A MODIFICATION OF THE VISCOSIMETRIC METHOD OF MICROESTIMATION OF THE HYALURONIDASE ACTIVITY OF BIOLOGICAL FLUIDS

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Laboratory and clinical studies of the hyaluronidase activity of biological fluids have often been limited by the lack of a simple and reliable micromethod for estimation of this enzyme. The existing macromethods require 1.5-2.0 ml of test fluid for the analysis. The micromethods so far described are either complicated or are designed for special purposes.

In the suggested micromodification of the viscosimetric method [7], the principle of the capillary type of viscosimeter [5] is used (see figure). The apparatus is placed in a thermostatically controlled water bath, for which purpose an aquarium or a glass accumulator case with a heating system may be used.

The investigations are carried out at a temperature of 34°. Into the funnel of a viscosimeter is poured 0.05 ml of the test solution, 0.05 ml of citrate buffer at pH = 4.6 and 0.2 ml of a 0.2 % solution of hyaluronic acid. The total volume of the mixture corresponds to the capacity of the working part of the viscosimeter – about 0.3 ml.

The enzymic activity of the test sample is judged by its ability to lower the viscosity of the original solution of hyaluronic acid in the course of 20 minutes. At the begining and end of the investigation, the time of flow of the mixture between the upper mark and the junction with the capillary tube is measured with a seconds counter with an accuracy of 0.2 seconds.

The enzymic activity of the test solution is calculated as the decrease in the viscosity of the substrate in percent. of the initial value. The conventional unit of activity is a decrease of 1% in the viscosity.

The calculation is made as follows. The time of flow of the mixture of test solution, buffer and hyaluronate at the begining of the experiment is designated t_1 and the time of flow after incubation for 20 minutes t_2. The difference $t_1 - t_2$ then gives the quantity of substrate losing its viscosity during the investigation.

The difference $t_1 - t_0$ will evidently give the initial value of the viscosity of the substrate, where t_0 is the time of flow of the test fluid and buffer without hyaluronic acid. In this series of investigations, t_0 is a practically constant value, and may be determined once only for each viscosimeter.

It is necessary to dwell in rather greater detail on this value t_0 which, in our opinion, must be affected also by products of total depolymerization of hyaluronic acid formed at the end of the investigation, which are not taken into consideration when the viscosimeter is used in the ordinary manner. We introduce the appropriate correction in such a way that, when determining t_0, a further equal volume of totally depolymerized 0.2% hyaluronic acid is added instead of distilled water to the buffer and the test fluid.

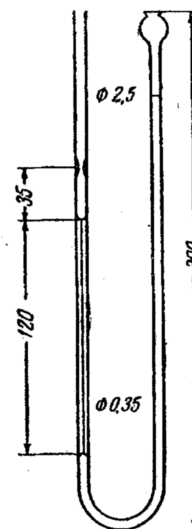
In accordance with what has been stated, the enzymic activity of the test fluid in conventional units is calculated according to the formula:

$$A = \frac{t_1 - t_2}{t_1 - t_0} \cdot 100.$$

When the results of the viscosimetric determination are being evaluated, it is imperative to bear in mind that the lowering of the viscosity of the substrate is not strictly specific for the action of hyaluronidase, but may be due also to other nonenzymic factors. In parallel with the experimental test, a control was therefore set up with the test solution which had been preliminarily heated on a boiling water bath for 30 minutes to inactive the enzyme. Subtraction of the results of the control test from the value obtained for the decrease in viscosity in the experimental test gives the true activity of the enzyme.

The nonenzymic depolymerization of hyaluronic acid in the control test may be so considerable that the correction equals or even exceeds the true enzymic effect. Under these circumstances the analysis as a whole becomes insufficiently reliable.

On this basis a number of authors conclude that the mucolytic activity of the urine, determined by a viscosimetric method, is due not to hyaluronidase but to nonspecific thermostable substances [3, 4]. If the comments of these authors are justified, then doubts may be expressed about the results obtained in A. G. Ginetsinskii's laboratory from investigation of the hyaluronidase in the urine providing a basis for the expression of definite views on the mechanism of action of the antidiuretic hormone [1, 2].



Scheme of the microviscosimeter (the dimensions are given in mm).

A special investigation of this problem showed the following. Many samples of urine, after boiling, actually gave rise to a considerable lowering of the viscosity of the hyaluronic acid. The "mucolytic activity" of the control, boiled samples of urine, however, is not determined by their content of unknown substances of other than enzymic nature, but by the properties of the substrate itself.

Since no sufficiently purified standard preparation of hyaluronic acid manufactured commercially is yet available, each research worker usually obtains it independently. The method of McClean and Hale [6] is very widely used for this purpose, consisting of drying human umbilical cords in acetone, shredding them and extracting the hyaluronic acid with water, precipitating it with alcohol, and then drying the precipitate in ether and over $CaCl_2$. The method is very simple and gives a considerable yield of highly polymerized product, although insufficiently purified.

In recent years papers have appeared describing methods of complete purification of hyaluronic acid. In the usual laboratory practice, however, it is difficult to apply these methods of preparation of the highly purified product because of their complexity. Hyaluronic acid, isolated by the method of McClean and Hale, fully satisfies the requirements of the viscosimetric method, although it does have one essential defect. In some cases the hyaluronic acid undergoes spontaneous depolymerization, even after the addition of the distilled water and buffer. It this factor is not taken into consideration, the erroneous conslusion may be reached that substances of a nonenzyme nature are present in the test samples, depolymerizing the substrate, as took place in the researches of E. P. Stepanyan and N. I. Gorbarenko [3] and E. I. Chazova [4].

The cause of spontaneous depolimerization very probably is the extraction of proteins of an enzyme nature from the umbilical cords. In any case this essential defect can be overcome by a simple method. For this purpose it is sufficient, before the experiment, to warm the hyaluronic acid solution on a boiling water bath for aproximately 20 minutes (the time is chosen empirically), in order to destroy the enzymes contained in the substrate itself.

In the table are given the results of a comparative determination of the depolymerizing effect shown by various fluids, when noninactivated solutions of hyaluronic acid and solutions inactivated by boiling were used as substrate.

The Effect of Heating on the Spontaneous Depolymerization of Hyaluronic Acid

Experiment No.	Test fluid	Activity, in conventional units					
		substrate					
		noninactivated hyaluronic acid			hyaluronic acid, inactivated by boiling		
		experiment	control	activity	experiment	control	activity
1	Distilled water	12	—	12	0	—	0
2	" " »	14	—	14	1	—	1
3	Rat's urine	13	16	3	1	1	0
4	" "	25	10	15	17	2	15
5	" "	37	14	23	23	2	21

The spontaneous depolymerization of this particular hyaluronic acid amounts to about 14 units, as can be seen from the experiment with distilled water. Roughly the same value is given by the control, using non-inactivated acid, during analysis of the enzymic activity of the urine. On treatment of the substrate by boiling the spontaneous depolymerization ceases and the control sample becomes stable. At the same time it must be remembered that the correction introduced into the unstable control nevertheless is adequate. In experiment No. 3, for instance, the urine shows no activity, but in experiment No. 4 it shows the same activity when tested with both substrates.

After inactivation of the substrate and the prevention of spontaneous depolymerization of hyaluronic acid, the viscosimetric method and its modifications are thus simple and reliable methods of investigation of the hyaluronidase activity of very small quantities of biological fluids. The results obtained by this method for the hyaluronidase activity of urine are perfectly reliable.

SUMMARY

A viscosimeter of capillary type was employed for the microdetermination of hyaluronidase in biological fluids. For analysis samples of 0.05 ml of the solution under study are taken.

The inconstancy of results obtained by viscosimetric method may be, in a great measure, due to the presence of spontaneous hyaluronic acid depolymerization. This defect is removed by heating the substrate. The above data prove the thermolabile enzymic nature of the mucolytic factor revealed in the urine.

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