Pharmacology of Procaine in the Horse: Procaine Esterase Properties of Equine Plasma and Synovial Fluid

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SUMMARY AND CONCLUSIONS

Procaine added to whole equine blood or diluted plasma was hydrolyzed with half times of approximately 9 and 12 minutes, respectively, at 37 C. This hydrolytic activity was sensitive to heating and physostigmine but did not affect procainamide. At pharmacologic concentrations of procaine the rate of the hydrolytic reaction depended directly on the concentrations of plasma or procaine in the system and was less in whole blood than in plasma. These properties are consistent with hydrolysis being due to plasma esterases operating at less than saturating procaine concentrations. These esterases were also inhibited by cooling, sodium fluoride, or arsenite. Synovial fluid had approximately 20% procaine esterase activity of plasma. Comparison of hydrolytic activities of plasmas from Thoroughbred, Standardbred, and other breeds of horses showed statistically significant differences in the rates at which individual plasmas hydrolyzed procaine. A frequency distribution of these rates showed unimodal distribution, indicating that all horses tested may be regarded as members of a single population.

The pharmacology of procaine in horses is of forensic importance because the presence of procaine in the blood or urine of racing horses is prohibited by most racing authorities. In a preliminary report on the pharmacology of procaine in horses, Tobin et al[14] observed a relatively short half-life of procaine after its intravenous injection in horses. This short plasma half-life correlated well with an observed capability of horse plasma to hydrolyze procaine in vitro, presumably by means of plasma esterases.

Because this procaine hydrolytic activity of equine plasma seemed sufficiently active to be an important pathway for termination of the pharmacologic actions of procaine in the horse, its properties were investigated. Further, in man these esterases are active enough to destroy rapidly other local anesthetics,[7] succinylcholine,[5] atropine,[11] opiates,[1] acetylsalicylic acid,[9] steroids, and organophosphorus compounds.[1] Similarly, Kalow[6] has listed more than 200 drugs which contain ester links, and many of these, as reserpine, are of considerable importance in equine pharmacology. Because of this wide spectrum of drugs affected by esterases, it is necessary to have effective methods for blocking their hydrolytic activity when collecting blood samples for pharmacokinetic or forensic investigations such as prerace blood testing. Further, since plasma esterases may be an important pathway for the in vivo termination of action of procaine, we measured the plasma esterase activity of a number of horses of different breeds in a search for breed or individual differences in the hydrolysis of procaine that might affect the in vivo pharmacokinetics of procaine or other drugs containing ester links.

Another reason for investigating the esterase activity of equine plasma is a recent proposal that the procaine moiety of procaine penicillin remains complexed with penicillin in vivo and sterically protects it against hydrolysis by plasma esterases.[a] Since this hypothesis concerning the "procaine-penicillin" complex has been presented as a forensic argument, we characterized equine plasma esterases as a preliminary step in the study of the pharmacologic features of the proposed "procaine-penicillin" complex.

Materials and Methods

Procaine base, procaine HCl, and physostigmine (eserine) were commercially obtained.[b] The procaine base and procaine HCl ran as single spots on thin-layer chromatography in methanol or chloroform:methanol 9:1 on silica gel or neutral alumina plates.[15] The Na+ arsenite and the authentic procainamide[d] were commercially obtained. All other reagents and chemicals were analytical grade.

All equine blood or plasma samples were from recently collected blood. Blood samples were collected into 15-ml heparinized tubes,[e] placed in an ice bucket, and cooled to 0 C. The samples were centrifuged at 5,000 × g for 10 minutes, and the plasma was separated and used within 1 hour. Synovial fluid samples were collected from the carpal (knee) and tarsal (hock) joints of recently killed (less than 5 min) horses. Synovial fluid which became contaminated with blood during withdrawal was discarded. Whole blood used for esterase activity assays was also freshly collected. Blood samples collected from animals some distance from the laboratory (for the experiments

[a] Monti, G. W.: Some Aspects of the Metabolism of Procaine Penicillin in the Dog. MSc Thesis, the Ohio State University, Columbus, Oh, 1975.

[b] Sigma Chemical Company, St Louis, Mo.

[c] K & K, Ltd, Miles Road, Cleveland, Oh.

[d] E. R. Squibb & Sons, Princeton, NJ.

[e] Vacutainer, Becton, Dickinson and Company, Rutherford, NJ.

using different breeds) were kept at 0 C in an ice bucket for up to 2 hours before assay.

The procaine esterase activity assay was done as follows unless otherwise noted: Recently prepared plasma (2 ml) or synovial fluid (2 ml) was diluted with an equal volume of 60 mM phosphate buffer (pH 7.4) and incubated at 37 C. The experiment was started by the addition of the required concentration of procaine HCl and stopped by the addition of 1 ml of equal parts of 50% sodium arsenite and 1 mM physostigmine. The samples were then analyzed for procaine as described by Tobin et al.[15] Unless otherwise stated, all experimental points are the means of at least 4 experimental determinations. All lines were fitted by eye, except for the data from different breeds which were fitted by the method of least squares.

Results

The rate at which procaine HCl added to unbuffered, undiluted, whole equine blood kept at 37 C was hydrolyzed is shown (Fig 1). The apparent half-life of procaine added to this system was approximately 9 minutes, and the time course of hydrolysis was log linear over approximately a 100-fold concentration range. This concentration range includes all plasma levels at which pharmacologic and toxic effects of procaine are observed in equine species in vivo.[13] Results indicated that procaine was relatively rapidly hydrolyzed in equine blood at body temperature. Because only approximately 66% of the procaine added to whole blood was recovered from the plasma, seemingly, procaine distributed unequally between erythrocytes and plasma. In other experiments, recoveries from whole blood were essentially complete (Fig 2).

To determine which fraction of equine blood was responsible for this hydrolytic reaction, the rates of hydrolysis in diluted equine plasma were compared with hydrolysis in similarly diluted whole blood and erythrocyte fractions. In this and subsequent experiments, the blood fraction studied was buffered to pH 7.4 with 50 mM phosphate buffer to maintain normal blood pH. Hydrolysis in whole blood was significantly slower than that in plasma, and a negligible rate of hydrolysis was observed in the erythrocyte fraction. The hydrolytic activity of blood was identified as a plasma activity. The effect of the erythrocytes may be simply to dilute out the plasma. Because of the large variability in erythrocyte fraction commonly found in equine packed cell volumes,[10] most of the remaining experiments were performed with the plasma fraction alone.

The hydrolytic activity was identified as a plasma esterase activity (Fig 3). Significant loss of procaine was not observed in plasma samples which had been heated to 100 C for 10 minutes or in plasma samples to which 1.25×10^{-4} M eserine had been added. Procainamide was not hydrolyzed by equine plasma. Thus, the hydrolytic activity of equine plasma was heat labile, blocked by eserine, and inactive against amide bonds. The rate of the hydrolytic activity was directly dependent on the concentration of procaine. In other experiments in this laboratory (data not presented), the products of the hydrolytic reaction were examined by thin-layer chromatography as described by Tobin et al.[15] In these experiments, 90 minutes of incubation of procaine (2 μg/ml) with 2 ml of plasma in the standard
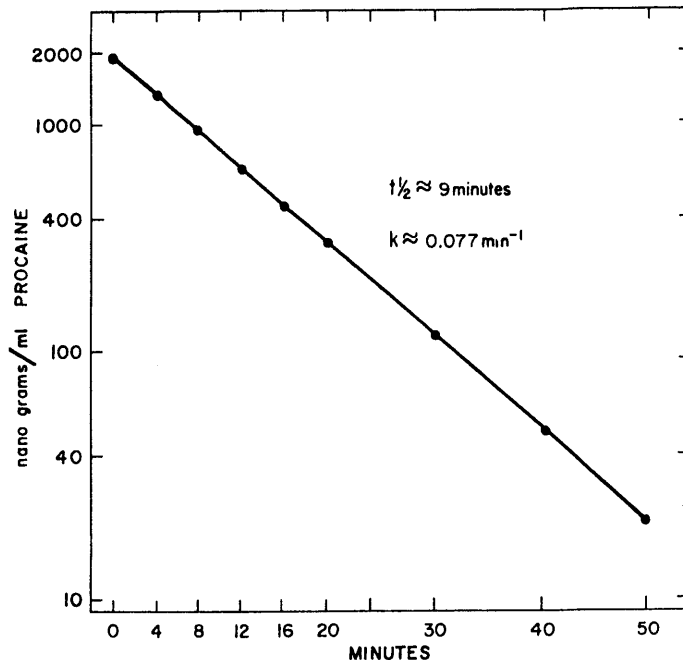

Fig 1—Hydrolysis of procaine by equine blood in vitro. Recently collected whole equine blood was incubated in 15-ml portions at 37 C with constant shaking. At indicated zero time, the reaction was started by the addition of 3 μg of procaine HCl per milliliter. The reaction was stopped when required by the addition of arsenite and eserine, the plasma was separated and the procaine concentrations in plasma was estimated as previously. All points are the means of 3 experimental determinations on different blood samples.
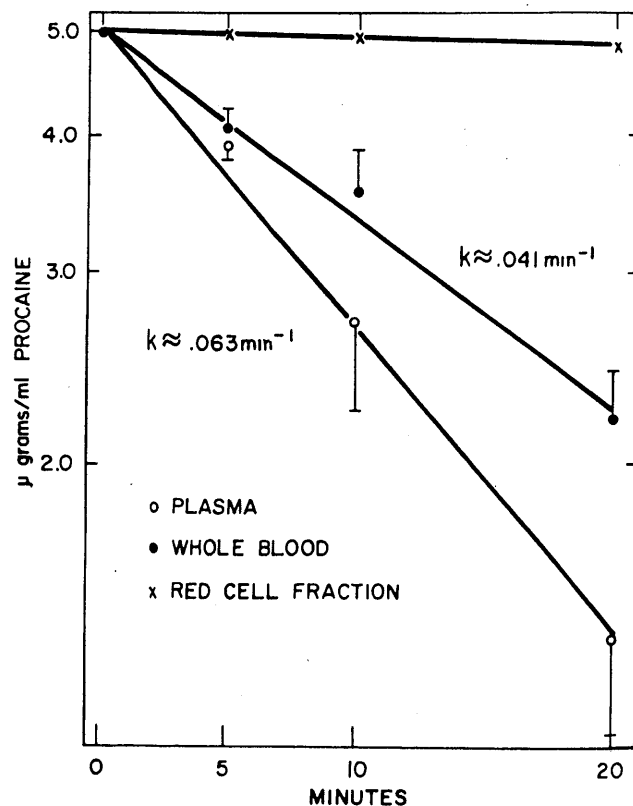

Fig 2—In vitro hydrolysis of procaine by equine plasma and whole blood. The open circles (O—O) show the rate of hydrolysis of procaine by 2 ml of equine plasma under standard conditions; the solid circles (●—●) show the rate of hydrolysis by 2 ml of diluted whole blood; and the crosses (×—×) show hydrolysis due to 2 ml of the packed red blood cell fraction. Each experimental point is the mean ± standard error of the mean (SEM) of experiments on 4 paired blood or plasma preparations, except for the red blood cell data which represent a single experiment.
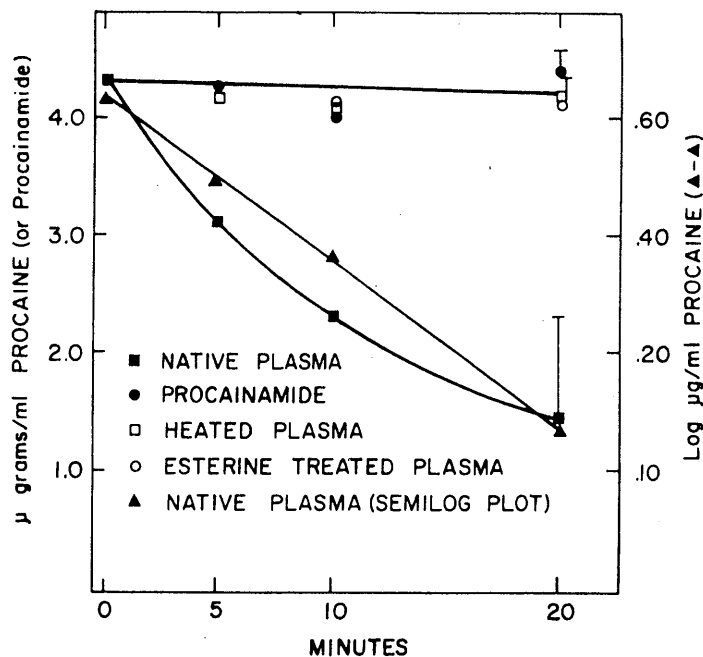
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Fig 3—Effects of eserine and heating on hydrolysis of procaine by equine plasma. The solid squares (■—■) show hydrolysis of procaine (5 μg/ml) by equine plasma when it was added to the standard incubation system at 37 C; the solid triangles (▲—▲) show the logarithms of these values plotted against time; the open circles (○—○) show hydrolysis of procaine by equine plasma in the presence of 1.25×10^{-4} M eserine; the open squares (□—□) show the hydrolysis of procaine by equine plasma which had been heated at 100 C for 10 minutes; the solid circles (●—●) show hydrolysis of procainamide by equine plasma under standard conditions. All data points are the mean of experiments on 4 equine plasmas, with the SEM plotted for the 20-minute points.
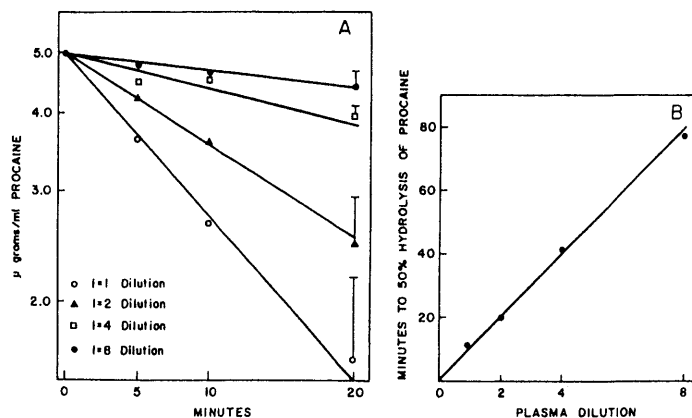

Fig 4—Effect of plasma dilution on hydrolysis of procaine by equine plasma. A—The open circles (○—○) show hydrolysis of procaine by 2 ml of equine plasma under the standard incubation conditions; the solid triangles (▲—▲), open squares (□—□), and solid circles (●—●), respectively, show hydrolysis of procaine by 1.0, 0.5, and 0.25 ml of equine plasma under standard conditions. All data points are the means ± SEM of experiments on 4 equine plasmas. B—The solid circles (●—●) show the half times for the in vitro hydrolysis of procaine by different concentrations of equine plasma obtained from Figure 4A plotted against plasma dilution.

assay system resulted in the disappearance of procaine from a basic plasma extract and the appearance of p-aminobenzoic acid (PABA) in an acidic extract of the plasma. Results indicated that the procaine hydrolytic activity of equine blood was due to plasma esterases and that p-aminobenzoic acid is one product of the reaction and thus of the metabolism of procaine in the horse.

The exponential nature of the procaine disappearance curve (Fig 1 and 2) was consistent with the procaine concentration in the system, being less than that required to saturate these esterases. Under these conditions, the rate of hydrolysis should follow simple mass action kinetics and be directly proportional to the concentration of either reactant in the system. Dilution of the amount of plasma in the assay system reduced the initial rate of hydrolysis of procaine (Fig 4A). The times for hydrolysis of 50% of the added procaine (obtained from Fig 4A) were directly proportional to dilution of the plasma (Fig 4B). Hydrolysis in equine plasma of pharmacologic concentrations of procaine followed mass action kinetics typical of enzymatic systems operating at substrate concentrations which were less than saturating. Since all in vivo blood levels of procaine are likely to be less than those used in the present experiment, the studies were continued with nonsaturating drug concentrations which were at the high end of the drug concentration range found in vivo.[13] Since the erythrocyte fraction was enzymatically inactive, the in vivo rate of hydrolysis of procaine could be calculated from in vitro data.
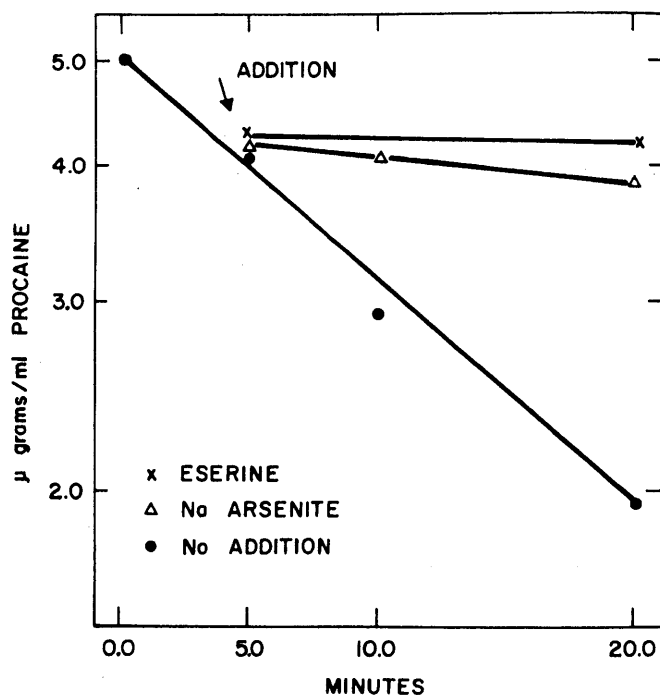

Fig 5—Onset of inhibition of esterase activity by arsenite or eserine. The solid circles (●—●) show the rate of procaine hydrolysis by 2 ml of equine plasma under standard conditions; the open triangles (△—△) and crosses (×—×) show the rate of procaine hydrolysis when 0.5 ml of 50% sodium arsenite or 1.25×10^{-3} M eserine were added after 5 minutes of incubation. All experimental points are the means of determinations on 3 equine plasmas.

If the plasma samples used in the experiments shown in Figure 4 contained any endogenous reversible esterase inhibitors, their presence would be indicated by a downward deviation in the curve of Figure 4B. Absence of such deviation indicates that reversible inhibitors of esterase activity were not present in these plasma samples.[2] Similarly, the data of Figure 1 and other experi-
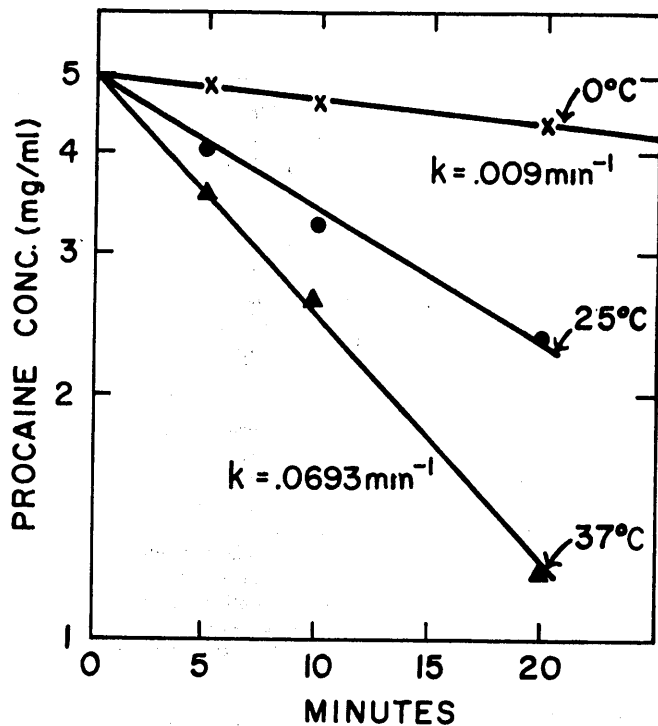
Fig 6—Effect of temperature on the rate of hydrolysis of procaine equine plasma. The solid triangles (▲—▲) show the rate of hydrolysis of procaine by 2 ml of equine plasma at 37 C; the solid circles (●—●) show the rate of hydrolysis at 25 C; and the crosses (✕—✕) show the rate of hydrolysis at 0 C. All points are the means of determinations on 3 plasma preparations. The Arrhenius activation energy for the reaction was calculated from the relationship: $Ea = [4.560 \cdot T_2 \cdot T_1 (\log K^2 - \log K^1)] (T_2 - T_1)$ and was determined to be 9.2 kCal/mole.
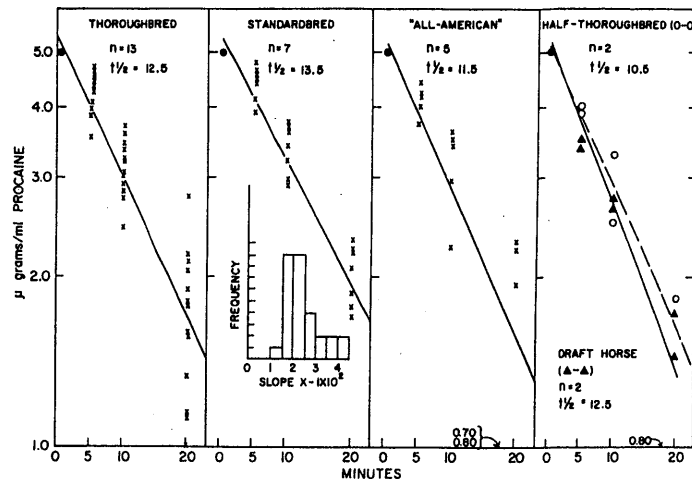

Fig 7—Rates of hydrolysis of procaine by plasma from different breeds of horses. Blood was freshly collected from horses of the indicated breeds, and the plasma was separated and added to the standard incubation system at 37 C. Each panel shows individual data points for the indicated breeds of horse, with a least squares regression line fitted to the mean values for each group at each time point. Three data points at 20 minutes plot off the figure, and these values are printed just above the horizontal axis. The designation "all-American" indicates horses of mixed or unknown background. An F test applied to these mean slopes showed that they were not significantly different at the $P < 0.05$ level. The inset panel shows a frequency distribution of the negative slopes of the least squares fit for the hydrolytic rates of each individual horse tested.

ments not presented show no change in the rate constant for hydrolysis when as much as 90% of the procaine added to the system was converted to products, indicating that these plasma esterases were relatively resistant to product inhibition.

In pharmacokinetic and forensic studies, it is important that blood levels of the drug measured are those which were actually present in the blood at the time the sample was collected. In the case of procaine, this requires prompt and effective inhibition of these esterases in blood samples. The effect of addition of 0.5 ml of 50% Na^+ arsenite or $1.25 \times 10^{-4}M$ physostigmine to the incubator system is shown (Fig 5). Both inhibitors seemed to act rapidly, and both reduced the rate of hydrolysis to close to zero; statistically significant differences were not seen between the 2 inhibitors. There was, however, a consistent tendency for a small rate of hydrolysis in the presence of arsenite, which could become significant over a longer period or in the presence of relatively active plasmas such as human or equine plasma. In other experiments not presented, high concentrations of fluoride also rapidly inhibited plasma esterase activity.

Another simple method of reducing the rate of an enzymatic reaction is cooling. Cooling the system to 0 C produced approximately an eightfold reduction in the initial rate of procaine hydrolysis (Fig 6). The Arrhenius activation energy[8] for the procaine esterase reaction was 9.2 kCal/mole.
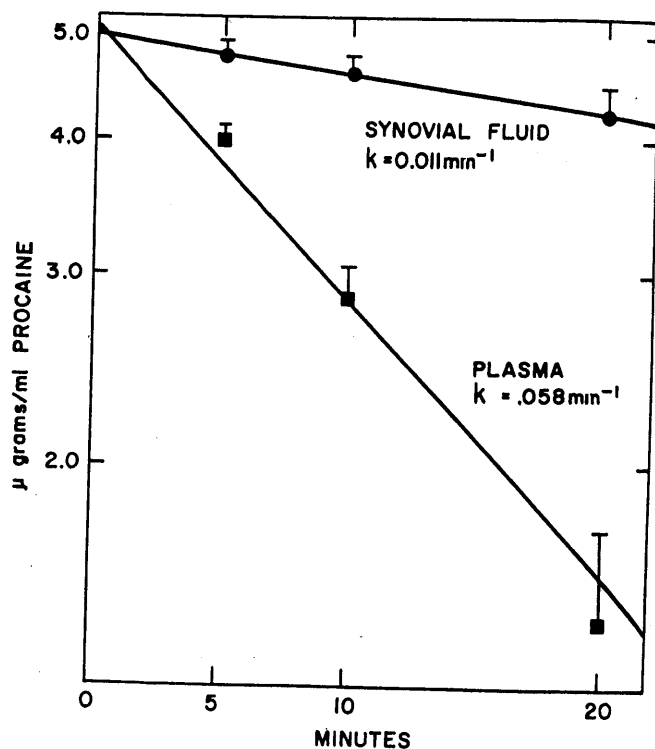

Fig 8—Hydrolysis of procaine by equine synovial fluid. Synovial fluid was collected from the knee and hock joints of recently killed horses, and 2 ml was added to the standard assay system. The solid circles (●—●) show the rate of hydrolysis of procaine by 9 synovial fluid samples; the solid squares (■—■) show hydrolysis by plasma from 4 horses; the vertical bars indicate the SEM.

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In preliminary studies[f] on the procaine esterase activity of equine plasma, marked differences were observed in the rates of hydrolysis of procaine by plasma samples from different racehorses. Because such differences would likely cause individual variations in the pharmacokinetics of procaine in equine species, the ability of recently prepared equine plasmas from a number of horses to hydrolyze procaine in vitro was investigated. Significant differences existed in the rates at which individual horses hydrolyzed procaine under conditions (nonsaturating kinetics in recently prepared plasma) which could be readily related to those occurring in vivo (Fig 7). A frequency distribution of these rates, however, indicated that this variability was that which might be expected within a single population.

A technique for alleviating joint lameness sometimes used in equine medicine involves direct injection of procaine into the affected joint; therefore, the ability of synovial fluid to hydrolyze procaine was tested. Synovial fluid hydrolyzed procaine at approximately 20% of the rate of plasma from the same horses (Fig 8).

Discussion

Equine blood or plasma hydrolyzed procaine in vitro at 37 C to para-amino benzoic acid and other products not identified. This hydrolytic activity was located in the plasma fraction and was sensitive to heating, physostigmine, arsenite, and fluoride, but did not affect procainamide. The rate of the hydrolytic reaction depended directly on the concentrations of plasma and procaine in the system, and the rate of hydrolysis in whole blood was less than that of plasma. The hydrolytic activity was also inhibited by cooling. In 30 horses of different breeds and ages, significant differences in the rate of hydrolysis of procaine by their plasmas were observed. Procaine was hydrolyzed by equine synovial fluid at approximately 20% the rate observed in plasma.

The characteristics of hydrolysis of procaine by equine plasma are consistent with it being due to plasma esterase activity. Plasma esterases have been studied in detail in many species.[3] Typically, plasma esterase activity consists of a mixture of nonspecific carboxylesterases (aliesterases), arylesterases (Are) and pseudocholinesterases (ψChe), but the proportions of each enzyme type varies from species to species. Studying mammalian plasma esterases, Ecobichon and Comeau[3] reported relatively high plasma pseudocholinesterase activity in man and horse, and in the horse this activity was classified as butyrlcholinesterase activity. These authors also identified arylesterase activity in equine plasma zymograms. Arylesterase activity is typically resistant to eserine inhibition, but in our hands physostigmine essentially completely inhibited the procaine esterase activity of equine plasma. Thus pseudocholinesterase appears to be the most important esterase component identified in equine plasma, and the importance of aliesterase and arylesterase activity remains unclear.

Because of the probable importance of plasma esterases as a mechanism for termination of the action of

procaine in vivo, it was of interest to investigate the kinetics of the reaction under conditions approximating those occurring in vivo. Thus, at plasma concentrations of 5 μg/ml and less, the rate of hydrolysis of procaine depended directly on the concentrations of procaine and plasma (Fig 1-5). Since the initial concentrations of procaine in the present experiments are generally somewhat greater than those found in vivo (Tobin et al, 1975),[14] it seems reasonable to conclude that saturation of the plasma esterase system will not occur in vivo. Results in other experiments[16] have shown that procaine administered to horses as IM procaine penicillin is hydrolyzed in blood isolated from these horses at the same rate as procaine added to blood in vitro under the conditions of the experiment of Figure 1. Thus, given a constant esterase activity in any individual horse, the rate of hydrolysis of plasma procaine in equine animals will depend directly on the concentration of the drug. The relative predictability of the plasma esterase activity seen in a large number of horses (Fig 7) indicates that fairly consistent and predictable pharmacokinetics may be expected for procaine in horses in vivo.

It seems reasonable to assume that the decrease in rate of the hydrolytic reaction (Fig 1 and 3) is due to decreasing saturation of the enzyme causing decreasing substrate concentrations. In this case, it is theoretically possible to fit progress curves such as those of Figures 1 and 3 to the integrated form of the Michaelas-Menten equation[2] and derive both the Vmax and Km values for the enzymatic system from data obtained at below saturating substrate concentrations. However, attempts to derive reasonable Km and Vmax values from the data of Figures 1 and 3, using the integrated Michaelas-Menten equation, were unsuccessful. Among possible reasons for this are the likelihood that the progress curve of Figure 1 represents the action of 1 or more blood esterases of differing Km values. Another complicating factor may be the binding of procaine in the erythrocyte fraction (Fig 1) which is presumably in equilibrium with, and slowly replaces, the pool of procaine available for hydrolysis. Thus, it has not been possible at this point to identify the kinetic criteria of the progress curve represented in the data of Figure 1 and which seems identical to the progress curve of procaine hydrolysis in the bloodstream of horses in vivo.[16]

The dependence of the rate of hydrolysis of procaine on the concentration of plasma presumably explains the slower hydrolysis of procaine observed in whole blood. Since little or no hydrolytic activity was associated with the erythrocytes, the diluting effect of the normally about 37% by volume of erythrocytes[10] on the rate of hydrolysis in plasma would be sufficient account for the observed 35.3% increase in the half-life of procaine in whole blood. This experiment also indicates that the half-time for procaine in whole blood at 37 C is likely to be 8 to 9 minutes, i.e., about half that of the 1:1 diluted blood used in the experiment of Figure 4. The observations made in diluted systems with buffered blood fractions are thus in good agreement with the data presented in Figure 1.

The experiment of Figure 2 also indicates that the rate of hydrolysis of procaine by a sample of equine

blood in vitro should vary with the packed cell volume. Since, in the horse, the packed cell volume can vary markedly, depending on the exercise status,[10] it is likely that the in vitro rate of hydrolysis of procaine by whole blood could vary, depending on the proportion of erythrocytes in the sample. It appears less likely that the half-life of procaine in vivo would vary much, depending on the exercise status of the animal, since there is no reason to suspect that the total amount of esterase present in an animal will vary with its exercise status. If the plasma esterases alone are predominant in the metabolism of procaine, the introduction of a large number of erythrocytes into the circulatory system associated with exercise could conceivably affect the rate at which procaine would be hydrolyzed in blood in vivo. However, procaine esterase activity in hepatic tissue has been reported by Yeary and Gerken,[17] and Terp[12] has shown considerable esterase activity in perfused rabbit hindquarters. Thus it is reasonable to assume that plasma esterase activity is only one of a number of esterase activities contributing to the decrease in plasma procaine concentrations, and small variations in the concentrations of plasma in the circulating system are unlikely to affect the overall plasma half-life of procaine in horses.

For pharmacokinetic study and forensic work with procaine and other drugs split by plasma esterases, the activity of these plasma esterases should be completely inhibited as soon as possible after blood samples are collected. For any given inhibitor, 2 principal sources of error exist, viz, (a) incomplete inhibition and (b) slow onset of complete inhibition. Figure 5 shows that the onset of inhibition due to either physostigmine or arsenite is rapid and, in the case of physostigmine, essentially complete. The inhibition due to arsenite was, however, less convincing and allowed an observable decrease in plasma procaine concentrations. In other experiments not presented,[f] similar incomplete inhibition due to arsenite in whole blood was observed. Another easily available technique for the stabilization of blood samples is cooling. As shown in Figure 5, cooling of the sample reduced hydrolysis of procaine in plasma eightfold. For these reasons, a combination of physostigmine, arsenite, and cooling was used wherever inhibition of these plasma esterases in blood or plasma samples was required.[14]

Because of the relative ease with which plasma procaine esterase activity can be measured, we tested equine plasmas for this activity. In this way, it was hoped that the probability of picking out breeds or individual horses which might react atypically to procaine would be increased. As shown in Figure 7, statistically significant differences in the rates of procaine hydrolysis between different breeds or species were observed. However, though statistically significant, these differences were small, and there were no significant differences in the mean rates at which the various groups hydrolyzed procaine. A frequency distribution of these data showed a unimodal distribution, indicating that all the horses tested comprised a single population as far as the hydrolysis of procaine in plasma was concerned. Therefore, on the basis of these observations, it may be expected that the in vivo pharmacokinetics of procaine in horses should be fairly consistent between different breeds and individual horses.

Because procaine is sometimes administered directly into joint capsules, we tested the procaine esterase activity of synovial fluid. Figure 8 shows that the esterase activity of synovial fluid is approximately 20% that of plasma. Since the synovial capsule is a highly vascular tissue[18] and the concentrations of drug introduced into the joint are likely to be relatively high, diffusion out of the joint is probably an important mechanism for the termination of action of intraarticularly administered procaine. It should thus be possible to detect measurable blood concentrations of procaine after intraarticular injection of procaine or other local anesthetics.

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