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The Kinetics of Ox Liver Arylsulphatase A

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The hydrolysis of 2-hydroxy-5-nitrophenyl sulphate catalyzed by ox liver arylsulphatase A was investigated by continued automatic titration of the liberated acid. It has been possible to keep the concentration of the substrate constant during an experiment, and the liberated sulphate has been precipitated as $BaSO_4$. It is shown that steady state is only reached after the course of more than one hour. A reaction mechanism is proposed involving slow, reversible inactivation of the enzyme.

Of the three arylsulphatases present in mammalian liver, arylsulphatase A has been found to exhibit anomalous kinetics when hydrolysing 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) [1–3]. The investigations reported in this paper have been performed with another technique than in the above mentioned investigations, in the hope that it would be possible to clarify some of the problems.

EXPERIMENTAL

Dipotassium nitrocatechol sulphate free from nitropyrogallol disulphate was prepared according to the method of Roy [4] and Dodgson and Spencer [5]. The equivalent weight determined by complete enzymic hydrolysis in a pH-state was found to be 347 (theoretical value for the dihydrate 347.3). A stock solution of nitrocatechol sulphate being 0.03 M and having pH 5.0 was prepared and stored in the refrigerator until use.

The enzyme was prepared from ox liver according to the method of Roy [2], with a few modifications. The acetone dried liver powder was extracted for 4 h with 0.1 M KCl at room temperature. After having performed the two fractionations with acetone the enzyme extract was heated at 65°C for 5 min and then centrifuged. The sulphatase activity was not affected by this procedure. The clear colourless supernatant was used without further purifications in the experiments described in this paper. It showed an enzyme activity towards nitrocatechol sulphate at about 10 000 units/mg N (one unit is defined as the amount of enzyme which liberates 1 μg of nitrocatechol from a 0.003 M nitrocatechol sulphate solution at pH 5.0 and 37°C in one hour [2]).

A diluted enzyme preparation was incubated at 20°C and pH 5.0. At suitable intervals samples were taken for determination of the enzyme activity. It was found that under these conditions the enzyme was stable for at least 48 h.

The hydrolysis of the nitrocatechol sulphate was followed by automatic titration of the liberated sulphuric acid in a pH-state. All experiments were performed at pH 5.0 and 20.0°C. A suitable amount of the enzyme preparation was diluted with so much 0.1 M

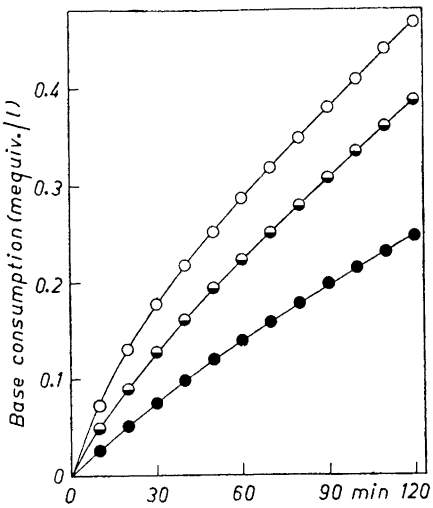

Fig. 1. Effect of addition of potassium sulphate on the enzymic hydrolysis of nitrocatechol sulphate. O—O—O: without sulphate, ◒—◒—◒: 0.3 mM K_2SO_4, ●—●—●: 1.5 mM K_2SO_4.

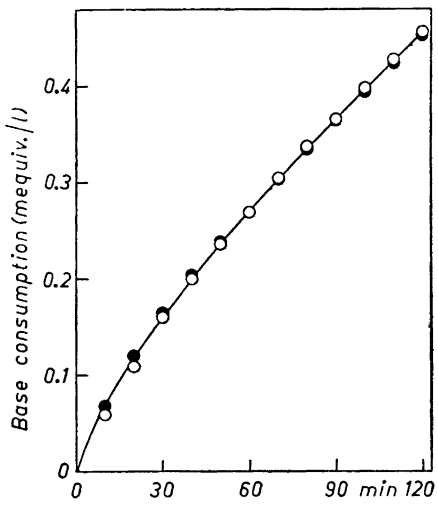

Fig. 2. Effect of addition of nitrocatechol on the enzymic hydrolysis of nitrocatechol sulphate. O—O—O: without nitrocatechol, ●—●—●: 3 mM nitrocatechol.

KCl that a final volume of 30 ml was obtained, and the pH was adjusted to 5.0. The reaction was started by adding the desired amount of substrate solution. The amount of base necessary to keep the pH constant was added automatically from a syringe, and corresponding values of time and amount of base added were read. The small dilution of the reaction mixture caused by this addition of base has not been considered in the calculations as it only amounts to a few per cent.

Roy[6] and Dodgson and Spencer[1] has shown that the reaction is influenced by sulphate ions. To overcome this complication the equivalent amount of $BaCl_2$ was added to the base in the syringe, so that the liberated sulphate would be precipitated as $BaSO_4$. It was found necessary to use exactly the equivalent amount of $BaCl_2$, as barium ions in excess inhibit the enzymic reaction. Before an experiment was started, a small amount of $BaSO_4$ was added to the reaction mixture to avoid supersaturation.

In most of the experiments reported the substrate concentration was kept constant. This was done by titrating with a mixture of equivalent amounts of base and substrate. The substrate is sufficiently stable in alkaline solution at room temperature to make such a procedure possible.

RESULTS

Experiments with added sulphate or nitrocatechol show that sulphate in a concentration of 0.0003 M inhibits the reaction (Fig. 1), and that nitrocatechol in a concentration of 0.003 M has very little influence on the reaction velocity (Fig. 2).

Experiments, where the substrate concentration was kept constant and where the liberated sulphate was precipitated as $BaSO_4$, showed that the reaction velocity decreased to about half the initial velocity during the first hour

Table 1. Measured (ξ_{exp}) and calculated (ξ_{calc}) values of the amount of substrate split at the time t. ξ_{exp} and ξ_{calc} are expressed in 10^{-6} M. E_{rel} is the relative enzyme concentration.

t	$E_{rel} = 1$		$E_{rel} = 1.7$		$E_{rel} = 3$		$E_{rel} = 4$		$E_{rel} = 6$	
min	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}
10	17	16	26	27	44	46	61	62	92	91
20	29	28	48	48	84	84	113	113	167	165
30	38	39	65	67	116	117	158	157	228	228
40	52	49	84	83	145	145	195	195	288	284
50	61	58	98	99	171	171	231	230	338	335
60	67	66	112	112	197	195	263	263	379	382
70	75	73	125	126	220	218	293	294	424	426
80	82	81	139	138	241	240	325	324	466	469
90	89	88	150	151	261	262	351	352	507	511
100	96	95	162	163	283	283	381	382	550	552
110	101	102	175	175	303	303	409	410	591	592
120	108	109	187	187	324	324	436	437	633	633

of the experiment, after which the velocity was constant for at least seven hours.

Incubation of the diluted enzyme for one hour at 20°C and pH 5.0 before starting the experiment has no inluence on the reaction progress. This indicates that the substrate is among the factors involved in the initial decrease of the reaction velocity.

Increasing the substrate concentration in an experiment when the reaction velocity had been constant for some time, showed that the velocity increased suddenly and then decreased slowly again for about one hour, when it again became constant at a higher level than before.

Experiments were performed at five different enzyme concentrations, the substrate concentrations being kept constant at 0.002 M nitrocatechol sulphate. The liberated sulphate was precipitated as $BaSO_4$. It was found that

Table 2. The absolute and relative values of the parameters A, B and C used to calculate the values of ξ_{calc} in Table 1.

E_{rel}	A 10^{-6} mole × l^{-1} min^{-1}	B 10^{-6} mole × l^{-1}	C min^{-1}	A_{rel}	B_{rel}	C_{rel}
1	0.684	27.4	0.038	1	1	1
1.7	1.170	46.8	0.038	1.71	1.71	1
3	2.03	81.9	0.038	2.97	2.99	1
4	2.74	109.5	0.038	4.01	4.00	1
6	3.94	162.0	0.038	5.76	5.91	1

Table 3. Measured (ξ_{exp}) and calculated (ξ_{calc}) values of the amount of substrate split at the time t. ξ_{exp} and ξ_{calc} are expressed in 10^{-6} M. a is the substrate concentration.

t min	a = 0.25 mM		a = 0.50 mM		a = 1.00 mM		a = 2.00 mM		a = 3.00 mM	
	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}	ξ_{exp}	ξ_{calc}
10	16	15	23	23	40	40	55	55	63	62
20	31	29	44	44	74	74	102	100	110	109
30	44	43	63	63	105	105	139	138	148	148
40	54	55	81	81	132	132	172	171	182	181
50	65	67	98	98	157	156	201	201	209	211
60	77	78	114	114	180	180	228	228	236	238
70	90	90	130	130	202	202	254	255	264	265
80	101	101	145	145	223	223	278	280	290	291
90	112	111	160	159	243	243	304	304	316	316
100	122	121	175	174	264	264	328	328	340	341
110	132	132	189	188	283	284	353	352	367	366
120	141	142	204	203	303	304	378	376	392	390

under these circumstances the progress of the reaction could be described by the expression:

$$\xi = At + B(1 - e^{-Ct})$$

where ξ is the amount of acid liberated at the time t, and A, B and C are parameters, the values of which were determined to give the best description of the reaction progress. The results are given in Tables 1 and 2. It is seen that A and B are proportional to, and C independent of the enzyme concentration.

Experiments were performed at five different substrate concentrations, and with the same enzyme concentration. The substrate concentrations were kept constant in the individual experiments, and the liberated sulphate was precipitated as $BaSO_4$. In these experiments too the reaction progress could be described by the expression:

$$\xi = At + B(1 - e^{-Ct})$$

as seen in Table 3. The values of the parameters A, B and C are given in Table 4.

Table 4. The values of the parameters A, B and C used to calculate the values of ξ_{calc} in Table 3.

a mM	A	B	C
	10^{-6} mole × l^{-1} × min^{-1}	10^{-6} mole × l^{-1}	min^{-1}
0.25	0.972	27.8	0.022
0.50	1.37	40.5	0.026
1.00	1.91	76.0	0.032
2.00	2.29	101.2	0.038
3.00	2.43	98.7	0.048

REACTION MECHANISM

To explain the results presented in this paper, it has been assumed that the active form of the enzyme can be transformed into an inactive form. This transformation proceeds slowly to an equilibrium, and the substrate seems to be necessary for this transformation.

The suggested reaction scheme can be represented in the following way:

$$\begin{aligned} X_1 + A &\rightleftharpoons X_2 && (\pm 1)\\ X_2 &\rightarrow X_1 + P && (+ 2)\\ X_2 &\rightleftharpoons X_3 && (\pm 3) \end{aligned}$$

where X_1, X_2 and X_3 are different forms of the enzyme, A is the substrate and P is the reaction products.

The two first reactions are the usual Henri-Michaelis scheme, and reaction ($\pm$ 3) is the slow, reversible inactivation of the enzyme here postulated.

If we assume steady state at any moment with respect to the two first reactions, we have

$$x_1 = (K_m/a)x_2 \tag{4}$$

where K_m is $(k_2 + k_{-1})/k_1$. Small letters are used to denote concentrations, and k_i is the velocity constant for reaction i.

Further we have for the total enzyme concentration

$$E = x_1 + x_2 + x_3 \tag{5}$$

and that

$$\mathrm{d}x_3/\mathrm{d}t = k_3x_2 - k_{-3}x_3 \tag{6}$$

From (4) and (5) we get

$$x_3 = E - (K_m/a + 1)x_2$$

and this together with (6) gives, if a is kept constant,

$$-(K_m/a + 1)\mathrm{d}x_2/\mathrm{d}t = (k_3 + k_{-3}(K_m/a + 1))x_2 - k_{-3}E \tag{7}$$

This equation can be solved with respect to x_2, and if we assume that the concentration of x_3 is zero at the beginning of the experiment, we get

$$x_2 = c_1 + c_2\mathrm{e}^{-\lambda t} \tag{8}$$

where $c_1 = k_{-3}E/(k_3 + k_{-3}(K_m/a + 1))$

and $c_2 = k_3E/(k_3 + k_{-3}(K_m/a + 1))(K_m/a + 1)$

and $\lambda = (k_3 + k_{-3}(K_m/a + 1))/(K_m/a + 1)$

The velocity of the reaction is

$$v = \mathrm{d}\xi/\mathrm{d}t = k_2x_2$$

hence

$$\xi/k_2 = c_1t + \frac{c_2}{\lambda}(1 - \mathrm{e}^{-\lambda t}) \tag{9}$$

This expression is of the same form as that determined from the results of the experiments:

$$\xi = At + B(1 - \mathrm{e}^{-Ct})$$

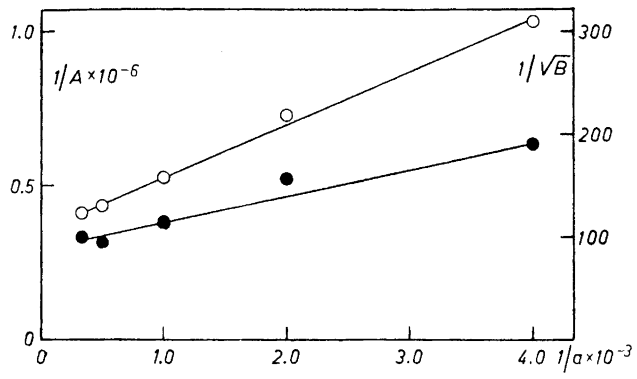

Fig. 3. The calculated values of 1/A and $1/\sqrt{B}$ plotted against the reciprocal substrate concentration.

In is seen that c_1 and c_2/λ are proportional to and λ is independent of the enzyme concentration, which is in accordance with the experiments. Moreover it can be found that there is a rectilinear relationship between $1/c_1$ and $1/a$ and between $\sqrt{\lambda/c_2}$ and $1/a$. In Fig. 3 the experimentally determined values of 1/A and $1/\sqrt{B}$ are plotted against $1/a$.

DISCUSSION

The experiments reported in this paper show that even when the substrate concentration is kept constant, and the liberated sulphate is precipitated as $BaSO_4$, the reaction velocity only reaches a constant value more than one hour after the beginning of the experiment. As it has been shown that the liberated nitrocatechol has only little influence on the reaction velocity, the cause of this phenomenon must be sought in the enzyme. A reaction mechanism has been proposed and shown to fit the experiments. The special feature of this reaction mechanism is a slow reversible inactivation of the enzyme, in which

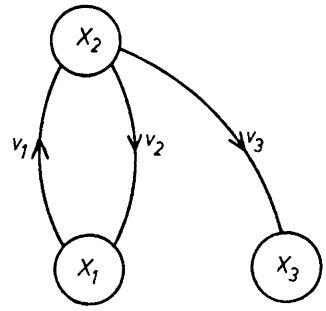

Fig. 4.

the substrate is involved. The reaction scheme can be expressed in the notation proposed by Christiansen [7,8] as in Fig. 4, where the circles symbolize the different enzyme forms.

It must be noted that there are some differences between the results obtained in other laboratories and those here reported. Roy [3] has found that free nitrocatechol causes an overall increase in the reaction velocity, and both Roy [3] and Dodgson and Spencer [1] report that there can be observed an increase in velocity after the initial decrease in the reaction velocity. Both groups have performed there experiments at 37°C and used an acetate buffer as medium, and they have followed the reaction spectrophotometrically. In this work 0.1 M KCl has been used as medium at 20°C, and the reaction was followed by titrating the liberated acid. Before more experiments have been performed it will not be possible to decide whether the cause of the discrepancies has to be sought in the different techniques used.

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REFERENCES

1. Dodgson, K. S. and Spencer, B. *Biochem. J.* **62** (1956) 30 p.
2. Roy, A. B. *Biochem. J.* **55** (1953) 653.
3. Roy, A. B. *Experientia* **13** (1957) 32.
4. Roy, A. B. *Biochem. J.* **53** (1953) 12.
5. Dodgson, K. S. and Spencer, B. *Biochim. et Biophys. Acta* **21** (1956) 175.
6. Roy, A. B. *Biochem. J.* **59** (1955) 8.
7. Christiansen, J. A. *Acta Chem. Scand.* **3** (1949) 493.
8. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.

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