was added to the mark, giving $^1/_{850}$ M solutions. A 10.0-ml. portion of this solution was placed in each of five sterile test tubes inoculated with 0.5 ml. each of a 24-hour nutrient broth culture of the following organisms: *Streptococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus stearothermophile*, and *Bacillus circulans*. The seeded test tubes were incubated for 24 hours at 37°. If growth was observed at the initial concentration of compound, no dilutions were made. If growth was not observed at this concentration, additional dilutions were tested by taking appropriate quantities of the stock solution of inhibiting compounds and adding sterile broth in the required amount. The tubes were examined visually for growth. Minimum inhibitory concentrations, expressed as 1/molarity are recorded in Table II.

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Effect of Procainesterase Levels on Duration of Procaine Local Anesthesia

By H. B. DANIELL*, A. E. WADE, and F. FORD MILLIKAN

This study originated with the observation that rabbit serums consistently exhibited low levels of cholinesterase activity while having variable levels of procainesterase activity. This finding enabled the authors to determine the effects of procainesterase on the duration of procaine anesthesia using a single species. On conducting double blind duration studies, an inverse correlation between serum procainesterase activity and duration of conduction anesthesia existed which was highly significant ($p < .001$). Atropinesterase activity was present in the serums of those rabbits that contained intermediate or high procainesterase activity but was not evident in the serums of rabbits exhibiting low procainesterase levels. This, coupled with the fact that atropine inhibited procaine hydrolysis, suggested that in rabbit serum procaine and atropine are hydrolyzed by the same enzyme.

WHILE THE LIVER is the chief site of enzymatic detoxication of drugs, other tissues in the body are capable of drug inactivation. Human blood serum contains an enzyme capable of hydrolyzing acetylcholine and certain other choline esters (1). This enzyme is also responsible for the hydrolysis of the local anesthetic procaine (2) and other noncholine esters (3). Serums of other species appear to contain esterases that differ from those in human serum (4–8). Sawyer (9) reported the presence of an esterase in guinea pig and rabbit liver which hydrolyzes benzoylcholine but is not concerned with acetylcholine hydrolysis. A similar enzyme has been found in the plasma of rabbits (6).

The serum of certain rabbits exhibits atropinesterase activity, while that of others does not (10). This enzyme is also capable of deacetylating some derivatives of morphine (11) and hydrolyzing several alkyl and aryl esters (12). It appears to be different from cholinesterase (12, 13).

Kalow (2) suggested that an inverse correlation existed between the duration of action of certain local anesthetics and their speed of hydrolysis *in vitro* by human serum cholinesterase. It was the purpose of this investigation to determine the correlation between serum procainesterase activity *in vitro* and duration of procaine local anesthesia *in vivo* and to determine if any relationship exists between the activities of procainesterase, acetylcholinesterase, and atropinesterase in the serum of rabbits.

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EXPERIMENTAL

Serum Cholinesterase Activity.—Serum cholinesterase activity at 25±1° was determined by the procedure of Michel (14) using a Beckman model G pH meter. The acetylcholine chloride substrate (0.165 *M*) was prepared daily by dissolving 100 mg. acetylcholine chloride (Merck & Co.) in 3.33 ml. of distilled water. The cholinesterase activity was expressed as corrected pH change per hour.

Blood was taken by heart puncture from guinea pigs (mixed breed) and rabbits (New Zealand) and from the cephalic vein in humans (Caucasian). The serums were removed from the clot, clarified by centrifugation at room temperature, and stored at −20°. Before use, the serums were diluted with distilled water so that each milliliter of the solution contained 0.02 ml. of serum.

Serum Procainesterase Activity.—The enzymatic hydrolysis of procaine was conducted in 1-cm. quartz cells at 25±1° by the method of Kalow (2). Two milliliters each of a 1:4 dilution of rabbit serum and 1×10^{-4} *M* procaine solution were mixed and added to the cell. The absorbance at 313 mμ was determined within 45 seconds of mixing the two solutions and at 5-minute intervals thereafter for 1 hour using the Beckman model DU spectrophotometer. The *M*/15 phosphate buffer of Kalow and Lindsay (15) was used as the diluent and the blank. Procaine hydrolysis at timed intervals was calculated from the decrease in absorbance and reported as micromoles of procaine hydrolyzed per hour per milliliter of serum.

Duration of Procaine Local Anesthesia.—The duration of procaine local anesthesia in the rabbit and guinea pig was determined by a modification of the intracutaneous wheal method of McIntyre and Sievers (16) and by other investigators (17–19). The animal's hair was clipped from the lumbar region of the back, and the sensitivity of the area was tested by six jabs with a dissecting needle at intervals of not less than 3 seconds. In all animals used, the six jabs elicited six flinches of the skin prior to drug administration. One-half milliliter of a 1.0% solution of procaine hydrochloride in isotonic sodium chloride solution was injected intracutaneously in the area predetermined to be sensitive, and the slight elevation in the skin produced by the injected solution was marked off. The duration of anesthesia was measured by testing the marked area in the manner described at 5-minute intervals until the end point was reached. The end point chosen for the duration of anesthesia was the reappearance of response to at least four of the six jabs in two successive trials. Since this procedure involved some subjective judgment on the part of the researcher, it was conducted "double blind."

Atropinesterase Activity.—Ing *et al.* (20) described a procedure for determining the presence of atropine involving the intraperitoneal injection of atropine into albino mice with the subsequent observation of the mydriatic effect. A modification of this method was used in this study to determine the presence of atropinesterase activity of serum.

Groups of five male albino mice (BALB/c strain) were used. A solution of 250 mcg. atropine sulfate in 5.0 ml. of rabbit serum was incubated in a water bath for 1 hour at 37°. One-half milliliter of this incubated solution (equivalent to 25 mcg. atropine sulfate) was injected intraperitoneally into each of five mice, and 0.25 ml. of the same solution was injected into five additional mice. This procedure was repeated using rabbit serums of different procainesterase activity. In addition, two groups of mice received intraperitoneal injections of 25 and 12.5 mcg. of atropine sulfate in normal saline solution, respectively. The diameter of the pupil of each mouse was measured prior to and 15 minutes after injection.

Inhibition of Procaine Hydrolysis by Atropine.—Rabbit serums with high procainesterase activity were used in this study. The effect of atropine sulfate on the rate of procaine hydrolysis by this serum was investigated by comparing the absorbances at 313 mμ of solutions of procaine in serum with those of a mixture of procaine and atropine in serum.

In assessing the rate of uninhibited procaine hydrolysis by the serum, an 8.4×10^{-5} *M* concentration of procaine hydrochloride was prepared. One milliliter of this solution diluted with 1.0 ml. of buffer was mixed with 2.0 ml. of a 1:4 dilution of rabbit serum. The rate of hydrolysis was measured as described previously. The rate of hydrolysis of the same dilution of procaine hydrochloride in the presence of atropine sulfate was measured by mixing 1.0 ml. of 8.4×10^{-5} *M* procaine hydrochloride with 1.0 ml. of 1×10^{-4} *M* atropine sulfate solution and allowing the mixture to equilibrate for 5 minutes. This mixture was then added to 2.0 ml. of 1:4 rabbit serum, and the procaine hydrolysis observed. This procedure was repeated using 1.0 ml. of 1×10^{-2} *M* atropine sulfate. The percentage of inhibition of procaine hydrolysis by atropine was calculated by use of the formula provided by Varley (21).

$$\%\ \text{Inhibition of Procaine} = 1 - \frac{\text{Change in O.D./hr. with Atropine}}{\text{Change in O.D./hr. without Atropine}} \times 100$$

Statistical Analyses.—All analyses were conducted by the University of Georgia Bureau of Statistics on standard I.B.M. electronic data processing equipment using standard statistical formulas. Levels of significance, where applicable, were determined using $n-2$ degrees of freedom (22).

Table I.—Serum Cholinesterase Activity

Species	Subjects Tested, No.	ChoE Activity[a] ± S. D.
Human	5	0.966 ± .12
Guinea pig	10	0.635 ± .10
Rabbit	22	0.256 ± .05

[a] Expressed as corrected pH change/hour.

RESULTS

Cholinesterase Activity.—The cholinesterase activity of human, guinea pig, and rabbit serums was relatively constant within each species tested, with human serum having the greatest activity and rabbit serum the least (Table I). This agrees with earlier findings of Mendel *et al.* (23). The human serum cholinesterase activity reported here falls within normal established limits (24).

Procaine Hydrolysis.—The ultraviolet absorption spectra of a 5×10^{-5} *M* solution of procaine hydro-

chloride in phosphate buffer and of a 5×10^{-5} M solution of p-aminobenzoic acid in buffer are shown in Fig. 1. Since the absorbance of p-aminobenzoic acid was negligible at 313 mμ, this wavelength was chosen for enzymatic hydrolysis studies. Although a greater difference in the absorbance of the substrate and its metabolized product exists at wavelength 300, this point could not be used because of the absorbance of serum at this wavelength.

The relationship between absorbance at 313 mμ and molar concentration of procaine is shown in Fig. 2. Since Beer's law is obeyed, a factor was calculated which allowed the conversion of decrease in absorbance to micromoles of procaine hydrolyzed. At this wavelength a change in absorbance of 0.078 occurred for each change of 1×10^{-5} M concentration. Since each milliliter of a 1×10^{-5} M concentration contains .01 μm. of procaine, a drop in absorbance of 0.078 would be the equivalent of .01 μm. of procaine hydrolyzed per milliliter of the solution.

Typical hydrolyses of procaine at timed intervals by human and guinea pig serums are shown in Fig.

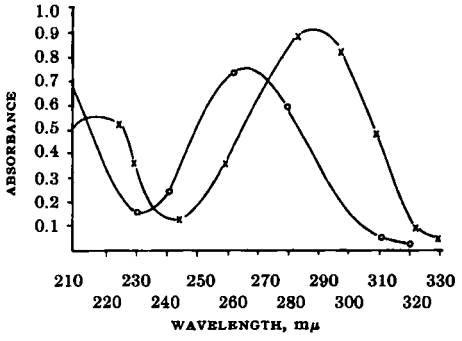

Fig. 1.—The ultraviolet absorption spectra of a 5×10^{-5} M solution of procaine hydrochloride in phosphate buffer (×) and a 5×10^{-5} M solution of p-aminobenzoic acid in buffer (○).

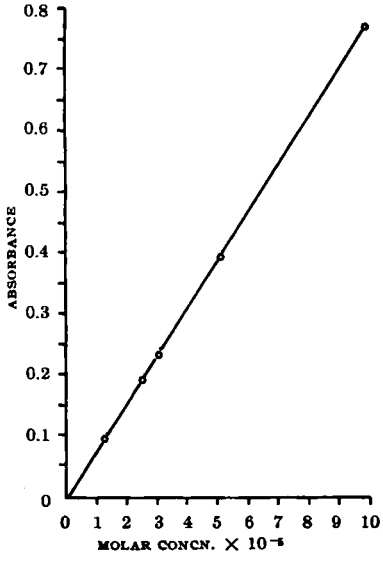

Fig. 2.—The relationship between absorbance at 313 mμ and molar concentration of procaine hydrochloride in phosphate buffer.

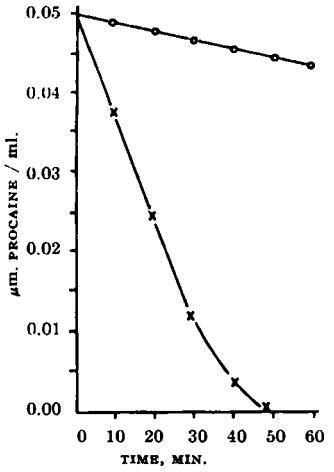

Fig. 3.—Typical hydrolyses of procaine at timed intervals by guinea pig serum (○) and human serum (×) as calculated from absorbance measurements at 313 mμ.

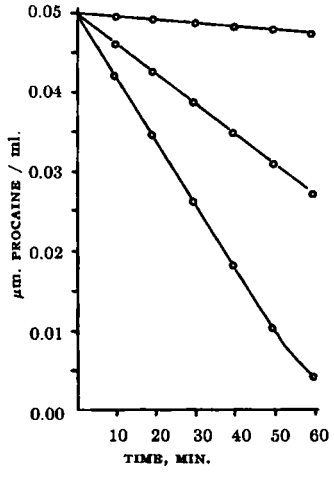

Fig. 4.—Typical hydrolyses of procaine at timed intervals by different rabbit serums as calculated from absorbance measurements at 313 mμ.

3. Human serum hydrolyzed procaine at a rate more rapid than that of either of the other two test species. In the presence of excess substrate, this hydrolysis was relatively constant until substrate concentrations became low, at which time the rate of hydrolysis decreased. Guinea pig serum, while fairly rich in cholinesterase activity, failed to hydrolyze procaine to an appreciable extent.

The amounts of procaine hydrolyzed by three different but typical rabbit serums is shown in Fig. 4; unlike the human and guinea pig serums tested, rabbit serums exhibit wide variations in procainesterase activity. The procainesterase activities of the serums from test rabbits were grouped at three levels of activity: low (0.0152 to 0.0592 μm./hour/ml. serum), intermediate (0.1232 to 0.1824 μm./hour/ml. serum), and high (0.248 to 0.368 μm./hour/ml. serum).

Duration of Procaine Local Anesthesia.—The duration of local anesthetic action due to the intracutaneous injection of 0.5 ml. of 1% procaine hydro-

TABLE II.—RELATIONSHIP BETWEEN CHOLINESTERASE AND PROCAINESTERASE ACTIVITY IN RABBIT SERUM TO DURATION OF ACTION OF PROCAINE *In Vivo*

Rabbit No.	ChoE Activity pH Change/Hr.	Procainesterase Activity Drop in O.D./Hr.	Procainesterase Activity μm. Hydrolyzed/Hr./ml. Serum	Duration of Anesthesia,[a] Min.
12	0.21	0.015	0.0152	95
20	0.23	0.016	0.0168	96
15	0.23	0.017	0.0176	120
18	0.31	0.020	0.0208	93
10	0.20	0.026	0.0264	120
2	0.26	0.027	0.0280	118
1	0.23	0.037	0.0376	100
6	0.35	0.040	0.0408	90
14	0.20	0.047	0.0480	115
16	0.23	0.050	0.0512	110
8	0.31	0.050	0.0512	80
7	0.16	0.057	0.0584	115
5	0.31	0.058	0.0592	126
17	0.21	0.120	0.1232	62
21	0.28	0.144	0.1480	62
3	0.24	0.175	0.1792	50
11	0.23	0.178	0.1824	60
19	0.24	0.242	0.2480	55
13	0.24	0.275	0.2824	86
22	0.26	0.334	0.3424	40
9	0.35	0.359	0.3680	45

[a] Due to intracutaneous injection of 0.5 ml. of 1% procaine hydrochloride in isotonic sodium chloride solution.

chloride in guinea pigs was 42.9±4.1 minutes. This was shorter than that observed in those rabbits having the highest procainesterase levels. Thus, it became apparent that the duration of local anesthesia in different species could not be explained on the basis of procainesterase levels in the serum.

Due to the wide variation of serum procainesterase activity between individual rabbits, this animal was used to compare procainesterase activity of serum *in vitro* to duration of procaine anesthesia *in vivo*. Table II illustrates this correlation. The correlation coefficient of procainesterase activity *versus* the reciprocal of duration of procaine local anesthesia was calculated to be 0.8539 ($p < .001$).

Atropinesterase Activity.—Ing *et al.* (20) reported that the extent of dilatation of the mouse pupil by atropine is directly correlated with the logarithm of the dose. In this study mice having pupil diameters of 0.43 mm. prior to atropine injection had pupil diameters of 1.1 and 1.9 mm. 15 minutes following 12.5 and 25 mcg. of atropine sulfate, respectively.

The comparison of procainesterase activity to atropinesterase activity of the individual rabbit serums is shown in Table III. Serum atropinesterase activity is indicated if atropine mydriasis is destroyed upon incubation of the drug with the serum. Each animal received an intraperitoneal injection of 0.5 ml. of rabbit serum that had been incubated for 1 hour with 25 mcg. of atropine sulfate. Rabbit serums having little procainesterase activity had little atropinesterase activity; however, rabbit serums having appreciable procainesterase activity were able to destroy 50 mcg. of atropine sulfate per milliliter of serum in 1 hour.

The inhibitory effects of atropine upon procaine hydrolysis by a rabbit serum are illustrated in Fig. 5. Approximately equal molar concentrations of the two drugs resulted in only a slight inhibition of procaine hydrolysis; but when 2.5×10^{-3} *M* (final dilution) atropine sulfate was incubated with 2.1×10^{-5} *M* (final dilution) procaine hydrochloride, procaine hydrolysis was inhibited 85.2% at the end of 1 hour.

TABLE III.—PROCAINESTERASE AND ATROPINESTERASE ACTIVITIES OF RABBIT SERUMS

Rabbit No.	Procainesterase Activity μm. Procaine Hydrolyzed/Hr./ml. Serum	Atropinesterase Activity[a] Mouse Pupil Size Before and After I. P. Injection Incubated Serum, mm. Before	After
20	0.0168	0.43	1.74
20	0.0168	0.43	2.17
20	0.0168	0.43	1.74
2	0.0280	0.43	1.96
2	0.0280	0.43	1.74
6	0.0408	0.43	1.74
6	0.0408	0.43	1.74
6	0.0408	0.43	1.74
3	0.1792	0.43	0.43
3	0.1792	0.43	0.43
3	0.1792	0.43	0.43
22	0.3424	0.43	0.43
22	0.3424	0.43	0.43
9	0.3680	0.43	0.43
9	0.3680	0.43	0.43

[a] Indicated by absence of atropine mydriasis after incubation of serum with 50 mcg. of atropine sulfate/milliliter for 1 hour.

DISCUSSION

The serum cholinesterase activity of humans, guinea pigs, and rabbits varies widely. As previously reported (23), human serum exhibits the greatest amount of cholinesterase activity, followed by guinea pig and then by rabbit serum. Optimum substrate concentrations exist for both pseudocholinesterase and true cholinesterase (23). The method employed for cholinesterase determinations in this study requires the use of high acetylcholine substrate concentrations. The inability of rabbit serum to hydrolyze acetylcholine to any appreciable extent at these high substrate concentrations lends support to the findings of Levine *et al.* (6) that acetylcholine hydrolysis by rabbit serum is largely a function of true cholinesterase. Similarly, the high activity of human serum toward acetylcholine in high substrate concentrations indicates that acetylcholine hydrolysis by human serum is a function of pseudocholinesterase.

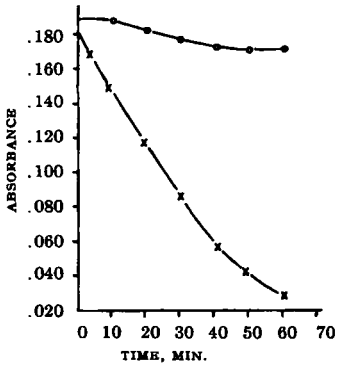

Fig. 5.—The hydrolysis of procaine by a rabbit serum when uninhibited (×) and when inhibited (○) by atropine.

Guinea pig serum, while exhibiting the characteristic action of pseudocholinesterase toward acetylcholine, failed to hydrolyze procaine at an appreciable rate. This finding indicates that acetylcholine hydrolysis is accomplished in the serum of guinea pigs in the same manner as it is in the liver, by true cholinesterase (9), or by a pseudocholinesterase which is distinct from the nonspecific enzyme found in human serum.

The human serum enzyme exerted the same high activity for the hydrolysis of procaine as toward the hydrolysis of acetylcholine. This was anticipated since it has been shown that in man both hydrolyses are accomplished by the same enzyme (2). This hydrolysis *in vitro* clearly shows that human serum cholinesterase has the ability to destroy considerable amounts of procaine when injected *in vivo*.

Rabbit serums varied widely in their ability to hydrolyze procaine *in vitro*. Three distinct levels of procainesterase activity were observed in the serums of individual rabbits. Procainesterase activity *in vitro* in the first group was essentially the same as that found in the serums of guinea pigs. This slow hydrolysis of procaine might be due to the action of some enzyme system not primarily concerned with substrates of the general molecular configuration of procaine. Procaine hydrolysis by the second and third groups is accomplished by an enzyme capable of actively hydrolyzing procaine, differing only in the level of enzymatic activity. No relationship was found between *in vitro* cholinesterase activity and *in vitro* procainesterase activity in rabbit serum; however, both are inhibited by diisopropyl phosphorofluoridate.

The duration of local anesthesia in guinea pigs was generally shorter than in rabbits. Since the serum of guinea pigs failed to hydrolyze procaine *in vitro*, it might be assumed that this short duration of action was due to a more rapid diffusion from the site of injection with or without subsequent destruction by the enzymes of some other organ of the body.

Many variables exist between different species of animals, such as rates of diffusion, skin sensitivity, and the presence of different enzymes in the serums. Therefore, attempts to correlate serum enzyme activity and duration of local anesthetic action between different species may easily lead to erroneous conclusions. Consequently, duration of anesthesia determinations *in vivo* were not conducted in humans. However, since rabbits have several procainesterase levels, they appeared to be the ideal test animal to study the relationship between quantity of serum procainesterase and duration of anesthetic action of procaine *in vivo*.

Studies in the rabbit imply that the duration of local anesthetic activity of procaine is indeed affected by the level of procainesterase activity in the serum. A correlation ($r = 0.8539$) existed between the procainesterase activity of serum and the reciprocal of anesthetic duration which was highly significant statistically ($p < 0.001$). Based on the results of these experiments in rabbits one may assume, therefore, that if an enzyme is found in the serum of a given species capable of destroying a given drug actively, the duration of action of that drug will be affected directly by the enzymatic level of the serum.

It was not within the scope of this investigation to quantitate the amount of atropinesterase activity but to determine if atropinesterase activity was present in the serum of those rabbits that had displayed procainesterase activity. Qualitative tests employing the mouse pupil as an indicator for the presence of atropine showed that atropinesterase activity occurred in rabbit serums that had procainesterase activity; conversely, this activity was absent in the serum of rabbits that did not hydrolyze procaine extensively.

Inhibition studies provided additional evidence that procaine and atropine are hydrolyzed by the same enzyme. Low substrate concentrations of atropine produced only a transient effect on procaine hydrolysis, while higher concentrations of atropine were able to inhibit hydrolysis of procaine by rabbit serum by as much as 85% for 1 hour.

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