Since in preliminary experiments we showed (Bertaccini and Impicciatore, unpublished) that also in stimulating pancreatic secretion the two naturally occurring methyl derivatives (MMH and DMH) are less potent than the mother substance, it may be suggested that gastric glands are probably the only area in which these *N*-methyl histamines are more potent than histamine at least in some animal species.

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Kininase activity in equine plasma

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THE KININOGENS and kallikreins derived from horse plasma have been thoroughly studied by Henriques *et al.* [1–5] No information is available, however, concerning the kininases of this plasma. Many reports have been published describing the kininases of other species, including humans.[6–10] This paper describes some of the characteristics of equine plasma kininases and compares them with the plasma kininases of the rat and guinea-pig.

Materials and methods

Bradykinin acetate was synthesized by the Peptide Center, Institute for Protein Research, Osaka University, Osaka, Japan; 2-bromo-*d*-lysergic acid diethylamide (BOL 148) and 1-methyl-lysergic acid butanolamide (UML 491), were kindly supplied by Sandoz Ltd., Basle, Switzerland; tranexamic acid was supplied by Daiichi Seiyaku Co., Ltd., Tokyo, Japan; 1,10-phenanthroline, was purchased from Wako Pure Chemical Industries, Ltd., Tokyo, Japan; soyabean trypsin inhibitor (SBTI), from Worthington Biochemical Co., U.S.A., hexesterol, from Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan and heparin sodium, from Sigma, Mo, U.S.A.

Plasma was used as the source of plasma kininases. Blood samples were drawn by venepuncture from the external jugular vein of unanaesthetized male thoroughbred horses, 4–9 years old, and

collected into polyethylene tubes containing either 2–5 units heparin, 3 mg sodium citrate or 1 mg disodium EDTA/ml blood. Blood samples from the external jugular vein of male guinea pigs (300–500 g) or Wistar rats (200–300 g) was taken under pentobarbital anaesthesia through polyethylene tubing into tubes containing heparin. The blood was centrifuged at 4500 *g* for 15 min, and the plasma collected in polyethylene tubes was used immediately or stored at −20° until use.

Kininase activity was measured by incubating 2 μg bradykinin for 5 or 10 min at 38° with 0·2 mg SBTI and 0·2 ml equine plasma (0·1 ml guinea-pig or rat plasma) in a final volume of 2·0 ml with either 0·2 M Tris or 0·2 M sodium phosphate buffer. SBTI was used to prevent kinin formation during incubation. The reaction was terminated by immersing the tubes in boiling water for 10 min. The residual bradykinin was measured on the isolated rat uterus suspended in Munsick's solution at 28° in the presence of BOL or UML (2×10^{-7} g/ml). Virgin rats for the uterus preparation were injected intraperitoneally with 10 mg hexesterol 24 or 48 hr before sacrifice.

Because of the simplicity of the statistical calculations, the kininase activity is expressed in terms of the rate constant (elimination constant) b rather than the half-life $T_{1/2}$. When the amount of bradykinin and the volume of plasma are chosen so that destruction of bradykinin follows a first order reaction, the following relationship holds:

$$bT_{1/2} = 1n\,2$$

where $1n\,2 = \log_e 2$.

When there is a linear relationship between the logarithmic concentration of bradykinin and the rat uterus contraction in a given range, the estimation of the rate constant b is rather simple. Three preparations are to be used for the estimation: (1) the standard preparation, which is the incubation mixture terminated at 0 time; (2) the diluted standard preparation, which is prepared by diluting the standard preparation m times; and (3) the test preparation, which is the incubation mixture in which the reaction has been terminated at T_p min. All three preparations are tested r times on the uterus to give the mean heights of contraction $\bar{Y}_o$, $\bar{Y}_m$ and $\bar{Y}_p mm$, respectively. The rate constant is given by

$$b = \frac{1n\,m}{T_p} \frac{\bar{Y}_o - \bar{Y}_p}{\bar{Y}_o - \bar{Y}_m}$$

with approximate variance of

$$\text{Var}\,[b] = \left[\frac{1n\,m}{T_p} \frac{s}{\bar{Y}_o - \bar{Y}_m}\right]^2 \frac{2}{r}\left[1 - \frac{\bar{Y}_o - \bar{Y}_p}{\bar{Y}_o - \bar{Y}_m} + \left(\frac{\bar{Y}_o - \bar{Y}_p}{\bar{Y}_o - \bar{Y}_m}\right)^2\right]$$

where s^2 is the error variance of the contraction. Variance b is derived according to the method described in the three-point slope ratio assay.[11]

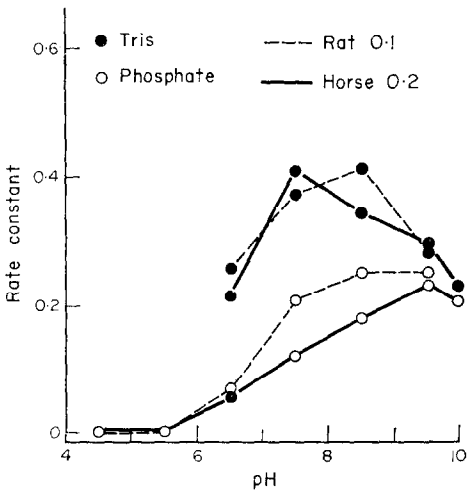

FIG. 1. Effects of pH on the kininase activity of equine and rat plasma. Plasma kininase activity is represented as the rate constant described in the text. Incubation mixtures contained rat plasma 0·1 ml or equine plasma 0·2 ml, bradykinin 2 μg, SBTI 0·2 mg and buffer to a final volume of 2·0 ml. The mixtures were incubated for 5 (rat plasma) or 10 min (equine plasma) at 38°. Each point is the mean of three to six samples.

Results

Figure 1 shows the effect of pH on the mean values of the kininase activity found in horse and rat plasma. In phosphate buffer, the highest kininase activity with equine plasma was obtained at pH 9·0. The activity decreased with increasing acidity and could not be detected below pH 5·5. On the other hand, in Tris buffer, the activity of the equine kininases was maximal between pH 7 and 8, and was higher than that found at any pH in phosphate buffer. The kininases in rat plasma followed a similar pattern, except that the activity of rat plasma was slightly higher than that found in the equine plasma. The standard deviation of the rate constants in Tris buffer was larger than in phosphate buffer, but the value did not exceed 0·19. It has been reported that human[7,9] and guinea-pig[10] plasma kininases are more active in the neutral and slightly alkaline pH range. This result is in agreement with the present studies only when Tris buffer is employed. With phosphate buffer, maximal activity of the equine and rat kininases had shifted to the alkaline pH range (Fig. 1). In the presence of $CoCl_2$, Erdös *et al.*[7,8] have shown that human kininases, when hippuryl-L-lysine or (*O*-benzoylglycl)-α-L-hydroxyl-δ-guanidino-*n*-valeric acid are used as substrates, do not always have the same optimal pH in Tris and phosphate buffer. However, the relative ability of the kininases to attack these synthetic substrates did not vary with the buffer employed. Also, in our studies, NaCl or KCl did not enhance kininase activity as reported by Aarsen and Kemp[12] who used acetone precipitates of guinea pig plasma in phosphate buffer. The discrepancy between the present study and those of others could be due to either differences in species or in methods.

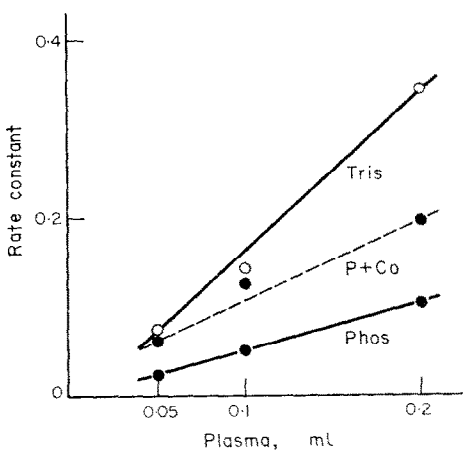

FIG. 2. Effects of plasma volumes on the kininase activity of equine plasma. Equine plasma 0·05, 0·1 or 0·2 ml in Tris buffer (pH 7·5) or in sodium phosphate buffer (pH 7·5) were incubated for 10 min. Other conditions of the incubation were the same as in Fig. 1. Effect of $CoCl_2$ (10^{-4} M) on the kininase activity in phosphate buffer is shown by the broken line (P + Co).

As shown in Fig. 2, the kininase activity in horse plasma increased linearly with increasing volumes of plasma at pH 7·5. Again, values of the rate constant in phosphate buffer were smaller than in Tris buffer. Furthermore, $CoCl_2$ (10^{-4} M) enhanced the activity in phosphate buffer as observed with kininases from other species.[6–8]

A comparison was made of the kininase activity in horse, rat and guinea-pig plasma (Fig. 3). The kininase activity in equine plasma was lower than that found in either rat or guinea-pig plasma regardless of the buffer employed. In each species, the kininase activity was higher in Tris buffer than in phosphate buffer at pH 7·5. Standard deviations of the rate constants for guinea-pig, rat and horse plasma were 0·15, 0·07 and 0·10, respectively.

The kininase activity of equine plasma in two other buffers, Krebs–Ringer (pH 8·0) and 0·1 M borate buffer (pH 7·6), was approximately the same as that found in Tris buffer. The mean rate constants were 0·37 (Krebs–Ringer) and 0·39 (borate).

The effects of various chelating agents and metals on the equine plasma kininases in phosphate buffer, pH 7·5, are given in Table 1. The results are approximately similar to those previously reported for human, guinea-pig and rat plasma.[7,12,13] $CoSO_4$ and $Co(NO_3)_2$, like $CoCl_2$, could increase the kininase activity of equine plasma. The rate constant was twice that obtained in the absence of Co^{2+}. Tranexamic acid did not inhibit horse plasma kininases.

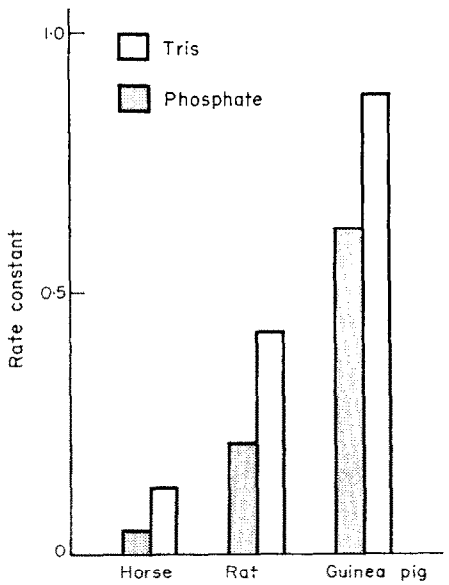

FIG. 3. Comparison of the kininase activity of horse, rat and guinea-pig plasma. 0·1 ml of plasma was used for all three species. Tris buffer (pH 7·5) or phosphate buffer (pH 7·5) were incubated for 5 (rat or guinea-pig plasma) or 10 min (equine plasma). Other conditions of the incubation were the same as in Fig. 1.

TABLE 1. EFFECT OF METAL IONS AND CHELATING AGENTS ON THE EQUINE PLASMA KININASE

Compounds	Concentration	Effect	Compounds	Concentration	Effect
$MgCl_2$	10^{-4} M	0	KCl	10^{-4} M	0
$NiCl_2$	10^{-4} M	0	NaCl	10^{-4} M	0
$ZnCl_2$	10^{-4} M	0	EDTA-Ca	10^{-4} M	—
$CaCl_2$	10^{-4} M	0	EDTA-Zn	10^{-4} M	—
$CoCl_2$	10^{-4} M	+	EDTA-Co	10^{-4} M	0
$CoSO_4$	10^{-4} M	+	EDTA-2Na	10^{-4} M	—
$Co(NO_3)_2$	10^{-4} M	+	Cysteine	10^{-4} M	—
$FeCl_3$	10^{-4} M	—	1,10-Phenanthroline	5×10^{-4}M	—
			Tranexamic acid	5×10^{-4}M	0

0; No effect, +; enhancement, −; inhibition.

Equine plasma collected in either sodium citrate or heparin had almost the same rate constant. Plasma collected in EDTA showed no kininase activity. Glass activation by shaking plasma 30 sec with glass ballotini (100 mg/ml) in the presence of soyabean trypsin inhibitor (10^{-4} g/ml) had no effect on equine kininase activity. Activation of Hageman factor, therefore, did not influence kininase activity. Equine kininase was stable to storage at 4° for 1 week, or to freezing at −20° for 1 month, even after repetition in freezing and thawing five times.

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Inhibition of development of tolerance to morphine by cycloheximide

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Cochin and Kornetsky[1] have stated that tolerance to narcotic analgesics resembles, in some respects, an immune response. In ensuing years, these authors and others[2–4] have attempted to shed light on this resemblance. In considering this phenomenon, we felt that it might be profitable to investigate the effect on tolerance of agents affecting the immune response. One of the drugs we have studied is cycloheximide, which inhibits protein synthesis, and thus, RNA synthesis. We are reporting some of this work in this paper.

Way *et al.*[5–7] have shown that cycloheximide, given chronically to mice, inhibits the development of tolerance to an implanted pellet of morphine sulfate and reduces the intensity of physical dependence precipitated by an injection of naloxone. They attribute their results to effects on brain serotonin levels rather than on any possible immune response. Our work involved rats, and cycloheximide was given weekly rather than daily, because we wished to avoid the possibility of obtaining the desired effect (increased latency of reflex response time relative to control animals) by some nonspecific depression or debilitation of the animals. We observed at the start of our work that rats are far more sensitive to cycloheximide than are mice. The dose used chronically in mice (20 mg/kg) by Way *et al.*[5–7] is fatal to rats on the first administration.

Male rats weighing about 275 g were randomly assigned by weight into two groups, one receiving cycloheximide and morphine sulfate and one receiving only morphine sulfate. All rats were injected subcutaneously once a week for the first 13 weeks of the experiment, with the injections of cycloheximide (1 mg/kg) given 1 hr before the injections of morphine sulfate (10 mg/kg).

On alternate weeks from 1 to 13, the animals were tested for analgesia immediately after receiving their weekly dose of morphine, using the hot-plate method of Eddy and Leimbach;[8] areas under the time-effect curve were calculated using a modification of the method of Winter and Flataker.[9] At the end of this 13-week period, we lengthened the dosage interval in order to observe any possible differences in the rates of disappearance of tolerance in the two groups.