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Inactivation of Thrombin by Means of Tetranitromethane *

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In view of the inhibitory effect of tetranitromethane (TNM) on the diphteria toxin-antitoxin precipitin reaction found by Ehrenberg, Fischer and Löfgren [1, 2] the influence of TNM on the clotting of fibrinogen was investigated.

Fibrinogen prepared from ox plasma (Bordet) as described by Astrup and Darling [3] was used. Ox thrombin (B) prepared after Astrup and Darling [4,5] was obtained from *Løvens kemiske Fabrik*, Copenhagen. Buffer: *N*/15 phosphate, pH 6.8.

The following solutions were prepared:

Th_1: 1 ml thrombin solution (100 units/ml) + 4 ml buffer.

Th_2: 1 ml thrombin solution (100 units/ml) + 4 ml buffer saturated with TNM.

Fi_1: 5 ml fibrinogen solution + 10 ml buffer.

Fi_2: 5 ml fibrinogen solution + 10 ml buffer saturated with TNM.

After standing for half an hour at room temperature Th_2 had developed a faint yellow colour, while Fi_2 was intense yellow. The following clotting times were found (in seconds):

0.2 ml Th_1 + 1 ml Fi_1: 45, 48; 0.2 ml Th_2 + 1 ml Fi_1: 155, 205.
0.2 ml Th_1 + 1 ml Fi_2: 55, 65; 0.2 ml Th_2 + 1 ml Fi_2: >240.
0.5 ml Th_1 + 1 ml Fi_2: 29, 30; 0.5 ml Th_2 + 1 ml Fi_2: 170, 210.

It is seen that the presence of TNM in the fibrinogen solution only increases the clotting time to a slight extent, while the thrombin solution containing TNM has lost most of its activity. TNM thus seems to inactivate thrombin while it is without influence on the clotting ability of fibrinogen even

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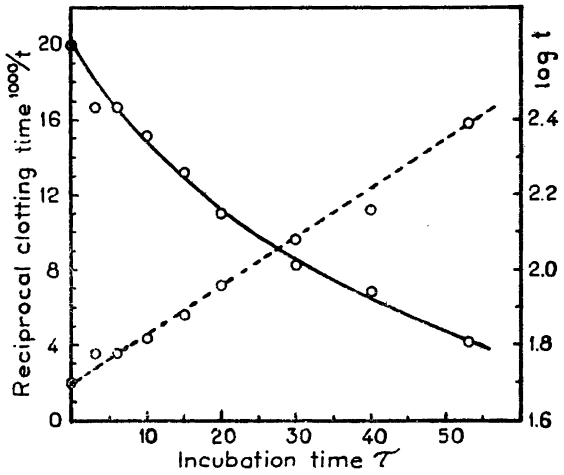

Fig. 1. Inactivation of thrombin with TNM at 20°. Abscissa: Incubation time τ. Ordinates: Full curve: Reciprocal clotting time $t \times 10^3$. Dotted curve: log t.

if the yellow colour is more intense in the latter case. This was confirmed in the following experiments:

To 2 ml thrombin solution (100 units per ml) was added 4 ml buffer saturated with TNM. 0.2 ml was removed after standing at room temperature for varying periods of time, and the clotting time of 1 ml fibrinogen solution (Fi_1) determined. Table 1 shows the results. As the reciprocal clotting times are a rather accurate measure of the potency of the thrombin solution these values are calculated and the corresponding curve drawn in Fig. 1.

When TNM is present in excess the inactivation can be described as a first order reaction:

Table 1. Inactivation of thrombin by TNM.

Incubation time τ min	Clotting time t s	$1000/t$	log t
0	50	20	1.699
3	60	16.7	1.778
6	60	16.7	1.778
10	66	15.2	1.820
15	76	13.2	1.881
20	91	11.0	1.959
30	120	8.3	2.079
40	145	6.9	2.161
53	245	4.1	2.389

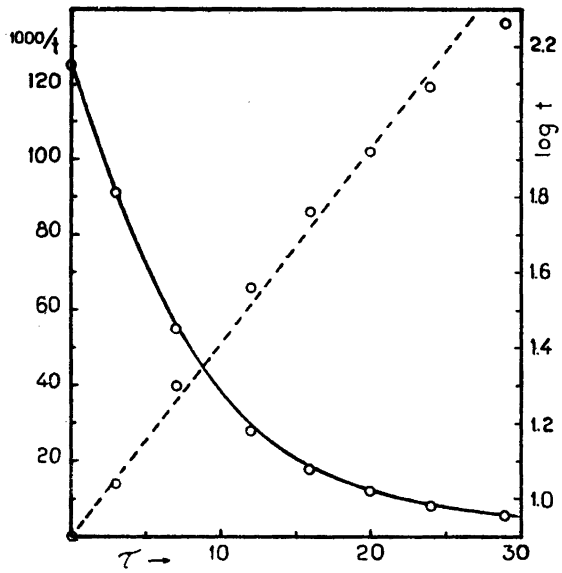

Fig. 2. Inactivation of thrombin with TNM at 37°, compare Fig.1.

$$dx = k \cdot (a—x) \cdot d\tau \tag{1}$$

where a is the original concentration of thrombin and x the amount inactivated at time τ. After integration equation (2) is obtained as $x = 0$ for $\tau = 0$:

$$\log \frac{a}{a—x} = \frac{k}{2.303} \cdot \tau \tag{2}$$

As the amount of unchanged thrombin $(a—x)$ is proportional to the reciprocal of the clotting time t:

$$t \cdot (a—x) = \text{const.} \tag{3}$$

and hence:

$$\log t = \frac{k}{2.303} \cdot \tau + \text{const.} \tag{4}$$

Thus there should be direct proportionality between $\log t$ and τ. In Fig. 1 is drawn a curve with the reaction time τ as abscissa and the logarithm of the clotting time t as ordinate. The curve is a straight line. The inactivation therefore proceeds as a first order reaction. At 37° the inactivation proceeds with a considerably increased velocity, an example is shown in Fig. 2.

Table 2. Incubation of fibrinogen with TNM. τ = incubation time (min); t = clotting time (s).

Room temperature			37°		
τ	control t	TNM t	τ	control t	TNM t
3	10	8	0	5	6
6	9	10	4	7	8
12	9	11	8	5	10
25	8	12	16	—	11
40	8	11	24	—	12
68	9	11	45	7	14
			60	7	17

From the curves the following velocity constants for the reaction and the periods of half-life ($\tau_{½}$) may be calculated:

20°: $k = 0.0005$ s^{-1}; $\tau_{½} = 23$ min.
37°: $k = 0.002$ s^{-1}; $\tau_{½} = 5.8$ min.

Similar values were found in other experiments.

From the Arrhenius equation (5) it is now possible to calculate the activation energy E of the reaction:

$$E = \frac{R \cdot T_1 \cdot T_2}{T_2 - T_1} \cdot \ln \frac{k_2}{k_1} \qquad (5)$$

Here R is the gas constant (1.987 cal per degree per mole), T_1 and the absolute temperatures and k_1 and k_2 the corresponding velocity constants. By inserting the above mentioned values we get:

$$E \cong 15.000 \text{ cal}$$

The activation energy thus found is considerably lower than the activation energy found for the heat inactivation and denaturation of enzymes and proteins, compare Sizer [6], in which, however, the heat of dissociation was found by Steinhardt [7] to furnish a large part of the activation energy as calculated in the usual manner, see La Mer [8].

A fibrinogen solution is only very slowly changed in the presence of TNM as evident from the following experiments.

Fi_1: 5 ml fibrinogen solution, 5 ml buffer, 5 ml H_2O.

Fi_2: 5 ml fibrinogen solution, 5 ml buffer saturated with TNM, 5 ml H_2O.

These mixtures are placed at room temperature and one ml clotted by adding to 0.2 ml of a thrombin solution containing 25 units per ml. A similar experiment was performed at 37°. The results are tabulated in Table 2. Even at 37° the ability of fibrinogen to clot is decreased only to a slight extent.

The inactivation of thrombin by TMN resembles the inactivation by the natural antithrombins found in plasma and serum and by trypsin, Glazko and Ferguson [9], Astrup and Darling [10], Volkert [11], processes which also proceeds much faster at 37° than at room temperature and with almost the same velocity as we found. But as the môde of inactivation is known neither for the natural antithrombins nor for tetranitromethane it is too early to speculate about the details of this process.

The only other enzymatic reaction so far studied in the presence of TNM is the action of amylase on starch, Ehrenberg *et al.* [1] In this reaction TNM had no effect. Thus it is probable that TNM is a reagent acting specifically on certain enzymes and not on others.

SUMMARY

In the presence of tetranitromethane the inactivation of thrombin proceeds as a first order reaction. The activation energy E is about 15.000 cal. Fibrinogen is not changed.

REFERENCES

1. Ehrenberg, L., Fischer, I., and Löfgren, N. *Svensk Kem. Tid.* **57** (1945) 303.
2. Ehrenberg, L., Fischer, I., and Löfgren, N. *Nature* **157** (1946) 730.
3. Astrup, T., and Darling, S. *Acta Physiol. Scand.* **4** (1942) 45.
4. Astrup, T. and Darling, S. *J. Biol. Chem.* **133** (1940) 761.
5. Astrup, T., and Darling, S. *Acta Physiol. Scand.* **2** (1941) 22.
6. Sizer, I. W. *Advances in Enzymol.* **3** (1943) 35.
7. Steinhardt, J. *Kgl. Danske Videnskab. Selskab., Mat.-fys. Medd.* **14** (1937) no. 11.
8. La Mer, V. K. *Science* **86** (1937) 614.
9. Glazko, A. J., and Ferguson, J. H. *J. Gen. Physiol.* **24** (1940) 169.
10. Astrup, T., and Darling, S. *Acta Physiol. Scand.* **4** (1942) 293.
11. Volkert, M. *Acta Physiol. Scand.* (1942) suppl. no. 15.

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