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The Influence of Nitrocatechol and Sulphate Ions on Ox Liver Arylsulphatase A at 40° C

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Experiments are reported which show that when ox liver arylsulphatase A hydrolyses nitrocatechol sulphate at pH 5.0 and 40°C there is a distinct fall in the reaction velocity during the first 10–15 min. Later the reaction velocity increases and, eventually, it reaches a constant value. It is shown that this increase in velocity is due to the liberated nitrocatechol, whereas the liberated sulphate ions tend to inhibit the reaction, if they are not precipitated. A reaction scheme is proposed and an expression is derived which can describe the reaction progress under the condition of the experiments.

In 1953 Roy[1] observed that the kinetics of the enzymatic hydrolysis of the potassium salt of nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate) by partially purified preparations of ox liver arylsulphatase A are of an anomalous type. Since then the kinetics of this system have been investigated by several groups,[2-6] but the problem cannot be considered as solved.

Both Roy [2] and Baum, Dodgson and Spencer [4] found that when the reaction takes place at 37°C and pH 5.0, the reaction progress can be divided in three stages. In stage I and stage III the velocity is rather high, but is low in stage II; the duration of the stages depends on substrate and enzyme concentrations. In contrast to this the present author has reported some experiments performed at pH 5.0 and 20°C, in which only stage I and II were found.[6] The cause of this discrepancy was not found. Experiments have now been performed at 40°C in an attempt to clarify this problem, and they are reported here together with a proposed mechanism, which explains the kinetics found at this temperature.

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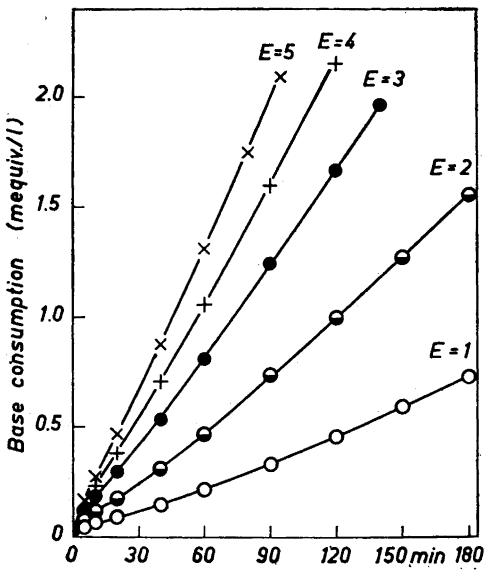

Fig. 1. Reaction progress of the enzymatic hydrolysis of nitrocatechol sulphate at pH 5.0 and 40°C. Substrate concentration was kept constant at 0.003 M in 30 ml 0.05 M $SrCl_2$. The relative enzyme concentrations are indicated on the figure.

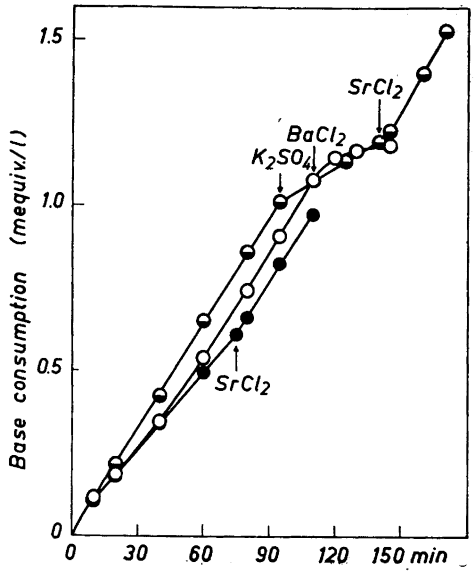

Fig. 2. Reaction progress of the enzymatic hydrolysis of nitrocatechol sulphate at pH 5.0 and 40°C. Substrate concentration was kept constant at 0.003 M in 30 ml 0.10 M KCl. ◒—◒—◒: 0.002 M nitrocatechol added before start of experiment, 1.0 ml M K_2SO_4 added at 95 min, 3 ml 0.5 M $SrCl_2$ added at 140 min. ●—●—●: 0.002 M K_2SO_4 added before start of experiment, 3 ml 0.5 M $SrCl_2$ added at 75 min. O—O—O: no addition before experiment, 20 mg $BaCl_2$ added at 110 min.

EXPERIMENTAL

The enzyme and the nitrocatechol sulphate were prepared as described earlier [6]. Nitrocatechol was prepared from its sulphate ester by acid hydrolysis.

The enzymatic hydrolysis of the sulphate ester was followed by continued automatic titration in a pH-stat, as already described [6]. The substrate concentration was kept constant during the experiments and the liberated sulphate was precipitated as $SrSO_4$ instead of as $BaSO_4$, since it has been found that excess Ba++ has a pronounced effect on the enzyme activity [7].

The experiments were performed at 40.0°C and at pH 5.0 in a medium, which consisted of 0.05 M solution of $SrCl_2$. A small amount of $SrSO_4$ was added to prevent supersaturation of this substance during the experiments. When the influence of sulphate ions was investigated 0.10 M KCl was used instead of 0.05 M $SrCl_2$.

RESULTS

The experiments in Fig. 1 were all performed at the same substrate concentration and at different enzyme concentrations. In contrast to the experiments at 20°C the three stages described by Roy and Dodgson are clearly seen

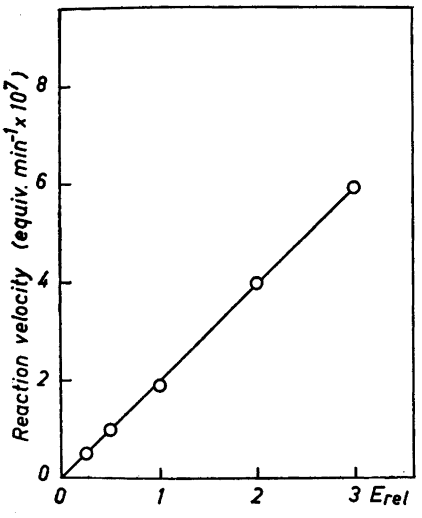

Fig. 3. Reaction velocity in stage III *versus* the relative enzyme concentration. Experiments with added nitrocatechol (0.002 M) and constant substrate concentration (0.003 M).

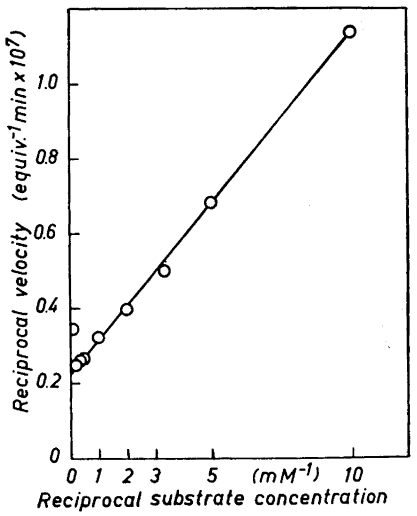

Fig. 4. Reciprocal reaction velocity in stage III *versus* reciprocal substrate concentration. Experiments with added nitrocatechol (0.002 M).

at 40°C. As the substrate concentration was kept constant during the experiments and the sulphate formed during the reaction was precipitated, the only factor known to change during the experiments is the liberated nitrocatechol. So nitrocatechol was added to the reaction mixture, before an experiment was started, in order to see, if it would have any influence on the reaction progress. The result was that stage II disappeared completely, so that stage I went directly into stage III. (◒—◒—◒ of Fig. 2). It made no difference to the reaction progress whether the enzyme was incubated for some hours with nitrocatechol, or it was added just at the beginning of the experiment.

Experiments with different enzyme concentrations and with 0.002 M nitrocatechol added were then performed and the reaction velocity in stage III determined. This velocity is plotted against the enzyme concentration in Fig. 3, and it is seen that under these circumstances the velocity is proportional to the enzyme concentration. Experiments with the same enzyme concentration but different substrate concentrations and added nitrocatechol were also performed. In Fig. 4 the reciprocal of the velocity in stage III is plotted against the reciprocal substrate concentration, according to Lineweaver and Burk [8]. This shows that below a substrate concentration of 0.005 M the kinetics of the reaction are in accordance with the Henry-Michaelis scheme with K_m equal to 0.0004 M. Inhibition occurred at the highest substrate concentration. The influence of sulphate ions can be seen in Fig. 2. 0.002 M nitrocatechol was added to one of the experiments and 0.002 M K_2SO_4 to another. The

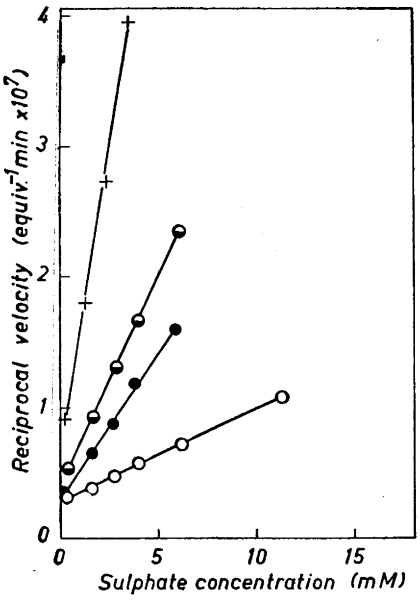

Fig. 5. Effect of sulphate ions on the reaction velocity in stage III in experiments with added nitrocatechol (0.002 M) and constant substrate concentration. O—O—O: 3 mM nitrocatechol sulphate, ●—●—●: 1 mM nitrocatechol sulphate, ◒—◒—◒: 0.5 mM nitrocatechol sulphate, +—+—+: 0.3 mM nitrocatechol sulphate.

reaction progress was followed for some time, whereafter it was tried, if addition of K_2SO_4, $SrCl_2$ or $BaCl_2$ would have any effects. It is seen that sulphate ions inhibit the reaction and that strontium ions can release the enzyme from this inhibition. Barium ions inhibit the reaction too. To investigate the influence of sulphate ions experiments were performed with excess of nitrocatechol and addition of varying amounts of K_2SO_4. The rate of the reaction was determined, and the concentration of SO_4^{--} was calculated as the sum of the sulphate added and the sulphate liberated during the reaction. The reciprocal velocity is plotted against the sulphate concentration for four different substrate concentrations in Fig. 5, which shows that the inhibition is of the competitive type.

REACTION MECHANISM

These experiments show that the kinetics of the enzyme at 40°C follow a simple scheme, when the reaction has reached a steady state, but that this is only the case when there is excess of nitrocatechol present, and when the liberated sulphate ions are removed. It has been shown previously that the kinetics at 20°C, where no reactivation was found, could be explained by supposing the following scheme:

$$A + X_1 \rightleftharpoons X_2 \qquad (\pm 1)$$
$$X_2 \rightarrow X_1 + P_1 + P_2 \qquad (+2)$$
$$X_2 \rightleftharpoons X_3 \qquad (\pm 3)$$

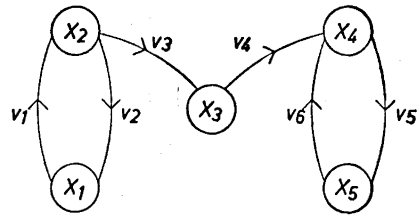

Fig. 6.

where X_1, X_2 and X_3 are different forms of the enzyme. A is the substrate, and P_1 and P_2 are the reaction products, nitrocatechol and sulphate ion respectively. The formation of X_3 is supposed to be rather slow.

To explain the reactivation found at 40°C the following reactions must be added to the scheme:

$$P_1 + X_3 \rightleftharpoons X_4 \qquad (\pm 4)$$
$$X_4 \rightarrow X_5 + P_1 + P_2 \qquad (+5)$$
$$A + X_5 \rightleftharpoons X_4 \qquad (\pm 6)$$

Nitrocatechol reacts in some way with the inactive form of the enzyme, X_3, and converts it to a new active form, X_4. The whole scheme is essentially two Henry-Michaelis schemes connected through the X_3-form. In the manner of Christiansen [9,10] it can be expressed as in Fig. 6.

If we make the simplifying assumption that reaction (3) only goes from the left to the right, so that there is no reverse reaction, then it is possible to derive from this scheme an expression for the reaction progress, which can be tested on the experiments. This assumption is not quite unjustified, as the equilibrium of the reaction must be far to the right. This follows from the results of experiments with very low enzyme concentration, where the velocity in stage II is less than a tenth of the initial velocity.

It is further assumed that the formation of X_3 is rather slow in comparison with all the other reactions in the scheme, so we have:

$$x_1 = (K_1/a)x_2 \qquad (7)$$
$$x_5 = (K_2/a)x_4 \qquad (8)$$
$$-\mathrm{d}(x_1 + x_2)/\mathrm{d}t = k_3 x_2 \qquad (9)$$

x_i is used for the concentration of the enzyme form X_i, and a and p_1 for the concentration of A and P_1, k_i is the velocity constant of reaction (i) and K_1 and K_2 are the Michaelis constants, $(k_{-1} + k_2)/k_1$ and $(k_{-6} + k_5)/k_6$, of the first and second cycle in the reaction scheme.

Further we have

$$E = x_1 + x_2 + x_3 + x_4 + x_5 \qquad (10)$$
$$x_3 p_1 = K_3 x_4 \qquad (11)$$

and

$$\mathrm{d}\xi/\mathrm{d}t = k_2 x_2 + k_5 x_4 \qquad (12)$$

where ξ is the amount of substrate split at time t. When no nitrocatechol has been added to the reaction mixture ξ will be equal to p_1. From (7), (8), (10), and (11) we get

$$E = (K_1/a + 1)x_2 + (K_3/\xi + K_2/a + 1)x_4 \tag{13}$$

and from (7) and (9) we get

$$x_2 = \frac{E}{(K_1/a + 1)} e^{-t/(K_1/a + 1)}$$

or setting $1/(K_1/a + 1) = \lambda$

$$x_2 = E\lambda e^{-\lambda t} \tag{14}$$

From (13) and (14) we can now get an expression for x_4

$$x_4 = E(1-e^{-\lambda t})/(K_3/\xi + K_2/a + 1) \tag{15}$$

Substitution of the values of x_2 and x_4 in (12) gives

$$d\xi/dt = Ek_2\lambda e^{-\lambda t} + Ek_5(1-e^{-\lambda t})/(K_3/\xi + K_2/a + 1) \tag{16}$$

This equation can be rewritten as

$$\left(\frac{K_3}{\xi} + \frac{K_2}{a} + 1\right) d\xi = \frac{Ek_2K_3\lambda e^{-\lambda t}}{\xi} dt + Ek_2\left(\frac{K_2}{a} + 1\right)\lambda e^{-\lambda t}dt + Ek_5(1-e^{-\lambda t})dt \tag{17}$$

This equation can be integrated directly apart from the term

$$Ek_2K_3\lambda(1/\xi)e^{-\lambda t}dt \tag{18}$$

but if we consider that this term will be insignificant in the later parts of the reaction when we no longer have small values of ξ and t, then we can transform it to an integrable form. We have for the first part of the reaction, before considerable amounts of x_4 have been formed:

$$\begin{aligned} d\xi/dt &= k_2x_2 = k_2E\lambda e^{-\lambda t} \\ \xi &= k_2E(1-e^{-\lambda t}) \end{aligned} \tag{19}$$

Substitution of ξ in (18) transforms it to

$$\frac{K_3\lambda e^{-\lambda t}}{1-e^{-\lambda t}} dt, \text{ or in integrated form } K_3\ln(1-e^{-\lambda t}) \tag{20}$$

Eqn. (17) can now be integrated:

$$K_3\ln\frac{\xi}{\xi_0} + \left(\frac{K_2}{a} + 1\right)\xi = K_3\ln\frac{1-e^{-\lambda t}}{1-e^{-\lambda t_0}} + E\left(\left(\frac{K_2}{a} + 1\right)k_2 - \frac{k_5}{\lambda}\right)(1-e^{-\lambda t}) + Ek_5t \tag{21}$$

As ξ_0 and t_0, the values of ξ and t at zero time, both are zero, ln ξ_0 and $\ln(1-e^{-\lambda t_0})$ become $-\infty$, but their difference has a finite value. From (19) we get

$$\begin{aligned}(1-e^{-\lambda t})/\xi &= k_2E \\ \ln(1-e^{-\lambda t_0}) - \ln\ \xi_0 &= \ln\ (k_2E)\end{aligned} \qquad (22)$$

Eqn. (21) can now be written

$$t + A(1-e^{-\lambda t}) + B\ln(1-e^{-\lambda t}) = C\xi + B\ln\ \xi - D \qquad (23)$$

where $A = (K_2/a + 1)k_2/k_5 - (K_1/a + 1)$
$B = K_3/Ek_5$
$C = (K_2/a + 1)/Ek_5$
$D = \dfrac{K_3\ln(k_2E)}{k_5E}$

That this expression is in close agreement with the experiments is shown in Table 1. The values of A, B, C, D, and λ which were used in the calculations are given in Table 2.

Table 1. The table indicates the amount of substrate, ξ, hydrolysed at the time t for different enzyme concentrations (E). The experimental conditions are given in the text. t is in min and ξ in mequiv/l. Δ is the difference between corresponding values of the terms $t + A(1-10^{-\lambda t}) + B\log(1-10^{-\lambda t})$ and $C\xi + B\log\xi - D$. These terms have been calculated from the values of A, B, C, D, and λ given in Table 2.

t	$E_{rel} = 1$		$E_{rel} = 2$		$E_{rel} = 3$		$E_{rel} = 4$		$E_{rel} = 5$	
	ξ	Δ	ξ	Δ	ξ	Δ	ξ	Δ	ξ	Δ
2	23	−1.3	33	2.2	53	0.7	67	0.2	77	0.1
5	41	−0.3	75	−0.3	116	−0.4	139	−0.1	161	−0.1
10	62	0.0	116	−0.9	185	−0.6	230	−0.4	274	−0.2
15	77	−0.5	146	−0.5	241	−0.5	304	−0.2	371	−0.1
20	89	−0.3	175	−0.1	295	−0.2	378	0.0	467	0.0
30	116	0.0	240	0.2	409	0.2	537	0.1	667	0.0
40	146	0.1	310	0.6	535	0.4	703	0.1	876	0.0
50	179	0.1	385	1.0	669	0.3	881	−0.1	1 097	−0.2
60	214	0.2	467	0.8	808	0.2	1 055	0.0	1 309	0.0
70	251	0.1	553	0.5	951	0.0	1 233	0.0	1 527	0.2
80	292	−0.4	641	0.2	1 096	−0.3	1 415	0.0	1 745	0.3
90	330	0.0	735	−0.7	1 241	−0.4	1 596	0.1	1 973	0.2
100	371	−0.1	825	−0.9	1 395	−1.0	1 779	0.2	2 087	0.1
110	413	−0.1	913	−0.7	1 531	−0.3	1 963	0.2		
120	455	0.0	997	−0.1	1 663	0.7	2 153	−0.1		
140	544	−0.3	1 186	−0.8	1 966	0.3				
150	590	−0.6	1 272	−0.1						
180	726	−0.7	1 554	−0.5						
210	865	−0.7	1 837	−0.6						
230	955	−0.1	2 019	0.0						
270	1 145	0.0								

Table 2. The values of the parameters A, B, C, D, and λ used to calculate the results in Table 1.

E_{rel}	A min	B min	C min/mM	D min	λ min^{-1}
1	8.6	40.7	194.0	54.2	0.12
2	3.4	22.6	100.8	23.0	0.12
3	4.0	15.8	63.8	13.8	0.12
4	3.6	11.5	51.5	9.0	0.12
5	3.8	9.36	42.5	7.1	0.12

A and λ are independent of the enzyme concentration, and B and C are inversely proportional to it as shown in Fig. 7. From Fig. 8 it can be seen that DE is linearly related to log E, which is in accordance with the derived expression.

DISCUSSION

The results presented in this paper show that when the reaction is performed at 40°C the reaction progress can be divided into three stages with different velocities in accordance with the results of Roy [2] and Baum, Dodgson and Spencer [4]. In contrast to these authors it is found that sulphate ions do not have any activating effect, but inhibit the enzyme at all concentrations investigated. This means that the activation from stage II to stage III is due only to the liberated nitrocatechol. Apart from this difference the scheme proposed

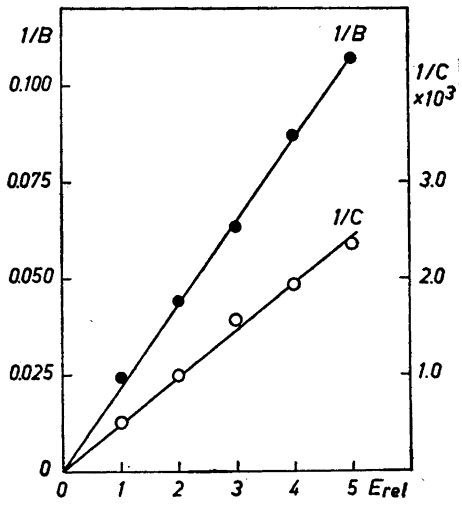

Fig. 7. Variation of the calculated values of 1/B and 1/C with the relative enzyme concentration.

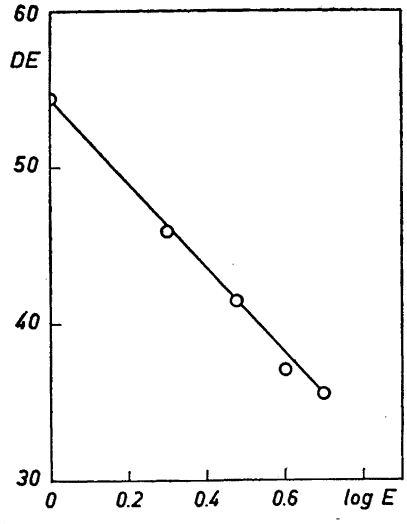

Fig. 8. Variation of the calculated value of DE with the logarithm of the relative enzyme concentration.

in this paper corresponds closely to the scheme proposed by Baum and Dodgson [5]. It is planned to investigate whether this difference could be due to acetate in the medium as used by the other groups, in contrast to chloride in this work.

The reaction scheme here proposed must only be regarded as tentative, though it is in accordance with the experiments. Minor variations in the reaction scheme are possible without the form of the final expression being effected. It is possible that reaction (3) should be $X_2 \rightleftharpoons X_3 + P_1 + P_2$, so that the inactive X_3-form of the enzyme does not contain substrate.
Then we should get further

$$X_3 + P_1 \rightleftharpoons X_4 \qquad (4a)$$
$$X_4 + A \rightleftharpoons X_5 \qquad (5a)$$
$$X_5 \rightarrow X_4 + P_1 + P_2 \qquad (6a)$$

This will give an expression for the reaction progress which will have the same form as (23), apart from the slight contribution to the reaction products from $X_2 \rightleftharpoons X_3 + P_1 + P_2$. But this contribution will be stoichiometrically equal to the enzyme concentration and far smaller than what can be measured.

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