

Pharmacology of Procaine in the Horse: Evidence Against the Existence of a "Procaine-Penicillin" Complex

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SUMMARY AND CONCLUSION

It has recently been suggested that procaine penicillin existed in solution in vitro and in vivo as a "procaine-penicillin" complex rather than as dissociated ions. In vivo, this complexed procaine was considered unavailable for hydrolysis by plasma esterases or for interaction with pharmacologic receptors for procaine. When procaine penicillin was intramuscularly given to horses, about 90% of the procaine in blood drawn from these horses was split at the same rate as authentic procaine or procaine penicillin added to equine blood in vitro. In vitro, procaine and procaine penicillin partitioned similarly from aqueous medium at physiologic pH into several organic solvents and were split at the same rate by blood or plasma esterases. Experiments on the time course of the partitioning of procaine from procaine penicillin into benzene showed no evidence for the existence of a "procaine-penicillin" complex within seconds after procaine penicillin was added to aqueous medium. Thin layer chromatography in 2 dimensions also yielded no evidence for the existence of this postulated complex. These results show no evidence in support of the "procaine-penicillin" hypothesis and argue against the physical and pharmacologic and forensic implications of this hypothesis.

The presence of local anesthetics in the blood or urine of racing horses is prohibited by most racing authorities because the local anesthetic or central stimulant actions of these agents may affect the outcome of a race. In the case of procaine, this ruling created considerable problems because long-acting penicillin preparations containing procaine are often given to horses for reasons unrelated to the local anesthetic or central stimulant actions of procaine. Thus, procaine was the

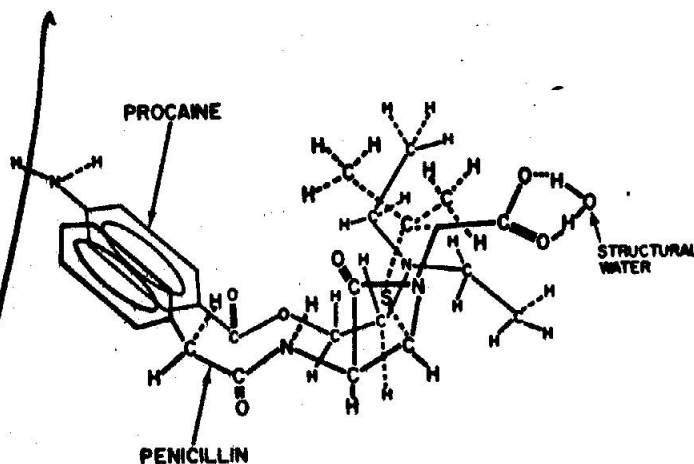


Fig 1—Proposed structure of procaine-penicillin salt.^a (Monti, 1975). The penicillin G moiety is based on the Drieding stereomodel of penicillin G.^b The diethylamino portion of the procaine molecule lies in the "valley" between the β -lactam and the thiazolidine rings. Thus, there are "electron clouds" at the 2 ends of the molecule with high electron densities and a relatively open area about the ester bond of procaine. Structural components of the penicillin molecule are represented by the heavier lines.

drug most commonly reported^a by the Association of Official Racing Chemists for the period 1949-1961, possibly, in some of these instances, procaine was administered as procaine penicillin.

Procaine penicillin is used clinically because the salt of procaine penicillin dissolves very slowly in water. When administered intramuscularly (IM), procaine penicillin is thought to dissolve slowly in the muscle fluid, and then the procaine and the penicillin distribute independently. Because the dissolution process is slow, blood concentrations of both procaine and penicillin are maintained much longer than if the 2 agents were injected alone.^a In this traditional interpretation of the actions of procaine penicillin, the rate-limiting process is the rate of dissolution of procaine penicillin.

Recently it has been suggested that procaine penicillin can exist in solution in vitro⁷ and in vivo^a as a "procaine-penicillin" complex and a molecular structure for this complex (Fig 1) has been proposed.^b This theory holds that after administration of procaine penicillin to animals, the predominant species present in the bloodstream is this molecular complex rather than the dissociated ions. In the bloodstream this molecular complex exerts the antibacterial action of penicillin by

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^a Monti, G. W.: Some aspects of the Metabolism of Procaine Penicillin in the Dog. MSc Thesis, Department of Veterinary Physiology and Pharmacology, Ohio State University, 1975.

^b Throughout this report, the hypothetical complex of procaine and penicillin described by Monti is referred to as the "procaine-penicillin" complex. Procaine plus penicillin refers to procaine and penicillin independently added to the same solution. Procaine penicillin refers to the crystalline salt of procaine-penicillin.

virtue of its unhindered β -lactam ring. The procaine portion of this molecule, however, is considered to be protected against the action of plasma esterases by the complexed penicillin molecule and, thus, escapes hydrolysis in the bloodstream. The intact complex is thought to be eliminated via the kidneys and to dissociate in alkaline urine to yield free procaine and penicillin.⁸

According to this theory, significant quantities of free procaine are not present in the animal which is given procaine penicillin. An extrapolation of this theory is that none of the pharmacologic actions of procaine may be expected after in vivo administration of procaine penicillin. Thus, the "procaine-penicillin" hypothesis and its pharmacologic extensions become attractive forensic arguments in doping cases.

In this communication, we studied the properties of procaine penicillin both in vivo and in vitro. In several systems, we failed to detect any differences between procaine and procaine penicillin. The results reported here deny the "procaine-penicillin" hypothesis of Monti⁸ and, therefore, its pharmacologic and forensic extrapolations.

Materials and Methods

Procaine base, procaine HCl, procaine penicillin, and physostigmine (eserine) were obtained from a commercial source.^{*} In our own tests, procaine base and procaine HCl ran as a single spot on thin-layer chromatography in methanol or chloroform:methanol (9:1) on silica gel or neutral alumina plates.¹² The procaine penicillin used for intramuscular injection in horses in vivo was procaine penicillin.⁸ Na⁺ arsenite was obtained from a commercial source.^{*} All other reagents and chemicals were analytical grade.

Blood samples were drawn into 15-ml heparinized tubes (partial vacuum) with added inhibitors (eserine and Na⁺ arsenite),^{9,10} placed in an ice bucket, and cooled to 0 C. They were centrifuged at $5,000 \times g$ for 10 minutes; the plasma was separated and used within 1 hour. For addition of procaine or procaine penicillin to aqueous environments the agent was dissolved in a small quantity of methanol:isopropanol (7:3) buffered to pH 5:1 as described by Monti.⁸ For thin-layer chromatography, this solution was spotted on A.G. F254 neutral alumina plates.⁷ These were then chromatographed in methanol:isopropanol (7:3). Procaine and penicillin spots were visualized by Blake's method, which consisted of overspraying with a freshly prepared 10% solution of KMnO₄ in 1 M HCl, gently heating, and spraying with bromocresol green.⁸ With this overspray, both procaine and penicillin gave powder blue spots. Unless otherwise noted, all experimental points are the mean \pm SEM of at least 4 separate experiments.

Results

Since the esterase activity of equine blood has been characterized previously,^{9,11} the simplest test of the "procaine-penicillin" hypothesis was to study the rate at which procaine penicillin was hydrolyzed in vitro by these esterases. The experiment illustrated (Fig 2) shows that diluted equine plasma hydrolyzed procaine

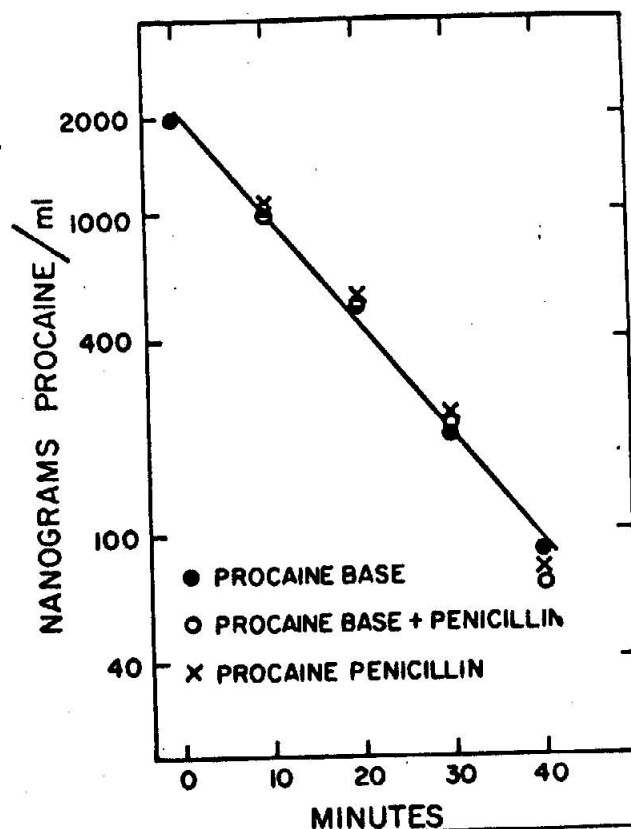


Fig 2—Hydrolysis of procaine added as procaine-penicillin by equine plasma in vitro. Procaine as procaine base, procaine penicillin, or procaine base plus penicillin were added to diluted equine plasma to a final concentration of 2,000 ng/ml and incubated at 37 C. Crosses (X-X) = time course of hydrolysis of the procaine added as procaine-penicillin; solid circles (●-●) = hydrolysis of procaine base; and open circles (○-○) = rate of hydrolysis of procaine base plus penicillin. Data points are the means of 4 separate determinations on different equine plasmas, while the points at 40 minutes are the means of 7 separate determinations. Analysis of the least squares fit to each of these lines showed that the slopes were not significantly different ($P > 0.25$).

added in vitro as procaine penicillin at the same rate as procaine base or procaine added as procaine plus penicillin. This experiment confirms and improves on preliminary experiments reported by Tobin et al.⁹

Because the mechanism by which procaine penicillin was introduced into the aqueous environment in vitro (Fig 2) is different from that occurring in vivo, we administered procaine penicillin by IM injection to horses and measured the rate of hydrolysis of plasma procaine. Thus, in the experiment (Fig 3), procaine penicillin 33,000 iv/kg was given IM to horses and plasma procaine levels determined at 2, 4½, and 7 hours post-dosing. Further, at these times blood samples were drawn into heparinized tubes, without added inhibitors, and held at 37 C. The hydrolysis of plasma procaine in these tubes was stopped at the indicated intervals after drawing by adding 1 ml of 50% Na⁺ arsenite and 0.5 ml of 1 mM eserine and the remaining plasma procaine determined. Procaine concentrations in the sample decreased rapidly (initial $t_{1/2} \sim 8$ minutes), suggesting rapid enzymatic hydrolysis of the procaine in the samples. In contrast, blood procaine concentration in these animals decreased slowly, with an apparent $t_{1/2}$ of about 8 hours.

* Sigma Chemical Company, St. Louis, Mo.

* Duracillin, Eli Lilly and Company, Ltd, Indianapolis, In.

* K & K Ltd, Cleveland, Oh.

* Brinkmann Instruments, Des Plaines, IL 60016.

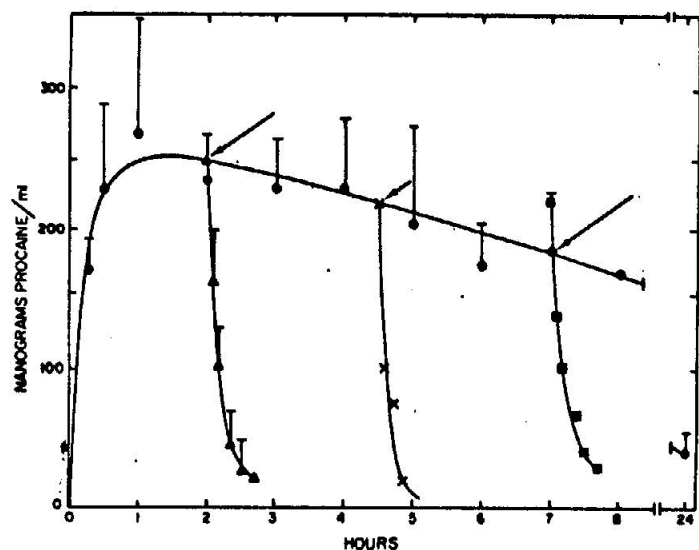


Fig 3—Hydrolysis by blood esterases of procaine administered by intramuscular (IM) injection to horses as procaine penicillin. Five Thoroughbred mares were given procaine penicillin (33,000 U/kg) IM in the side of the neck. The solid circles (●—●) show plasma procaine concentrations at the indicated times after injection. At given times (arrows) blood samples were collected and immediately incubated at 37 C. At the indicated times the esterase reaction was stopped by the addition of arsenite and physostigmine and the plasma procaine concentration was estimated. Plasma procaine values are the means \pm SEM of 5 experimental determinations in different horses. The hydrolysis curve at 2 hours (solid triangles, Δ — Δ) represents 4 separate experiments \pm SEM. The hydrolysis curves at 7 (solid squares, \blacksquare — \blacksquare) and 4.5 hours (crosses, \times — \times) represent triplicate and single experimental determinations, respectively.

This rapid hydrolysis of procaine in equine blood samples indicated that procaine administered in vivo as procaine penicillin was readily available for hydrolysis by plasma esterases. If this were so, the rate of hydrolysis observed in the experiment of Figure 3 should be similar to that of authentic procaine added to equine blood in vitro. Rates of hydrolysis of added authentic procaine and procaine penicillin in equine blood (in vitro) shown (Fig 4) were similar and compared well with the rate of hydrolysis of procaine which entered the blood of the horse with the IM injection of procaine penicillin (in vivo). The experiment showed that the rate of hydrolysis of procaine in blood was the same whether or not it was added to the blood as procaine or procaine penicillin. Deviation from the linearity was observed with procaine penicillin when more than 90% of the added procaine was hydrolyzed. The nature of this resistant material was not determined, but the amount constituted a relatively small percentage (less than 3%) of the material giving the color reaction for procaine.

The decrease of the plasma procaine concentration in blood samples drawn at 2 hours in the experiment of Figure 3 are shown (Fig 4A, open circles). The initial rate of hydrolysis was statistically significantly different from, but closely approximated, the rate observed for added procaine penicillin in equine blood (in vitro). Further, the rate of hydrolysis decreased as the reaction proceeds in a manner similar to that observed for authentic procaine penicillin in vitro.

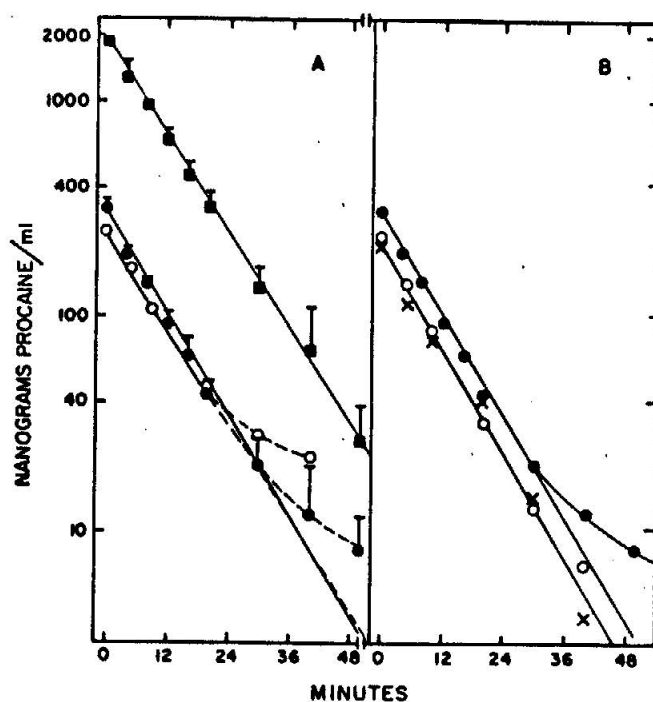


Fig 4—Rates of hydrolysis of procaine and procaine penicillin by equine blood in vitro and in vivo. Panel A—Procaine ($3\mu\text{g/ml}$) or procaine penicillin ($0.5\mu\text{g/ml}$) was added to 15 ml of equine blood in vitro at 37 C. Solid squares (■—■) = time course of disappearance of procaine from the plasma fraction of these samples with added procaine; solid circles (●—●) = disappearance of procaine penicillin; and open circles (○—○) = rate of decline of the plasma procaine concentrations in the blood samples taken at 2 hours from the horses in the experiment of Figure 3. Data points are the means \pm SEM of 4 individual experiments except that the SEM on the 2-hour in vivo hydrolysis study are omitted (for clarity). Because of the deviation from linearity in hydrolysis at low concentrations, points obtained after 36 minutes with procaine penicillin in vitro and in vivo were not included in calculation of the slopes of these curves. Least squares fit to these curves indicated the rates of hydrolysis of added procaine and added procaine penicillin in blood (in vitro) were not significantly different ($P < 0.05$). However, rate of hydrolysis of the procaine penicillin given in vivo is significantly slower than the rate of procaine penicillin added in vitro ($P < 0.01$). Panel B—Rates are shown at which the blood procaine concentrations in the samples drawn from the horses of Figure 3 decreased when corrected for the presence of 18 ng of nonhydrolyzable material/ml (crosses (\times — \times) 7-hour samples). Solid circles (●—●) = rate of hydrolysis of added procaine penicillin in horse blood (in vitro) replotted from panel A. (All lines fitted by visual observation).

Hydrolysis curves obtained for procaine penicillin both in vitro and in vivo were consistent with the presence of a small amount of material resistant to hydrolysis by plasma esterases. If this resistant material were assumed to be about 18 and 26 ng/ml in the 2- and 7-hour hydrolysis curves, respectively, and that if these values were deducted out (curve peeling), the rates of hydrolysis in vitro and in vivo were similar (Fig 4B). Thus, about 90% of the material giving the color reaction for procaine in vivo was susceptible to hydrolysis at the same rate as authentic procaine but that a small portion of the material is resistant to hydrolysis (Fig 3 and 4). In other experiments,¹⁰ a similar reduction in the rate of hydrolysis of procaine was seen after the IM injection of authentic procaine HCl; thus, this resistant portion does not appear to be unique to procaine penicillin.

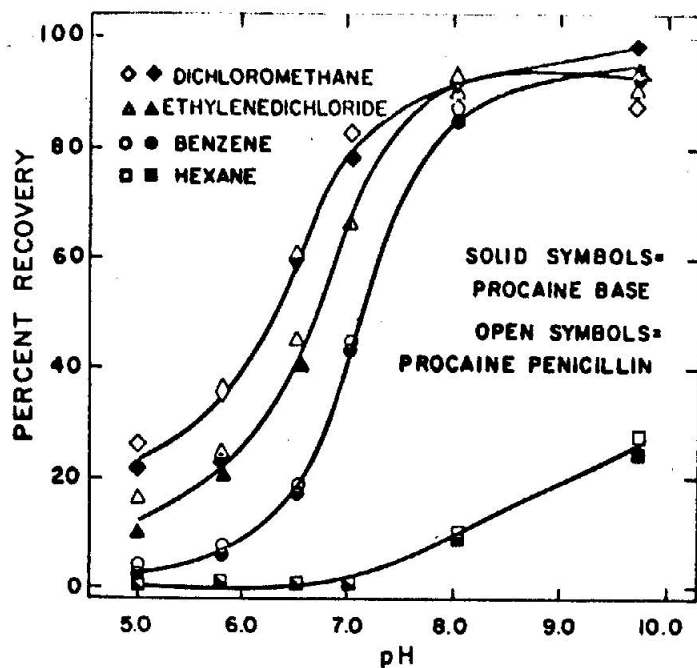


Fig 5—Partitioning of procaine or procaine · penicillin into polar solvents. Procaine (7 μ g) as either procaine base or procaine penicillin was dissolved in methanol:isopropanol (7:3) and added to 7.0 ml of 10 mM phosphate buffer of the indicated pH values. An equal volume of the organic phase was then added to the system, and the whole was shaken for 15 minutes. The organic phase was then decanted and its procaine content was estimated. Solid circles (\bullet — \bullet), squares (\blacksquare — \blacksquare), triangles (\blacktriangle — \blacktriangle), and diamonds (\blacklozenge — \blacklozenge) show the partitioning of procaine base into benzene, hexane, ethylene dichloride, and dichloromethane, respectively. Open circles (\circ — \circ), squares (\square — \square), triangles (\triangle — \triangle), and diamonds (\diamond — \diamond) show the partitioning of "procaine · penicillin" into the same solvents. Experimental points are single determinations except those for benzene, which are the means of 3 individual experiments.

The results of the experiments (Fig 2, 3, and 4) showed that almost all (90% or more) of the procaine of procaine penicillin was readily available for hydrolysis by plasma esterases either in vitro or in vivo. Since procaine complexed with penicillin may be just as susceptible (though unlikely) to hydrolysis by plasma esterases as free procaine, results reported so far do not rule out the existence of the proposed "procaine · penicillin" complex. Therefore, the approach of comparing the partition coefficients of procaine and procaine penicillin was taken. Because of the different molecular structure of the "procaine · penicillin" complex and particularly because of the presence of a free carboxyl group on the penicillin portion of the complex, the distribution into organic solvents of the "procaine · penicillin" complex should be different from that of free procaine.² The data (Fig 5), however, showed no differences in the partitioning of procaine and procaine penicillin between aqueous environments and 4 different polar solvents over a 5-unit pH range. These observations indicated that procaine in each case distributed independently of penicillin.

A comparison of the 2-dimensional thin-layer chromatographic patterns of procaine penicillin and a solution of equivalent concentrations of free procaine and penicillin is shown (Fig 6). About 6 μ l (300 μ g) of each solution was first spotted on the plate at the 100%

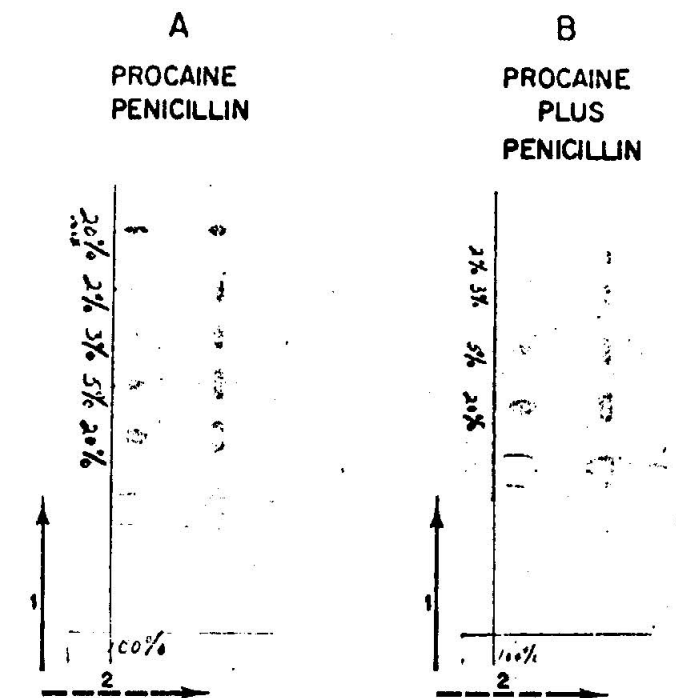


Fig 6—Two-dimensional thin-layer chromatography of procaine penicillin and procaine plus penicillin. Panel A—Two-dimensional thin layer chromatogram of procaine penicillin. About 300 μ g of procaine penicillin freshly dissolved in methanol-isopropanol (as described in Materials and Methods) was spotted at the point indicated as 100%. This material was then chromatographed in methanol-isopropanol system in the direction indicated by the solid arrow for about 7 minutes. The plate was then removed from the tank and air-dried, and the procaine spot at R_f 0.9 visualized under ultraviolet light. Amounts of procaine penicillin corresponding to 20, 5, 3, and 2% of the original amount of procaine penicillin were then spotted beside the procaine spot. Also, a mixture of procaine base plus penicillin in methanol-isopropanol, corresponding to 20% of the original quantity of procaine penicillin in the system, was spotted parallel to the procaine spot. The plate was then re-chromatographed at right angles to the original direction (dotted arrow). After about 7 minutes of chromatography, the plate was air-dried and sprayed. Panel B—A repetition of this experiment except that all spots are from equivalent concentrations of procaine base and the penicillin dissolved in the methanol-isopropanol system.

point and chromatographed in the direction of the solid arrows. This produced 2 distinct spots, one with the R_f of penicillin (about 0.1) and the other with the R_f of procaine (about 0.9). These data are in agreement with those reported by Monti.⁴ However, on the basis of autoradiographic studies, Monti⁴ considered that about 25% of the material at R_f 0.9 in his system was a labile "procaine · penicillin" complex. Therefore, plates in the present study were re-chromatographed at right angles to the original direction to determine whether the material at R_f 0.9 would again yield the 2 spots typical for procaine penicillin in this system. No distinct spot corresponding to the 0.1 R_f of penicillin was seen on these plates, although quantities of procaine penicillin as small as 2% of the amount of original material spotted could be detected. Further, the mixture of procaine and penicillin could not be distinguished from the "procaine · penicillin" preparation by this method.

In the experiment shown in Figure 7, study was made of the rates at which freshly prepared aqueous

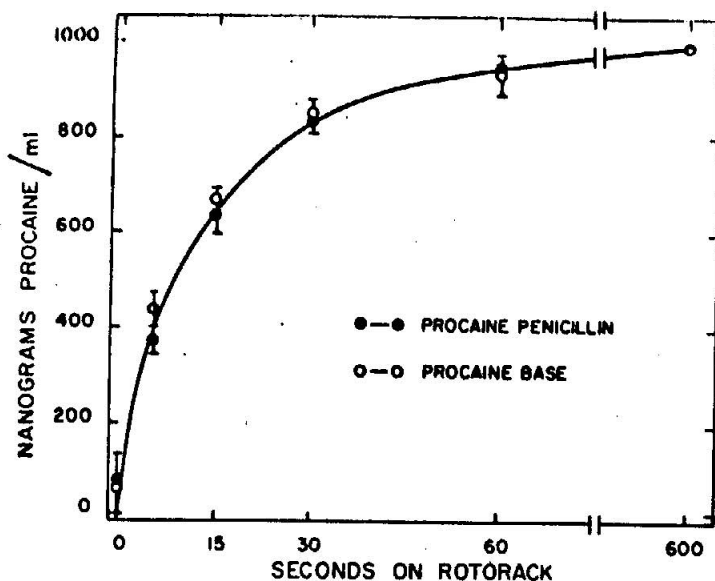


Fig 7—Rate of partitioning of procaine or procaine penicillin from aqueous solution into benzene. Procaine base or procaine penicillin was freshly dissolved in methanol:isopropanol and amounts equivalent to 7 μ g of procaine added to 7.0 ml of 10 mM phosphate buffer (pH 7.0). Immediately after addition of the procaine, 7 ml of benzene was layered on top of the aqueous phase and the tubes shaken for the indicated number of seconds. The tubes were allowed to stand for 3 minutes to permit the phases to separate, and the amount of procaine in the benzene layer was then estimated. Each experimental point is the mean \pm SEM of 4 experimental determinations.

solutions of procaine and procaine penicillin partitioned into benzene. Since partitioning occurred rapidly it should be capable of detecting the existence of a relatively short-lived "procaine·penicillin" complex. Significant differences in the rates of partitioning of procaine or procaine penicillin were not observed.

Discussion

Equine plasma esterases hydrolyzed procaine from procaine penicillin at the same rate as they hydrolyzed authentic procaine *in vitro*. Equine blood hydrolyzed authentic procaine and procaine penicillin at the same rates *in vitro*, and this rate was similar to the rate at which more than 90% of the procaine administered as procaine penicillin *in vivo* was hydrolyzed. Distributions of procaine penicillin from aqueous medium into several organic solvents were not different from those of free procaine, and the rates at which this distribution equilibrated in a benzene water system for both procaine and procaine penicillin could not be distinguished. Results of 2-dimensional thin-layer chromatography of authentic procaine penicillin could not be distinguished from those of a mixture of procaine and penicillin. Thus, 6 experimental approaches in 3 experimental systems offered no evidence for the existence in aqueous medium at physiologic pH of the postulated "procaine·penicillin" complex.

In vivo, 90% or more of the procaine present in the bloodstream of horses treated with procaine penicillin was available for hydrolysis by plasma esterases. Similarly, procaine and procaine penicillin was hydrolyzed at same rates when added *in vitro* to either whole equine blood or diluted equine plasma. When procaine peni-

cillin was used as the substrate *in vitro*, a small portion (less than 3%) of the material added to the system gave the color reaction for procaine but was resistant to hydrolysis by the esterases. In other experiments, a similar slow hydrolysis of residual amounts of procaine in equine blood were observed when authentic procaine was given IM to horses.

Though the bulk of the procaine added to equine blood either *in vitro* (Fig 3) or *in vivo* (Fig 2) was fully accessible for hydrolysis by plasma esterases, this alone did not prove that the procaine present in these systems was free procaine. It is possible (though unlikely) that procaine remained complexed with the penicillin molecule and that procaine was hydrolyzed *in situ* on the penicillin molecule. All this would require is that the configuration in which the procaine molecule is held by penicillin (Fig 1) be close to that required for a good fit on the esterase-active sites and that bound penicillin not interfere with either binding or hydrolysis. Therefore, to obtain more information on the problem whether "procaine·penicillin" remained complexed in aqueous solution for any significant length of time, the partitioning of procaine penicillin from aqueous solution into a series of organic solvents was studied.

Although the esterase experiments can be interpreted on the basis of only one particular aspect of the "procaine·penicillin" complex coming into contact with the esterase, such interpretations were not possible in partitioning experiments where the entire molecule is moved from one environment to another. Therefore, partitioning ratios are sensitive to small structural and polarity changes in a molecule, and this method can be used to distinguish between parent drugs and their metabolites.² Since penicillin was poorly soluble in nonpolar solvents and since the "procaine·penicillin" complex contained a free acidic group which would be largely ionized at neutral pH, it is highly unlikely that the hypothetical "procaine·penicillin" complex (Fig 1) would partition in the same way as procaine. The results of the experiments (Fig 5) which showed no differences between the partitioning of procaine and procaine penicillin into 4 different organic solvents over a 5-unit pH range, make it highly unlikely that significant amounts of a "procaine·penicillin" complex were present in the aqueous environment.

The principal experimental evidence for the postulated "procaine·penicillin" complex was the chromatographic behavior of procaine penicillin.⁴ Monti⁴ grew procaine penicillin crystals, using radiolabeled penicillin, dissolved the resulting crystals in methanol:isopropanol, and chromatographed the resulting solution on neutral alumina plates. He found that 25% of the counts migrating in this system ran with an R_f value of about 0.9, similar to that of procaine. Monti concluded that his "procaine·penicillin" complex was unstable, that about 75% of it had broken down, and that the 25% of the counts at R_f 0.9 represented his "procaine·penicillin" complex.

A repetition of these experiments is shown (Fig 6), using unlabeled procaine penicillin. Procaine penicillin was freshly dissolved in methanol:isopropanol and then rapidly chromatographed (time = 7 minutes) in

one direction under the conditions described by Monti.⁴ The plate was then dried, appropriate control concentrations of procaine penicillin were spotted, and plate was re-chromatographed at right angles to the original direction of chromatography. The rationale behind this approach was that if a substantial percentage of the material migrating to R_f 0.9 was indeed a labile "procaine-penicillin" complex as described by Monti,⁴ dissociation of about 75% of the penicillin would occur at this point and appear at R_f 0.1 on the second run. From Monti's data⁴ and interpretations, the present material should have been at least 20% of the amount originally spotted. Evidence for any significant quantity of slowly migrating material originating from the R_f 0.9 spot was not observed, even though the method used was sufficiently sensitive to detect about 2% of the amount of penicillin spotted. In other experiments, microbiological assays¹ did not show evidence for the presence of penicillin at R_f 0.9 on these plates. The experiments (Fig 7) show that if the "procaine-penicillin" complex does exist, its half-life must be in the order of seconds. Thus, as a practical matter, the results described in this communication offer no theoretical or experimental support for the hypothesis that procaine administered as procaine penicillin exists as a unique "procaine-penicillin" complex possessing all of pharmacologic properties of penicillin but none of the pharmacologic properties of procaine.⁴

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