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THE KINETIC DETERMINATION OF OXYTOCINASE. USE OF THE "ENZYRATOR"

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Summary

The present methods for the determination of oxytocinase show fairly large deviations from the desired zero order kinetics. These irregularities were studied and accordingly the procedure was improved.

Introduction

The determination of oxytocinase is based on the hydrolysis of *S*-benzyl-1-cystein-*p*-nitroanilide. The use of this substrate gives rise to several difficulties, which have been extensively described by van Oudheusden [1] and by Tovey et al. [2].

These difficulties are for the greater part due to the low solubility of this substrate in water, which is the reason that one has to have recourse to the use of mixtures with organic solvents or surface-active agents.

Such procedures may make the determination practicable; however, one should be aware of other, non-physiological, phenomena which may influence the reaction.

Materials and methods

Instrumentation

For the determination of the kinetic activity we used the "Enzyrator" (Marius, Utrecht), an enzyme reaction-rate analyzer which has been developed in our laboratory.

In principle it is a photometer with a very high sensitivity and stability. It determines the absorbance of the test solution 12 times every 20 seconds. From these values the ΔA/min for this period is calculated automatically and, after multiplying by an appropriate factor the activity (in I.U.) is displayed on nixie-tubes, recorded on a strip chart recorder and/or printed out. Because

nearly every reaction has a short period of instability immediately after mixing, a lag phase of 1 cycle (20 seconds) is built in, but thereafter the reaction may be followed at will.

The time graphs which are obtained are in fact the first derivative of the well known absorbance-time graphs.

True zero order reactions are displayed as straight horizontal lines, first order reactions as straight lines, the slope of which is a measure of the K-value.

Deviations from these true types are easily discernible in this mode of display.

Polyethylene pipets ("Sanz" type) were used to deliver reagents and samples into clear polystyrene tubes, which were incubated in an aluminum thermostatted block prior to placing them in the "Enzyrator" (thermostatted at the same temperature).

Mixing was done during 2 seconds on a "Vortex"-type mixer.

Reagents

The substrate, *S*-benzyl-1-cystein-*p*-nitroanilide, was obtained from Boehringer, Mannheim; the other reagents were from Merck, Darmstadt, pure analytical grade.

Samples

Serum samples were obtained from the department of Gynecology, Academic Hospital, Utrecht.

Methods

28 μl of serum were added to 500 μl phosphate buffer (0.1 M pH 7.5) and incubated for 5 minutes at 30°C. Subsequently 85 μl substrate solution (3.5 M in methoxyethanol) was added (prewarmed to the same temperature) and the course of the activity followed for 6—7 minutes.

Results

Carrying out the determinations as described, which is, apart from the temperature, identical to the method described by van Oudheusden [1], we obtained results as given in Fig. 1.

These activity vs time graphs for 4 sera with increasing activities, show 3 deviations from the ideal zero-order reaction, which, as mentioned, should give a horizontal line.

These irregularities are:

(1) a decreasing activity during the first 2—3 minutes;

(2) a real zero order part of the curve, followed by a decreasing activity;

(3) the onset of the decrease in activity, as well as the steepness of decrease, is dependent on the activity of the enzyme.

The fact that the decrease in activity as mentioned under (2) and (3) is practically linear points to an inhibition which develops during the progress of the reaction. From a study of the absorbance of the reaction mixture at the time of the onset of the fall in activity we concluded that at this moment the concentration of *p*-nitroaniline was about 0.03 mM. Addition of known con-

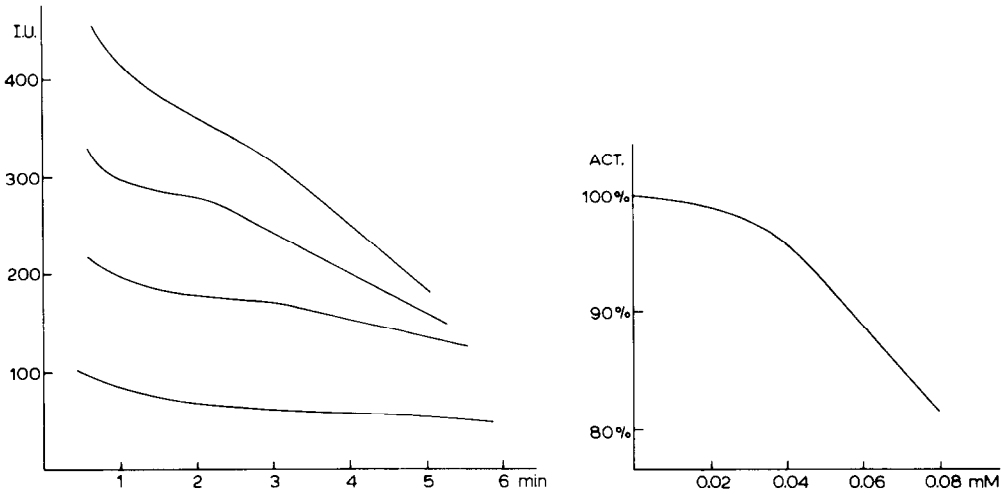

Fig. 1. Activity — time curves for 4 different sera.

Fig. 2. Inhibition of oxytocinase by *p*-nitroaniline, added to the reaction mixture.

centrations of *p*-nitroaniline to the reaction mixture showed an inhibitory effect, which was especially pronounced above this concentration (Fig. 2).

Identical experiments with *S*-benzylcystein showed no effect, as is also mentioned by van Oudheusden [1].

A minor part of the non-linearity will be the result of the decreasing concentration of the substrate, which is perhaps not optimal, but nearly so. The determination of the optimal concentration however is doubtful, since with higher substrate concentrations turbidities develop during the reaction, thus obscuring the real absorbance increase.

As the speed with which the concentration of *p*-nitroaniline increases is dependent on the activity of the enzyme, it is clear that the point of onset of this decrease is dependent on this activity.

Thus, to be able to make valid reaction-rate determinations it has to be done with enzyme concentrations as low as possible and at not too high a temperature.

The irregularity mentioned under (1) was the most puzzling. Eventually we remembered that heat is generated when alcohols are diluted. Measuring the temperature in the reaction mixture after addition of the normal amount of

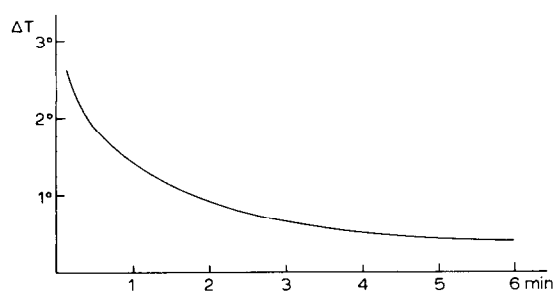

Fig. 3. Temperature rise in the reaction cuvette due to mixing 85 µl methoxyethanol with 500 µl buffer.

TABLE I

DECREASE OF VARIATION COEFFICIENT WITH TIME

Each value represents the mean of 10 determinations on 5 consecutive days.

	1′			2′			3′			4′		
V.C.	6.5	4.7	2.8	2.9	2.6	1.9	1.5	1.5	1.2	1.1	0.9	0.9

methoxyethanol gave a temperature-time curve as shown in Fig. 3. This rise in temperature, in connection with a Q_{10} which we determined as 2.1, completely explains the phenomenon.

The remedy against this disturbing influence was to add the necessary amount of methoxyethanol to the buffer and to dissolve the substrate in an acid medium, containing the same concentration of methoxyethanol, so that on mixing the temperature would not be influenced.

The optimal concentration, which is dependent on working temperature and the amount of serum used, was determined as being 12%. At 30°C and with 20 µl serum no turbidities occurred.

It should however be realized that the addition of a substrate which is dissolved in a partly organic solvent, and the progress of an enzymatic reaction in such a medium might be seriously hampered. This is reflected in the large fluctuations in the activities which are found during the first 2 minutes: the coefficient of variation gradually decreases to about 2% after 3 minutes (Table I).

Proposed method

Buffer: 0.1 M phosphate buffer, pH 7.5, containing 12% methoxyethanol.

Substrate solution: 3 mg substrate is dissolved in 120 µl methoxyethanol and made up to 1 ml with 0.1 *N* HCl.

Dispense 500 µl buffer in a cuvet, add 20 µl serum, incubate 3—5 minutes at 30°C.

Add 30 µl substrate solution, warmed to 30°C and measure the ΔA/min at 406—410 nm.

Up to 300 I.U. the reaction follows a zero-order progress for 5 minutes. It is advised to dilute sera with higher activities with a non-active serum. Not with saline.

References

1 van Oudheusden, A.P.M. (1973) Thesis, University of Utrecht
2 Tovey, J.E., Dawson, P.J.G. and Fellowes, K.P. (1973) Clin. Chem. 19, 756—761