanisms for the hydrolysis of the diester, both eventually leading to a mixture of choline and succinic acid. These are

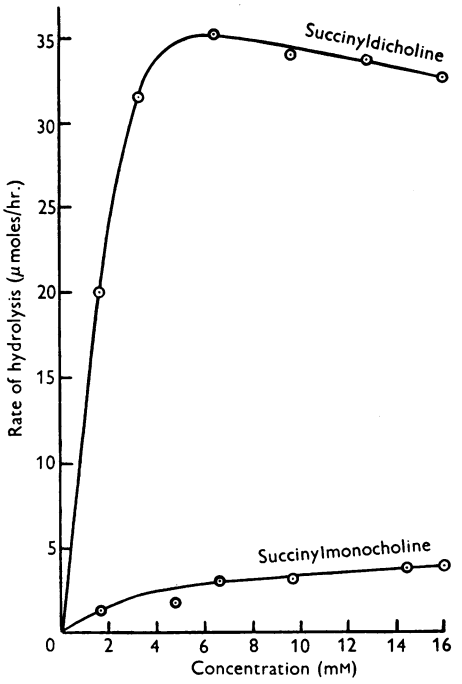

Fig. 1. Effect of substrate concentration on rates of hydrolysis of succinyldicholine and succinylmonocholine. Initial velocities measured over 5 min. and expressed as μmoles/hr.

(*a*) hydrolysis of both links simultaneously; (*b*) hydrolysis of one ester link with the formation of succinylmonocholine and choline followed by a slower hydrolysis of succinylmonocholine to choline and succinic acid.

Time course of hydrolysis of succinyldicholine

From Fig. 2, curve *A*, it will be seen that there is a marked change in the direction of the curve as 50% hydrolysis is reached, suggesting a two-stage reaction. The rate attained is equal to that obtained with an equimolar mixture of choline and succinylmonocholine (each 10 mM) (curve *B*); further, mixtures of succinyldicholine, succinylmonocholine and choline corresponding in composition to the reaction mixture at 25 and 40% hydrolysis on mechanism (*b*) give hydrolysis curves (Fig. 2, curves *C* and *D*) which

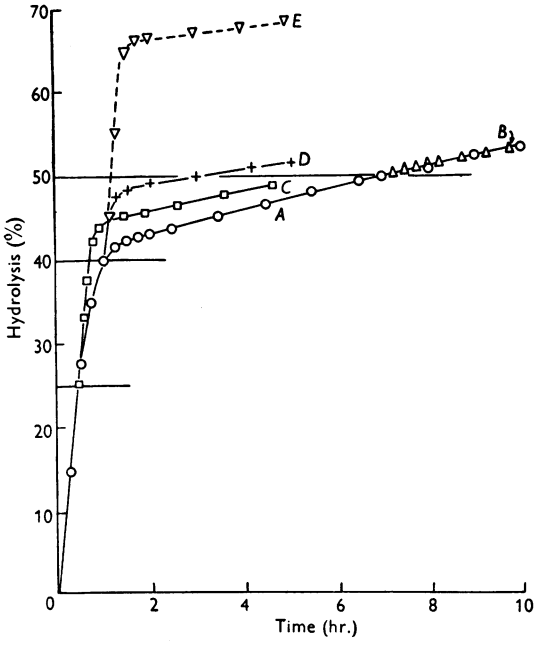

Fig. 2. The time course of hydrolysis of succinyldicholine (SCh_2) alone and mixed with succinylmonocholine (SCh), choline (Ch) and succinic acid (S). *A*, ○, 10 mM-SCh_2; *B*, △, 10 mM-SCh + 10 mM-Ch; *C*, □, 5 mM-SCh_2 + 5 mM-SCh + 5 mM-Ch; *D*, +, 2 mM-SCh_2 + 8 mM-SCh + 8 mM-Ch; *E*, ▽, 6 mM-SCh_2 + 8 mM-Ch + 4 mM-S.

follow the original curve fairly closely. By contrast, a mixture of succinyldicholine, choline and succinic acid corresponding to 40% hydrolysis on mechanism (*a*) is hydrolysed too fast to be consistent with the original hydrolysis curve (curve *E*). Curves *B*–*E* have been superimposed on curve *A* to show this more clearly. It seems likely, therefore, that the first stage in the hydrolysis of succinyldicholine is hydrolysis to succinylmonocholine and choline and that the rate which hydrolysis assumes at the 50% hydrolysis point is that of succinylmonocholine in the presence of choline.

Chromatographic analysis of the products of hydrolysis of succinyldicholine

For chromatographic analysis of the reaction mixture at different stages, five identical hydrolysis mixtures were set up. Hydrolysis was arrested in each at times corresponding respectively to 0, 10, 35, 50 and 60% hydrolysis of succinyldicholine by adding 0·1 ml. eserine solution to the Warburg flasks, giving a final concentration of 0·1 mM. Fig. 3 shows that this concentration of eserine stops the reaction in under 5 min. The reaction mixtures were adjusted to pH 7 by the cautious addition of 0·1 N-HCl, and 20 μl. of each solution (equivalent to approx. 100 μg. succinyldicholine) were pipetted on to the paper. Fig. 4 is a tracing of the resulting chromatograms. It will be seen by comparing chromatograms *a*–*e* that the succinyldicholine spot decreases in size and has disappeared at 50% hydrolysis while choline and succinylmonocholine, initially absent, are present in increasing amounts up to 50% hydrolysis. Thereafter, succinylmonocholine begins to break down. At 60% hydrolysis, the choline spot is larger than at 50% hydrolysis and succinic acid has now made its appearance on the chromatogram. Chromatogram *a* shows that protein and eserine do not interfere in the amounts present.

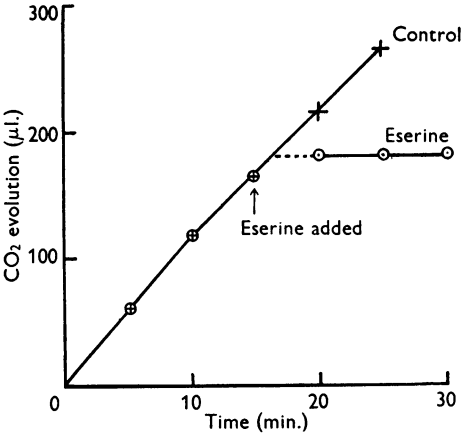

Fig. 3. The effect of 0·1 mM-eserine on the hydrolysis of 10 mM-succinyldicholine.

The slightly lower R_F values obtained with the constituents of the hydrolysates may be due to the presence of chloride ion introduced when the solutions were neutralized. It is known that chlorides have lower R_F values than the corresponding perchlorates (Whittaker & Wijesundera, 1952).

The enzymic breakdown of succinyldicholine in the presence of horse serum cholinesterase is thus clearly a two-stage process involving first the hydrolysis of one ester link with the formation of succinylmonocholine and choline and then the slower breakdown of succinylmonocholine to give another molecule of choline and succinic acid. Presumably the greater affinity of the dicholine ester for the enzyme assures its almost complete destruction before succinylmonocholine is attacked.

This is in marked contrast to the non-enzymic hydrolysis of succinyldicholine observed on omitting enzyme from the system used here. If this is allowed to occur to the extent of 20%, succinic acid as well as succinylmonocholine and choline are present in the hydrolysate (Fig. 4*g*).

THEORETICAL ANALYSIS OF THE HYDROLYSIS CURVE

An attempt has been made to give a theoretical treatment of the time course of hydrolysis of succinyldicholine by assuming (*a*) that the rate of hydrolysis of each ester is proportional to the concentration of the corresponding enzyme-substrate complex; (*b*) that the assumptions of the Michaelis theory hold good throughout the reaction and that, in consequence, the concentration of each enzyme-substrate complex is defined by the affinity of the various components of the reaction mixture for the enzyme; (*c*) that the succinate ion has a negligible affinity for the enzyme; (*d*) that inhibition by excess succinyldicholine (cf. Fig. 1) is negligible.

$$k_3=(e_0-e_1-e_2-e_3)\,z/e_3, \tag{4}$$

$$-\mathrm{d}x/\mathrm{d}t=ke_1, \tag{5}$$

$$-\mathrm{d}y/\mathrm{d}t=k'e_2-ke_1, \tag{6}$$

where k's are constants.

Eliminating e_2 and e_3 from eqns. 2, 3 and 4, we obtain

$$e_1=\frac{xe_0}{k_1(1+x/k_1+y/k_2+z/k_3)},$$

and replacing z by the right-hand side of eqn. 1, we have

$$e_1=\frac{xe_0/k_1}{1+2x_0/k_3+x(1/k_1-2/k_3)+y(1/k_2-1/k_3)}. \tag{7}$$

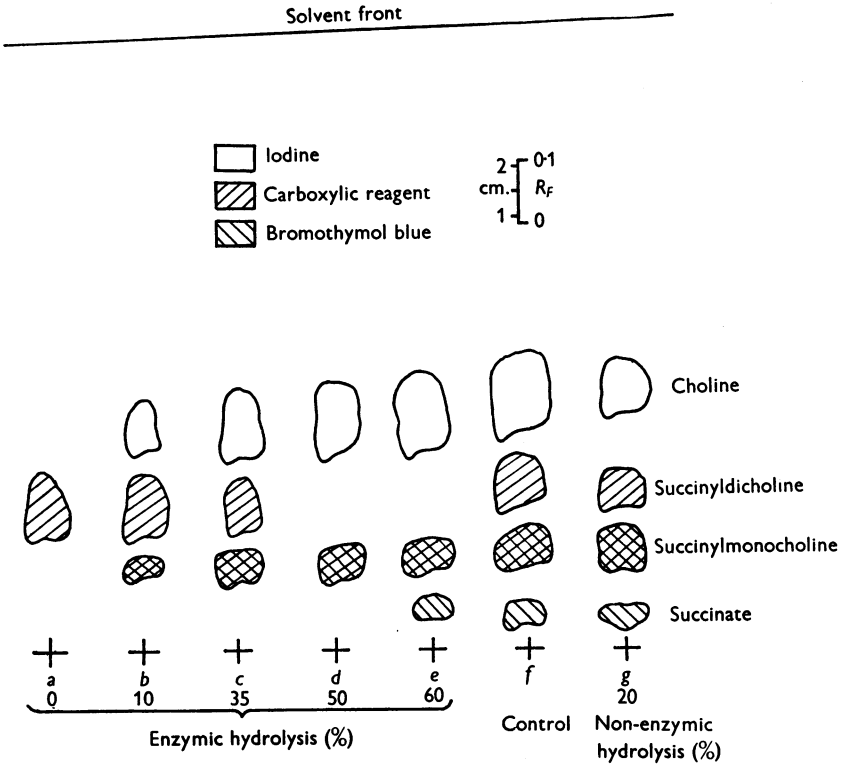

Fig. 4. Chromatogram of (*a–e*) succinyldicholine at various stages of enzymic hydrolysis; (*f*) a mixture of succinyldicholine and its hydrolysis products; (*g*) 100 μg. succinyldicholine after non-enzymic hydrolysis.

If e_0, e_1, e_2, e_3 denote the total concentration of enzyme, and the concentration of its complexes with succinyldicholine, succinylmonocholine and choline respectively, and if x_0, x, y, z, denote the initial concentration of succinyldicholine and the instantaneous concentrations of succinyldicholine, succinylmonocholine and choline respectively, the system is defined by the equations:

$$z=2x_0-2x-y, \tag{1}$$

$$k_1=(e_0-e_1-e_2-e_3)\,x/e_1, \tag{2}$$

$$k_2=(e_0-e_1-e_2-e_3)\,y/e_2, \tag{3}$$

Similarly,

$$e_2=\frac{ye_0/k_2}{1+2x_0/k_3+x(1/k_1-2/k_3)+y(1/k_2-1/k_3)}. \tag{8}$$

By dividing eqn. 6 by eqn. 5, replacing e_1 and e_2 by the right-hand side of eqns. 7 and 8 and integrating, y may be obtained as a function of x, thus,

$$y=\frac{x_0^{1-k'k_1/kk_2}}{1-k'k_1/kk_2}x^{k'k_1/kk_2}-\frac{x}{1-k'k_1/kk_2}. \tag{9}$$

Eqns. 7 and 9 allow us to obtain e_1 as a function of x and using this value of e_1 in eqn. 5, we obtain a differential

equation which can be integrated without difficulty. Thus we find

$$t=\frac{k(k_1k_2-k_2k_3+k_1k_3)+k'k_1(k_3-2k_1)}{e_0kk_3(k'k_1-k_2k)}(x_0-x)$$
$$-\frac{kk_2(k_3-k_2)}{e_0k'k_3(k_1k'-k_2k)}\frac{x_0^{k_1k'/k_2k}-x^{k_1k'/k_2k}}{x_0^{(k_1k'/k_2k-1)}}$$
$$+\frac{k_1(k_3+2x_0)}{e_0kk_3}\ln(x_0/x).$$

From Fig. 1, $k_1=1{\cdot}5$ mM, $k_2=3{\cdot}5$ mM, $e_0k=36$ units, $e_0k'=4{\cdot}2$ units (maximum initial velocities of succinyldicholine and succinylmonocholine hydrolysis respectively);

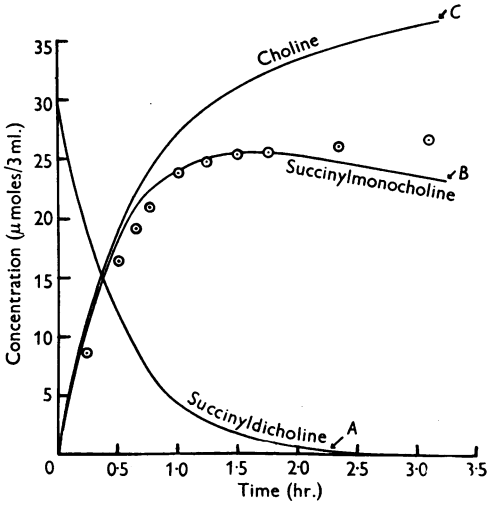

Fig. 5. Calculated concentrations of succinyldicholine, succinylmonocholine and choline as a function of time. ⊙, choline liberated in experimental hydrolysis of succinyldicholine.

also $k_3=3{\cdot}5$ mM, $x_0=30$ μmoles/3 ml. Using these values the third term only of the right-hand side of eqn. 10 need be considered; the function is expressed graphically in Fig. 5 (curve *A*). From eqns. 9 and 1, values of y and z corresponding to various values of x may be obtained; in Fig. 5 these are plotted against the values of t corresponding to these same values of x in order to obtain y and z as a function of t (curves *B* and *C*).

It will be seen that the curve of choline liberation, while not, as it should be, identical with the experimental hydrolysis curve, has the same general shape, with two sections corresponding to the hydrolysis of the dicholine and monocholine esters respectively and with a sharp change of direction between the two sections at a point where hydrolysis has proceeded to about 50 %. The discrepancy between the theoretical and experimental curves may well be accounted for by inaccuracies in the values selected for the numerical coefficients and in the initial assumptions of the theory.

SUMMARY

1. The hydrolysis of succinyldicholine by horse serum cholinesterase has been studied kinetically and by paper chromatography of the hydrolysis products.

2. A solvent system has been found which will effect the separation on filter paper of all four of the constituents of succinyldicholine hydrolysates, i.e. the di- and monocholine esters of succinic acid, free succinic acid and choline.

3. Succinylmonocholine has been identified as a constituent of the reaction mixture. Succinic acid does not make its appearance in detectable amounts before 50 % hydrolysis.

4. It is concluded that succinyldicholine is hydrolysed enzymically via succinylmonocholine. This is confirmed by the diphasic character of the hydrolysis curve and by kinetic studies.

5. The kinetics of such two-stage enzymic reactions is analysed mathematically.

We are grateful to Prof. D. Bovet for suggesting this problem and for hospitality to one of us (V.P.W.) at the Laboratorio di Chimica Terapeutica, Istituto Superiore di Sanità, Rome, where a preliminary study of the problem was made. We are also grateful to the Medical Research Council for a grant (V.P.W.) and to the University of Ceylon for a travelling fellowship (S.W.).

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