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26. Komei Miyaki, Isamu Mochida, Tadao Wada, and Takeko Kudo : Activation of Hyaluronidase by Spermine and Related Diamines.

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It has been known from earlier times that spermine and spermidine are distributed in the animal tissues but their physiological significance has not been elucidated as yet. The human semen is especially rich in spermine, containing 3300 γ/g. semen, but its role in reproduction is not clear.

Evans, *et al.*[1] found that spermine, like barbiturates, inhibited the oxidation of glucose, lactate, and pyruvate in the brain tissue. Jeffree[2] reported that spermine and spermidine increased the phosphatase action. Rosenthal, *et al.*[3] showed that spermine is nephrotoxic, while Tabor, *et al.*[4] further examined such action and pharmacological effect of its enzymatic oxidation products. These various reports indicate that the toxicity of spermine and spermidine is related to renal tubular necrosis. Besides these, spermine has antiheparin action similar to protamine and it is known to affect blood coagulation.

During studies on elucidation of physiological role of spermine and spermidine, they were found to strengthen the hyaluronidase of bovine testicle and the present report concerns this action. In addition, comparative examinations were also made on related mono- and diamines.

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Experimental

Method of Measurement—The determination of hyaluronidase activity *in vitro* was made by McClean's viscosity reduction method[5] and Rapport's method of measuring reductivity.[6] The latter was measured by the Park-Johnson method.[8] Measurement of the activity *in vivo* was made by intracutaneous diffusion using a rabbit.

Materials—Potassium hyaluronate and hyaluronidase were kindly supplied by the Mochida Pharmaceutical Company's laboratory and used without further treatment. The amines used were the hydrochlorides of $MeNH_2$, Me_2NH, Me_3N, ethylenediamine, tetramethylenediamine (putrescine), pentamethylenediamine (cadaverine), hexamethylenediamine, spermine, agmatine, and histamine; hydrobromides of ethanolamine and trimethylenediamine; phosphate of spermidine; carbonate of guanidine, and sulfate of methylguanidine.

Measurement—1) Viscosity Reduction Method : To small test tubes of 10-cc. capacity, *a* and *b*, a mixture of 4 cc. of 0.1% aqueous solution of potassium hyaluronate and 1 cc. of 0.1 *M* citrate buffer (pH 6.3) is placed in *a*, and a mixture of 0.5 cc. of hyaluronidase (1000 units/cc.**) and 0.5 cc. of amine solution is placed in *b*. The test tubes are incubated in a thermostat of 30° for 10 mins., the two solutions are mixed rapidly, and 2.5 cc. of this mixture is placed in the Ostwald viscosimeter. The time required for flow is measured and compared to the control which is the time required until the specific viscosity decreases to one-half (Fig. 1).

2) Measurement of Reductivity : In the same manner as for the viscosity reduction method, the substrate, enzyme, and amine solution are reacted, the potassium hyaluronate being a 0.05% solution in this case. From this reaction mixture, 0.2 cc. of the solution is diluted to 3 cc. with

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2) G. M. Jeffree : Biochem. J., **58**, 37(1954).
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** This unit indicates the commercial one.

distilled water, 1 cc. of ferricyanide solution (0.5 g. of potassium ferricyanide dissolved in 1 L. of water), and 1 cc. of carbonate-cyanide solution (5.3 g. of Na_2CO_3 and 0.65 g. of KCN dissolved in 1 L. of water) are added, and the mixture is boiled for 15 mins. After cool, 5 cc. of ferric ion reagent (1.5 g. of iron alum and 1 g. of sodium monolaurylsulfate dissolved in 1 L. of 0.05N H_2SO_4 solution) is added and the solution is submitted to colorimetry at 690 mμ. Standard is a glucose solution. In this case, enzymatic potency was found to be proportional to initial velocity of reduction and the results were compared by initial velocity.

3) Enzymatic Oxidation of Spermine: Enzymatic oxidation of spermine by oxidase of goat serum was measured manometrically by Warburg flask containing 1 cc. of serum, 0.2 cc. of 0.01M spermine solution, 0.8 cc. of 0.1M phosphate buffer of pH 7.2 (total volume of reaction mixture in the main compartment: 2.0 cc.) and 0.2 cc. of 20% KOH in the center well. As shown in Fig. 2, approximately 2 moles of O_2 is consumed for each mole of spermine supplied. This reaction mixture is added in place of the amine solution described in Method 1. In this experiment, the equivalent amount of goat serum is added in all procedures for comparison.

4) Intracutaneous Diffusion Method: 0.05 cc. of a solution containing Chinese ink, 200 units of hyaluronidase, and 0.05 μM of spermine is injected intracutaneously into a rabbit, and area of its diffusion is compared to that of the control, prepared without the hyaluronidase or the amine.

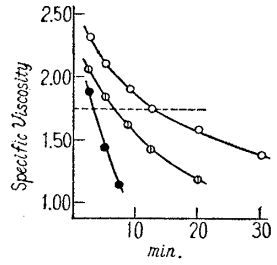


Fig. 1. Relationship between the Activity of Hyaluronidase and Reductivity
—○—: hyaluronidase 100 units
—①—: 〃 200 〃
—●—: 〃 400 〃

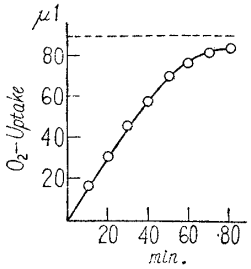


Fig. 2. Enzymatic Oxidation of Spermine by Spermine Oxidase of Goat Serum

Results

The present series of experiments revealed that spermine markedly increases the lowering of hyaluronate viscosity by hyaluronidase. The same effect was observed in spermidine and putrescine, and to a lesser degree in methylamine, about the same as that of the ammonium ion (using NH_4Cl) (Fig. 3).

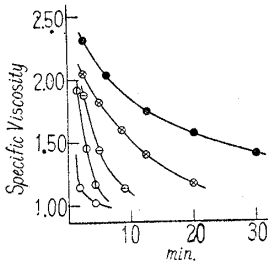


Fig. 3. Effect of Amines on the Hyaluronidase Activity (Measured by viscometry)
—○—: 0.001M spermine
—①—: 0.001M spermidine
—⊖—: 0.001M putrescine
—⊗—: 0.001M methylamine or ammonium chloride
—●—: control

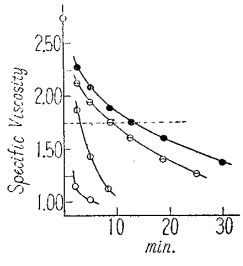


Fig. 4. Effect of Spermine at Various Concentration on the Hyaluronidase
—○—: 0.001M spermine
—①—: 0.0001M spermine
—⊖—: 0.00001M spermine
—●—: control

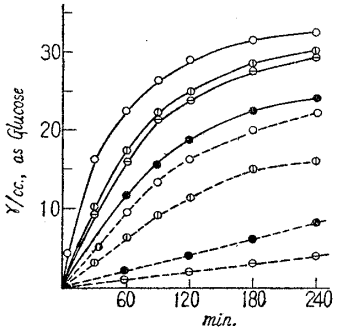


Fig. 5. Effect of Amines ($10^{-3}M$) on the Hyaluronidase Activity (Measured by reductometry)
—○—: spermine
—①—: histamine
—⊖—: hexamethylenediamine
—●—: putrescine
---○---: methylamine
---①---: ammonium chloride
---⊖---: guanidine
---●---: control

As indicated in Fig. 4, the fortifying action of spermine against hyaluronidase is far stronger than that of the other amines and even showed a strong effect in $10^{-4}M$ concentration.

The same tendency was observed in the case of measuring the reductive activity (Fig. 5) but in this case, the effect of coexistent β-glucuronidase is not eliminated and determined values are not very reliable.

Taking the value of control as 1, based on the results of measurement by the two methods, values of the amines calculated are indicated in Table I. Spermine has the strongest activity, followed by spermidine, histamine, and trimethylenediamine, in that order. These were followed, in the descending order, by hexamethylenediamine, trimethylamine, ethylenediamine, tetramethylenediamine (putrescine), and pentamethylenediamine (cadaverine). It should be noted that, in contrast to the promoting effect in these amines, guanidine showed a suppressive effect.

In the intracutaneous diffusion test with rabbit, spermine and spermidine also showed a promoting effect on hyaluronidase activity. It was observed that this promoting effect of spermine disappeared completely by enzymatic oxidation using goat serum (Fig. 6). In this case, it was considered that a spermine oxidase in the serum was responsible and the consumption of oxygen (by the Warburg manometer) was 2 moles for 1 mole of spermine. This result indicates that the promoting effect is due to spermine itself and not to other substances contaminated in spermine.

Table I. Comparative Ratio of Effect of Various Amines on Hyaluronidase

Amines ($10^{-3}M$)	Ratio	
	Viscometry	Reductometry
Methylamine	2.0	5.2
Dimethylamine	—	6.2
Trimethylamine	5.0	9.2
Trimethylamine N-oxide	—	2.6
Ethanolamine	2.1	—
Ethylenediamine	5.0	—
Trimethylenediamine	8.0	—
Tetramethylenediamine	4.0	6.0
Pentamethylenediamine	4.5	8.0
Hexamethylenediamine	6.0	9.0
Spermidine	9.0	—
Spermine	16.0	65.0
〃 ($10^{-4}M$)	8.0	—
〃 ($10^{-5}M$)	1.5	—
Histamine	9.0	10.0
〃 ($10^{-4}M$)	2.0	—
Guanidine	0.5	0.5
Methylguanidine	1.0	—
Agmatine	1.5	1.6
Ammonium chloride	2.0	2.5
Control	1.0	1.0

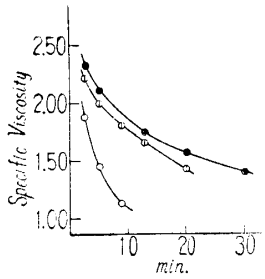
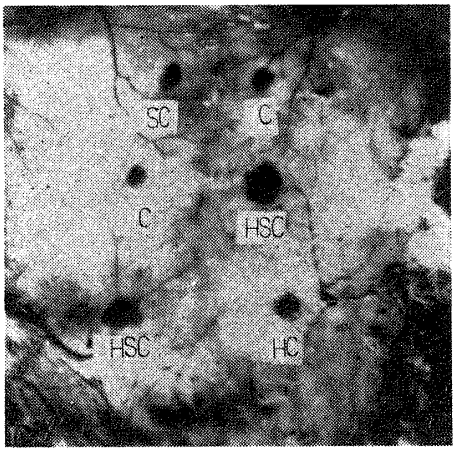


Fig. 6. Variation in the Activity of Spermine against Hyaluronidase by Spermine Oxidase of Goat Serum

—○— : 0.0001M spermine

—①— : 0.0001M spermine treated with spermine oxidase

—●— : control



C : Chinese ink injected alone

SC : Chinese ink containing 0.05 μmole spermine injected

HSC : Chinese ink containing 0.05 μmole spermine and 200 units hyaluronidase injected

HC : Chinese ink containing 200 units hyaluronidase injected

Photo. 1. Photograph which shows the Stimulation of Hyaluronidase by Spermine using Rabbit being Injected Intracutaneously

Discussion

It has already been pointed out that hyaluronidase present in semen plays an important role in fertilization.[9] Therefore, it is interesting that spermine promotes the hyaluronidase activity. Spermine and spermidine are distributed widely in the animal tissue[10] and the question of what kind of physiological or pathological activity increases this hyaluronidase action would be worth further study. It is still impossible to draw any conclusion as to why such an effect is found in spermine.

Heparin and some mucopolysaccharides have been cited as the inhibitor of sperm hyaluronidase. According to Tabor, *et al.*[4] spermine has antiheparin action, like protamine, and it may promote hyaluronidase action by removing this kind of inhibitor. As was found in the present series of experiment, the same effect is found in low molecular amines, such as trimethylamine and ethylenediamine, and this assumption cannot be accepted until further experimental examination had been made.

Tabor, *et al.* have reported that ethylenediamine and trimethylenediamine possess nephrotoxic action as spermine, while such an effect is not found in monoamines and the longer-chain diamines. This fact is in parallel with observations made on the effect of these amines in the present experiment and is rather interesting.

It has been found in the present work that histamine also promotes the hyaluronidase action and it may be assumed that this promoting effect plays some role in the case of inflammation, when local concentration of histamine has become larger.

Summary

Spermine was found to be the powerful activator of bovine testicular hyaluronidase at $10^{-4}M$ concentration. The enzyme activity was determined either *in vitro* by viscometric and reductometric methods or *in vivo* by intracutaneous diffusion method. Oxidation of spermine oxidase originating in goat serum resulted in the loss of activation of the enzyme activity.

Several amines, especially polymethylenediamines related to spermine, were also found to exhibit similar activating effect on hyaluronidase, although the effect is less than that of spermine. The activity of these amines at $10^{-3}M$ concentration decreases in the following order: Spermine, spermidine, trimethylenediamine, histamine, hexamethylenediamine, putrescine, cadaverine and ethylenediamine.

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