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SOME CHARACTERISTICS OF PLASMA AND URINE 'HYALURONIDASE'

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According to Ginetzinsky (1958) and Ginetzinsky, Krestinskaya, Natochin, Sax & Titova (1960) the antidiuretic hormone acts on the collecting tubules of the kidney by liberating hyaluronidase, which by depolymerizing intercellular mucopolysaccharides allows an enhanced transfer of water through the tubule wall. Dicker & Eggleton (1960, 1961) observed that antidiuresis in man was accompanied by excretion of hyaluronidase in the urine, and that in cases of nephrogenic diabetes insipidus the administration of vasopressin was not followed by an excretion of hyaluronidase in the urine. On the other hand, Ginetzinsky (1960) found that intravenous injection of commercial hyaluronidase had no effect on the urine of dogs, and Leaf (1960) found that commercial hyaluronidase added to the fluid bathing the isolated bladder of the toad had no effect on its permeability to water.

The purpose of the present investigation was to see whether the viscosity-reducing activity found in the urine by Ginetzinsky (1958), by Dicker & Eggleton (1960) and by Knudsen & Koefoed (1961) was due to an enzyme and if so whether it was identical with commercial hyaluronidase.

METHODS

The viscosity-reducing activity of urine and of plasma of human subjects and of rats was compared with that of commercial hyaluronidase by incubation with hyaluronic acid at 37° C. Five parts of 0·3 % buffered solution of hyaluronic acid were added to one part of either hyaluronidase or biological material. During the incubation, samples of 0·5 ml. were withdrawn from the bath at fixed time intervals, immediately cooled to 2° C and their flow time estimated at that temperature with a pre-cooled Poiseuille-type viscometer of a capacity of 0·3 ml. To obtain a measure of the relative viscosity (η) the average of four estimations of flowtime (t_u) for each sample was compared with that of a solution of the buffer alone (t_l);[$\eta = t_u/t_l$].

Two different buffer systems were used: veronal-acetate buffer (Michaelis, 1931) and citric-acid-phosphate buffer (McIlvaine, 1921). The ionic strength was calculated as

$$I = \tfrac{1}{2}\Sigma\,(m.Z^2),$$

where m = concentration of ions, and Z = valency (Bayliss, 1959). The ionic strength chosen was $I = 0{\cdot}17$; to ensure a constant value of I, buffers were adequately diluted, when necessary.

The hyaluronic acid used was either a purified preparation free of amino acids and proteins (L. Light and Co.) or a crude preparation of polysaccharides extracted from human umbilical cords. As no appreciable differences were observed whether one or the other preparation was used, the results will be presented as those obtained with hyaluronic acid without reference to its purity. The commercial hyaluronidase (L. Light and Co.) prepared from sheep testes according to the British Pharmacopoeia, 1958, was labelled as containing 550 i.u./mg. Plasma was separated from blood taken from the antecubital vein of man or from decapitated rats, collected in heparinized tubes (1 mg/ml.). Overnight urine samples were concentrated by dialysis against carbowax (Kohn, 1959) and treated as described in detail by Dicker & Eggleton (1960, 1961).

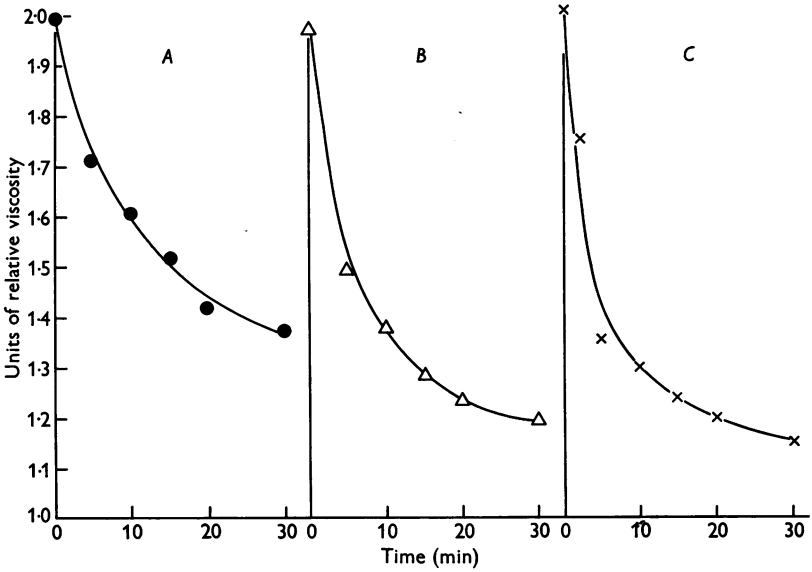

Fig. 1. Comparison between rates of depolymerization of hyaluronic acid by commercial hyaluronidase, urine and plasma. *A*, the effect of 16 i.u./ml. hyaluronidase; *B*, urine (dilution 1:6); *C*, plasma (dilution 1:60).

RESULTS

Figure 1 shows the decrease of the viscosity of a solution of hyaluronic acid incubated at 37° C with commercial hyaluronidase (*A*), urine (*B*) or plasma (*C*), in the presence of veronal-acetate buffer. A comparison of the three curves suggests that the viscosity-reducing activity of plasma or urine resembles that of hyaluronidase. The enzymic nature of the reaction is indicated further by the observation that there is no reduction of the viscosity of hyaluronic acid by urine or plasma in the presence of an anti-hyaluronidase (Suramin, B.P.) in the concentration of 0·001 M. Further comparisons between urine or plasma and commercial hyaluronidase are shown in Figs. 2–5. Figure 2 shows the effect of temperature and Fig. 3 the effect of different amounts of urine, plasma and commercial hyaluronidase in acting on the same amount of hyaluronic acid. In these figures no

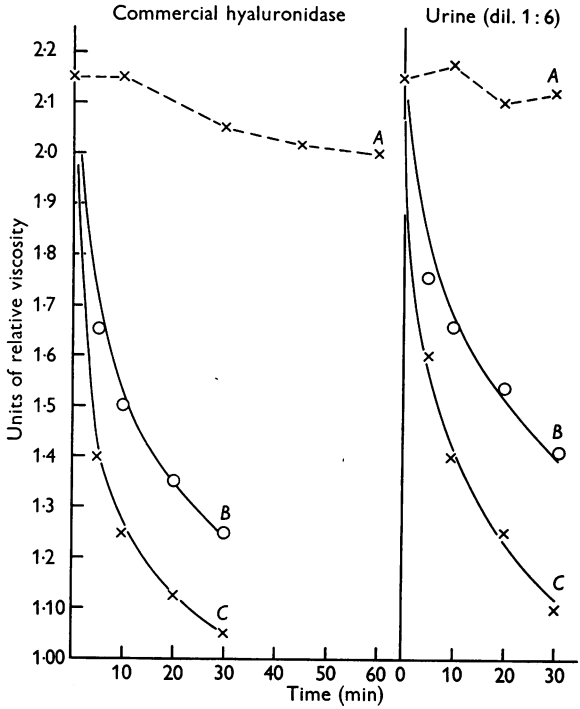

Fig. 2. The effects of temperature on the rate of depolymerization of hyaluronic acid by commercial hyaluronidase and urine (dilution 1:6). *A*, at 2° C; *B*, at 20° C and *C*, at 35° C.

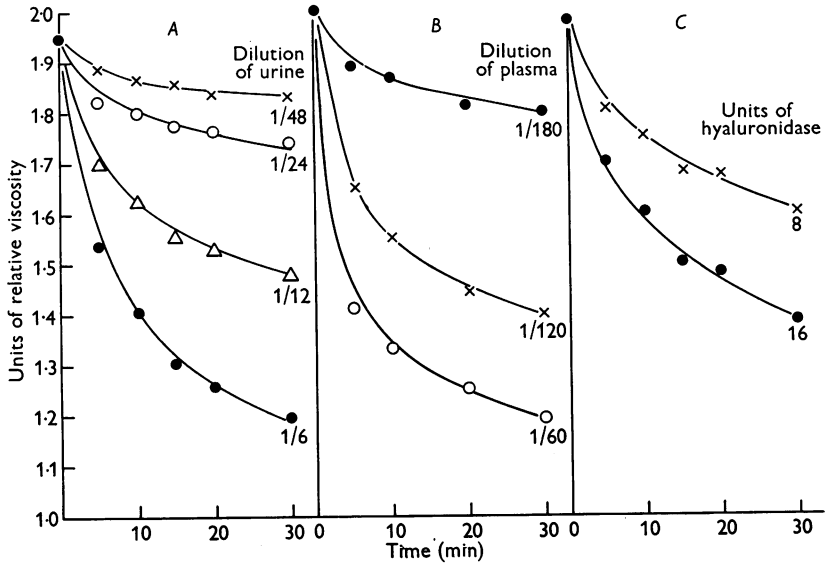

Fig. 3. The effect of different amounts of urine (*A*), plasma (*B*) and commercial hyaluronidase (*C*) incubated with the same amount of hyaluronic acid.

difference is apparent between the activity in urine, plasma and commercial hyaluronidase.

The activity of an enzyme is exercised only over a restricted range of pH values. Within this range the activity passes through a maximum at some particular pH, the optimum pH, which is characteristic of a given enzyme. By comparing the pH–activity curves of hyaluronidase, urine and plasma incubated with hyaluronic acid in the presence of veronal-acetate buffer, it was found that the optimum pH of hyaluronidase is 6·6–6·8, whereas that of plasma and urine is 4·1–4·2. This strongly suggests a difference between the two systems of enzymes investigated (Fig. 4).

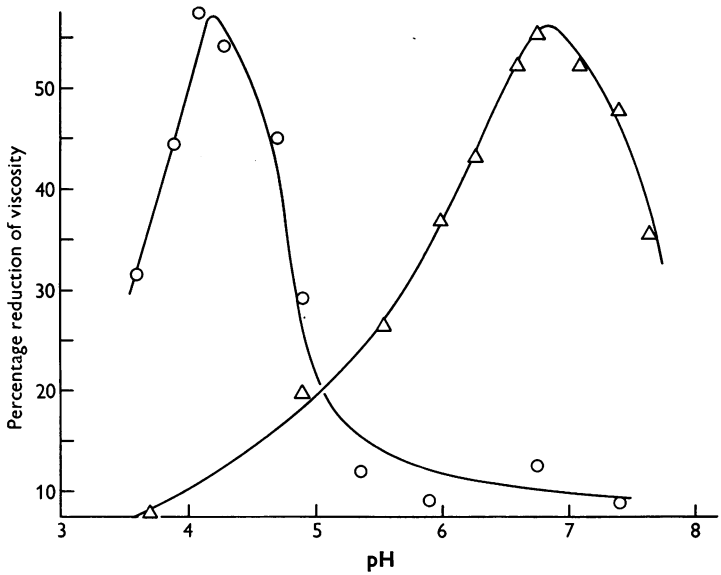

Fig. 4. Comparison between the depolymerization of hyaluronic acid by urinary and commercial hyaluronidase at different pH values in veronal-acetate buffer. ○—○ urinary hyaluronidase; △—△ commercial hyaluronidase. The ordinate shows the percentage reduction of viscosity after incubation for 10 min.

The use of veronal-acetate buffer has the advantage of a fairly constant ionic strength through the range of pH values investigated, but also the possible disadvantage that its high concentration of barbitone (20·62 g/l.) may interfere with the enzymic activity. To check this, experiments were repeated with the McIlvaine citric-acid-phosphate buffer. The ionic strength of this buffer, however, varies appreciably with the pH, being much greater at higher than at lower pH values. It was thus necessary to dilute the buffer so that at all pH values used its ionic strength remained similar to that of the veronal-acetate buffer solution, i.e. $I = 0{\cdot}17$. Use of the citric acid-phosphate instead of the veronal-acetate buffer does not

affect the optimum pH values of hyaluronidase, plasma and urine, which remain unchanged at 6·6 and 4·1, respectively. The rate of inactivation of hyaluronic acid by urine or hyaluronidase is, however, markedly influenced by the choice of the buffer system. For urine, at pH 4·1–4·2, and with an ionic strength of $I = 0{\cdot}17$, the rate at which the viscosity is reduced is appreciably greater in the presence of the citric-acid-phosphate than with the veronal-acetate buffer (Fig. 5). For commercial hyaluronidase, however, it is the opposite: at pH 6·5, but similar ionic strength ($I = 0{\cdot}17$)

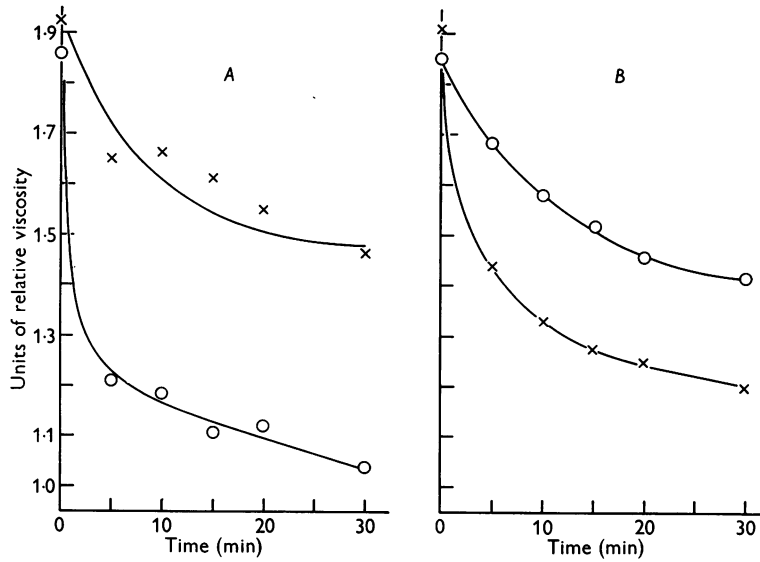

Fig. 5. The effects of two different buffer systems on the rate of depolymerization of hyaluronic acid by commercial and by urinary hyaluronidase. *A*, commercial hyaluronidase, pH 6·5; *B*, urinary hyaluronidase, pH 4·2. ○—○ incubation in the presence of veronal acetate buffer; ×—× incubation in the presence of citric-acid-phosphate buffer.

the rate of inactivation is greater with veronal-acetate than with citric-acid-phosphate buffer (Fig. 5). In contrast the viscosity-reducing activity of plasma is much the same with both buffer systems. These observations suggest that the viscosity-reducing property of urine may be due to an enzyme (or enzymic system) different from that of commercial ovine hyaluronidase and may not be identical with that of plasma.

DISCUSSION

The role of hyaluronidase in the mechanism of urine concentration has not yet been elucidated satisfactorily. On one side there are the observations of Ginetzinsky (1958), of Dicker & Eggleton (1960) and of Thorn,

Knudsen & Koefoed (1961), according to which there is some correlation between antidiuresis and the presence of hyaluronidase in the urine. On the other side there are the observations of Leaf (1960) on the permeability of the isolated toad's bladder, and of Ginetzinsky (1960) and of Cort (1960) on the perfused dog's kidneys, which are all negative. All the latter authors have used commercial hyaluronidase without reference to the pH values of their preparations. From the methods used by Leaf (1960) it is probable that the pH of the isolated preparation was well below the optimum pH of commercial hyaluronidase. According to the present investigation the activity of commercial hyaluronidase is practically negligible at pH 5·5–5·0 (Fig. 4). As for the negative results observed during kidney perfusions, neither Ginetzinsky (1960) nor Cort (1960) mentioned whether the infused commercial hyaluronidase was excreted in the urine or not. In this respect it is of interest to note that Thorn *et al.* (1961) were able to produce an antidiuresis only in those rats in which an infusion of hyaluronidase was accompanied by the excretion of hyaluronidase activity in the urine. Finally, it should be mentioned that the rate of inactivation of arginine- or lysine-vasopressin incubated with kidney slices at pH 7·1 is markedly enhanced in the presence of commercial hyaluronidase (Smith, 1962).

It would appear from the present investigation that urinary hyaluronidase may differ from the commercial hyaluronidase by at least one important characteristic, the optimum pH; it is 4·1–4·2 for the urinary hyaluronidase and 6·6–6·8 for commercial hyaluronidase. This would suggest that the viscosity-reducing activity of urine and of the commercial preparation are due to two different enzymes, a fact which might explain the negative results observed by authors who used testicular hyaluronidase.

Hyaluronidase is not the name of one enzyme; it is a general term applied to a group of enzymes hydrolysing hyaluronic acid. According to Malmgren (1953) it is possible to separate from bovine testes at least three different preparations which all have the properties of hyaluronidase. Pure hyaluronidase has a molecular weight of about 11,000 (Malmgren, 1953). The fact that the viscosity-reducing activity of either commercial hyaluronidase, plasma or urine appear in the first collection samples after gel filtration through Sephadex G-75 suggests that it is attached on a larger molecule which may act as a carrier (Knudsen & Koefoed, 1961).

It was not possible to express the 'hyaluronidase' activity of urine or plasma in terms of International Units of hyaluronidase B.P., owing to the different optimum pH of the preparations. The results in this paper have therefore been presented in the figures by showing the change in viscosity of a solution of hyaluronic acid.

SUMMARY

1. The inactivation of hyaluronic acid by urine and plasma is of enzymic nature and resembles that produced by commercial hyaluronidase.

2. The optimum pH value for commercial hyaluronidase is 6·6–6·8, whereas the optimum pH value for urine and plasma is 4·1–4·2.

3. The rate of inactivation of hyaluronic acid when incubated with commercial hyaluronidase at pH 6·6, is greater in the presence of veronal-acetate than in the presence of citric-acid-phosphate buffer. In contrast, the rate of inactivation of hyaluronic acid when incubated with urine at pH 4·1 is greater with citric-acid-phosphate than with veronal-acetate buffer.

4. These observations suggest that the viscosity-reducing activity of urine and plasma is due to an enzyme different from that of commercial hyaluronidase.

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