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Received September 19, 1957. P.S.E.B.M., 1957, v96.

Effect of Urea and Methylamine on Plasmin.* (23584)

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Plasmin, partially purified from human plasma, is unstable in neutral solutions, as demonstrated by rapid loss of proteolytic activity at 37°C. The experiments presented here show the lability to be due to self-digestion of plasmin and describe inhibition of this process by urea and methylamine. This study was undertaken after the report of the preserving effect of urea on trypsin(1), which also undergoes auto-destruction at neutral reaction. The proteolytic activity of trypsin is completely inhibited in 8 M urea, thus accounting for the ability of that concentration of urea to prevent auto-digestion of trypsin(2). The preserving and inhibiting effect is entirely reversible on sufficient dilution, indicating that trypsin does not undergo permanent change upon exposure to urea(1). With plasmin a similar situation can be shown to exist not only with urea but also with methylamine, another highly soluble simple amine.

Methods and materials. The caseinolytic assay previously described was used for determination of plasmin and plasminogen(3). The method has been modified from that originally described in that 15% rather than 10% trichloroacetic acid was added for precipitation of undigested casein. Slightly more consistent results are obtained by the use of the higher concentration of acid. *Plasmin.* The inactive precursor, plasminogen, was prepared from human plasma fraction III† by the method of Kline(4), and dried with alcohol and ether as described by Cliffton and Cannamela(5). Several lots of 400 mg were converted to plasmin as follows: The dry powder was dissolved in 30 ml distilled water with the aid of a few drops of N HCl. The volume was brought to 200 ml with 0.05 M borate-saline buffer, pH 7.4, with the formation of a finely divided precipitate. 3.0 mg commercial streptokinase‡ was added and the mixture was incubated at 25°C for 30 minutes with occasional stirring. 200 ml 2 M NaCl was added and the reaction adjusted to pH 2.0 with N HCl. As reported by Troll and Sherry, active plasmin was precipitated by this technic and streptokinase lost(6). The precipitate was separated by centrifugation for 10 minutes at 1,500 rpm and washed twice with 1 M NaCl adjusted to pH 2.0 with N HCl. The precipitate was dried by washing twice with 95% alcohol and 3 times with anhydrous ether. About

* This work was supported by contract with Chemical Corps, Fort Detrick, Frederick, Md.

† Kindly furnished by E. R. Squibb & Sons Corp., through the courtesy of Dr. J. N. Ashworth, Amer. Natl. Red Cross.

‡ Varidase, a lyophilized mixture of streptokinase, streptodornase and other proteins with phosphate buffer, kindly furnished by Lederle Laboratories Division, Amer. Cyanamid Co., Pearl River, N. Y.

TABLE I. Loss of Plasmin Activity at pH 7.4 and 37°C.

Time (t) (min.)	Residual activity (units $\times 10^{-3}$/ml)	Fraction of initial activity (fA)	K
0	18.7	1.0	
5	12.2	.65	.0058
10	9.8	.52	.0049
15	7.5	.40	.0054
20	6.2	.33	.0054
25	5.4	.29	.0052

2 mg plasmin dissolved in 1 ml 0.0025 M HCl and 7 ml warmed buffer added at 0 time. 1 ml aliquots removed for proteolytic determinations. If diluted similarly with 0.0025 M HCl the activity of the plasmin solution was 22.8 $\times$ 10^{-3} units/ml.

200 mg of a fine white powder was recovered from each 400 mg lot; it contained about 95% of the potential protease of the starting material. Thus, a 2-fold concentration of activity was achieved. The preparation was free of streptokinase activity, and, like partially purified plasminogen, poorly soluble at neutrality but highly soluble and stable in 0.0025 M HCl. A pool of several plasmin preparations was used for all experiments described and had a specific activity of 91.2 x 10^{-3} units/mg. For each experiment a concentrated solution of the enzyme in 0.0025 M HCl was made and, at the beginning of incubation, was diluted with 5 to 10 times its volume of appropriate solution buffered at pH 7.4 with borate.

Urea and methylamine. C.P. urea and methylamine hydrochloride were dissolved in 0.05 M borate-saline buffer, pH 7.4, at 10 M and 8 M respectively and adjusted to pH 7.4 with N HCl or N NaOH. Dilution to the desired strength was made with additional borate buffer.

Results. When a solution of plasmin in 0.0025 M HCl was brought to pH 7.4 by addition of borate buffer an immediate reduction of activity was observed due to a partial reversible denaturation of protein. This was followed by a slower temperature dependent and irreversible destruction of proteolytic activity. These facts are illustrated by the experiment in Table I. The relatively slow and decreasing rate of the second reaction suggests that it may be enzymatic in nature. According to Kunitz and Northrop(7) a bimolecular reaction involving the digestion of reversibly denatured enzyme by the active enzyme should fit the formula $\frac{1}{fA} - 1 = K A_o t$ where fA is the fraction of active enzyme remaining at time t and A_o is the activity at the beginning of the experiment. Calculated values for K show good agreement and indicate that the formula fits the data.

The effect of various concentrations of urea and methylamine upon the rate of inactivation was tested. Because the urea and methylamine concentrations to be used were also inhibitory to proteolytic activity, it was necessary to allow auto-digestion to proceed in a solution with a 10-fold concentration of plasmin, remove a 0.1 ml aliquot and add it to the 1.0 ml substrate along with 0.9 ml buffer in order to dilute the urea or methylamine to a non-inhibitory level. As might be expected, concentrated solutions of plasmin digested themselves more rapidly and had higher values for K than dilute solutions. Also the rate of reaction tended to decrease after 10 to 20 minutes, a phenomenon observed for trypsin by Kunitz and Northrop (7) and explained by them as inhibition of the enzyme activity by products of digestion. For this reason, the values for K given here were determined during the first 10 minutes of incubation when inhibition was minimal.

The ability of similar concentrations of urea and methylamine to inhibit the proteolytic activity of plasmin for casein was also tested. In order to minimize the denaturing effect of these compounds on casein, they were added to the substrate just 30 seconds before plasmin was added for digestion. Despite this precaution, casein was rendered somewhat more digestible by urea, so that the values for urea given are in percent inhibition of the maximum digestion in the presence of urea.

In Fig. 1 the auto-digestion and inhibition of plasmin at various concentrations of urea is shown and an inverse relationship between inhibition and auto-digestion of plasmin demonstrated. Although at 6 M urea the value for K was extremely low, there was a slow loss of activity demonstrable during incubation over an hour or more at 37°C.

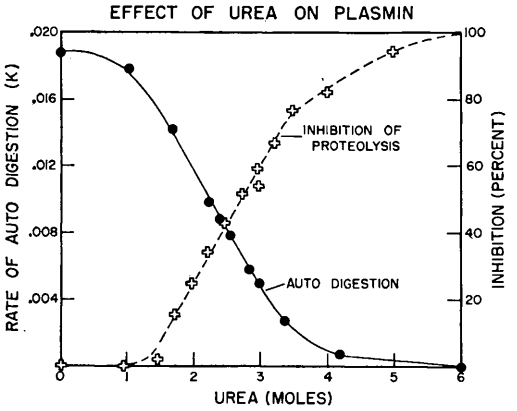

FIG. 1. Effect of urea on auto-digestion of plasmin (●). 0.1 ml 0.0025 M HCl containing 1.6 mg plasmin added to 0.6 ml buffered urea solution at 37°C. 0.1 ml aliquots removed at 1, 4, 7, and 10 min. and added to 1.0 ml 4% casein and 0.9 ml buffer for protease determination. K is an average from the 4 determinations. Inhibition of caseinolysis by urea (+). 1 ml warmed buffered urea solution added to 0.5 ml 8% buffered casein solution. 30 seconds later 0.5 ml 0.0025 M HCl containing 0.15 mg plasmin added. Activities expressed as percent of the activity in 1 M urea.

In Fig. 2 the same experiment with methylamine is shown and a similar but less perfect inverse relation is seen. Inhibition and preservation of plasmin were less complete with this compound, and at 5 M methylamine a slight increase in the rate of autolysis was found, probably due to irreversible denaturation by a high concentration. An effective preservation was seen, however, at rather low levels of methylamine.

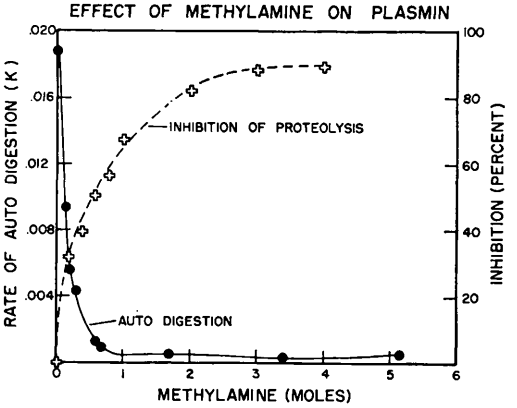

FIG. 2. Effect of methylamine on auto-digestion of plasmin (●). Inhibition of caseinolysis by methylamine (+). Activities expressed as percent of activity in buffer.

Discussion. These experiments indicate that 2 substances which inhibit proteolysis by plasmin also inhibit autolysis to about the same degree and are evidence that the lability of plasmin at neutrality is actually due to proteolytic digestion of plasmin by itself. Prevention of this process, while accomplished by urea or methylamine, can be achieved much more completely by lowering the pH. Plasmin in 0.0025 M HCl (pH 2.5) is entirely stable for many hours at 37°.

Inhibition and preservation by all 3 means (urea, methylamine, and low pH) can be correlated roughly with the ability of these conditions to make soluble this preparation of plasmin. In each case the usually poorly soluble enzyme becomes highly soluble and makes a clear solution.

In experiments with trypsin Viswanatha and Liener(1) showed that intermediate concentrations of urea actually hastened autodestruction, presumably because the denaturing effect of urea made more reversibly denatured enzyme available to destruction by the remaining native material. The lack of a similar effect with our preparation of plasmin suggests that it may already be somewhat denatured by the process of purification even though it is fully active. Its insolubility may also be due to partial denaturation.

Conclusions. 1. Preparation of partially purified and highly active human plasmin free of streptokinase is described. 2. In neutral solutions plasmin undergoes rapid auto-digestion at 37°C. 3. Autolysis of plasmin can be inhibited by concentrations of urea and methylamine which inhibit digestion of protein by plasmin.

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