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KINETIC PROPERTIES OF THE ARYLSULPHATASE A FROM HUMAN KIDNEYS

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SUMMARY

A mathematical correlation between the reaction velocity and the incubation time is derived for the anomalous kinetics of the enzymic reaction of the arylsulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) from human kidneys.

With the aid of this correlation the influence of monovalent anions (formate, acetate, propionate, and Cl^-) on the enzymic hydrolysis of nitrocatechol sulphate is studied. The anions cited above inhibit arylsulphatase A. Furthermore, they exert a concentration dependent influence on the rate with which the enzyme is inactivated by its substrate and on the velocity of the enzymic reaction after reactivation of the inactivated enzyme by SO_4^{2-}.

The rate of inactivation of the enzyme also depends on the substrate concentration, being lower the lower the substrate concentration is.

The apparent Michaelis constant of the arylsulphatase A depends both on the concentration of monovalent anions and on the incubation time.

On the basis of these results a mechanism of the enzymic reaction of the arylsulphatase A is proposed.

INTRODUCTION

The arylsulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) of vertebrates is an acid hydrolase of lysosomal origin[1], which catalyses the degradation of arylsulphuric esters. The physiological substrates of the enzyme are the sulphatides[2]. Upon degrading nitrocatechol sulphate and related substrates the enzyme exhibits anomalous kinetics which were noted first by Roy[3] for the arylsulphatase A from ox liver. Similar kinetic anomalies, which result in a progressive inactivation of the enzyme associated with its catalytic activity[4], have been confirmed for the arylsulphatase A from other vertebrates[5–7]. Detailed studies of these anomalies have been made by Baum and Dodgson[8] and by Nicholls and Roy[9]. According to their interpretation of the mechanism of the enzymic reaction the arylsulphatase A is slowly modified when degrading its substrates. The modified form of the enzyme is

assumed to expose a second binding site which can bind either substrate or the reaction products or certain other anions (like PO_4^{3-} and $P_2O_7^{4-}$). The resulting complex between the modified enzyme and substrate ions is inactive while the activity is at least partially restored when either of the reaction products (nitrocatechol and SO_4^{2-}) or PO_4^{3-} or $P_2O_7^{4-}$ is bound to the second binding site. The influence of monovalent anions on the kinetics of arylsulphatase A has not yet been studied in detail, though acetate and Cl^- are widely used as constituents of the assay system.

EXPERIMENTAL

Enzyme

Non-pathological human kidneys from male and female persons of various ages were obtained within 24 h *post-mortem*. The kidneys were stored at −20 °C. The enzyme was purified according to the method Bleszynski[10] has developed for the isolation of arylsulphatase A from bovine brain. A 20 000-fold enrichment was achieved. During the isolation the enzyme activity was determined according to Baum *et al.*[11] (incubation period: 5 min). The protein concentration was measured by the method of Lowry *et al.*[12]. Under these conditions the final enzyme preparation had a specific activity of 30 units per mg, one unit being defined as the amount of enzyme which hydrolyses 1 μmole of nitrocatechol sulphate per min at 37 °C. Electrophoresis on 7.5% standard gel (Canal. Co., Bethesda, Md., U.S.A.). in Tris–glycine buffer (pH 8.3, I 0.005) with a current of 5 mA for 1 h revealed no major impurities. Only a single band of protein was obtained. The enzymic activity was located in this band.

Assay conditions for the studies of the kinetic properties of the arylsulphatase A

The enzyme solution was dialysed 6 times for 2 h against distilled water and diluted to a final concentration of 25 μg per ml. The incubations were performed with dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) (Sigma Chem. Co., St. Louis, Mo., U.S.A.) as substrate. The pH of the 40 mM substrate solution had been adjusted to 5.3 at 21 °C with 0.1 M formic, acetic or propionic acid, corresponding to the electrolyte used in the assay. The amount of acid necessary for the pH adjustment raised the buffer concentration in the incubation mixture by 5 mM at 5 mM substrate concentration. Sodium formate, sodium acetate and sodium propionate solutions of the concentrations 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 M were adjusted to pH 5.3 at 21 °C. For the assay in the presence of Cl^- a 3.2 M solution of NaCl, and for those in the presence of Ba^{2+} or SO_4^{2-} 0.1 M solutions of $BaCl_2$ and Na_2SO_4 were prepared. The reagent system contained 0.2 ml of sodium formate, sodium acetate or sodium propionate solution, appropriate volumes of enzyme solution, substrate solution, distilled water, and, in some cases, NaCl, Na_2SO_4 or $BaCl_2$ solutions to give a total incubation volume of 0.4 ml. The incubation temperature was 37 °C. After a preincubation period of 5 min at 37 °C the enzymic reaction was started by the addition of the substrate solution. After an appropriate incubation time it was stopped by adding 0.5 ml 1 M NaOH. The amount of the nitrocatechol liberated was colorimetrically determined with a Zeiss spectrophotometer at 515 nm ($\varepsilon_{515\ nm}$ of nitrocatechol: 12 400).

The 10-fold of the volumes given above was incubated for the determination

of the progress curves. Samples (0.4 ml) were withdrawn at suitable time intervals and added immediately to 0.5 ml 1 M NaOH.

The pH of the reagent system was controlled before and after the incubation (before the addition of NaOH) with a micro-pH-meter of Radiometer Kopenhagen. It was 5.3 $\pm$ 0.02 in all cases.

RESULTS

Relation between the incubation time and the activity of the arylsulphatase A

A definition of the mathematical relation between the incubation time and the enzymic activity renders the possibility of an exact determination of parameters characterizing the enzymic reaction under specific conditions. The determination of this relationship was based on the following considerations: The time dependent variation of the activity of the arylsulphatase A is caused by the progressive inactivation of the enzyme during the reaction[8,9]. The inactivation results from an effect of the substrate on the enzyme[13]. The reaction velocity v (nmoles nitrocatechol released per μg protein per min) therewith progressively decreases from V_0 at the incubation time $t = 0$ (min) (initial velocity) and asymptotically approaches $V_\infty = 0$ which is reached when the enzyme in the assay system is completely inactivated and when the enzymic activity has not been restored by any activator.

The reaction velocity v_x at the time t_x is equal to the slope of the progress curve at the time t_x. The relation between the reaction velocity and the incubation time therefore is given by the differentiation quotient du/dt of the equation describing the progress curve, that is the relation between the turnover u of the reaction (nmoles of nitrocatechol released per μg protein) and the incubation time t (min).

Graphical evaluations of the experiments give linear relations between the reciprocals of the turnover u and the incubation time t (Figs 1b and 3b). The progress curve may therefore be described by Eqn 1:

$$\frac{1}{u} = A + B\frac{1}{t} \tag{1}$$

Eqn 1 shows a formal analogy to the equation describing the dependence of the reaction velocity on the substrate concentration in cases of normal enzymic reactions. Accordingly the constant A can be calculated by inducing the limiting condition $\lim_{t \to \infty}$ into Eqn 1, while B is derived by solving Eqn 1 for u, differentiating and inducing the limiting condition $\lim_{t \to 0}$. These operations lead to the following results:

$$A = \frac{1}{U_{max}} \tag{2a}$$

where U_{max} is the maximum turnover the enzyme can achieve under the specific conditions at infinite incubation time without being reactivated, and

$$B = \frac{1}{V_0} \tag{2b}$$

where V_0 is the initial velocity of the enzymic reaction under the specific conditions. From Eqns 1, 2a and 2b Eqn 3 is derived:

$$\frac{1}{u} = \frac{1}{U_{max}} + \frac{1}{V_0 t}$$

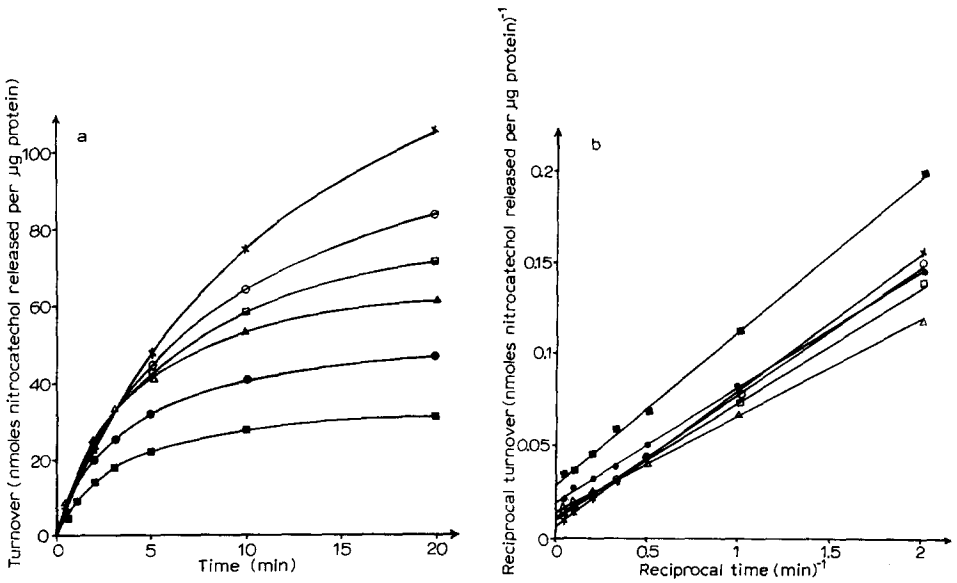


Fig. 1. (a) Progress curves of the enzymic reaction at different formate concentrations. Assay conditions as described under Experimental; 1.25 µg/ml protein; 5 mM nitrocatechol sulphate; sodium formate concentrations: ×—×, 0.805 M; ○—○, 0.405 M; □—□, 0.205 M; △—△, 0.105 M; ●—●, 0.055 M; ■—■, 0.03 M. The maximum amount of product formed is 2.75% of the initial substrate concentration. (b) Reciprocal plot corresponding to (a).

$$u = \frac{U_{max} V_0 t}{U_{max} + V_0 t} \tag{3}$$

The differentiation quotient du/dt of Eqn 3 gives the dependence of the velocity of the reaction catalyzed by arylsulphatase A on the incubation time:

$$\frac{du}{dt} = v = \frac{U_{max}^2 V_0}{(U_{max} + V_0 t)^2} \tag{4}$$

The half-life $\tau_{\frac{1}{2}}$ (min) necessary for the reaction velocity to fall to half its initial value is calculated as follows:

$$\frac{V_0}{2} = \frac{U_{max}^2 V_0}{(U_{max} + V_0 \tau_{\frac{1}{2}})^2}$$

$$\tau_{\frac{1}{2}} = 0.414 \frac{U_{max}}{V_0} \tag{5}$$

The variation of the reaction velocity must be proportional to the variation of the concentration of the active enzyme in the assay system. The half-life of the reaction velocity should therefore be an indication of the rate at which the enzyme is inactivated.

In what follows the influence of certain monovalent anions, of SO_4^{2-}, and of substrate on the kinetic properties of the arylsulphatase A is studied with the aid of the relations deduced above.

TABLE I

EFFECT OF THE FORMATE CONCENTRATION ON SOME PARAMETERS OF THE ENZYMIC REACTION

Assay as described under Experimental; 1.25 μg/ml protein; 5 mM nitrocatechol sulphate.

Sodium formate concn (M)	*Half-life* $\tau_{\frac{1}{2}}$ *(min)*	*Maximum turnover* U_{max} *(nmoles nitrocatechol released per μg protein)*	*Initial velocity* V_0 *(nmoles nitrocatechol released per μg protein per min)*
0.805	5.6	168	12.3
0.405	3.9	117	12.7
0.205	2.7	89	13.4
0.105	2.0	74	14.8
0.055	1.9	55	12.0
0.03	1.7	36	8.8

Influence of the sodium formate concentration in the assay system on the activity of the arylsulphatase A

The progress curves of the enzymic reactions catalysed by the arylsulphatase A at various sodium formate concentrations and at 5 mM substrate concentration are given in Fig. 1a. They show an increase in the rate of inactivation of the enzyme which results in a decrease of the maximum turnover as the formate concentration is lowered. The reciprocal plots corresponding to Fig. 1a are given in Fig. 1b, the maximum turnovers, the initial velocities, and the half-lives of the enzymic reactions calculated from Fig. 1b are listed in Table I. The initial velocities appear to show a more complex dependence on the formate concentration: When the formate concentration is lowered it slowly rises, to reach a maximum at 0.105 M formate, after which it falls off sharply.

Similar experiments performed in the presence of 1 mM $BaCl_2$ to precipitate

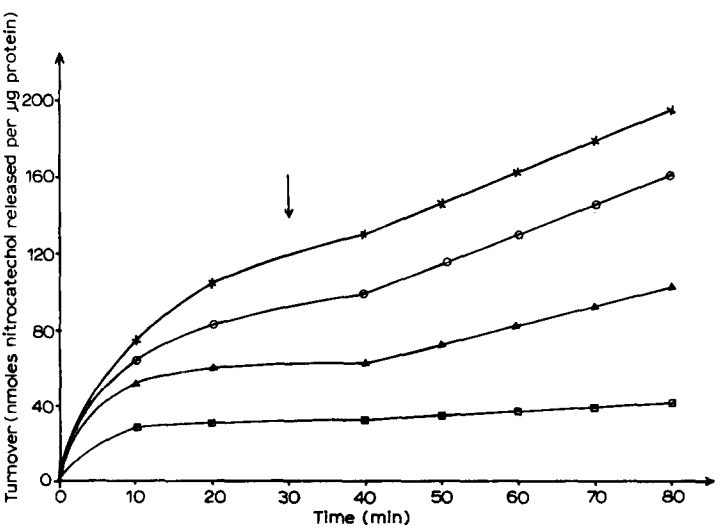


Fig. 2. Progress curves at different sodium formate concentrations under the influence of SO_4^{2-}. Assay conditions as outlined under Fig. 1a. After 30 min incubation time 333 μl Na_2SO_4 solution were added to 3 ml incubation mixture to give a SO_4^{2-} concentration of about 10 mM.

the liberated SO_4^{2-} resulted in virtually the same values for the reaction parameters. It was thus demonstrated that the SO_4^{2-} liberated by the hydrolysis of the nitro catechol sulphate had no effect on the initial phase of the enzymic reaction.

Influence of the sodium formate concentration on the activity of the arylsulphatase A reactivated by SO_4^{2-}

Arylsulphatase A is largely inactivated after an incubation period of about 30 min. The addition of Na_2SO_4 to give a SO_4^{2-} concentration of 10 mM caused a reactivation of the enzyme which led to constant reaction velocities[9]. The resulting reaction velocities depended on the sodium formate concentration in the assay system, being higher the higher the formate concentration (Fig. 2, Table II). The small amount of SO_4^{2-} released by the hydrolysis of the substrate gave rise to a minor variation of the SO_4^{2-} concentration in the assay systems. These variations could be neglected, however, as the following experiment indicated:

After an incubation period of 30 min an assay system (0.405 M sodium formate) was divided into two portions. To both portions Na_2SO_4 was added to give SO_4^{2-} concentrations of 9.5 and 10 mM, respectively. The resulting constant reaction velocities were 1.57 and 1.56 (nmoles of nitrocatechol released per μg protein per min), respectively.

TABLE II

VELOCITY OF THE ENZYMIC REACTION AFTER REACTIVATION BY SO_4^{2-}

Assay as described under Experimental; 1.25 μg/ml protein; 5 mM nitrocatechol sulphate; after an incubation period of 30 min addition of 0.333 ml 0.1 M Na_2SO_4 solution to 3 ml incubation mixture.

Sodium formate concn (M)	*Effective SO_4^{2-} concn (mM)*	*Reaction velocity after reactivation (nmoles nitrocatechol released per μg protein per min)*
0.805	10.3	1.63
0.405	10.25	1.57
0.105	10.17	1.015
0.03	10.1	0.216

Influence of the substrate concentration on the activity of the arylsulphatase A

Fig. 3 shows the time dependent variation of the activity of arylsulphatase A at various substrate concentrations in the presence of 0.105 M sodium formate. The initial velocity of the hydrolytic reaction increases with the substrate concentration as should be expected from the Michaelis–Menten theory. The same holds true for the rate of enzyme inactivation, while the maximum turnover does not depend on the substrate concentration (Table III). At 0.1 and 0.05 mM substrate concentration the theoretical maximum turnover turned out to be higher than the amount of nitrocatechol sulphate present in the assay system. In these cases the reaction was only followed over an incubation period of 2 min. During this period the reciprocals of the turnover and the incubation time were linearly correlated with each other. At longer incubation times the turnover became progressively less than expected because of the depletion of substrate in the assay system. The independence of the maximum turnover from the substrate concentration was also observed in assay systems containing 0.805 M sodium formate.

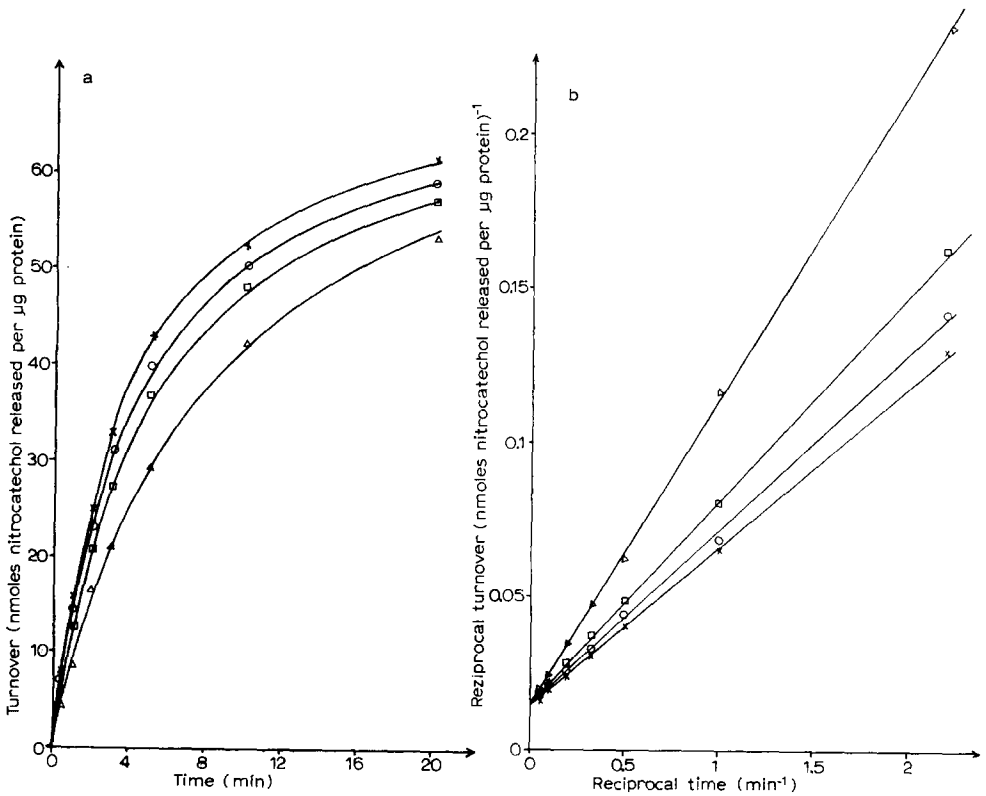


Fig. 3. (a) Progress curves at different substrate concentrations. Assay conditions as described under Experimental; 1.25 µg/ml protein; 0.105 M sodium formate concentration; nitrocatechol sulphate concentrations: ×—×, 5 mM; ○—○, 2 mM; □—□, 1 mM; △—△, 0.5 mM. (b) Reciprocal plot corresponding to (a).

TABLE III

EFFECT OF SUBSTRATE CONCENTRATION ON SOME PARAMETERS OF THE ENZYMIC REACTION

Assay conditions as described under Experimental; 1.25 µg/ml protein; 0.105 M sodium formate.

Substrate concn (mM)	*Half-life* $\tau_{\frac{1}{2}}$ *(min)*	*Maximum turnover* U_{max} *(nmoles nitrocatechol released per µg protein)*	*Initial velocity* V_0 *(nmoles nitrocatechol released per µg protein per min)*
5	1.5	74	20.0
2	1.7	74	17.4
1	2.0	74	14.8
0.5	3.0	74	10.0
0.1	10.2	74	3.0
0.05	19.1	74	1.6

Apparent Michaelis constants of the arylsulphatase A

To establish the Michaelis–Menten curves of the enzyme the velocities of the enzymic reaction were determined at incubation periods of 1, 5, and 10 min as described under Experimental. The velocities referred to in this chapter therefore are not initial but instantanous velocities.

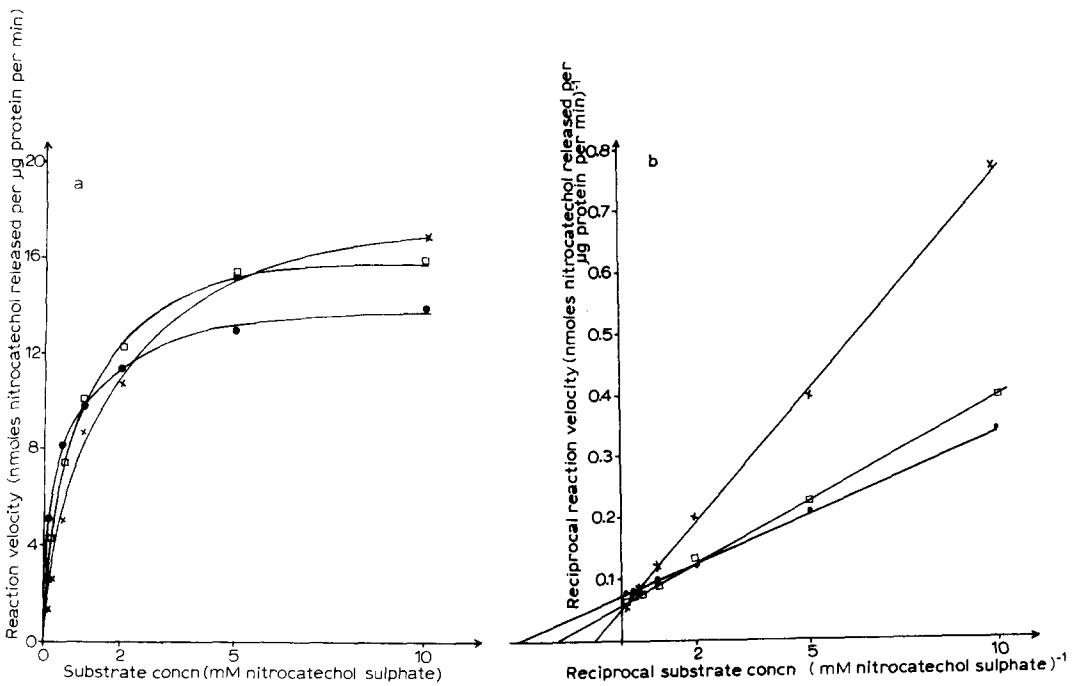


Fig. 4. Michaelis–Menten curves. Assay conditions as described under Experimental; incubation time 1 min, 6.15 µg/ml protein; sodium formate concentrations: ×—×, 0.805 M; □—□, 0.205 M; ●—●, 0.055 M. (b) Lineweaver–Burk plots corresponding to (a).

Fig. 4a gives a typical example of the variation of the Michaelis–Menten curves with the sodium formate concentration; the corresponding Lineweaver–Burk plots are shown in Fig. 4b. It can be seen from Fig. 4 that the apparent Michaelis constants decrease and the reaction velocities at low substrate concentrations increase, when the formate concentration in the assay system is lowered. From this evidence an inhibition of arylsulphatase A by formate is obvious.

The enzyme is inactivated at different rates depending on the formate concentration. Therefore the maximum velocity of the enzymic reaction must also depend on the formate concentration. For this reason the variation of the maximum velocities gives no evidence on the mode of inhibition of the enzyme by formate. The apparent Michaelis constants K_p calculated from the Lineweaver–Burk plots increase proportionally to the formate concentration in the assay system (Fig. 5). According to Dixon and Webb[14] this proportionality indicates a competitive mode of inhibition, and the apparent Michaelis constant in the absence of the inhibitor K_s as well as the inhibitor constant K_i may be calculated from Eqn 6:

$$K_p = K_s \left(1 + \frac{c_{\text{sodium formate}}}{K_i}\right) \tag{6}$$

The evaluation of the Michaelis–Menten curves is further complicated by the fact that due to the inactivation of the enzyme during the reaction the constants K_p, K_s and K_i also depend on the length of the incubation period (Table IV).

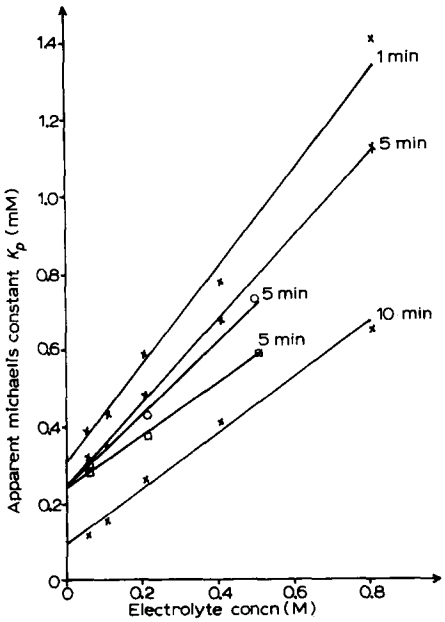


Fig. 5. Variation of apparent Michaelis constants K_s and K_p with electrolyte concentration and incubation time. Assay conditions as outlined under Fig. 4a. Electrolyte: ×—×, sodium formate; ○—○, sodium acetate; □—□, sodium propionate. Incubation times are noted in the figure.

TABLE IV

APPARENT MICHAELIS AND INHIBITOR CONSTANTS OF ARYLSULPHATASE A IN THE PRESENCE OF FORMATE AND SO_4^{2-} AT DIFFERENT INCUBATION TIMES

The competitive inhibition of arylsulphatase A by SO_4^{2-} is well known[13]. Data on the inhibitor constant for the human enzyme are not available, however. The incubations were performed in 0.405 M sodium formate, therefore the apparent Michaelis constant K_s still reflects the inhibitory effect of formate. The same must be assumed for the inhibitor constant K_i. It is clear, however, that the K_i of SO_4^{2-} is very much smaller than the K_i of formate.

Electrolyte	*Incubation time (min)*	K_s *(mM)*	K_i *(mM)*
Sodium formate	1	0.3	238
Sodium formate	5	0.24	221
Sodium formate	10	0.09	124
Sodium sulphate	1	0.67	8.7

Influence of other monovalent anions on the kinetic properties of the arylsulphatase A

The effects of sodium acetate and sodium propionate on the arylsulphatase A are very similar to those of sodium formate: they inhibit the enzymic reaction (Fig. 5) and at the same time exert a concentration dependent influence on the rate of enzyme inactivation. On comparison the inhibitor constant increases with the chain length of the monocarboxylic acid, while the half-life of the enzymic reaction decreases at the same instance (Table V).

The effect of NaCl, which is often used in the assay system, could only be studied in the presence of sodium acetate or formate as buffer components. Any in-

TABLE V

INHIBITOR CONSTANTS AND HALF-LIFE OF THE ENZYMIC REACTION IN THE PRESENCE OF FORMATE, ACETATE, AND PROPIONATE

Incubation time for the determination of the inhibitor constants: 5 min. 0.405 M electrolyte concentration; 1.25 μg/ml protein; 5 mM nitrocatechol sulphate in the assay systems for the determination of half-lives of the enzymic reaction.

Electrolyte	K_i *(mM)*	*Half-life* $\tau_{\frac{1}{2}}$ *(min)*
Sodium formate	221	3.9
Sodium acetate	256	3.4
Sodium propionate	362	2.3

hibitor constant or half-life would therefore reflect the influence of two distinct kinds of anions on the kinetic properties of arylsulphatase A and cannot be compared directly to the data given above. The progress curves of two assays in the presence of 0.03 M sodium formate as well as 0.8 and 0.08 M NaCl, respectively (Fig. 6), show, however, that the effects of Cl^- on the arylsulphatase A are much the same as those of formate, acetate, and propionate.

From corresponding experiments with KCl and RbCl exactly the same progress curves were obtained, thus showing that it were indeed the anions of the electrolytes cited above which influenced the kinetic properties of arylsulphatase A.

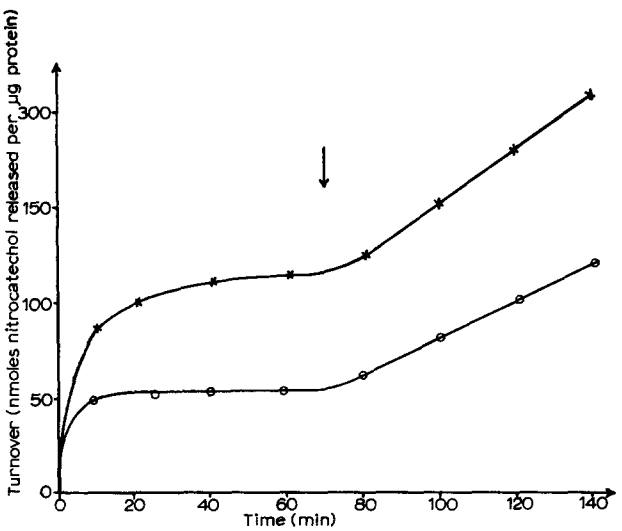


Fig. 6. Progress curves in the presence of NaCl. Assay conditions as outlined under Experimental; 1.25 μg/ml protein; 5 mM nitrocatechol sulphate; 0.03 M sodium formate; NaCl concentration: ×—×, 0.8 M; ○—○, 0.08 M. After an incubation period of 70 min 222 μl 0.1 M Na_2SO_4 solution were added to 2 ml incubation mixture to give a SO_4^{2-} concentration of 10 mM.

DISCUSSION

The effects of formate, acetate, propionate, and Cl^- anions on the kinetic properties of the arylsulphatase A are qualitatively alike. In what follows they will be

discussed with regard to formate only. It must not be forgotten, however, that other monovalent anions show similar effects. Arylsulphatase A is inhibited by formate anions. From the linear correlation between the apparent Michaelis constant K_p and the formate concentration (Fig. 5) a competitive mode of inhibition is assumed. Regarding, however, the complexity of factors affecting the Michaelis–Menten curves this assumption is by no means unambiguous. A comparison between the apparent Michaelis constant in the absence of formate K_s and the inhibitor constants K_i of formate and of SO_4^{2-} shows that the affinity of the enzyme to nitrocatechol sulphate and to SO_4^{2-} is much higher than to formate (Table IV).

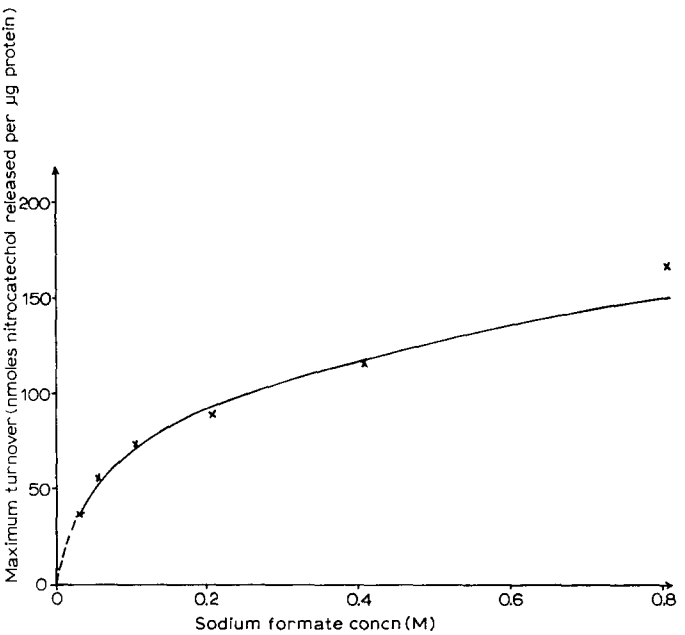
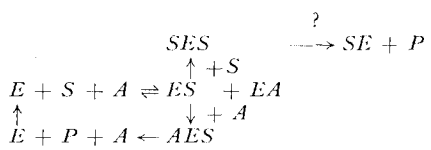


Fig. 7. Variation of maximum turnover U_{max} with sodium formate concentration. Maximum turnovers are obtained from Table I.

Despite their inhibitory effect formate ions significantly enhance the maximum turnover arylsulphatase A achieves before total inactivation (Table I). In fact, the maximum turnover seems to be not much different from zero in the absence of formate, as an extrapolation of the curve in Fig. 7 (a graphical representation of the correlation between the formate concentration and the maximum turnover) to zero formate concentration indicates. In the absence of monovalent anions like formate the arylsulphatase A seems to show hardly any activity. Direct evidence for this assumption could not be obtained, because the assay system had to contain at least that amount of formate which was necessary to adjust the pH of the substrate solution to 5.3 with formic acid .On the basis of the proposed reaction mechanism[8,9] these results may be explained as follows: By the formation of the enzyme–substrate complex with the substrate ion at the active sites (ES) a second binding site of the enzyme molecule is exposed which can bind either a second substrate ion to form an inactive SES complex, or a formate anion (A) to form an active AES complex. The

latter reacts to yield product *(P)*, formate *(A)*, and native enzyme *(E)*. Whether the *SES* complex reacts to yield product and an enzyme species with substrate bound to its second binding site *(SE)* or whether it is completely inactive cannot be decided. If, however, the SE complex is formed, it should be unable to degrade further substrate ions and thus stay inactive.

From the competitive inhibition of the enzyme by formate the existence of an enzyme–formate complex with formate at the active site of the enzyme *(EA)* must also be expected. It is ,however, doubtful whether a complex of the form *AEA* exists, since the kinetic properties of the enzyme cannot be altered by preincubation with formate alone. The proposed reaction scheme is represented in the following diagram, in which the stoichiometric relations are not taken into account:



According to this scheme the initial velocity of the enzymic reaction should be proportional to the concentration of *AES* in the assay system. This concentration is influenced by the formate concentration via two different reactions of opposite effects: an increase in the formate concentration leads (a) to an increase in the amount of EA and thus to a decrease in *ES* ($E + A + S \rightleftharpoons ES + EA$), and (b) to an increase in the amount of *AES* formed from the existing *ES* ($ES + A \rightarrow AES$). Whether Reaction a or b predominates in the variation of the initial velocity with the formate. concentration depends on the substrate concentration. At low substrate concentrations Reaction a predominates at least from 0.03 M formate concentration on, thus leading to a decrease in the initial velocity with increasing formate concentrations (Fig. 4a). At 5 mM substrate concentration Reaction b controls the variation of the initial velocity up to a maximum at 0.105 M formate concentration, whereafter Reaction a becomes predominant.

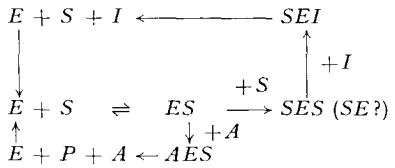
If this explanation of the reaction mechanism is correct, then the relation of the affinities to substrate and formate must be different for the first and second binding sites. The ratio of the affinities of substrate and formate to the first binding site must be much lower than the corresponding ratio of the affinities to the second binding site.

The same result is obtained from a comparison of the progress curves at different substrate concentrations (Fig. 3 and Table III), which shows that the maximum turnover is not affected by this item. That means that at any substrate concentration each enzyme molecule on the average hydrolyses the same number of substrate ions prior to its inactivation. The percentage of *SES* formed from the existing *ES* therefore must be largely independent from the substrate concentration, and the affinity of the second binding site to substrate must hence be much higher than to formate.

The relation between the maximum turnover and the substrate concentration furthermore explains the decrease of the apparent Michaelis constant with increasing incubation times. Since the turnovers at different substrate concentrations approach each other when the incubation time is increased, the apparent Michaelis

constant of the non-reactivated enzyme must decrease and should become zero at infinite incubation times.

Formate not only affects the reaction of the native arylsulphatase A but also the activity of the enzyme species, which has been reactivated by the addition of SO_4^{2-}. The constant reaction velocity, which is achieved after the addition of SO_4^{2-} to an assay system containing the inactivated enzyme, decreases with decreasing formate concentrations (Fig. 2, Table II). Formate accordingly also participates in the reaction of the reactivated enzyme, and the effect of activators like SO_4^{2-} (I) may merely result in a removal of the blockage of the enzyme by the substrate:



This diagram again does not take the stoichiometric relations into account.

Since SO_4^{2-} and formate are inhibitors of the enzyme, the enzyme–inhibitor complexes EI and EA should also exist. They have been omitted from the above scheme, however, because they do not change it basically. After the reactivation of the inactivated enzyme by SO_4^{2-} an equilibrium between the different enzyme species is slowly achieved which leads to a constant reaction velocity. Nicholls and Roy[9] have shown that in the presence of SO_4^{2-} the reactivated enzyme exhibits a mode of substrate inhibitions which indicates the existence of an inactive ES_2 species, as should be expected from the above reaction scheme. According to the law of mass-action the substrate concentration at which the inhibition becomes manifest should depend on the SO_4^{2-} concentration. Substrate inhibition cannot be expected, however, in the initial phase of the reaction, in which the enzyme is progressively inactivated. In this phase the reaction does not reach an equilibrium, and an elevation of the substrate concentration merely enhances the rate of inactivation and does not alter the maximum turnover (Table III).

The proposed scheme of the enzymic reaction of arylsulphatase A is by no means complete and unambiguous .To explain the effect of activators like SO_4^{2-} on the inactivated enzyme further studies are necessary, especially since Nicholls and Roy[9] have shown that at pH 6 and in the absence of substrate the rate of conversion of the inactivated to the active form of the enzyme is not affected by the presence of SO_4^{2-}. Up to now it is furthermore only ascertained that the enzyme is inactivated by the action of its substrate. Whether the inactivated form really contains any substrate ions we hope to elucidate by incubating native arylsulphatase A with double-labelled nitrocatechol sulphate.

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APPENDIX (Received March 20th, 1972)

KINETIC PROPERTIES OF ARYLSULPHATASE A—THEORETICAL TREATMENT

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In the foregoing paper it was shown empirically that the relationship between the amount of nitrocatechol produced *(u)* by the action of sulphatase A on nitrocatechol sulphate in time *(t)* had the following form:

$$\frac{1}{u} = A + B\frac{1}{t} \tag{1}$$

Eqn 1 accounts for the inactivation of sulphatase A which occurs during its reaction with its substrate. A relationship of this form can be rather simply derived if the enzymic reaction is assumed to be

$$E + S \rightleftharpoons ES \xrightarrow{k_3} E + \text{products} \tag{2}$$

$$ES \xrightarrow{\alpha k_3} \text{inactive enzyme} \tag{3}$$

In this scheme α is small: k_3 is approximately 30 s^{-1} (Roy, unpublished observations) whereas the half-time for the inactivation of sulphatase A is about 5 min, corresponding to a velocity constant of about 0.003 s^{-1} so that α must be of the order of 10^{-4}. When the substrate concentration (*s*) is much greater than K_m, then

$$v_0 = k_3 e' = k_3 e_0 \tag{3a}$$

where v_0 is the initial velocity of the enzymic reaction and e_0 and e' the initial total enzyme concentration and the concentration of the enzyme–substrate complex *(ES)*, respectively.